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Antibiofilm activity of lichen secondary metabolites

Alaa Sweidan

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ANNÉE 2017

THÈSE / UNIVERSITÉ DE RENNES 1
sous le sceau de l'Université Bretagne Loire

pour le grade de

DOCTEUR DE L'UNIVERSITÉ DE RENNES 1

Mention : Biologie et Sciences de la Santé

Ecole doctorale Vie-Agro-Santé

présentée par

Alaa Sweidan

Préparée dans les unités de recherche (UMR INSERM 1241, UMR CNRS 6226)
(Equipe CIMIAD, NUMECAN/ Equipe CORINT, ISCR)
UFR Sciences Pharmaceutiques, Université de Rennes 1

Antibiofilm activity of lichen secondary metabolites

Thèse soutenue à Rennes le 20 juillet 2017

devant le jury composé de :

Pierre Germon
Chargé de Recherches INRA, HDR, INRA de Tours/*rapporteur*

Olivier Grovel
Maître de conférences des Universités, HDR, Faculté des Sciences et Techniques, Université de Nantes/*rapporteur*

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IINTRODUCTION

A- Introduction

The following thesis presents a multidisciplinary work where chemistry has served to find new antibiotic agents against the oral bacteria. Four directors have contributed to this successful co-directional project between Lebanon and France. The French directors were Drs Latifa Bousarghin, Sophie Tomasi, and Pierre van de Weghe alongside Dr. Ali Chokr who was the Lebanese counterpart. It is worth to mention that I was working with the U-1241 INSERM-INRA, CIMIAD Team, formerly EA 1254, where Dr. Latifa Bousarghin was the direct supervisor. In addition, we had a strong collaboration with UMR CNRS 6226, Institut des Sciences Chimiques de Rennes, Equipe CORINT, mainly with Dr. Sophie Tomasi who was the second direct supervisor taking care of the chemistry part and doing a weekly meeting with me and Dr. Bousarghin to discuss the work progression.

Being EA 1254 working with the oral microbiota and studying the periodontal disease, our project aimed to find a new antibiotic that combats the oral infection resulting from this disease. We have chosen the two oral bacteria, *Streptococcus gordonii* and *Porphyromonas gingivalis*, for this study as being one of the best identified interspecies combinations [1]. *S. gordonii* is an eminent member of the viridans streptococci large category [2]. In the oral cavity, *S. gordonii* adheres to the salivary pellicle which coats the teeth, proliferates and excretes an extracellular polysaccharide matrix protecting its developing microcolony on which secondary colonizers will adhere [3]. *P. gingivalis* which is a dangerous late colonizer as it has been considered the etiological agent of periodontal diseases binds the sites provided by *S. gordonii* forming a highly pathogenic microbial community [1,4]. Not only does this biofilm have local effects, but also can lead to systemic infections and complications [5,6]. Hence, *S. gordonii* as a pioneer initial colonizer initiates the formation of dental plaques contributing in turn to the onset of periodontal diseases as well as their progression [7], [8].

The usages of antibiotics on a large scale alongside their misapplication have led to the emergence of resistant pathogenic bacteria [9]. Both, the infection of these re-emergent strains which has increased the global mortality rate to be a growing concern and the global reduction in antibiotics production open a new era where other potent candidates should be found to fight against bacteria [10], [11]. Throughout the last 2 decades, plants are becoming a famous rich source of antimicrobial substances [12]. This green treasure has provided more than 300 natural antimicrobial metabolites between 2000 and 2008, however, many promising drug sources still need to be explored [10]. Lichens which are symbiotic organisms comprising a fungus and a photosynthetic alga and/or cyanobacterium constitutes a potential source of over 1000 distinct secondary metabolites [13]. They comprise antitumor, antiviral and antimicrobial activities [13–15]. Concerning their antibacterial properties, sensitive as well as several multi-drug resistant bacterial strains were shown to be susceptible to their potency [13].

To address the antibiotic crisis in one of its fields, the oral cavity, lichen metabolites were screened for efficient antibiotics against two oral bacteria, *S. gordonii* and *P. gingivalis*. Two main tracks have been followed:

1- Inhibiting *S. gordonii* and the early plaque thereby preventing the complex biofilm to form.
2- Targeting *P. gingivalis* to prevent the developing biofilm from progressing into a more advanced stage.

STATE OF ART

B- State of art

As a bibliographical introduction, it will be worth to start with a brief anatomy part which will draw the oral cavity focusing on the jaw structure to know the characteristics of the teeth and to compare the healthy with the diseased status. The diseases attributed to the bacteria in this oral niche involve a sessile lifestyle of the latter called the biofilm. If we wanted to combat the oral bacteria, we would first understand their behavior in this organized community. This has pushed us to explain a little bit about the biofilms in general to reach the dental plaque which is our interest in this project. The dental biofilms don't only have local effects, but also can cause systemic complications which make the issue very urgent to find some compounds capable of preventing or treating the infections of these dangerous biofilms.

Despite the fact that there are many compounds already described in the literature, several factors have helped the bacteria to develop resistance against them until reaching a post-antibiotic era where the resistance has touched all the antibiotics discovered to date. "What are these factors?", "How do the antibiotics kill the bacteria? i.e., what are their bacterial targets?" and "How do the bacteria resist their modes of action?" are all important questions we tried to answer in the following sections to discuss after that the reasons behind choosing lichens organisms for our antibiotic searching journey.

I- Oral cavity

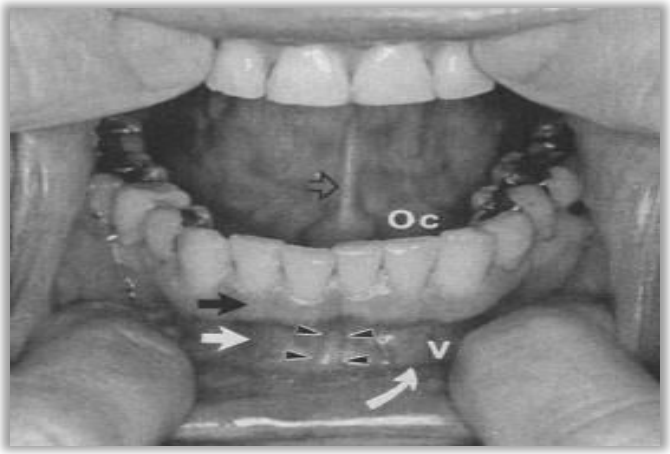
Many distinct ecological niches colonized by microorganisms exist in the human body [1]. The oral cavity is one of these important sites as it reflects the health of this complex organism [16]. Oral microbes or microbiome, as defined by Joshua Lederberg, can reside in there utilizing various habitats like cheek, lips, hard and soft palates, tongue, attached gingiva, gingival sulcus, and teeth. In addition, they can inhabit the mouth neighboring extensions reaching the distal part of the esophagus [17]. The prevalent members are the bacteria alongside minorities of Fungi, Mycoplasma, Protozoa, and Archae [18].

It has been said before that the mouth is the mirror of the body's health. This section will dissect the regions of this oral niche where the bacteria can assemble and form communities to disturb the oral health and consequently the whole body health. It's an indispensable introductory section for the coming chapters to be clear. In microbiology words, this chapter is like an early colonizer forming a platform in the reader's brain and the other chapters need this basis to bind and form a complex understanding community.

A brief dentition-focused anatomy of the oral cavity

The upper part of the aerodigestive tract constitutes of the oral cavity and oropharynx [19]. Inside the oral cavity we have the dentition structure or jaw which is composed of 32 teeth

divided in half into a maxilla and a mandible. The teeth are fixed firmly, deeply and separately in bony sinuses in an osseous rib named the alveolar process where the periodontal ligament is responsible for their anchoring. This process divides the oral cavity into a central part comprising the tongue and a peripheral oral vestibule part constituted of the lips and the cheeks. Reflecting onto the alveolar process, the mucosa lines the oral vestibule creating a groove named the fornix vestibuli. Another mucosa coats the alveolar process to be split up into alveolar mucosa below the fornix and gingiva above it. The free boundary of the alveolar



process neighboring the teeth is covered by the gingiva (Figure 1) [20].

Figure 1: The vestibule and the oral cavity. The aveolar process and teeth separates the vestibule (V) from the oral cavity (Oc). Curved arrow refers to fornix vestibuli, black arrow refers to gingiva, white arrow refers to alveolar mucosa, open arrow refers to lingual frenum, and arrowheads refer to labial frenum [20].

The exposed part of each tooth is called the anatomical crown and when the gingiva recesses with age, it is named the functional crown. The other part fixed in the alveolar process is called the root and it is framed by a dense cementum. The crown is composed of enamel and an underlying dentin. An area called the pulp is found beneath the dentin and is constituted of connective tissue, hosting nerves and blood vessels. The border separating the crown from the root is the cementoenamel junction, or cervical constriction or neck. The tooth sinus is lined with a dense cortical bone named the lamina dura where the periodontal ligament resides between it and the root cementum (Figure 2) [20].

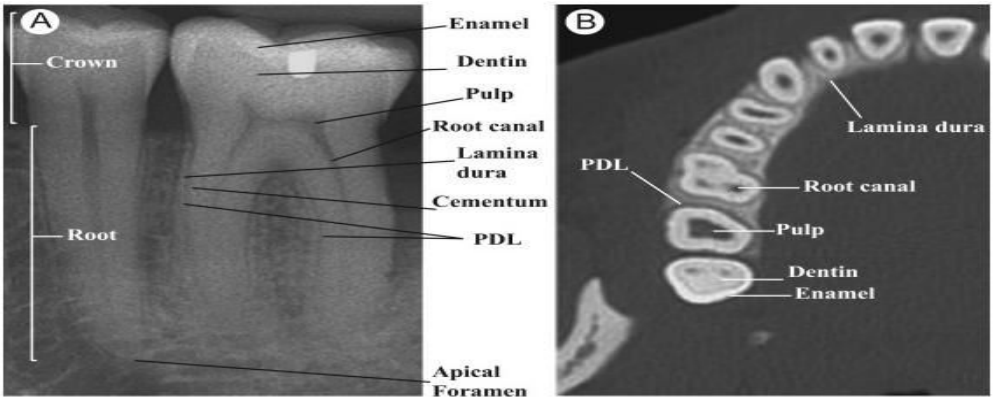


Figure 2: Radiography showing the tooth anatomy. Intraoral radiograph is shown in A, however, B displays an axial computed tomography (CT) image. Sclerotic lamina dura is displayed as a white region surrounding the teeth and in between the two there exists a thin radiolucent line or the periodontal ligament (PDL). Cementum which lines the root doesn't appear on radiographs. An extremely radiodense enamel appears a cap above an opaque softer dentin consisting most of the tooth. Inside the dentin, radiolucent chambers connected to radiolucent canals form the pulp and root canals, respectively. The deepest end of the tooth is the root apex [20].

The gingival part loosely bound and nearest to the tooth crown is called the free gingiva. It constitutes a collar around each tooth leaving a potential space in between called the gingival crevice or sulcus. Its clinical healthy depth can extend from about 1 into 3 mm (Figure 3) [21].

Figure 3: Inserting the periodontal probe tool between the tooth and the free gingiva to measure the depth of the gingival sulcus.

II- The sessile microbial lifestyle; the biofilm

The biofilm theory hasn't grown up until 1978 and since that time the scientific world is trying to understand as much as possible this universal microbial lifestyle whose existence has touched aquatic and industrial water systems along with a numerous number of environments and medical devices pertinent to public health.

The historical time line of developing the biofilm definition and the formation steps needed by the free-swimming bacteria to form this organized agglomeration will begin this chapter. They will be followed by the impact of this lifestyle on several fields finishing on the medical one. After the latter, the reader will be ready to enter the oral cavity and discover the dental biofilm and its attribution to the periodontal disease. The chapter will then complete the story with the local and distant complications of this biofilm. Finally, the periodontal diseases classification will be briefly discussed to finish with a description of two important bacterial strains implicated in the oral infection and related to the systemic complications.

a. Definition

Growing of the bacteria in a matrix-enclosed biofilm wasn't immediately accepted in medical and dental areas. However, when the scientists have admitted the absence of a complex nervous system in the bacteria to locate themselves in comparison to the animal body, they have concluded that these microorganisms utilize certain basic survival strategies by forming biofilms. Defining this lifestyle has developed with time as new characteristics being discovered (Table 1) [22].

Table 1: The development of biofilm definition with time was described by Donlan et al, 2011 [22].

Year	***Author***	***Facts found***
1976	Marshall	Very fine extracellular polymer fibrils anchor bacteria to surfaces.
1978	Consterton et al	Bacteria are enclosed in glycocalyx matrix of polysaccharide nature and helps in adhesion.
1987	Consterton et al	(Biofilm) is an assembly of single cells and microcolonies embedded in a highly hydrated, predominantly anionic exopolymer matrix.
1990	Caraklis and Marshall	Spatial and temporal heterogeneity characterizes this biofilm whose matrix contains also abiotic and inorganic substances.
1995	Conserton et al	Biofilms attach to surfaces, interfaces and to each other. The definition mentioned also microbial aggregates, floccules and populations adherent in the pore spaces of porous media.
	Consterton and Lappin-Scott	The attachment stimulated the expression of genes involved in generating components which aid adhesion and biofilm formation.

In summary, the complete definition that the scientists have determined till now for a biofilm will be summarized as a microbial fixed community containing cells which have adhered irreversibly to a surface, interface, or to each other. They are embedded in an extracellular polymeric matrix they have generated and differ at the level of growth rate and gene transcription [23].

a. Biofilm formation process

Regardless of the relatively high cell growth and reproduction rate that the planktonic bacteria have, three main reasons can push the latter to transfer from the planktonic lifestyle into the sessile counterpart:

1- The biofilm can protect the bacteria from the harsh environmental conditions where they can withstand strong and repeated shear forces such as washing away by water flow or blood stream via adherence to a certain tissue or surface.
2- The extracellular polymeric matrix engulfs the bacteria deeply in its layers forming a barrier against antimicrobial agents whose diffusion will be limited.
3- The sessile community will limit the bacterial mobility and increase their density facilitating genetic exchange by conjugation whose rate is reported to be significantly higher than that between planktonic cells. The risky consequence is that this horizontal gene exchange can transfer resistance-coding genes [24].

This switching into the new fixed habitat occurs in mainly 5 sequential stages (Figure 4) [25].

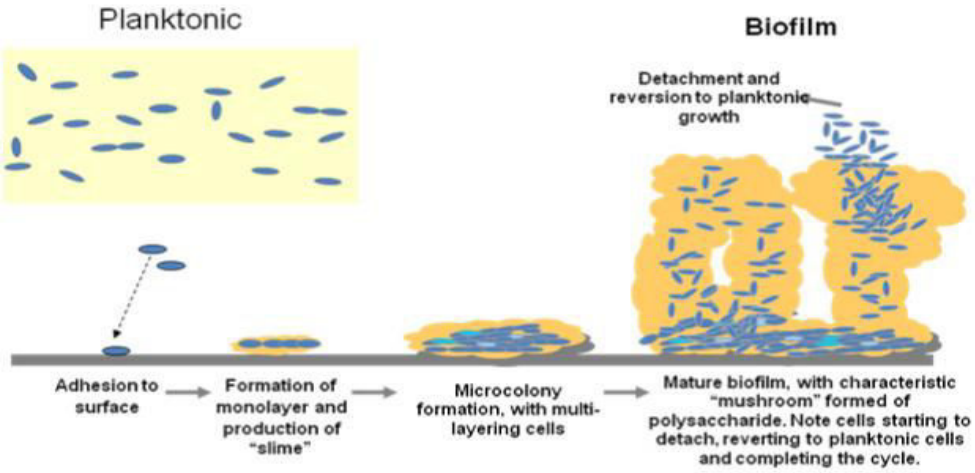


Figure 4: The 5 sequential stages of biofilm formation: a) adhesion to surface, b) formation of monolayer and production of slime, c) microcolony formation with multi-layering cells, d) formation of a mature biofilm, and e) detachment and reversion to planktonic growth which can adhere to the surface in another place and start a new biofilm formation process in a distinct site [25].

The factors which control the growth potential of a biofilm include nutrients availability and their diffusion power to the cells alongside the excretion of waste products. Moreover, pH, organic sources, oxygenation and osmolarity can influence its maturation. It is worth to mention here that the maturation in its turn also modifies the micro-environment enclosing the bacteria regarding their population density, oxygen and nutrients diffusion, and pH. In addition, different environments can result in heterogeneity regarding the biofilm cells functionalities in term of metabolism and reproduction [26].

A mature biofilm will constitute of a matrix encompassing the microbes with organic and inorganic materials in its lower layer coated with a fragile and indeterminate shape layer which extends into the surrounding medium. On the surface, a fluid layer exists bordering the whole community and comprising dynamic and static sub layers [23].

b. Impact of biofilm on diverse fields

The impact of the biofilm has spanned from distinct branches of industries into the clinical field. These biological deposits which form on any surface and known as biofouling have their considerable implications in many branches of industries including water systems and medical and process ones [27].

In food industry, biofilms attach rapidly to food-processing surface and cause serious microbial contamination leading to food deterioration and disease transmission. These sessile cells are reported, according to the microbes identity, to be more resistant than their planktonic counterparts to biosides, aqueous sanitizers, cleaning agents and disinfectants comprising iodine, chlorine, ozone, trisodium phosphate, peracetic acid, hydrogen peroxide and quaternary ammonium compounds, in addition to organic acids, ethanol and sodium hypochlorite [28].

Another important site for biofilm formation is the paper mill process waters. The abundant quantities of biodegradable matter from wood, starch and other raw materials along with a temperature range between 25 and 50°C found in these industries set very suitable conditions permitting a fast growth of microorganisms which can gain unrestricted access to the system by water, air, or with the raw materials. The microbes can form flocs or films in wastewater treatment plants, soils, and surface waters and can cause serious damage as clogging filters or perforating the papers [29].

On the other side, the clinical consequences of these stubborn communities may also exceed that of the industrial counterparts. The biofilm is reported to be responsible for 80% of human infections in the United States. They resist phagocytosis, innate and adaptive immune defense system, antibiotics and disinfectant chemicals thereby colonizing numerous surfaces in the human body leading to serious medical complications. Some examples of the organs that could be infected by biofilms are shown in figure 5 [30], [31], [32].

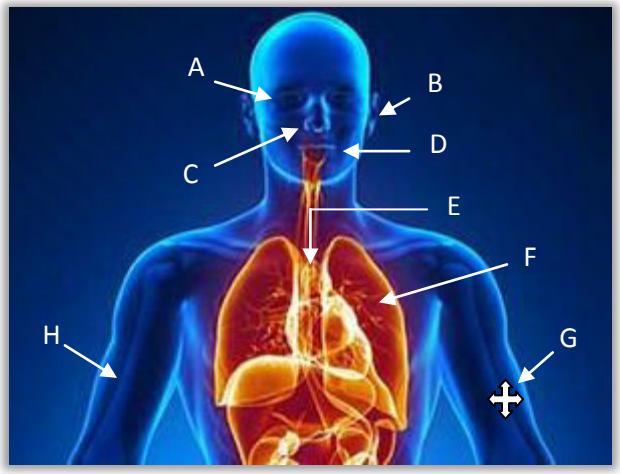


Figure 5: The biofilm can form on the contact lenses leading to corneal and ocular infections in the eyes comprising microbial keratitis, contact lens-related acute red eye, contact lens peripheral ulcer and infiltrative keratitis (A), or in the ear (chronic and secretory otitis media) (B), nose (chronic rhinosinusitis) (C), mouth (dental plaque and resulting periodontal diseases) (D), heart valves (endocarditis) and blood vessels on intravenous catheters or stents (E), lungs (cystic fibrosis causing chronic bronchopneumonia) (F), bones (chronic osteomyelitis and prosthetic joint infections) (H), and chronic wounds (G) [30], [31], [32].

c. Dental biofilms

The surfaces of the oral cavity can be colonized by several associations of about 700 bacterial species [33]. The complexity increased with Ji et al. who mentioned that these 700 species can just colonize the gingival sulcus comprising 10^3 bacteria. This number increases to be 10^8 bacteria in the periodontal pocket [34]. These oral microbial communities reside majorly in biofilms on saliva-coated surfaces. Their everyday life starts right after cleaning the teeth which will be coated rapidly with a salivary pellicle. The adsorption of its components relies on the composition of the surface where each substratum will expose different receptors [35].

Saliva has a pH ranging between 6.25 and 7.25 and affecting intensely the buccal ecology whereby it fosters the growth of microorganisms. One of its actions impacting oral bacteria is by forming a layer and coating the teeth permitting microbial attachment. Other important roles can be summarized by facilitating microbial clearance through their agglomeration, presenting a major nutrients source and intermediating killing or inhibiting the microbes [36]. In addition to saliva which provides proteins and glycoproteins, two additional nutrients sources are available for the oral microbiota. Since the teeth anchored to the jaw grow out of the gingiva, serum proteins released in the gingival sulcus form the second source. The third one constitute of the dietary food comprising proteins, carbohydrates, and lipids [37].

Some bacteria called the primary colonizers will bind these receptors selectively depending on their surface adhesins. As a result, the more versatile strains in receptor binding due to the

expression of several adhesins possess a major selective advantage over those which have less binding capabilities [35]. There exists a balance between the attachment and the removal factors including: a) mastication, nose blowing and swallowing, b) oral hygiene, and c) washing out by the fluids present (nasal, salivary, and crevicular fluids). The survivor species can only bind the shedding surfaces of the soft tissue or the non-shedding ones of the hard counterpart such as teeth [38]. The non-shedding surface such as tooth surface supports more the growth and maturation of the biofilm [37]. The resulting early biofilm contains only between 1 and 20 layers [39].

The early colonizers are also called the pioneer bacteria and include many species of *Streptococcus* such as *Streptococcus gordonii* which can bind, beside the salivary pellicle, to host cells and exposed root dentine. This genus constitutes more than 60% of the strains in the enamel early communities. The other genera include *Actinomyces*, *Veillonella* and *Neisseria* [35]. Specificity appears again in the next step where it characterizes the following recruitment of the late colonizers such as *Porphyromonas gingivalis* controlled by the interspecies co-adhesive proteins. Not only does the early streptococcal plaque recruit bacterial strains to develop their biofilm but also it coadheres with *Candida albicans*, an opportunistic fungal pathogen, forming a fungal-bacterial community with a risk to develop candidiasis [40].

This assembly is of two types due to the presence of same and different species. Autoaggregation describes the attachment of same species, whereas coaggregation exists between different ones. The latter results in distinct architectures such as Corncobs [23] formed of filamentous Gram-positive coated with Gram-positive cocci, bristle brushes constituted of big filaments surrounded by short ones or Gram-negative rods, or rosettes which are coccal bacteria coated with small curved Gram-positive rods (Figure 6 [41]).

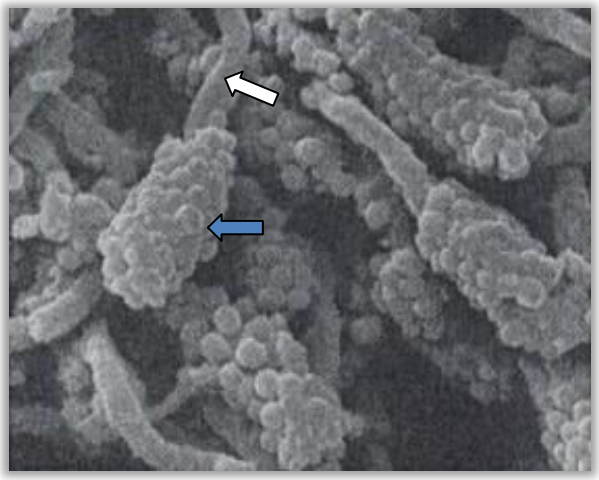


Figure 6: Scanning electron micrograph displaying the corncob structure. White arrow refers to the filamentous Gram-positive, and the blue arrow refers to the Gram-positive cocci coating [41].

The dental plaque can develop by accumulation of additional organisms or by growth and cell division. Each microorganism can adjust some transcriptional or proteomic features as an adaptive response maximizing its ability to increase its numbers in the developing biofilm. In addition, signal transduction networks and transcriptional regulation of one species can ease the colonization of other species. For instance, the so called, BrfAB, two-component signaling system of *S. gordonii* whose interaction with the saliva results in several genes upregulation comprising those that encode antigen I/II

family adhesions. Streptococcal surface protein A (SspA) and streptococcal surface protein B (SspB) antigen I/II proteins induce coagregation of this species with Actinomyces and *P. gingivalis* which may improve the following colonization of streptococcal platform by these species leading to diversity in the biofilm [35].

The future dental plaque can form at stationary sites existing between the teeth (approximal surfaces), on the occlusal surfaces of molars and pre-molars (within the pits and fissures) or in the gingival crevice (Figure 7). Each site develops a distinct biofilm with distinct risks. The approximal community becomes a cariogenic biofilm predominated by streptococci and lactobacilli. With respect to the gingival sulcus, the supragingival plaque is characterized with high availability of Gram-positive bacteria predominated with streptococci species [42]. Alongside saliva, a fluid that nourishes the microbes and has an immune role adjusting the existing microflora is produced in this crevice and called the gingival crevicular fluid (GCF) [18].

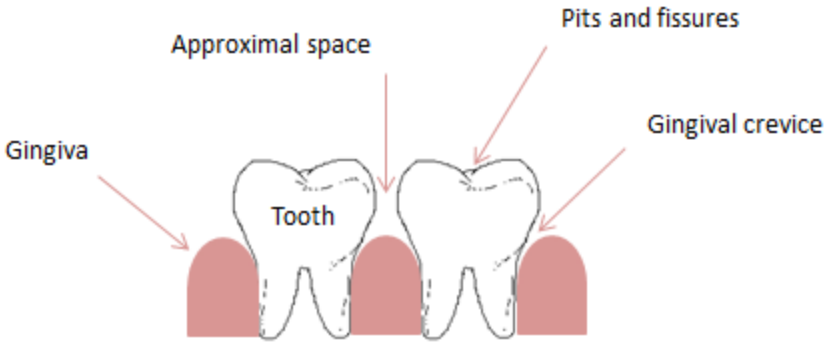


Figure 7: The sites of dental plaque formation.

The bacterial species which form the dental plaque below the gum line were molecularly studied by Socransky et al. They have taken such plaque samples from the mesial aspect of every tooth of 185 subjects having a mean age of 51 ± 16 years including 160 subjects with periodontitis and 25 without. An evaluation of the inter-connections between these species was done resulting in five main complexes: red, orange, green, yellow, and purple complexes (Figure 8). Moreover, some of which and some of their members were effectively related to the clinical conditions of inflammation and periodontal diseases. Both the orange and red complexes members were related to pocket depth and bleeding on probing. Existence of such relation can propose that the therapy that targets one species of these groups can affect as well another related member within them. Consequently, realizing these connections can diagnose the clinical condition and orient the periodontal therapy [43]. Haffajee et al. in 2008 have addressed the relations among the species found above the gum line. They have examined the microbial communities of supragingival plaque samples taken from 187 subjects of age between 22 and 74 years; only 38 of which were periodontally healthy. Interestingly, a similar

clustering with few minor variations was found compared to the subgingival plaque. In addition, the same complexes, orange and red, were related to inflammation [44].

Inspite of the continuous air flow throughout the mouth, the aggregation of bacteria in the plaque makes the region rapidly anaerobic favoring the growth of anaerobic strains. This dental plaque recruits planktonic bacteria to attach irreversibly to a stratum or interface and produce an extracellular polymeric matrix which will host also abiotic components. This new life pattern has a dramatic change in the microbial physiology including growth rate and gene expression profile exhibiting an inherent resistance to antibiotics [45].

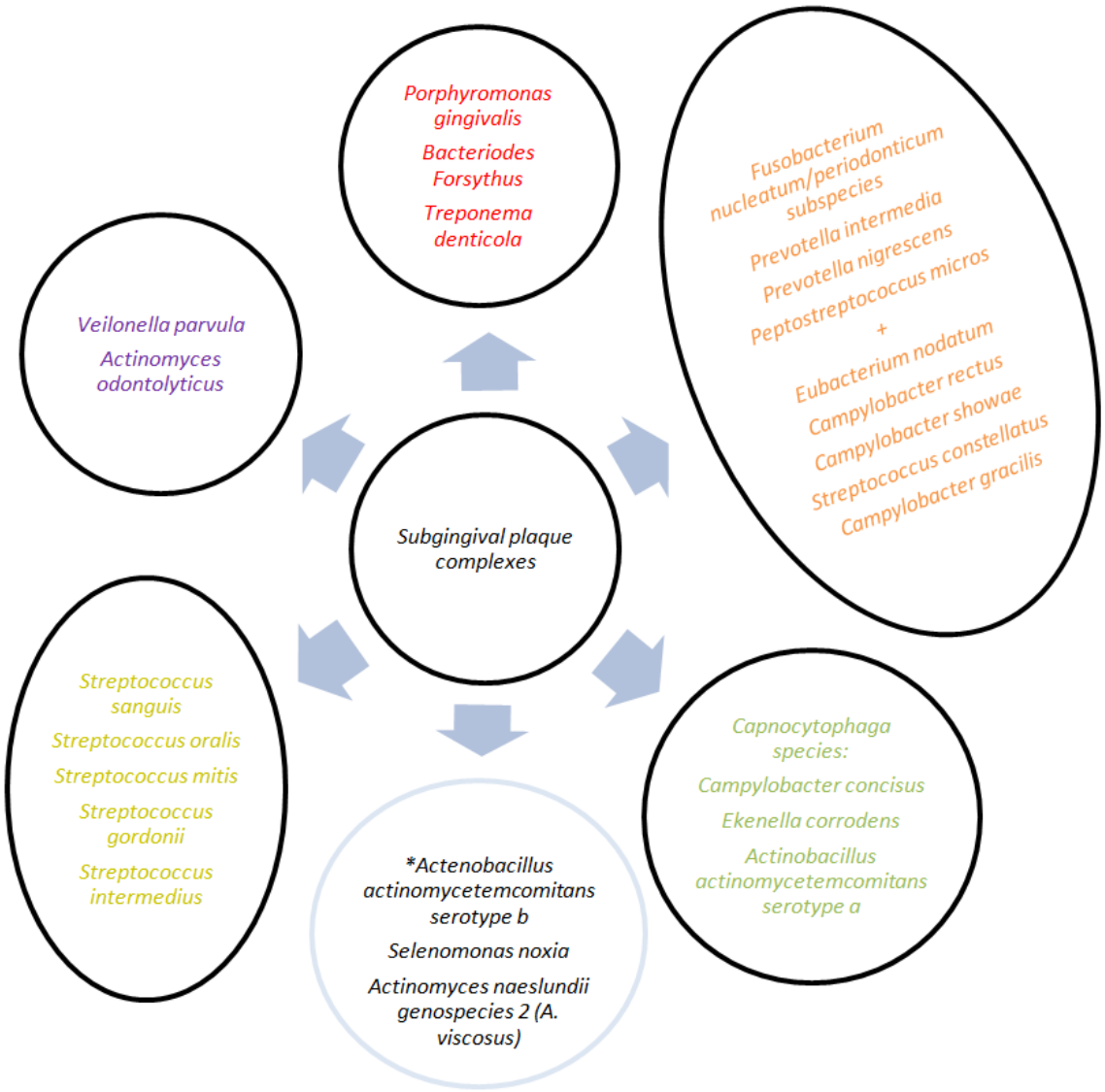


Figure 8: The five main bacterial complexes (red, orange, green, yellow, and purple complexes) written by their corresponding color. *: Socransky et al. had obtained little relation of these strains to each other and to other groups [43].

An interesting fact exists in the way the bacteria organize their places in the biofilm. When the planktonic cells lunch their initial colonization on a surface such as tooth surface, their physiological status determines their positions in this multi-layered biofilm. The cells constituting the biofilm surface resemble the planktonic cells regarding their physiological status where they can easily receive oxygen and nutrients and excrete metabolic wastes. In contrast, as the biofilm internal zone is deprived of oxygen, the cells in there respire utilizing nitrate and inorganic substances which serve as final electron acceptors [26].

i. The periodontal diseases

Numerous oral pathologies are biofilm related such as periodontal disease [39]. The disease-causing risk increases as the plaque remains more on the teeth causing gingivitis defined as the inflammation of the gums [46]. In this clinical status, the biofilm becomes an organized community of about 100-300 layers where the embedded species are arranged according to metabolism and aerotolerance [39].

The biofilm will launch the inflammation as the pathogenic bacteria are capable to spread beyond the primary infection site [47]. Despite the fact that the epithelial cells defend themselves against the attacking bacteria by their continuous turnover and shedding, these invading pathogens can double in a time short enough to diffuse beyond this physical barrier which needs between 41 and 57 days as a turnover interval [48]. The inflamed gum will have a red color, swell, and can easily bleed. This mild gum disease can be treated with daily teeth brushing accompanied by dental flossing with the aid of regular dentist cleaning. It can be reversed without any bone, tissue or eventually teeth loss which will mark a more advanced stage of inflammation if gingivitis is kept untreated [46].

Although the clinicians do their best, many patients will not spend the required time in brushing their teeth and most of them won’t or can’t floss one time a day. These facts result in gingivitis in more than 50% of adults in a population. Then, gingivitis may or may not progress to a more serious stage called periodontitis depending on several factors listed in table 2. These factors can influence the onset, progression rate, and severity of periodontitis as well as response to therapy. This will provide the clinician the capacity to constitute an accurate diagnosis, prescribe an optimal plan for the patient’s treatment, and provide correct maintenance schedule [49].

Table 2: Risk factors for developing periodontitis [49].

1. Heredity as determined by genetic testing and family history
2. Smoking including frequency, current use, and history
3. Hormonal variations such as those seen in
a. pregnancy in which there are increased levels of estradiol and progesterone that may change the environment and permit the virulent organisms to become more destructive
b. menopause in which the reductions in estrogen levels lead to osteopenia and eventually osteoporosis
4. Systemic diseases such as
a. diabetes (the duration and level of control are important)
b. osteoporosis
c. immune system disorders such as HIV
d. hematologic disorders such as neutropenias
e. connective tissue disorders such as Marfan's and Ehlers-Danlos syndromes
5. Stress as reported by the patient
6. Nutritional deficiencies that may require a dietary analysis
7. Medications such as
a. calcium channel blockers
b. immunomodulatory agents
c. anticonvulsants
d. those known to cause dry mouth or xerostomia
8. Faulty dentistry such as overhangs and subgingival margins
9. Excessive occlusal loads
10. Poor oral hygiene resulting in excessive plaque and calculus
11. History of periodontal disease
12. Additional risk factors including hyperlipidemia and possibly arthritis

Periodontitis was reported by epidemiological studies to be present in about 5 to 20% of the general population [49]. Quirynen et al. has mentioned three main reasons standing behind the activation of periodontitis including the host susceptibility, existence of pathogenic species, and deprivation of the beneficial ones [38]. The latter factor added by this author can be supported by the low microbial diversity and richness in the healthy status compared to the diseased status (Figure 9, [37]). For instance, certain bacterial strains were proposed as protective or beneficial to the host such as *Streptococcus sanguinis* and *Veillonella parvula*. They exist in high numbers in healthy sites and low numbers in diseased ones. They may have a protection role by preventing the pathogenic species from colonization and proliferation. This has been supported also by the clinical studies that demonstrated the high numbers of these beneficial strains where there is a greater gain in periodontium attachment after therapy [50]. While progressing to periodontitis, the transit stage is accompanied with halitosis, bleeding gums, and gingival swelling [51]. In the late phase of the disease, the free gingiva will start detaching from the tooth increasing the depth of the gingival sulcus forming pockets. As the plaque develops and spreads subgingivally, the body's immune system will combat the bacteria. This fight is highly destructive as it will destroy the teeth supporting tissues, bone and connective tissues, loosening the teeth which will be lost after that [46].

Many research papers have reported that the bacteria are only responsible for destroying the periodontium by releasing enzymes and toxins. However, recent results have proved that the host's immune system response plays a considerable role in this destruction procedure. They commence by stimulating the immune system via lipopolysaccharides of the bacteria leading to cytokine release. These inflammatory mediators induce the fibroblasts and epithelial cells which release in turn prostaglandins (PGE2) and matrix metalloproteinase. Prostaglandins stimulate alveolar bone resorption while matrix metalloproteinase or collagenase deteriorates the connective tissue or the periodontium-supporting collagen. Also, interleukin-1β and tumor necrosis factor-α are additional inflammatory mediators implicated in the periodontium destruction [51].

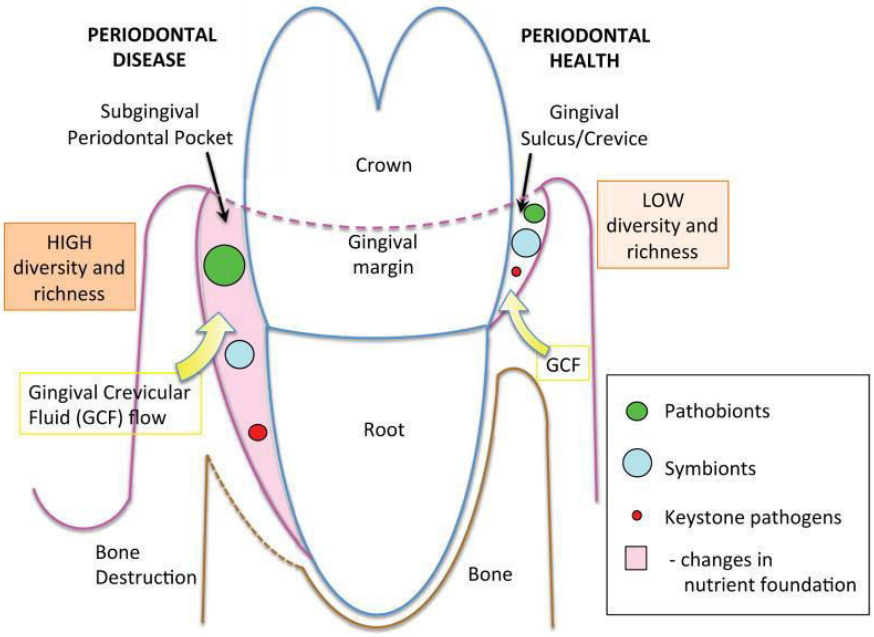


Figure 9: Periodontal disease and periodontal health status [37].

After these infections that lead to cytokine release and inflammatory, immune and autoimmune responses, several processes commence. They comprise endothelial dysfunction, lipid deposition, monocyte migration, smooth muscle proliferation and release of platelets and reactant plasma proteins. These blaze a trail into atherosclerosis, thrombosis and cardiovascular disease [5]. Furthermore, periodontal diseases drive other complications such as bacteremia, endotoxemia, adverse pregnancy outcomes, nonalcoholic liver diseases, rheumatoid arthritis, osteoporosis, respiratory lung infections, pancreatic and oral cancers, obesity and type 2 diabetes [52].

Moreover, the systemic infections can alter the host's immune response to the periodontal bacteria and their by-products and this may increase the periodontal disease incidence and

severity. This will enter the patient in a closed cursed cycle where periodontal diseases enhance systemic diseases and the vice versa [53].

ii. The periodontal diseases classification

The periodontal diseases classification has been developing with time by the American Academy of Periodontology (AAP). This has relied on the research results and the cases encountered.

Two categories in 1977 became 4 in 1986 and then 5 in 1989. Finally, an international workshop was hold in 1999 hosting participants from Europe, Asia, and North America, has recommended a new classification (Table 3) which has been approved by AAP [54].

Distinguishing between the types of periodontal diseases is still difficult between some of them as stated by some studies [55]. The following brief description will try to give as much as possible some differential marks concerning the bacterial species present and some clinical signs.

1) Gingivitis development due to dental plaque has been broadly studied and the following observations were realized:
    a. Following a period of 8 hours without oral hygiene, the bacteria were 10^3 to 10^4 per millimeter square of the tooth surface. They started to increase in a factor 100 to 1000 in the 24 hours. When 36 hours have passed, a visible plaque appeared. Then, inflammatory changes marked evidently the transition into gingivitis where Gram-negative rods and filaments started to appear followed by spirochetal and motile microorganisms.
    b. It is marked with equal proportions of Gram-negative (44%) and Gram-positive species (56%) and facultative (59%) and anaerobic (41%) organisms.
    c. Sometimes, gingivitis never advances into tissue destruction [50].
2) Periodontitis is distinguished from gingivitis by periodontium detachment and alveolar bone loss, however, we have numerous forms of periodontitis:
    a. Chronic periodontitis exists in adults as distinct forms regarding its progression rate which is relatively slowly (0.05 to 0.3 mm tissue attachment loss per year) as its gradual model and response to therapy. When followed over short time intervals, it showed short phases of tissue destruction separated by inactive durations. Also, it can be seen that some sites improve and others advance. Regarding the microbiota, this type will comprise 90% of anaerobes and 75% of Gram-negative species. In addition, viral infection (herpes viruses: EBV-1 and hCMV) is associated with chronic periodontitis where it contributes to

periodontal pathogenesis [50]. It can be localized or generalized as described in table 3 [54].

b. Aggressive periodontitis which is marked by a fast and severe attachment loss and can exist as localized or generalized (Table 3). Localized aggressive periodontitis is formerly known as localized juvenile periodontitis (LJP) which appears around puberty age in females more than in males. It is uniformly encountered in patients with defective immune regulation, often with defective neutrophil function. Its microbiota is predominated with Gram-negative, capnophilic and anaerobic rods. Herpes virus types, EBV-1 and hCMV, were also associated with the localized type. Without treatment, it can advance into the generalized form accompanied with severe attachment loss in numerous sites. The generalized form is formerly known as early-onset periodontitis, or rapidly progressive periodontitis. It appears in a young age ranging from 20 to 40 years. It is highly similar in its microbiota to the localized form.

c. Necrotizing periodontal disease is characterized by an acute gingival inflammation and necrosis at the level of the marginal gingival tissue and interdental papillae. It is associated clinically with stress and HIV infection and has the following signs: i) malodor, ii) pain, and possibly iii) systemic symptoms as lymphadenopathy (disease in the lymph nodes), fever and malaise (altered consciousness or intense feeling of discomfort of the patient). Its microbiota includes Gram-negative anaerobic rods and filaments.

d. Periodontal abscesses are acute lesions leading to a very fast periodontal tissue destruction. They can appear in patients who didn't treat the periodontitis or in those in the maintenance stage after scaling and root planning of deep pockets, in the absence of periodontitis as when some foreign bodies (popcorn kernel, dental floss) are impacted or with endodontic problems. Their clinical symptoms are: i) pain, ii) bleeding on probing, iii) swelling, iv) suppuration, and v) movement of the concerned tooth. Systemic attribution can be seen by the cervical lymphadenopathy and elevated white blood cell count. Gram-negative anaerobic rods and filaments constitute its microbiota [50].

Table 3: Developing of periodontal diseases classification [54].

1977	*1986*	*1989*	*1999*
1) Juvenile Periodontitis **2) Chronic Marginal periodontitis**	**1) Juvenile periodontitis** a. Prepubertal b. Localized Juvenile periodontitis c. Generalized Juvenile Periodontitis **2) Adult periodontitis** **3) Necrotizing Ulcerative Gingivo-Periodontitis** **4) Refractory Periodontitis**	**1) Early-Onset periodontitis** a. Prepubertal Periodontitis i. Localized ii. Generalized b. Juvenile Periodontitis i. Localized ii. Generalized c. Rapidly progressive Periodontitis **2) Adult Periodontitis** **3) Necrotizing Ulcerative Periodontitis** **4) Refractory Periodontitis** **5) Periodontitis Associated with Systemic Disease**	**1) Gingival Diseases** a. Dental plaque-induced gingival diseases b. Non-plaque-induced gingival lesions **2) Chronic Periodontitis** (slight: 1-2 mm clinical attachment loss (CAL); moderate: 3-4 mm CAL; severe: > 5 mm CAL) a. Localized b. Generalized (> 30% of sites are involved) **3) Aggressive Periodontitis** (slight: 1-2 mm CAL; moderate: 3-4 mm CAL; severe: > 5 mm CAL) a. Localized b. Generalized (> 30% of sites are involved) **4) Periodontitis as a Manifestation of Systemic Diseases** a. Associated with hematological disorders b. Associated with genetic disorders c. Not otherwise specified **5) Necrotizing Periodontal Diseases** a. Necrotizing ulcerative gingivitis b. Necrotizing ulcerative periodontitis **6) Abscesses of the Periodontium** a. Gingival abscess b. Periodontal abscess c. Pericoronal abscess **7) Periodontitis Associated With Endodontic Lesions** a. Combined periodontic-endodontic lesions **8) Developmental or Acquired Deformities and Conditions** a. Localized tooth-related factors that modify or predispose to plaque-induced gingival diseases/periodontitis b. Mucogingival deformities and conditions around teeth c. Mucogingival deformities and conditions on edentulous ridges d. Occlusal trauma

iii. Two important strains implicated in the oral infection

Two bacterial strains, *Streptococcus gordonii* and *Porphyromonas gingivalis*, of different Gram type, morphology and contributions to the oral and consequent systemic infections are worth to be described.

S. gordonii, an oral commensal bacterium, is a Gram-positive viridans streptococci member [7] (Figure 10). Its name is derived from the british microbiologist, Mervyn H. Gordon, who has pioneered the classification of viridians streptococci [56]. It belongs to one of the three groups into which the early streptococci are distributed. They were classified into pyogenic, mitis and mutans groups [57], where *S. gordonii* falls in the mitis one due to 16S rRNA gene sequencing tests [56,57]. *S. gordonii* coccoid cells, isolated from the oral cavity and pharynges; grow in short chains in serum broth. On blood agar, it produces α-hemolysis, and on chocolate agar it appears in green. Lys-Ala is its peptidoglycan type. Many strains were included under this species: SK3, ATCC 10558, CCUG 25608, CCUG 33482, CIP 205258, DSM 6777, LMG 14518, NCTC 7865. In 1989, Kilian et al. have distinguished three biovars within this species differing biochemically regarding the fermentation abilities and the production of extracellular polysaccharides. Biovar 1 was able to produce acid from melibiose, rafinose, and inulin and polysaccharides, however, biovars 2 and 3 couldn't ferment rafinose and melibiose. Biovar 2 was able to ferment inulin whereas biovar 3 could produce extracellular polysaccharides [56].

Figure 10: *S. gordonii* colonies on Columbia blood agar.

S. gordonii as a commensal oral bacterium may look not attractive as the species associated with diseases were the ones which took the lead in the extensive researches carried out by the scientists. However, this strain is among the primary colonizers which protect the host by occupying habitats and secreting substances toxic to the pathogens, and also by inducing the activation of the host immune system towards antigens shared among them and other pathogens. As a result, studying the commensal oral bacteria must constitute a considerable research zone in the biology of oral bacteria [58].

Moreover, *S. gordonii* didn't remain commensal, but, it has been reported as an agent of septic arthritis as well as a colonizer of damaged heart valves representing the major causative agent of subacute bacterial endocarditis. Hence, *S. gordonii* stands conspicuously as a dangerous bacterium inducing serious medical complications [2].

The early streptococcal plaque formation depends on several gene products. *S. gordonii* attaches primarily via Ssp surface adhesion proteins, SspA and SspB [59,60]. This attachment depends also on the enzyme, α-amylase, which exists in abundant proportion in the human saliva. *S. gordonii* binds this protein with high affinity through surface receptors called α-amylase binding protein, *abpA* [61]. After binding, *S. gordonii* can sense their environment and population density by the quorum sensing regulation system composed of the *com* regulon. The latter contains several genes and operons [62]. A biofilm-defective *S. gordonii* mutant had been shown to have an insertion within the *comD* gene that encodes for histidine kinase acting as an environmental sensor [63,64]. In addition, it has been suggested that *S. gordonii* produces an autoinducer-2 signaling molecule or LuxS serving as an intercellular communicator essential for biofilm formation between non-growing cells of *P. gingivalis* and *S. gordonii* [65].

With respect to the second strain; *P. gingivalis* is a Gram-negative species possessing short-rod or coccobacilli morphology (0.3-1 x 0.8-3.5 µm). It is obligately anaerobic, immobile and doesn't form spores. On blood agar, it forms brown-black colonies cause of protoheme production (Figure 11). Many strains of *P. gingivalis* were registered: 2561, ATCC 33277, CCUG 25893, CCUG 25928, CIP 103683, DSM 20709, JCM 12257, NCTC 11834, W83. Sequencing of several strains from different geographical territories has shown high genetic variation among them. Infected dental root canals, periodontal pockets and other oral sites can be the source of this bacterium. It has been shown to be susceptible to many antimicrobial agents used for the treatment of anaerobic infections including amoxicillin-clavulanic acid, piperacillin-tazobactam, ampicillin-sulbactam. However, in 2005, it has registered a resistance against ciprofloxacin [66].

Figure 11: *P. gingivalis* black colonies on Columbia blood agar.

P. gingivalis has been extensively studied as being the causative agent of periodontal diseases [67,68]. It is a maestro in the host's immune system evasion where it has been shown to register several capabilities from secreting gingipains which renders its resistance to complement destruction, into its adherence to erythrocytes serving as a safe transport mechanism without being detected by the circulating phagocytes. In addition, this smart bacterium can modify the structure of lipid A in LPS as an escaping mechanism in gingival tissues leading to the pathogenesis of periodontal diseases [69].

For the monospecies *P. gingivalis* biofilm to form, Mfa and FimA fimbriae were suggested to be required for autoaggregation where the expression of the long fimbriae, FimA, is controlled by the FimS-FimR two-component system [70]. UspA, the universal stress protein, is also involved in its development as shown before in microtiter plate assays and in flow cells [71]. Alongside, some gene products were found to be inhibitors of this homotypic biofilm accumulation such as GalE, UDP-galactose 4-epimerase, and their loss enhanced its growth [72,73].

S. gordonii is an essential partner for the pathogenesis of *P. gingivalis*. In addition to the fact that the latter needs *S. gordonii* as its binding platform leading to a complex biofilm, it can't also for instance penetrate the dentinal tubules in pure culture, but, it can invade the dentine attaching to *S. gordonii* which has the capacity of penetration for ≥0.2 mm in several days [57].

Binding of *P. gingivalis* to *S. gordonii* is one of the best identified interspecies combinations. Since *S. gordonii* reside as well below the gum line, two scenarios are possible. *P. gingivalis* can bind first to the streptococcal substrate supragingivally on the tooth surface to dislodge after that into the subgingival area or bind directly to the early plaque subgingivally [1].

These interrelated strains behave depending on the other in a concerted and coordinated fashion making them and their life interesting to be studied and dissected.

III- Controlling the oral bacteria

The inflammation is restricted in the initial stage of the disease or gingivitis to the gingiva. Later on, it migrates deeper in the tissues leading to bleeding and swelling of the gingiva as well as bad odor. In the late stage of the disease, the periodontium will be destroyed, the alveolar bone will be resorbed, and the gingiva will recede forming pockets. These different phases of the disease will require distinct treatment strategies which include surgical intervention, mechanical method, and the use of pharmacological agents [51].

Concerning the antimicrobial agents, they have various modes of actions by which they can inhibit or kill the bacteria thereby preventing or treating the oral bacterial complications. However, the bacteria were always challenging these antibiotics by developing resistance mechanisms which rendered these antibiotics ineffective.

This chapter will display the treatments available for the oral infection to focus finally on the antibiotics pathway and its developing difficulties. The targets of the antibiotics along with the bacterial resistance mechanisms will be explained in nutshell to pave the way for the next chapter.

a. Treating the oral infection

Several strategies and approaches have been described for controlling the oral infections. Five strategies have been followed: i) inhibiting bacterial adhesion and colonization, ii) inhibiting bacterial growth and metabolism, iii) eradicating the formed biofilm, iv) interfering with the biofilm biochemistry, and v) modifying the biofilm ecology.

The detailed clinical approaches for these strategies can be summarized as i) mechanical, ii) chemical (including the usage of antibiotics), iii) photodynamic, and iv) surgical methods. They can comprise both, the preventive and the curative approaches [23,74].

i) The mechanical means to control the oral biofilm can be the preventive everyday hygiene techniques such as toothbrushes, dental floss, wooden tips, and interdental brushes. They can use clinical ways to remove the calculus plaques or tartars (biofilms calcified with minerals) as well including scaling and root planning.

ii) The chemical pathway involves the usage of chemical agents. Some of them are only described by research studies and need further investigations and approval to be introduced into the market and some of them have graduated from the clinical trials and they are now prescribed in the clinics and used by the patients as an actual treatment. The latter two types will be discussed in the next part. They include antibiotics (doxycycline, ampicillin), natural products (sanguinarine, usnic acid [75]),

inorganic elements (zinc, copper), enzymes (dehydrated pancrease, mucinase), or other surfactants (sodium lauryl sulfate) [23]. These medications can modify the microbiota in the diseased site or modulate the host response by reducing the excess of enzymes, cytokines, or prostaglandins and osteoclast (bone resorbing cell) activity [51].

iii) The photodynamic pathway which has been used since 1900 when Oskar Raab has introduced it as an antimicrobial method. But, after the penicillin discovery by Sir Fleming, utilizing the light-stimulated disinfection was strongly inhibited to be used more in the cancer therapy. As the bacterial resistance has developed against antibiotics, the scientists started to search for new approaches where photodynamic therapy was one of these approaches. In nutshell, this therapy destroys the pathogens by the Reactive Oxygen Species (ROS) generated from the interaction of a photosensitizer (light-sensitive substance), light of a specific wavelength, and oxygen. This method is still in the clinical trials whose outcomes are inconsistent, and the authors confess that further studies are needed to set an optimized protocol combining this method with mechanical debridement to obtain good treatment outcomes [74].

Before advancing into the surgical approach, the therapies proposed above can interfere in the stages shown in figure 12 [51].

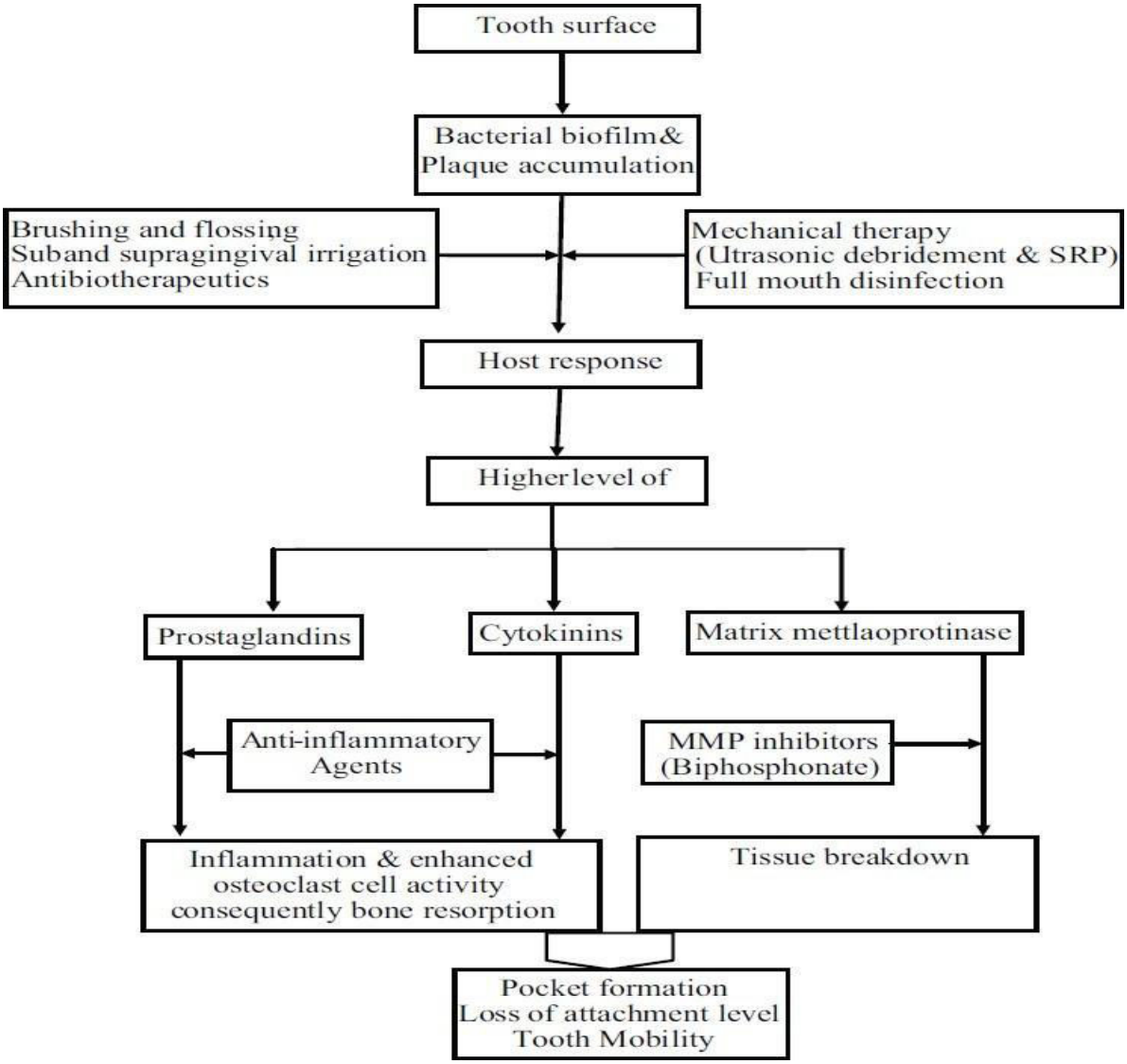


Figure 12: The non-surgical therapies intervention stages [51].

iv) Surgical intervention includes two types: a) flap surgery, or b) bone and tissue grafts.

a) Flap surgery may be required if inflammation and deep periodontal pockets remain after mechanical cleaning and taking medications. Briefly, the gums will be lifted for the tartar to be removed and then the gums are returned back to heal and fit more firmly around the teeth. The latter can become sometimes longer.

b) Bone and tissue grafts surgeries are suggested by the dentist to regenerate the lost bone or gum tissues. Concerning the bone, natural or synthetic bone is grafted in the area of bone loss thereby inducing bone growth. Also, synthetic or

natural tissue from other places in the mouth can be used as a graft to be inserted in the area where the tooth roots are exposed [76].

b. Antibiotics described in the literature for the oral bacteria

The compounds targeting the oral bacteria can be divided into synthetic and natural ones. The synthetic antibiotic can be an inorganic mineral, peptide or other organic compound. On the other hand, the natural antibiotic can be an extract from different plant parts, a pure secondary metabolite isolated from a plant extract, or a microbial extract. Some examples of these antibiotics are listed in Table 4.

Table 4: List of some different types of antibiotics described in the literature alongside their targeted oral bacteria [77–81].

Antibiotic	***Type of the antibiotic***	***The activity along with the targeted oral bacteria***
Ethanol extracts of Thai traditional herb [77]	Natural, from plants	**Antibacterial activity** against 5 Gram positive cariogenic bacteria, *Enterococcus faecalis* ATCC 19433, *Lactobacillus fermentum* ATCC 14931, *Lactobacillus salivarius* ATCC 11741, *Streptococcus sobrinus* ATCC 33478 and *Streptococcus mutans* ATCC 25175, and 2 Gram negative periodontopathogenic bacteria, *Aggregatibacter actinomycetemcomitans* ATCC 33384 and *Fusobacterium nucleatum* ATCC 25586. **Antibiofilm activity** was found against *S. mutans* ATCC 25175 and *A. actinomycetemcomitans* ATCC 33384.
Pediococcus pentosaceus FB2 and *Lactobacillus brevis* FF2	Lactic acid bacteria	**Antibacterial activity** against *Streptococcus salivarius* B468. **Antibiofilm activity** against

[78]		*Bacillus cereus* ATCC14579 and *S. salivarius* B468.
Mouthrinses containing Cetylpyridinium chloride and sodium fluoride [79]	Inorganic minerals	**Antibacterial activity** against *Streptococcus mutans* and salivary bacteria. **Antibiofilm activity** against the latter.
Ambroxol [80]	Synthetic	**Antibacterial and anibiofilm activities** against *Aggregatibacter actinomycetemcomitans* and *Streptococcus mutans*.
Antibacterial peptides [81]	Synthetic but its origin is the human epithelial cells	**Antibacterial activity** against several oral bacteria: *Actinobacillus actinomycetemcomitans* (20 strains), *Porphyromonas gingivalis* (6), *Prevotella intermedia* (7), *Fusobacterium nucleatum* (7), *Streptococcus mutans* (5), *Streptococcus sobrinus* (5), *Streptococcus salivarius* (5), *Streptococcus sanguis* (4), *Streptococcus mitis* (2) and *Lactobacillus casei* (1).

c. Antibiotics prescribed for the treatment of orally-infected patients

Will the antibiotics have significant beneficial effects on periodontal-diseased patients as a stand-alone therapy or combined with other approaches as obtained in the research studies? In addition, the patient can have any of the periodontal disease categories described before; will the latter require distinct antibiotics? Numerous studies have tried to answer these questions utilizing different antibiotics and patients' cases.

In order to support the conventional mechanical periodontal treatment or the host defense system, periodontal antibiotic therapy is used since some subgingival pathogens can remain

after the conventional therapy. A portion of these pathogens are out of the reach of periodontal instruments, others can reside in the biofilm section attached to epithelial cells of the periodontal pocket as the red complex including *P. gingivalis* where the oral hygiene efforts of the patients can't reach them. Another group of pathogens can survive due to the poor host defense mechanisms. Hence, the antibiotics are used to inhibit or kill these remnant pathogens. However, there are certain guidelines that should be followed to use these antibiotics. A clinical diagnosis of the patient can obligate the usage of the antibiotics such as the case if the disease activity has continued or returned to activation. Microbial samples from subgingival sites should be examined at different stages to detect the pathogens residing in the patients' sites and then the concerned species will be targeted by the antibiotics. In addition, the antibiotics have been demonstrated to possess a beneficial value in reducing the need for surgeries. Finally, the biofilm as discussed before increase the resistance of the bacteria where the concentration of the antibiotics needed to inhibit some pathogens in their fixed lifestyle will be increased to reach 500 times more than the systemic therapeutic dose. As a result, disrupting the biofilm physically will be essential for the antibiotic therapy to reach and inhibit the pathogens [82].

The medications prescribed for periodontal diseases can wear several dresses. They can be: i) antimicrobial mouthrinses, ii) antiseptic chips, iii) antibiotic gels, iv) antibiotic microspheres, v) enzyme suppressants, or vi) oral antibiotics [76].

i) Antimicrobial mouthrinses contain antibiotics such as chlorhexidine and they are used as regular mouthwashes to control bacteria when treating gingivitis and following gum surgeries.
ii) Antiseptic chips are tiny gelatin pieces filled with an antibiotic as chlorhexidine. They can be used after root planning by inserting them in the periodontal pockets where the medication will be slowly released with time. They help in controlling the bacteria and reducing the size of the pockets.
iii) Antibiotic gels are gels containing antibiotics as doxycycline. They are used in the same way as chips and for the same aim.
iv) Antibiotic microspheres are very tiny round particles comprising antibiotics as minocycline and used for the same purpose and in the same way as the chips and gels.
v) Enzyme suppressants exist in tablet form and utilized as an adjunct for scaling and root planning. They are used to control the body's enzyme response evading gum tissue breaking down by those enzymes. A low dose of doxycycline can serve as an enzyme suppressant.

vi) Oral antibiotics which are provided as tablets or capsules. They are used to treat acute or locally persistent periodontal infection [76]. Amoxicillin is one of the oral antibiotics used [83].

Since there is a broad panel of agents; several factors can decide which one should be used: i) patient age, ii) renal and hepatic failure, iii) existence of local factors as pH, pus and secretions, or necrotic material and foreign body which will influence the antibiotic action, iv) drug allergy, v) impaired host defense, vi) pregnancy, vii) type of the targeted organism, and viii) drug factors which can be summarized in its spectrum of activity, type of activity, organism sensitivity, relative toxicity, pharmacokinetic profile, route of administration, evidence of clinical efficacy and cost of the drug [82].

Each disease type and its details from clinical signs into the microbiota present require distinct antibiotics [82]:

1) Chronic periodontitis : Tetracycline, Doxycycline, Metronidazole, Clindamycin, Amoxicillin + Clavulinic acid (Augmentin), Azithromycin, Metronidazole + Amoxicillin, Spiramycin.
2) Aggressive periodontitis : Tetracycline, Doxycycline, Minocycline, Metronidazole, Amoxicillin + Clavulinic acid (Augmentin), Metronidazole + Amoxicillin
3) Necrotising periodontal disease : amoxicillin, metronidazole and combination of amoxicillin+metronidazole
4) Periodontal abscess: Amoxicillin, and in case the patient has an allergy to β-lactam drugs, azithromycin or clindamycin is used.

It is worth noting that despite the fact that the oral bacteria are sensible to many antibiotics, no single antibiotic at the concentration reached in the body fluid can inhibit all the putative pathogens, hence, a combination of antibiotics is proposed to be essential to clear all the pathogens from some diseased sites. Each of these antibiotics used has its own characteristics and activity profile and uses [83]:

Doxycycline: several facts provide this antibiotic with a high importance as an oral drug including: i) the higher availability of doxycycline in the gingival crevice which can reach between 7 to 20 times greater than any other drug, and iii) the multiple capabalities in modulating the host properties this antibiotic possesses alongside its antibacterial activity: 1) anti-inflammatory, 2) anticollagenase, 3) reducing bone resorption, 4) induces periodontium reattachment, 5) concept of low dose of doxycycline known as LDD, and 6) chemically modified tetracycline (CMT). Doxycycline acts by targeting the ribosomes thereby inhibiting protein translation.

Metronidazole: Utilizing this antibiotic alone is a poor choice, so, it should be combined with root planning, surgery, or other antibiotics. It has been reported that consuming metronidazole by subjects has significantly reduced more the pocket depth and led to greater reattachment in diseased sites having pockets of ≥6 mm depth in comparison to those receiving doxycycline. Inhibiting DNA synthesis is the mode of action of metronidazole.

Amoxicillin: Because it is a β-lactamase sensitive penicillin, it is not recommended to be received alone and sometimes it may also speed up the periodontal degeneration. For this reason, it is used combined with a β-lactamase inhibitor, clavulanic acid, under the form Augmentin. This combination has been also reported to suppress periodontal pathogens and increase the reattachement in some tissue regeneration surgeries [82].

d. Antimicrobial resistance of oral bacteria

The antimicrobial resistance is defined simply by the gained resistance of a microorganism against a drug which was formerly able to cure its caused infections. This microorganism can be a bacterium, fungus, virus or parasite [84].

Sir Alexander Fleming didn't only uncover the first antibiotic, penicillin, but also he set a priceless hypothesis which should be written in every pharmacy or a center where the antibiotics are sold. This hypothesis is probably more important than penicillin itself. He clearly warned in an interview with *The New York Times* in 1954 that the misuse of penicillin could result in the selection of the resistant or mutant forms of *Staphylococcus aureus* which can therefore lead to more dangerous infections not only in the host but also in the people who were in contact with him/her. He warned but nobody has taken his words into consideration as the widespread use of this antibiotic has told us. Within only one year of this inappropriate spreading of penicillin, a large number of *S. aureus* resistant strains have appeared reaching more than 50% a few years later [85].

S. aureus was the first strain to register its resistance against penicillin and sulfonamide between 1930 and 1940. This was followed by *Neisseria gonorrhoeae* which displayed resistance to penicillin alongside *Haemophilus influenzae* which was shown to produce β-lactamase in the 1970s. Then, between 1970 and 1980, methicillin-resistant *Staphylococcus aureus* (MRSA) and the multi-drug resistant (MDR) *Mycobacterium tuberculosis* appeared. After that, various common enteric and non-enteric Gram-negative bacterial strains joined the resistance panel between 1980 and 1990, for instance: *Shigella spp.*, *Salmonella spp.*, *Vibrio cholerae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*. Some of which were resistances developed due to the usage of antimicrobial agents in the animals consumed by humans. The number of active antibiotics continued to

decrease with the years until reaching now the antibiotic crisis where the microbes have developed resistance against all the antibiotics discovered to date [86–103]. A more recent example is the report of World Health Organization (WHO) which stated that a progressive evolution of resistance against HIV drugs in 2012 has occurred. After one year, new 480 000 multidrug-resistant tuberculosis (MDR-TB) incidents were registered. Alongside, extensively drug-resistant tuberculosis (XDR-TB) was characterized in 100 countries in the same year, 2013 [84].

Focusing on the bacterium will narrow our term to be called the antibiotic resistance. WHO mentioned in 2015 in its fact sheet number 194 that the bacterial resistance exists in high ratios in the common infections such as blood stream infections. The new resistant bacterium causes more complicated infections compared to the wild strain. It will put the patient in front of augmented hazard of more serious and unpleasant clinical circumstances which may even lead to death [84].

The oral bacteria have developed resistance as well long time ago. In 1950, the enterococci which were present in 6 to 8% of the infected dental root canals cases have been shown to resist penicillin and streptomycin *in vivo* [104]. In 1993, *Streptococci* (*S. mitis*, *S. salivarius*, *S. sobrinus*, *S. mutans*) were shown to be more resistant to mercury (5-40 µg/mL) than *Actinomyces* (*A. naeslundii* genospecies 1 (ATCC 12104)) (< 5-30 µg/mL) [105]. Many resistance cases and the mechanism of action of oral bacteria against many antibiotics (lincosamides, streptogramins, trimethoprim, sulfonamides, aminoglycosides, and chloramphenicol) have been reported by Roberts in 1998 [106]. Recently, several oral bacterial isolates have displayed variable resistance against ampicillin, kanamycin, gentamicin, and tetracycline, where the most resistant ones were two species, *Chryseobacterium culicis* and *Chryseobacterium indologenes*, treated with 32 µg/ml of chlorhexidine and had the ability to grow as planktonic cells or biofilms [107].

Moreover, Haenni et al. have mentioned that after 36 passages of *S. gordonii* with penicillin, the MIC augmented to more than 100-fold, from 0.008 into 2 µg/mL [108]. Itzek et al. have monitored its resistance danger also. They have mentioned that the H_2O_2 produced by this strain is not only a simple toxic metabolic by-product, but also a necessary environmental signal smoothening the way of strain evolution by genetic information transfer and mutation rate increase [109]. With respect to the other strain, *P. gingivalis* was also shown to be resistant to tetracycline and/or erythromycin in 55% of 47 infected children [110]. Furthermore, this bacterium has shown a resistance to antimicrobial peptides of human and nonhuman origins. However, it is worth to mention the finding of Bachrach et al. who found that *P. gingivalis* ATCC 33277 resistance to the antimicrobial peptides they tested is protease independent suggesting the low affinity of the latter to the strain [111].

e. The causative factors of the universal bacterial resistance

Numerous factors have served in developing the bacterial resistance against the antibiotics: i) the usages of antibiotics on a large scale alongside their misapplications, ii) vertical and horizontal genetic transfers, iii) spontaneous mutations, and iv) sub-inhibitory concentrations.

i) The usages of antibiotics on a large scale alongside their misapplications have led to the emergence of resistant pathogenic bacteria [9]. This is mainly encountered in countries where the antibiotics are cheap and can be bought over the counter. Even some countries which regulate their antibiotics, made it possible to buy them online. This abuse of such drugs and its resulting resistance will divide the microbial community into sensitive and resistant groups to a certain antibiotic. The latter will eliminate the sensitive strains conserving the resistant ones to reproduce as a natural selection [112].

ii) The resistance can be developed also through vertical and horizontal genetic transfers by inheritance from relatives or acquirement via mobile elements like plasmids from non-relatives, respectively [112]. Biofilms are very well known sites for having increased rates of genetic transfer where some genes can be resistant [113].

iii) Spontaneous mutations which are selectively favorable for the bacteria can result in the formation of resistant genes and consequently resistant strain [112]. These novel resistant genes can pass into other strains by genetic transfer as described in the second reason.

iv) Sub-inhibitory concentrations: These concentrations constitute a main aspect of the antibiotic crisis since the rationale antibiotic dosing is to sustain the highest antibiotic concentration in the concerned body region without having cytotoxicity for a duration long enough to remove the infection. This excellent theory is poorly applied due to the usage of weak drugs, poor drug dosing regimens and pharmacokinetics, in addition to patients' disobedience. Hence, the aimed plan fails soon from reaching its aim and the bacteria in that body region will be exposed to concentrations lower than the MIC. As a result, the targeted strains will be weakly inhibited. Moreover, the bacteria can be exposed to these sub-inhibitory concentrations in different ways and in different environments as shown in figure 13 [114]. Some antibiotics at this concentration induce antibacterial effects where the bacterial cells will have lower growth rate and distinct morphology compared to the

cells grown in drug-free medium as registered by cephalosporin and ampicillin antibiotics, respectively (Figure 14, [115])[116].

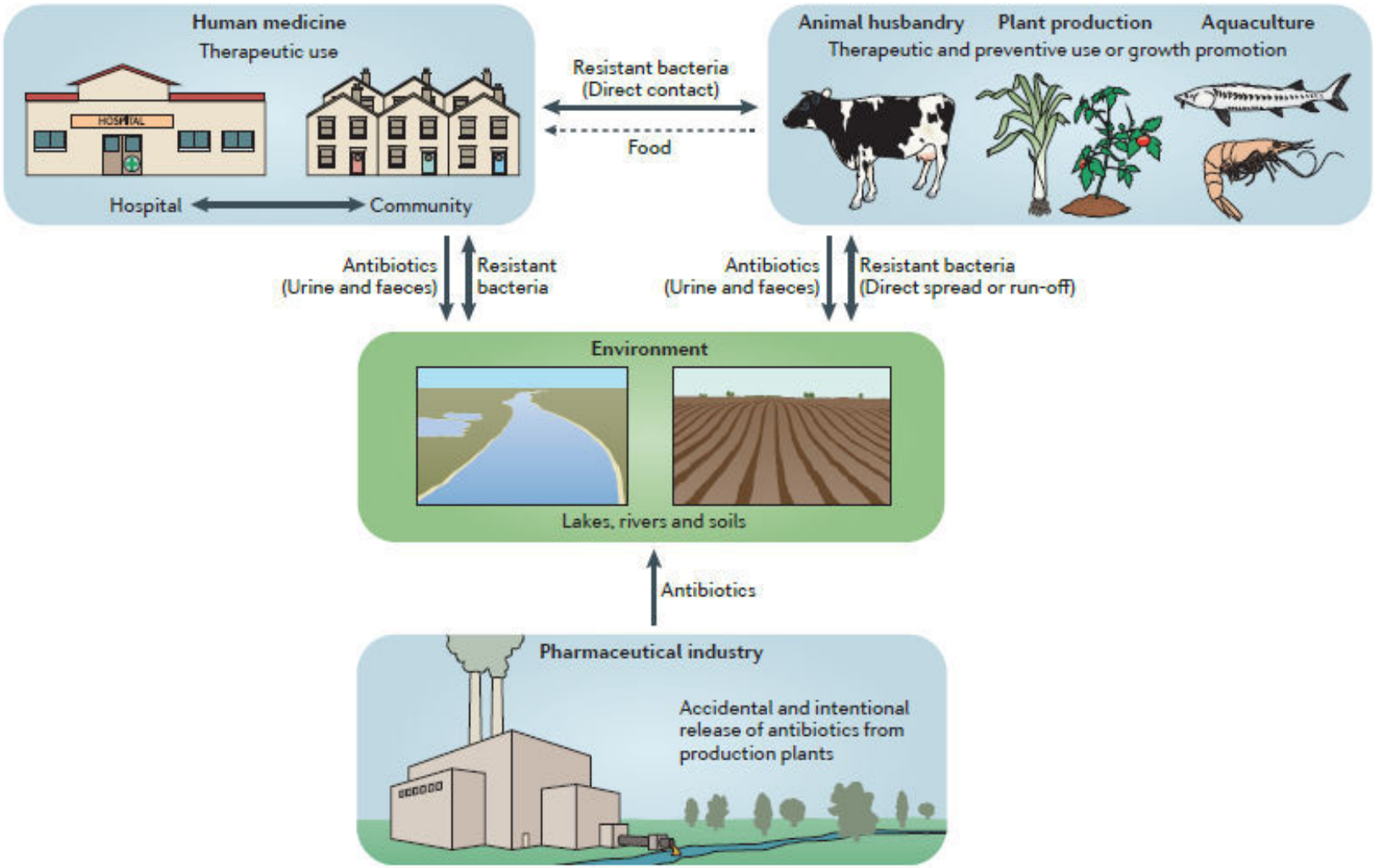


Figure 13: The drugs cycle between different environments including medical centers, agricultural settings, aquacultural environments, the pharmaceutical industry and the bigger environment. About 20 to 80 % of the antibiotics utilized worldwide are released into the environment in their active forms in the excreted urine and feces or via accidental or intentional release. Then, the drugs will exert a selective pressure on the bacteria selecting resistant strains which can pass on from one environment to another transmitting the antibiotic resistance genes with them [114].

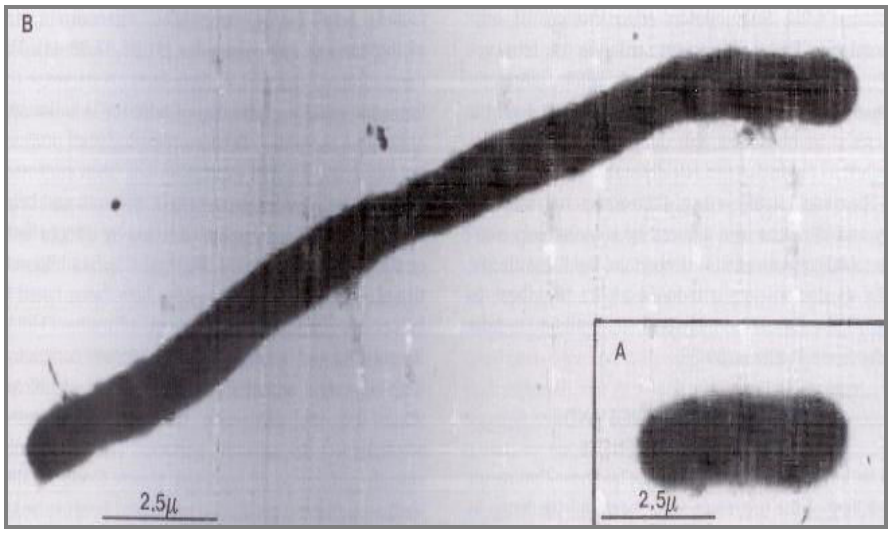


Figure 14: An electron micrograph showing 2 *Escherichia coli* cells grown differently. A) *E. coli* cell grown in a normal Mueller-Hinton broth medium; B) A long filamentous *E. coli* cell exposed to MIC/2 of ampicillin antibiotic for 2 hours [115].

f. The antibiotics modes of actions versus the bacterial resistance mechanisms

To begin, antibiotics can either block the bacterial growth (bacteriostatic) or kill the bacteria (bactericidal) [117]. They can exert their effects at several levels in the bacterial cell (DNA, RNA, or proteins) using various mechanisms. The most efficient antibiotics target the ribosomes, cell wall, or DNA topoisomerase (Figure 15, [118]). The antibiotics can:

i) Interfere with the bacterial cell wall by preventing the transfer of peptidoglycan monomers synthesized in the cytoplasm across the plasma membrane and inhibiting the transpeptidase enzyme which links the peptide units for example β-lactams as amoxicillin. They can also inhibit both, transpeptidase and transglycosidase where the latter links the sugar units such as glycopeptides [119].

ii) Modify the bacterial plasma membrane disrupting it and increasing its permeability as the interaction of cationic peptides of polymixin with the bacterial membrane. This antibiotic has been demonstrated by several authors to be associated with nephrotoxicity where the incidence rate can reach about 60% according to the authors definition of nephrotoxicity [120].

iii) Interfere with the translation process by binding to the ribosome subunits. Those which bind the 30S subunit prevent the binding of tRNA as tetracyclines and aminoglycosides. However, the second group binds the 50S subunit closing the ribosome exit tunnel as macrolides and clindamycin [117,119]. it has been reported with respect to their cytotoxicity that major differences exist between the eukaryotic and bacterial ribosomes, however, only one nucleotide or amino acid can

affect the drug selectivity influencing protein translation [121]. Many drugs used as a therapy for the oral infection were shown to target the ribosomes including macrolides and tetracyclines as doxycycline or minocycline [118,122]. For instance, minocycline was reported to have more toxicity than doxycycline and tetracycline against human gingival epithilioid S-G cells depending on their concentrations and the time of exposure [123].

iv) Block the replication of nucleic acids via inhibiting topoisomerases ubiquitous enzymes involved in the DNA supercoiling and entanglements making them essential for transcription and replication. These important enzymes exist in eukaryotes, archaebacteria, and eubacteria, where in human there are six types versus generally 4 types in bacteria. However, topoisomerase inhibitors are highly selective and utilized as targeted therapies explaining their lower cytotoxicity [124]. Quinolones such as ciprofloxacin which is used as an oral antibiotic acts by this mode of action [83,118].

v) Bind RNA polymerase enzyme thereby inhibiting the transcription of DNA into mRNA as rifampicin antibiotic [117,119].

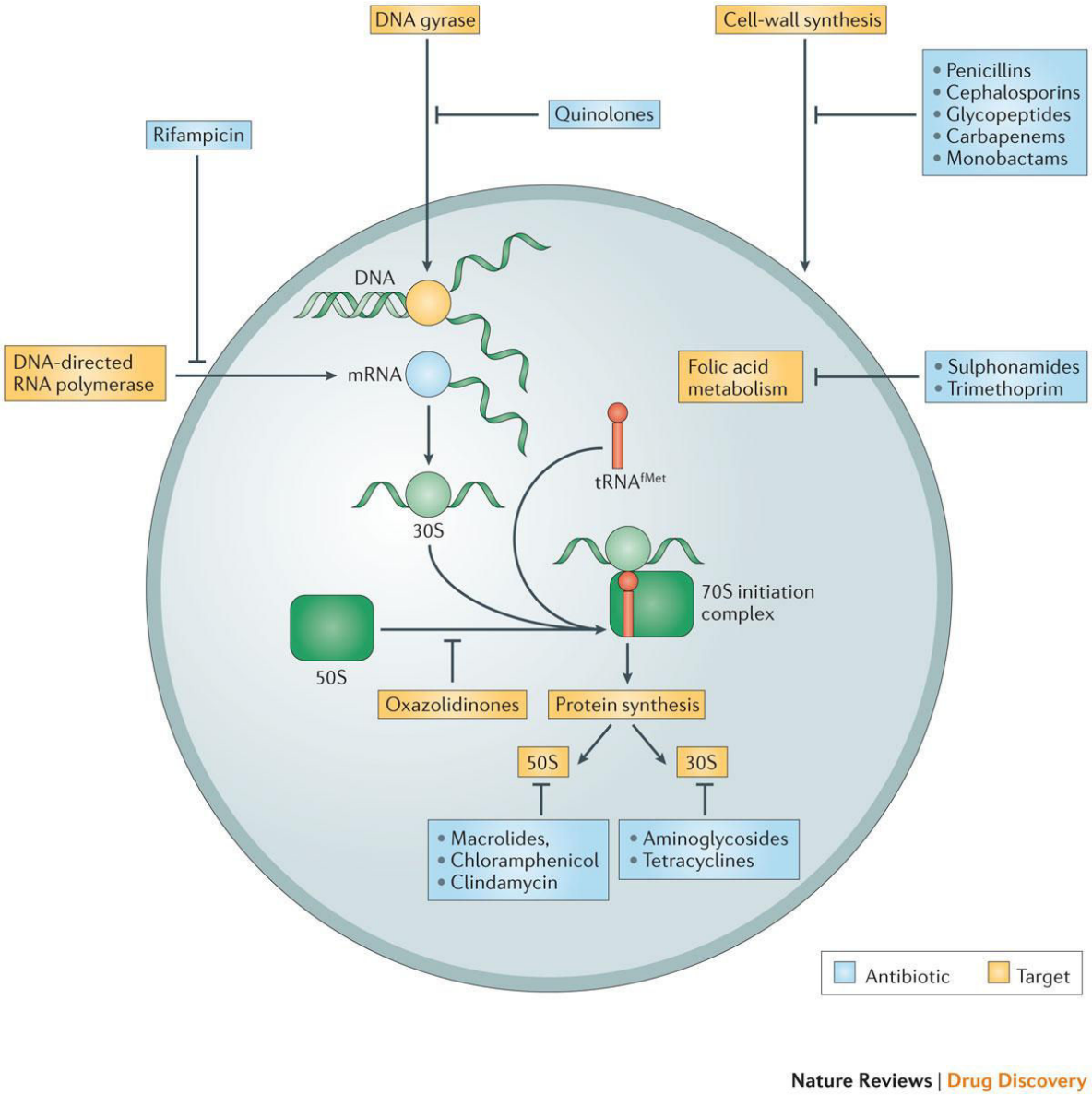


Figure 15: The targets of antibiotics in a bacterial cell along with some examples of the antibiotics utilizing the corresponding mechanisms [118].

Four criteria can characterize the more efficient antibiotic: i) acting via one of the three most efficient mechanisms mentioned above or a new one (it is worth noting that the new mechanism can be less efficient than the already discovered ones but as it is a new mechanism involving a new target, then no resistance has been developed against it till now. This provides any new discovered mechanism the best efficiency), ii) possessing lower cytotoxicity, iii) the rate of appearance of resistant bacteria which can be determined from the combined rates of horizontal gene transfer of resistance determinants and *de novo* mutation [125], iv) affecting the bacteria in its different growth rates such as the cells that have a significantly reduced growth when arranged in a biofilm [22], and v) the hydrophobicity of the antibiotic structure,

for instance, the more lipophilic is the antibiotic, the more efficient in penetrating the extracellular polymeric substances of the biofilms [126].

On the other hand, as a natural selection procedure, the bacteria have developed its characteristics following several modes of action to resist the antibiotics (Figure 16, [127]). In addition, the bacteria have the capacity to use an arsenal of more than one mechanism to concur the antibiotic. For instance, mutations in the genes encoding the DNA topoisomerase IV as the target site, up-regulation of efflux pumps which eject the antibiotic outside, and protection of the target protein by another protein are three different mechanisms used by the bacteria at the same time to resist fluoroquinolones antibiotics. It is also worth to mention that different bacteria can preferably choose to follow different resistance routes against the same antibiotic as the case of β-lactams whose target site (penicillin binding protein, PBP) is modified in Gram-positive bacteria, however, the Gram-negative counterpart produce β-lactamases instead [128]. Briefly, the bacteria can resist the antibiotics by:

i) Alteration of the antibiotic target molecule by either introducing chemical modifications decreasing the affinity of the drug to its target and thus increasing the MIC value or by destroying the target where the drug will be unable anymore to interact with its target [128].

ii) Reducing antibiotic influx by modifying the porins structures leading to their impairment, switching into another type, or changing in their expression level. This mechanism is often correlated with the next one [128].

iii) Overexpression of efflux pumps which can belong to 5 major classes: a) the major facilitator super family or MFS, b) the resistance-nodulation-cell division family or RND, c) the ATP-binding cassette family or ABC, d) the small multidrug resistance family or SMR, or e) the multidrug and toxic compound extrusion family or MATE. Each of which has a distinct energy source, structure, substrate specificity range, and distribution among bacterial species [128].

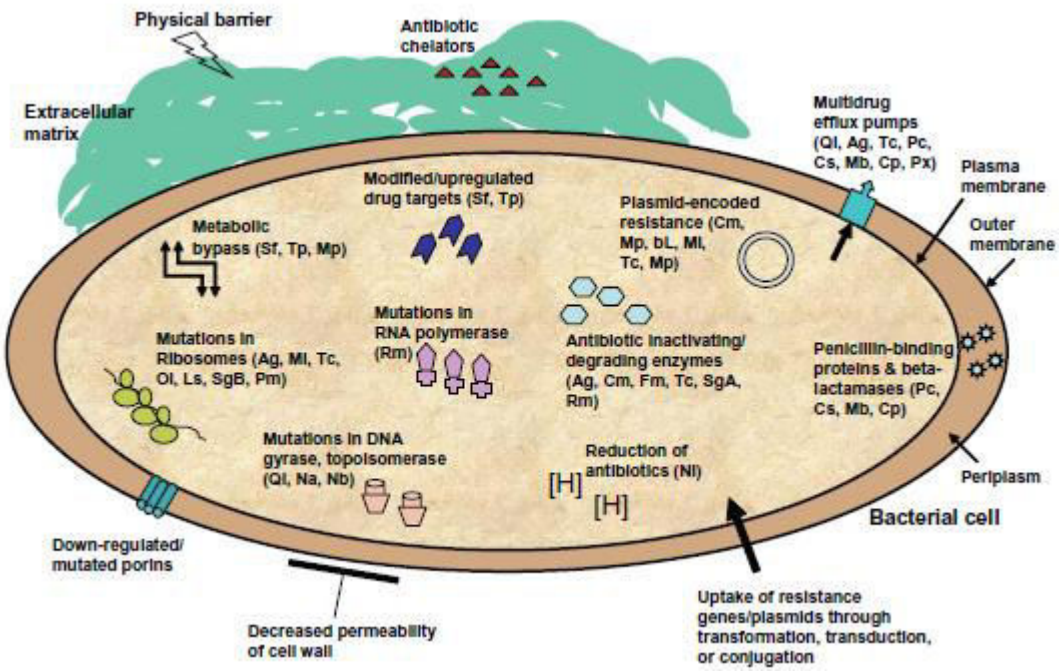


Figure 16: The bacterial resistance mechanisms in addition to some examples of the antibiotics using the corresponding mechanisms. Ag, aminoglycosides; As, antiseptics; bL, beta-lactams; Bt, bacitracin; Cs, cephalosporins; Cp, carbapenems; Cm, chloramphenicol; Fa, fusidic acid; Fm, fosfomycin; Ls, Lincosamides; Mb, monobactams; Ml, macrolides; Mp, mupirocin; Na, nalidixic acid; Nb, novobiocin; Ni, nitroimidazoles; Ol, Oxazolidinones; Pc, penicillins; Pm, Pleuromutilins; Px, polymyxins; ; Ql, quinolones; Rm, rifamycins; SgA, Streptogramin A; SgB, Streptogramin B; Sf, sulfonamides; Tc, Tetracyclines; Tp, trimethoprim [127].

iv) Forming fixed complex agglomerations or biofilms which attach to biotic or abiotic surfaces and will be surrounded by an extracellular matrix comprising polysaccharides, proteins, and DNA. This protective layer constitutes a defense barrier against drugs and hosts environmental promoters which stimulate biofilm growth. The enhanced resistance acquired by the biofilm life style can be attributed to several mechanisms as the case of dental plaque shown in figure 17 [129]. Gene transfer also occurs among bacteria within a biofilm gaining new genes; some of which are concerned with resistance. This explains the scarcity of success reached by the host defense mechanisms to treat biofilm infections even in patients with perfect immune system. The antigens secreted by the sessile bacterial cells induce the host to produce antibodies which are not capable of reaching the matrix-enclosed cells and eliminate them. On the contrary, this may lead to undesired immune reactions damaging the supporting tissues. In addition, the functions of immune cells that work as engulfing structures will be impaired. As a result, not only do the biofilms resist the antibiotics, but also they can escape the host defense system [113].

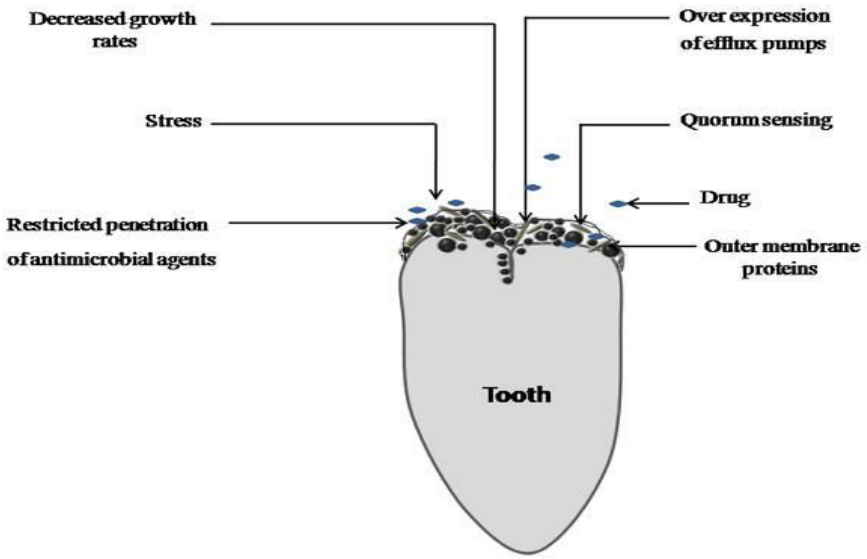


Figure 17: Resistance strategies followed by a biofilm: the dental biofilm [129].

Nevertheless, bacterial resistance has sorrowfully touched all the antibiotics discovered to date. Frightening reports issued from Centers for Disease Control and Prevention (CDC) in 2013 claiming that the human has went beyond the antibiotic age. This has been followed in 2014 by another declaration from WHO warning that the antibiotic resistance catastrophe is atrocious. A real and terrible example is the fact that multi-drug resistant *Stapylococcus aureus* (MDSA) has a larger mortality rate each year than Parkinson's disease, HIV/AIDS, emphysema, and homicide rates taken together [112].

Nevertheless, new commercialized antibiotics should not be prescribed rapidly by physicians, but kept in the drawer and replaced by other older drugs of similar efficiency. They should represent the last resort in serious illnesses; otherwise we will collide with strains that have developed resistance to them [112].

This worsening crisis supported by the diminishing in newly discovered antibiotics (Figure 18, [112]) should be faced with all the possible efforts to surpass the bacterial resistance and to have some new antibiotics capable of fighting the present threatening infections, otherwise, we will be in an era similar to that before 1928 i.e the date when ampicillin was introduced and the bacteria will then harvest millions of lives again. Hence, other potent candidates should be found to fight against bacteria [11]. Throughout the last 2 decades, plants are becoming a famous rich source of antimicrobial substances [12]. Furthermore, many other promising drug sources still need to be explored as the lichens [10,13].

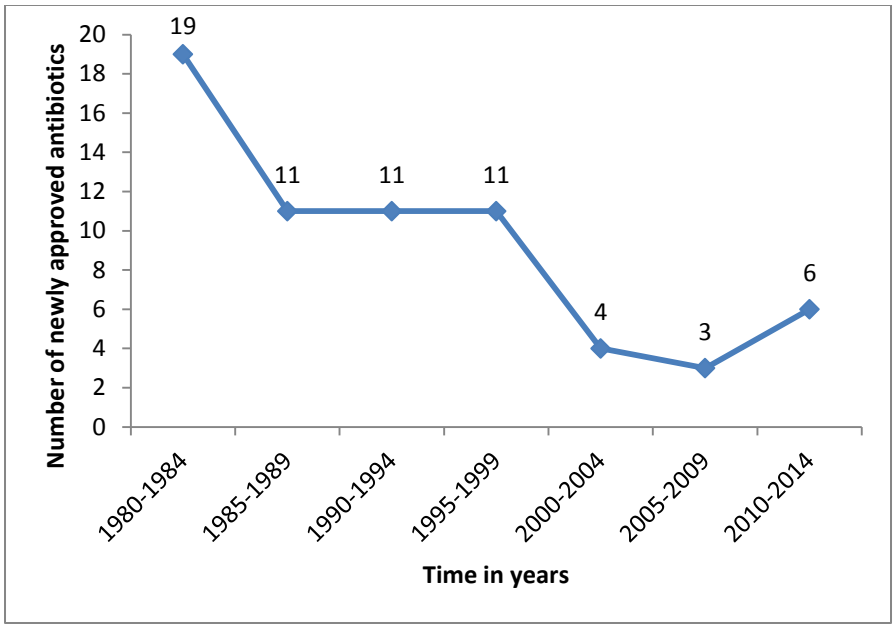


Figure 18: The number of newly discovered and approved drugs as a function of year intervals [112].

VI- Lichens

For more than thousand years and across several civilizations, herbal medicine has been used to treat ailments. *Ocimum sanctum* is one example of the plants used in the old ages for medicinal purposes. Nowadays, plants represent a major actor in the health care therapeutic movie in the developing countries, since their phytochemicals constitute an important framework for the development of drugs in the modern medicine. Hence, the main objective today by the scientists is to uncover plants or other organisms with promising active compounds. Lichens were our interest zone due to their powerful secretions capable of curing several diseases [15].

A clear definition of the lichens taking all the scientists points of view into consideration alongside the types of this organism and its secretions constituted the introductory paragraph in this chapter. They are followed with a historic demonstration of their usages in several fields to end as a rich source of antibacterial agents and consequently a resort for the antibiotic crisis.

a. Lichen, an interesting organism

This small 6-letter-length organism, Lichen, has been estimated to cover 6% of the earth's surface. Its name was introduced the first time before 300 B. C. by Theophrastus [130]. Lichen is a symbiotic association comprising a mycobiont and a photobiont form. The mycobiont is a filamentous fungi whereas the photobiont is its photosynthetic partner which can be a eukaryotic algae and/or cyanobacterium and in some cases non-photosynthetic bacterium [13]. One-fifth of all fungi adopt this lifestyle comprising not less than 40% of ascomycetes and a few basidiomycetes. This apparently successful symbiosis dominated by the mycobiont has been traditionally classified as a fungal life-form. Around 18500 distinct lichen species have been characterized globally and they adapt a drastic array of ecological conditions. They can exist either in very cold and dry places or in tropical rainforests; however, they can harshly live in non-native sites [15].

A broad spectrum of morphologies, colors, and sizes were registered for Lichens species. They may possess tiny leafless branches, flakes lying on the surface, flat leaf-like structures, granular or powdery forms or other numerous growth shapes [130]. Lichens can be subdivided on the morphology basis into three main groups, fruticose, foliose, and crustose, but according to the traditional classification, intermediate forms can be added. Moreover, the gelatinous and the hairlike or filamentous lichens constitute two additional groups since they can't be included in the others [131]. Some examples of the lichen structures are present in table 5.

Table 5: Some examples of the lichen structures along with their images [132–136].

Lichen Species	Thallus Type	Image	Reference
Roccella fuciformis	Fruticose		[132]
Squamarina cartilaginea	Foliose		[133]
Ochrolechia parella	Crustose		[137]

Since lichens grow very slowly in places with low resources, they are able to defend themselves by producing a wide array of chemicals where they have been found to secrete more than 1000 distinct secondary metabolites [138] (Figure 19). In addition, lichens produce primary metabolites which differ in their synthesis source, structures, and functions from the secondary metabolites (Table 6).

Table 6: The metabolites manufactured by lichens and their characteristics [139–143].

Metabolites	***Production source***	***Functions***	***Compounds***
Primary metabolites	Both partners [139]	-Morphological function -Role in cellular metabolism [139]	Chitin, lichenin, isolichenin, hemicellulose, pectins, disaccharides, polyalcohols, amino acids, enzymes, pigments like algal chromophores: chlorophyll and, β-carotenes, xanthophylls, etc [139]
Secondary metabolites	-Fungi [140] -Cyanobacteria (produce some metabolites in some circumstances) [141,142]	-Regulate the lichens metabolism -Defense against microbes, animal predators, plant opponents, environmental stress (ultra-violet irradiation, dryness) [143]	Three chemical pathways produce a wide variety of compounds (Figure 19)

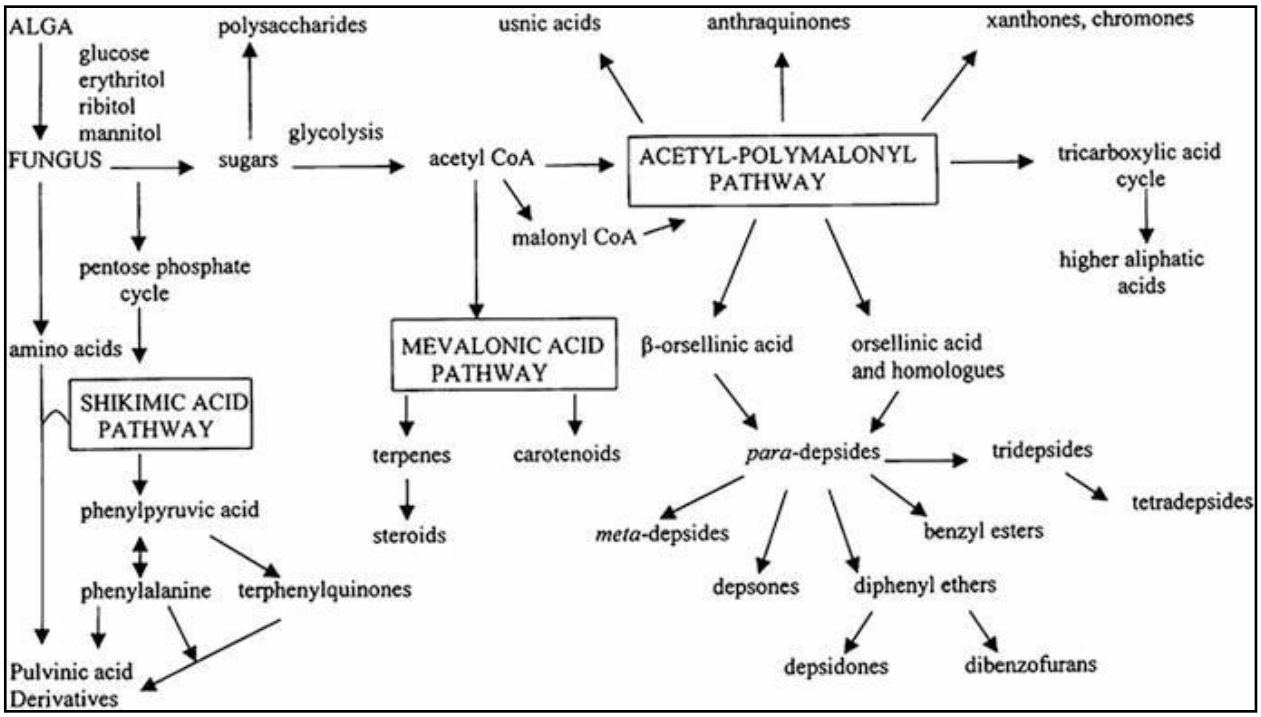


Figure 19: The chemical pathways that synthesize the secondary metabolites in lichens [144].

Recently, new studies have also demonstrated the presence of a third partner associated with lichens. High diverse bacterial communities of more than 800 bacterial species were reported as specific, stable, ubiquitous, abundant, and structurally integrated symbiont of the lichen association. They have been shown to contribute to various indispensable functions: 1) nutrient supply, mainly nitrogen, phosphorous and sulfur, 2) resistance against biotic and abiotic stress factors, 3) supporting photosynthesis by providing vitamin B12, 4) supporting fungal and algal growth by providing hormones, 5) metabolites detoxification, and 6) degeneration of older parts of the lichen thallus. [145]. In addition, these colonizing bacterial communities have been shown to produce interesting metabolites of biological potencies such as uncialamycin [146].

Due to the fact that lichen secondary metabolites have all these protective roles, in addition to the pharmacophores of these compounds, they have gained the scientists interests to be traced pharmacologically [147]. Hence, lichens stood prominently in the medical field as a very rich source of promising drugs.

b. Usages of Lichens

Lichens have been utilized in folk medicine for centuries where their biological potencies have been realized by several civilizations [148]. In addition to remedies, they were used to extract dyes and perfumes since Egyptians.

Starting with dyes, *Roccella* sp. was a lichen species known to secrete a purple pigment called orchil which was used alongside crotall, a brown pigment from *Parmelia*, *Ochrolechia* and

Everenia sp., by the Romans to dye their togas. Jumping into the 18th century, the textiles dyed with lichens reached a prominent economic importance as in the Canary Islands. In addition, lichen fermentation has produced a blue pigment named litmus used for textiles and beverages. This coloring matter was extracted likewise by water from *Roccella* sp. and used to impregnate paper strips becoming pH indicators utilized in the laboratories from the old ages till now. Secondly, some species of lichens such as *Everenia prunastri* were used to extract some molecules constituting contents of perfumes. Finally, lichens were used to treat humans and animals as well. Long pendulous species of *Usnea* lichen were used by New Zealand Moari for nappies and sanitary pads. This very lichen was used moreover in Europe, Asia and Africa to control fever and relieve pain. In the same way, *Usnea densirostra* or "Barba de la piedra", was utilized as a versatile treatment for several diseases in Argentina's folk medicine. Finland also used *Ramalina thrausta* to treat wounds, athlete's foot or other skin diseases as well as to relieve toothache and sore throat. Furthermore, many other species were used to treat several other ailments such as cough, dyspepsia, diabetes, blood and heart diseases, bleeding piles, pulmonary tuberculosis, bronchitis, and spermatorrhoea [149]. As a result, the scientists have uncovered important lichen compounds diagnosed as promising future versatile drugs. They included antibiotics, anti-proliferatives, antioxidants, anti-HIVs, anti-cancers, and anti-protozoans [13].

Nevertheless with new researches are being done every day discovering new things about lichens, some of their compounds were found helpful in ultraviolet radiation B protection. More interesting discoveries also are the antifreeze proteins for frozen foods, capacity of bioplastic degradation and prevention of desertification reported recently and added to the unique biological profile of lichens [150].

c. Lichens, a resort for the antibiotic crisis

After the antibiotic discovery diminishing and the universal bacterial resistance as described in the previous section, scientists started to search for new solutions to this developing problem to face the danger of the resistant bacterial strains. Lichens, according to its antibiotic reputation, constitute one of the resorts to this crisis. This reputation was built by Burkholder et al. who were the first team to examine the antibiotic potency of lichens. *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* were the strains used to test their sensitivity to extracts of 42 lichen species. They found that 27 lichens were able to inhibit *S. aureus* and/or *B. subtilis* where *E. coli* displayed resistance to all extracts [151].

After building this promising basement, many researchers started constructing a solid building designed with many hypotheses windows. Not only lichen extracts or isolated compounds have been evaluated, but also some scientists tried to mix lichen compounds with known antibiotics searching for a better activity driven by synergism. Sensitive as well as multi-drug resistant

bacterial strains have been tested and found sensible to lichen compounds to introduce new antibiotics into the market. For instance, Segatore et al. in 2012 have tested the efficiency of several combinations of a lichen compound, usnic acid, with known antibiotics against 20 different methicillin resistant clinical isolates of *S. aureus*. They found that usnic acid was able to inhibit 50 and 90 % of all *S. aureus* strains with 2 and 4 µg/mL, respectively. In addition, synergism was registered between it and gentamicin, whereas an antagonism was found with levofloxin and finally, no difference with erythromycin. However, combining usnic acid with clindamycin and oxacillin yielded variable results [152]. In the same year, Manojlovic et al. have tested crude extracts from the lichen, *Umbilicaria cylindrica* against several strains (*B. subtilis*, *S. aureus*, *E. coli*, *Proteus vulgaris*, *P. mirabilis*, and *Klebseilla pneumonia*) which have been found all sensitive including *E. coli* which was very resistant as shown by Burkholder et al. and that's why it was worth to mention this example [153]. Furthermore, Honda et al. in 2010 have tested various lichen compounds against *Mycobacterium tuberculosis* which was shown to be sensible also [154]. Nevertheless, some lichen compounds have been also reported acting as antibacterial agents against various bacteria such as evernic acid [155], hybocarpone [156], lichesterinic acid [157], norlichexanthone, protocetraric acid [158], physodic acid [159,160], secalonic acids [161], vulpinic acid [162,163], or usnic acid [164], the latter being the more studied. More recently, the antibacterial activity against the oral pathogens *Streptococcus mutans* and *Porphyromonas gingivalis* of various diphenylethers and lobaric acid, a depsidone, isolated from *Stereocaulon paschale* has been described [165]. This antibiotic potency was proved by many other studies as well against different bacterial strains of different sensitivity, Gram types, and respiration styles [162,166–169].

A hypothesis has been reported by Grube et al., suggesting that the periodic hydration exerts a selective pressure leading to enrichement of specific and stress-tolerant bacteria. Although the secondary metabolites of distinct lichen species have antibacterial activity, a plenty of bacteria have been found on the surfaces and among crystals of these antibacterial compounds. They explained this by a single suggestion that is the bacteria have different sensibility to the antibacterial agents and this can be considered as another factor of bacterial selection in lichens. The colonizing bacterial species have been shown to possess considerable number of multi-drug resistance efflux pumps and contigs of genes encoding products which process complex and cyclic carbohydrates thereby degrading the fungal secondary metabolites. In conclusion, the phylogenetically old lichen symbiosis could constitute a natural reservoir of bacterial resistance mechanisms [145]. Nevertheless, the other partners of this symbiosis will most likely counteract this problem trying to regulate their bacterial populations by producing new effective agents for example.

Alongside this promising antibiotic reputation of lichens, they undergo a limited utilization in the modern medicine due to certain drawbacks. But, despite the fact that lichens have a slow

growth rate and the scientists collide with challenges concerning the lichen *in vitro* propagation, recent technological advancements have been surpassing these difficulties. Enhancements in lichens culturing, alternative molecular genetic techniques for exploring the biosynthetic pathways in lichens, introducing lichen genes into a surrogate host with good fermenting ability and characterized endogenous chemical profile like *E. coli* to yield large quantities of lichen metabolites, synthesizing the lichen compounds, and enhancing the methods of lichen metabolites solubilization were all efficient ways to exploit this biological important resort [13].

THESIS OBJECTIVES

C- The thesis objectives

The misuse and over use of antibiotics is one of the primary reasons behind the bacterial resistance developing globally [170]. Facing this public health concern, more effective antimicrobial candidates compared to the current antibiotics were studied. The new drugs, which are of natural origin, are capable to surpass the bacterial resistance mechanisms and the most important is that they can affect the bacteria inside their biofilms [171]. Among the natural sources is the association of fungus and alga and/or cyanobacterium forming a symbiotic organism named lichen which produce more than 1000 distinct secondary metabolites. They include depsones, depsidones, depsides, dibenzofurans, phenolic compounds, lactones, quinones and derivatives of pulvinic acid possessing antitumor, antiviral and antimicrobial activities. They were shown to be effective against sensitive and several multi-drug resistant bacterial strains [13,14].

The cost of dental care is the fourth highest one of all diseases and consuming between 5 and 10% of all health care resources. Among the oral complications defined clinically, periodontal diseases stand prominently due to their prevalence, notable effects on individuals and society as well as the required high cost to treat [172]. They can be identified as an infectious inflammation of the teeth-supporting tissues caused by the oral pathogens residing in dental biofilms. A streptococcal layer will form above the salivary pellicle and constitutes a recruitment site on which late pathogenic colonizers can bind. The latter include the etiological agent of this disease, *Porphyromonas gingivalis*. The inflammation commences mildly and can worsen if infections were left untreated destroying the tissues with time and leading to teeth loss [67].

As described previously, *P. gingivalis* is a Gram-negative rod and late colonizer compared to *S. gordonii* which is a Gram-positive coccus and early colonizer. This diversity provides this project with a multifaceted aim regarding various scopes. First, the project tests the ability of novel antibacterial agents to interfere positively in the oral infection status of the patient as either early or advanced, second, the study has followed a multi-route strategy for combating the oral infections by testing the butyrolactones ability of preventing the biofilm formation and thereby preventing the infection to be launched or targeting the late pathogen, *P. gingivalis*, after the infection has already commenced, and third, the study evaluates these butyrolactones on two bacterial strains possessing distinct Gram type, morphology, and attribution to differential systemic infections.

As the early plaque constitutes a base on which other late colonizers such as *P. gingivalis* can bind and lead inflammatory actions. Two pathways have been utilized in this project. The first pathway was to target and inhibit the predominant bacterial strain, *S. gordonii*, from forming the early plaque. This will be a proactive effort preventing the future complications rather than

treating an already existing biofilm. The second pathway is for a more advanced stage where the compounds were used to attack the periodontitis etiological agent, *P. gingivalis*.

In order to uncover a new antibacterial agent from the lichen source to fight against the oral bacteria, *S. gordonii* and *P. gingivalis*, we have selected a panel of lichen compounds belonging to different classes of structures and spanning from linear into cyclic and aromatic features. Some of them possess close structures to those of already known antibacterial lichen compounds e.g. roccellic acid, an opening form of lichesterinic acid [157], the four depsidones, and two depsides close to protocetraric [158] and/or physodic [160] or lobaric acids [165], and evernic acid [155]. To our knowledge, this study (Article 1) presents for the first time the activities of these lichen compounds against the targeted bacterial strains. Vulpinic acid and (+)-erythrin have been evaluated against other bacteria [162,173] and are tested herein as controls.

After that, since lichesterinic acid was the most active compound, it has been elected to synthesize some butyrolactone derivatives based on its parental structure trying to enhance the activity. The most active compounds will be evaluated for their cytotoxicity against gingival epithelial cells and macrophages and for their antibiofilm activity. The design and synthesis of the derivatives, evaluating their activity against *S. gordonii*, as well as the cytotoxic effect of the best compounds were published in article 2. The antibiotiflm activity will be demonstrated in article 3.

Finally, the underpinning mechanism of action will be tried to be deciphered to find the bacterial target as discussed in article 4.

RESULTS

D- Results

This part will introduce the results obtained in the present project as small resumes followed with the corresponding articles. The order by which the latter are presented doesn't depend on the publication time; however, it relies on the logical thinking followed in this project.

The first article entitled: "Antibacterial activity of natural lichen compounds against oral bacteria" is under revision in the Fitoterapia journal.

The second article entitled: "Design, synthesis, and biological evaluation of potential butyrolactone analogues" has been published in the Bioorganic and Medicinal Chemistry journal, 2016.

The third article entitled: "Antibiofilm activity of lichen butyrolactones against oral bacteria" is under preparation and will be submitted soon to the Applied Microbiology and Biotechnology journal.

The last or fourth article entitled: "Lichen butyrolactone derivatives disrupted the cell wall of oral bacteria" is under preparation and will be submitted soon to the Journal of American Chemical Society.

I- Screening of natural lichen compounds; article 1

The present study started by searching for efficient natural antibiotics extracted from antibacterial potent organisms, lichens, since it has been reported that the natural compounds are efficient antibacterials. They can surpass the bacterial resistance mechanisms and the most promising is that they can affect the bacteria inside the biofilms [171].

Screening a panel of lichen compounds belonging to different classes of structures and spanning from linear into cyclic and aromatic features for their antibacterial activity against the oral bacteria, *S. gordonii* and *P. gingivalis*, by broth microdilution method is described in article 1 that follows. The results of the natural lichen compound, lichesterinic acid, weren't included in this article, but kept aside to be focused on in the second one.

ARTICLE 1

Manuscript Details

Manuscript number	FITOTE_2017_513
Title	Antibacterial activities of natural lichen compounds against Streptococcus gordonii and Porphyromonas gingivalis
Article type	Research Paper

Abstract

The oral bacteria not only infect the mouth and reside there, but also travel through the blood and reach distant body organs. If left untreated, the dental biofilm that can cause destructive inflammation in the oral cavity may result in serious medical complications. In dental biofilm, Streptococcus gordonii, a primary oral colonizer, constitutes the platform on which late pathogenic colonizers like Porphyromonas gingivalis, the causative agent of periodontal diseases, will bind. The aim of this study was to determine the antibacterial activity of eleven natural lichen compounds belonging to different chemical families and spanning from linear into cyclic and aromatic structures to uncover new antibiotics which can fight against the oral bacteria. The compounds were screened by broth microdilution assay. Three compounds were shown to have promising antibacterial activities where the depsidone core with certain functional groups constituted the best active compound, psoromic acid, with MICs= 11.72 and 5.86 µg/mL against S. gordonii and P. gingivalis, respectively. The compounds screened had promising antibacterial activity which might be attributed to some important functional groups. These results introduce new compounds having potent antibacterial activities against oral pathogens causing serious medical complications.

Keywords	Screening; lichen;antibacterial activity; Streptococcus gordonii; Porphyromonas gingivalis

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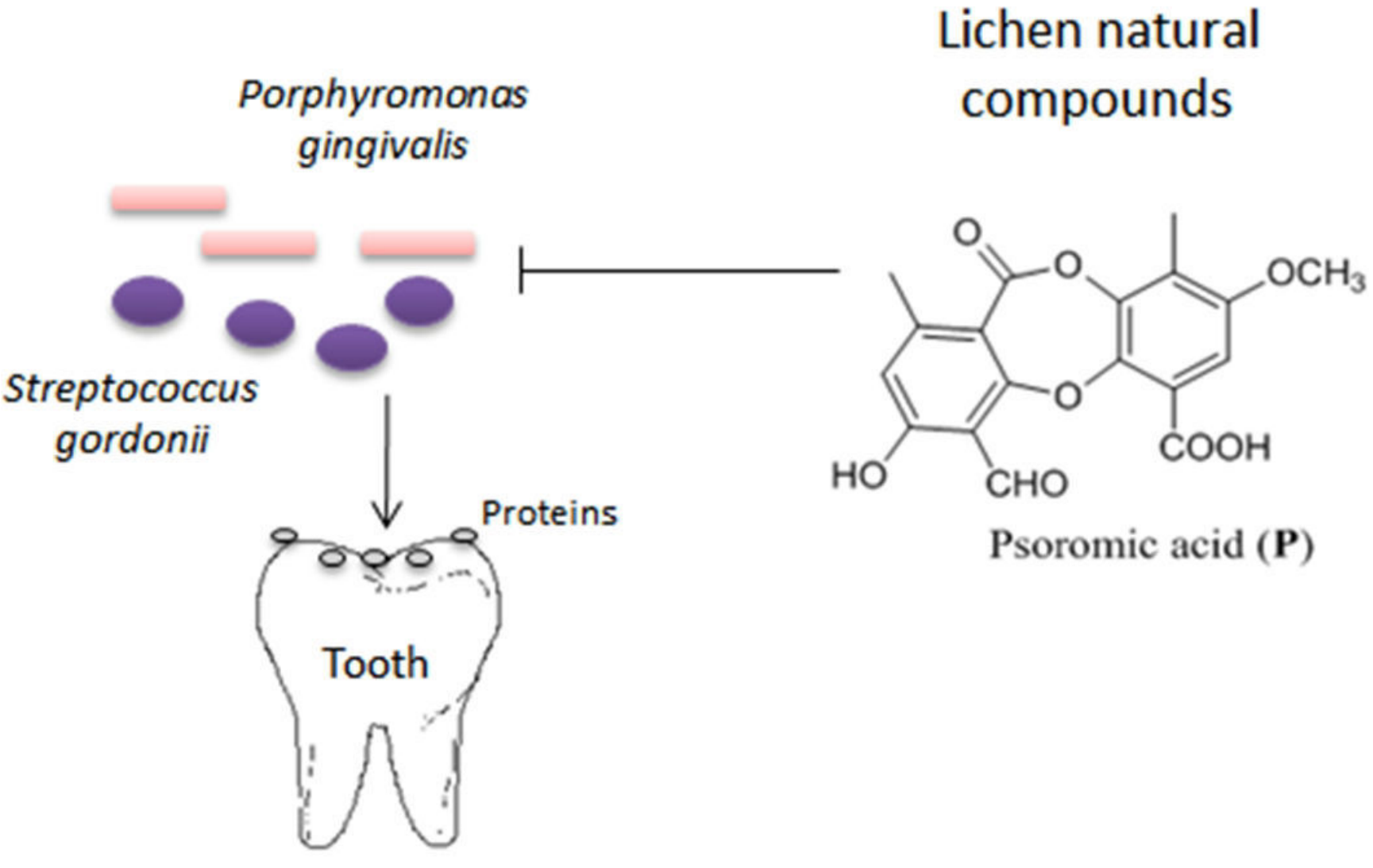
Lichen natural compounds
Porphyromonas gingivalis
Streptococcus gordonii
Proteins
Tooth
O
O
O
OCH3
HO
CHO
COOH
Psoromic acid (P)

Antibacterial activities of natural lichen compounds against Streptococcus gordonii and Porphyromonas gingivalis

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Abstract

The oral bacteria not only infect the mouth and reside there, but also travel through the blood and reach distant body organs. If left untreated, the dental biofilm that can cause destructive inflammation in the oral cavity may result in serious medical complications. In dental biofilm, *Streptococcus gordonii*, a primary oral colonizer, constitutes the platform on which late pathogenic colonizers like *Porphyromonas gingivalis*, the causative agent of periodontal diseases, will bind. The aim of this study was to determine the antibacterial activity of eleven natural lichen compounds belonging to different chemical families and spanning from linear into cyclic and aromatic structures to uncover new antibiotics which can fight against the oral bacteria. The compounds were screened by broth microdilution assay. Three compounds were shown to have promising antibacterial activities where the depsidone core with certain functional groups constituted the best active compound, psoromic acid, with MICs= 11.72 and 5.86 µg/mL against *S. gordonii* and *P. gingivalis*, respectively. The compounds screened had promising antibacterial activity which might be attributed to some important functional groups. These results introduce new compounds having potent antibacterial activities against oral pathogens causing serious medical complications.

Keywords: Screening, lichen, antibacterial activity, *Streptococcus gordonii, Porphyromonas gingivalis*

Chemical compounds studied in this article

Methyl-beta-orcinocarboxylate (**M**) (PubChem CID: 78435); Psoromic acid (**P**) (PubChem CID: 23725); Conhypoprotocetraric acid (**C**) (PubChem CID: 101282317); Demethylbarbatic acid (**D**) (PubChem CID: 10450302); Hypoprotocetraric acid (**H**) (PubChem CID: 627044); Variolaric acid (**Var**) (PubChem CID: 12444681); Vulpinic acid (**Vul**) (PubChem CID: 54690323); (+)-Erythrin (**E**) (PubChem CID: 12308905); Lepraric acid (**L**) (PubChem CID: 12304992); (+)-acetylportentol (**A**) (PubChem CID: 101282317); (+)-Roccellic acid (**R**) (PubChem CID: 11449446)

1. Introduction

The early treatment of infections with antibiotics reduces morbidity; however, the erroneous or unsuitable antibiotic prescription reaches 20-50% in hospitals. This misuse and over use of antibiotics is one of the primary reasons behind the bacterial resistance developing globally [1]. The world is registering a substantial increase of the bacterial resistance against the discovered drugs where this resistance has almost touched all the human pathogens. Facing this fact, organizations like World Health Organization has alerted of being very close to the post-antibiotic age where the antibiotic treatments will be dramatically ineffectual against the infectious pathogens. This coincides with the concept of a position paper published by the Infectious diseases Society of America in 2009. It has reported the critical and expeditious need for developing new antibacterial agents to face this serious health crisis [2].

Facing this public health concern, more effective antimicrobial candidates compared to the current antibiotics were studied. The new drugs, which are of natural origin, are capable to surpass the bacterial resistance mechanisms and the most important is that they can affect the bacteria inside their biofilms [3]. Among the natural sources is the association of fungus and alga and/or cyanobacterium forming a symbiotic organism named lichen which produce more than 1000 distinct secondary metabolites. They include depsones, depsidones, depsides, dibenzofurans, phenolic compounds, lactones, quinones and derivatives of pulvinic acid possessing antitumor, antiviral and antimicrobial activities. They were shown to be effective against sensitive and several multi-drug resistant bacterial strains [4,5]. Some lichen compounds have been already reported acting as antibacterial agents against various bacteria such as evernic acid [6], hybocarpone [7], lichesterinic acid [8], norlichexanthone, protocetraric acid [9], physodic acid [10,11], secalonic acids [12], vulpinic acid [13,14], or usnic acid [15], the latter being the more studied. More recently, the antibacterial activity against the oral pathogens *Streptococcus mutans* and *Porphyromonas gingivalis* of various diphenylethers and laboric acid, a depsidone, isolated from *Stereocaulon paschale* has been described [16].

The cost of dental care is the fourth highest one of all diseases and consuming between 5 and 10% of all health care resources. Among the oral complications defined clinically, periodontal diseases stand prominently due to their prevalence, notable effects on individuals and society as well as the required high cost to treat [17]. They can be identified as an infectious inflammation

93 of the teeth-supporting tissues caused by the oral pathogens residing in dental biofilms. A
94 streptococcal layer will form above the salivary pellicle and constitutes a recruitment site on
95 which late pathogenic colonizers can bind. The latter include the etiological agent of this disease,
96 *Porphyromonas gingivalis*. The inflammation commences mildly and can worsen if infections
97 were left untreated destroying the tissues with time and leading to teeth loss [18].

98 Being the primary colonizer of the oral cavity, an agent of septic arthritis as well as a colonizer
99 of damaged heart valves representing the major causative agent of subacute bacterial
100 endocarditis, *S. gordonii* stands conspicuously as a dangerous bacterium inducing serious
101 medical complications. Alongside, *P. gingivalis*, a maestro in the host's immune system evasion,
102 has been shown to register a lot of capabilities from secreting gingipains which renders its
103 resistance to complement destruction, into its adherence to erythrocytes serving as a safe
104 transport mechanism without being detected by the circulating phagocytes. In addition, this smart
105 bacterium can modify the structure of lipid A in LPS as an escaping mechanism in gingival
106 tissues leading to the pathogenesis of periodontal diseases [19].

107 Not only dental extraction, periodontal surgery or tooth scaling, but even tooth brushing and
108 flossing can disrupt the barrier between the oral bacterial biofilm and the blood circulation which
109 can vehicle these bacteria so far to reach distant body organs. Recently, periodontal disease has
110 been shown to be related with the cause of Alzheimer's disease [19]. Moreover, periodontal
111 diseases seriousness extends to many dangerous systemic complications like type 2 diabetes and
112 oral and pancreatic cancers [17].

113 Against this public oral health burden, we have evaluated the antibacterial activity of eleven
114 natural lichen compounds (Figure 1) against *S. gordonii* and *P. gingivalis.* We have selected a
115 panel of lichen compounds belonging to different classes of structures and spanning from linear
116 into cyclic and aromatic features. Some of them possess close structures to those of already
117 known antibacterial lichen compounds e.g. roccellic acid, an opening form of lichesterinic acid
118 [8], the four depsidones, and two depsides close to protocetraric [9] and/or physodic [11] or
119 lobaric acids [16], and evernic acid [6]. To our knowledge, this study presents for the first time
120 the activities of these lichen compounds against the targeted bacterial strains. Nevertheless,
121 vulpinic acid has been evaluated against other bacteria [13] and is tested herein as a control. Our

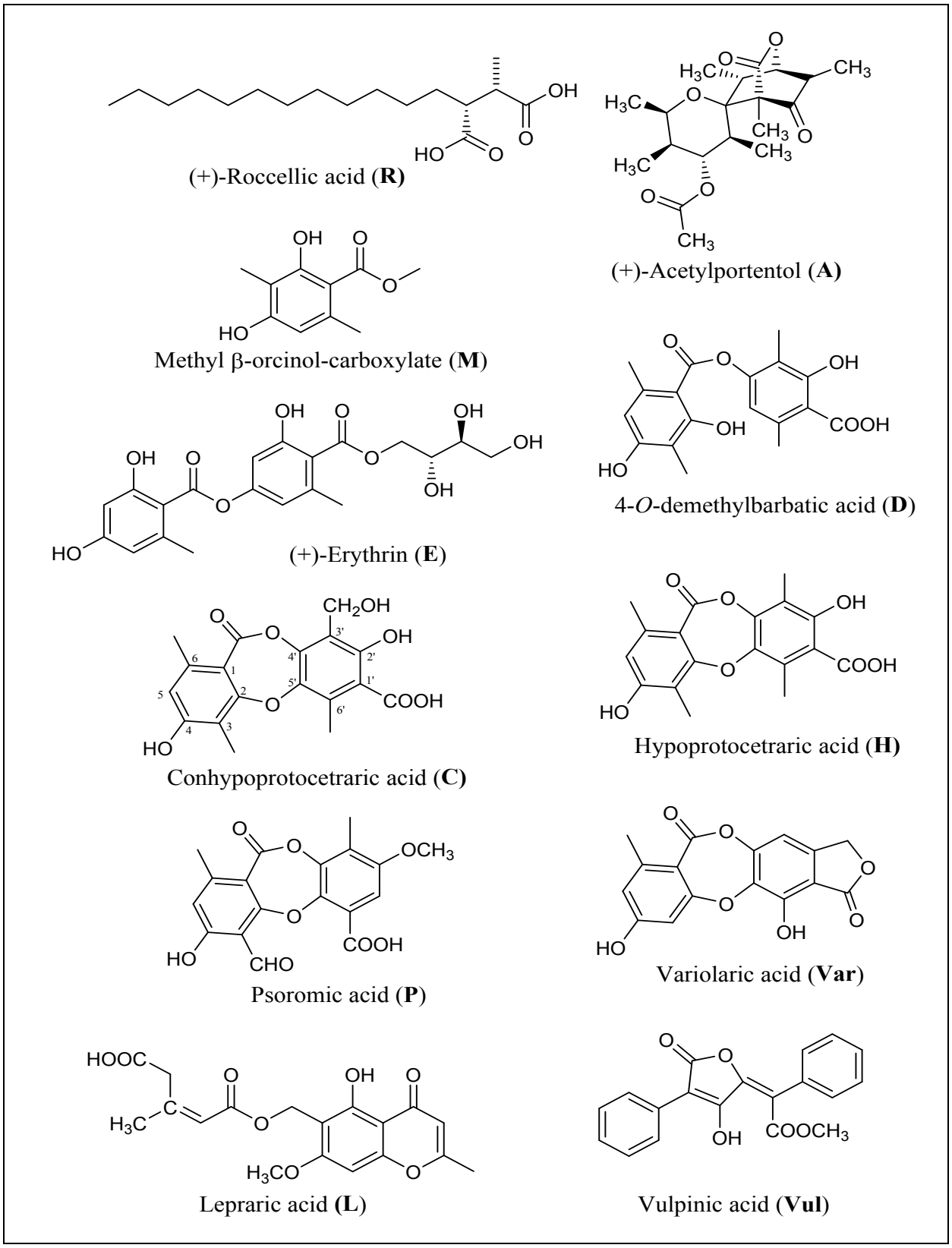
122 promising results introduce new antibiotics that might be able to prevent and treat the periodontal
123 diseases.

124

125 **2. Materials and methods**

126 *2.1. Chemical Compounds*

127 Methyl-beta-orcinocarboxylate (**M**) and Psoromic acid (**P**) were purchased from Sigma Aldrich
128 (France) and Extrasynthèse (France). The other compounds were obtained from UMR CNRS
129 ISCR 6226, CORINT, France, and their spectroscopic data were reported in literature [20,21].
130 Conhypoprotocetraric acid (**C**) was isolated from *Ramalina siliquosa* var. x, demethylbarbatic
131 acid (**D**) from var. *druidarum* and hypoprotocetraric acid (**H**) from var. *zopfii* [20], variolaric
132 acid (**Var**) from *Ochrolechia parella* [22] and vulpinic acid (**Vul**) from *Letharia vulpina* [23].
133 While (+)-Erythrin (**E**), lepraric acid (**L**) and (+)-acetylportentol (**A**) were isolated from *Roccella*
134 *fuciformis*, (+)-roccellic acid (**R**) was extracted from *Roccella phycopsis* [21]. Their calculated
135 Log P along with their lichen sources and the solvents used to prepare the initial concentrations
136 are listed in Table 1. Al the tested compounds (Figure 1) were checked for their >95% purity by
137 HPLC (data not shown).



138

139

140 **Fig. 1.** Chemical structures of the tested lichen compounds listed according to their structural similarities.

141 **Table 1**
142 List of the natural lichen compounds with their lichen species source, along with the solvents used to prepare the
143 solutions and their initial concentrations.
144

Lichen compounds and their derivations	Lichen Species	Solvents used (%)	Initial concentration prepared (µg/mL)
(+)-**A**cetylportentol (**A**)	*Roccella fuciformis* [21]	Methanol (100)	2000
Conhypoprotocetraric acid (**C**)	*Ramalina siliquosa var. x* [20]	Methanol (100)	1400
Demethylbarbatic acid (**D**)	*Ramalina siliquosa* var. *druidarum* [20]	Acetone (100)	1400
(+)-**E**rythrin (**E**)	*Roccella fuciformis*, *Roccella phycopsis* [21]	DMSO/Methanol (50/50)	3000
Hypoprotocetraric acid (**H**)	*Ramalina siliquosa* var. *zopfii* [20]	DMSO/Methanol (50/50)	1000
Lepraric acid (**L**)	*Roccella fuciformis* [21]	DMSO/Methanol (50/50)	2500
Methyl-beta-orcinocarboxylate (**M**)*	Various lichens [23]	Acetone/Methanol (50/50)	3000
Psoromic acid (**P**)*	*Squamarina cartilaginea* [23]	DMSO/Methanol (50/50)	3000
(+)-**R**occellic acid (**R**)	*Roccella phycopsis* [21]	Methanol (100)	3000
Variolaric acid (**Var**)	*Ochrolechia parella* [22]	DMSO/Methanol (50/50)	3000
Vulpinic acid (**Vul**)	*Letharia vulpina* [23]	Chloroform/Methanol (50/50)	3000

145 * Purchased

146

147 *2.2. Bacterial strains*

148 *Streptococcus gordonii* DL1 and *Porphyromonas gingivalis* ATCC 33277 were grown
149 anaerobically (N_2-H_2-CO_2 [80:10:10]) at 37°C according to Sweidan et al [8]. Brain-heart
150 infusion (BHI) medium (DIFCO, France) and blood Columbia agar plates (BioMerieux, France)
151 supplemented with hemin (5 µg/mL) and menadione (1 µg/mL) (Sigma Aldrich, France) were
152 prepared as advised by the manufacturer and utilized for bacterial growth.

153

154 *2.3. Broth microdilution*

155 According to Clinical and Laboratory Standards Institute (CLSI) [24], the compounds were 1:2
156 serially diluted in BHI in a sterile 96-well plate (untreated, flat bottom, with lid, Evergreen

157 Scientific) starting from their initial concentrations (Table 1). Each well was then inoculated with
158 $3x10^7$ CFU/mL of *S. gordonii* and incubated for 24 hours or *P. gingivalis* and incubated for 48
159 hours. The solvents used to prepare the compounds were also tested on the bacteria. After that,
160 the minimal inhibitory concentration (MIC), defined as the minimal concentration able to inhibit
161 the visible bacterial growth, was determined as the clear well having the smallest concentration.
162 All the clear wells were then plated on blood Columbia agar and incubated for 24 hours as
163 needed by *S. gordonii* or for 5 days as required by *P. gingivalis*. Finally, the minimal bactericidal
164 concentration (MBC), corresponding to the lowest compound concentration killing the bacteria
165 in the well, is determined from the Petri-plate showing no colonies and inoculated from the well
166 with the lowest compound concentration. The experiments were repeated three times.

167

168 **3. Results and Discussion**

169 We have tested the antibacterial activity of some natural lichen compounds due to the potent
170 antibacterial reputation of lichen compounds as reported by several authors against different
171 bacterial strains of different sensitivity, Gram types and respiration styles [4]. The set of lichen
172 compounds used here has shown promising antibacterial activities against two bacterial strains
173 differing in their Gram type, *S. gordonii* as a Gram-positive strain and *P. gingivalis* as a Gram-
174 negative counterpart. All of them were found active except **A** and **L** on *S. gordonii*, which
175 registered more resistance, compared to *P. gingivalis* (Table 2).
176 The activity alternates with the compounds structures reflecting their ability to inhibit and/or kill
177 the bacteria. The structure spanned from linear chains into aromatic and cyclic compounds. Both,
178 their chemical structure and the bacterial type (Gram-positive or Gram-negative) have defined
179 their antibacterial potency.
180 Concerning *S. gordonii*, the least active compound was **E** with MIC = 750 µg/mL and MBC =
181 3000 µg/mL. The bacteriostatic activity increased to register MIC = 46.9 and 21.8 µg/mL for **R**
182 and **D**, respectively. Then, it reached the maximum with **P** having MIC = 11.72 µg/mL. On the
183 other side, **A**, **C** and **L** have shown no bactericidal potency. The lowest killing activity was
184 shown for **E** and **P**. Then, it increased to display MBC = 750 µg/mL for **R** and then to reach the
185 maximum with a MBC = 700 µg/mL for **D** (Table 2).

Regarding *P. gingivalis*, which was shown to be more sensitive than *S. gordonii*, **A** was the least active compound with MIC = 1000 µg/mL and MBC = 2000 µg/mL. The inhibitory activity increased to display MIC = 46.9 µg/mL for **R** and then MIC = 10.94 µg/mL for **D**. It continued enhancing to reach the best value of MIC = 5.86 µg/mL for **P**. However, with respect to the bactericidal activity, this strain needed 3000 µg/mL to be killed by the weakest compound, **Var**. The MBC value decreased to be 175 µg/mL for **D** and finally reached the maximum with 11.72 µg/mL displayed by the strongest compound, **P**.

Table 2
The antibacterial activity of the natural lichen compounds against *S. gordonii* and *P. gingivalis* and their calculated Log P.

Compound	MIC (µg/mL)		MBC (µg/mL)		Log P[#]
	S. gordonii	*P. gingivalis*	*S. gordonii*	*P. gingivalis*	
A	>i	1000	>i	2000	2.18 (± 0.66)
C	700	175	>i	700	2.19 (± 0.37)
D	21.8	10.94	700	175	3.55 (± 0.67)
E	750	375	3000	1500	1.43 (± 0.44)
H	250	62.5	1000	500	3.49 (± 0.47)
L	>i	625	>i	2500	1.95 (± 0.40)
M	375	93.75	750	375	2.07 (± 0.23)
P	11.72	5.86	3000	11.72	2.68 (± 0.47)
R	46.9	46.9	750	375	5.28 (± 0.64)
Var	375	375	1500	3000	2.18 (± 0.33)
Vul	187.5	375	1500	375	2.96 (± 0.72)

>i, greater than the initial concentration. [#] calculated by ALOGPS 2.1

Three compounds were shown to have promising antibacterial activities and can be listed from the least into the most active as **R**, **D** then **P**, whereas their Log P value, the coefficient describing their relative lipophilicity, decreases from **R** to **P** but remains high.

Starting with compound **R**, it showed the same MIC value, 46.9 µg/mL, against both bacterial strains, suggesting that it may have the same bacterial target in the two Gram types. As the butyrolactones, it has the same long chain and the carboxyl group suggested to be involved in the antibacterial activity by Sweidan et al [8]. This compound appears to be the most lipophilic regarding its Log P value.

208 The Gram-negative strain was more sensitive to **D** than the other bacterium. It displayed a strong
209 inhibition effect against both bacteria, but a weak killing potential.
210 *S. gordonii* was also more resistant against **P** than *P. gingivalis*. Alongside its strong inhibition
211 against both strains, its killing effect was weak to need 3000 µg/mL to kill *S. gordonii* compared
212 to 11.72 µg/mL needed to kill *P. gingivalis*. Regarding their lipophilic character, **D** being more
213 lipophilic than **P**, this parameter seems not to have influenced their antibacterial activity.
214 Compound **Vul** was reported to be active against several bacterial strains. Its best MIC was 4
215 µg/mL against *Propionibacterium acnes* [13]. We have found in this study that it is active
216 against *S. gordonii* and *P. gingivalis* but to a much less efficiency than what Lauterwein et al
217 have found.
218 Among the compounds we can find 5 compounds that possess close structure, **C**, **D**, **H**, **P** and
219 **Var** (Table 2). Compounds **C**, **H** and **Var** were less active than **D** and **P**. Regarding **C** and **Var**,
220 they showed different activity regarding the Gram type of the bacteria. **C** was more effective
221 against *P. gingivalis* (Gram-negative) whereas **Var** was more active to kill *S. gordonii* (Gram-
222 positive). This result is in accordance with those of protocetraric and lobaric acids which showed
223 a good activity against *Salmonella Typhi* [9] and *P. gingivalis* [16], respectively. Then, we can
224 conclude that some functional groups have a selective antibacterial activity that will target a
225 certain type. CH_3, CH_2OH, OH and COOH groups at carbons 3, 3', 2', and 1', respectively, in
226 compound **C** were absent in **Var** which had a 5-membered ring at carbons 1' and 2'. Also, CH_3
227 at carbon 6' in **C** was replaced with OH in **Var**. Then, if we compare the depsidones **C** and **H** to
228 the depside **D,** we find that one or two structural changes have taken place: substituting CH_2OH
229 at carbon number 3' in compound **C** instead of CH_3 in compound **D** and the presence of ether
230 linkage in **C** and **H** at C-5'. These changes have weakened the antibacterial activity and showed
231 the importance of CH_3 group at C-3'. The most important activity of **D** could be related to its
232 flexibility around the ester linkage. In comparison with the most active compound **P**, two CH_3
233 groups at carbons 3 and 6' in **D** were replaced with aldehyde and carboxyl groups, respectively.
234 In addition to the ether linkage between C-2 and C-5', the carboxyl group of **D** at C-1' was lost
235 in **P** and the hydroxyl group attached to C-2' was replaced by a methoxy group.
236 Summarizing the structural differences, we can conclude the importance of the following groups
237 in depsidone core to obtain the best antibacterial activity: a) An aldehyde group at carbon 3, b) A

238 methyl group at carbon 3' instead of CH_2OH, c) A hydroxyl or methoxy group at carbon 2' and
239 d) presence of a carboxyl group.
240 The lipophilicity of compounds can play an important role in their antibacterial properties since
241 the bacterial lipid membrane is lipophilic. Nevertheless, other physicochemical properties such
242 as pKa could be an important parameter to determine the partition coefficient of these lichen
243 compounds as already mentioned by Honda et al [25]. All the active compounds possess a
244 carboxylic group indicating that these compounds are mostly ionized at pH 7. Our results are in
245 agreement with those reported previously [25]. Further investigations will be carried out to
246 determine the means they used to penetrate bacterial cells and to precise the mechanism of action
247 of these compounds.
248

249 **4. Conclusion**

250 The natural lichen compounds screened had promising antibacterial activity against the oral
251 bacteria, *S. gordonii* and *P. gingivalis*. Compounds (+)-Roccellic acid (**R**), Demethylbarbatic
252 acid (**D**) and Psoromic acid (**P**) had the highest activity with **P** being the best compound.
253 Chemically, some structural changes among the compounds have shown some important sites
254 that might be involved in the antibacterial activity. However, this activity seems not to be
255 attributed to their Log P values. These results introduce new compounds having potent
256 antibacterial activities against oral pathogens causing serious medical complications.

257 **Conflict of interests**

258 None to declare

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265

266

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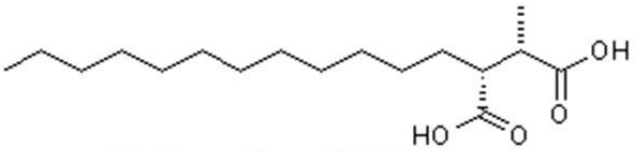

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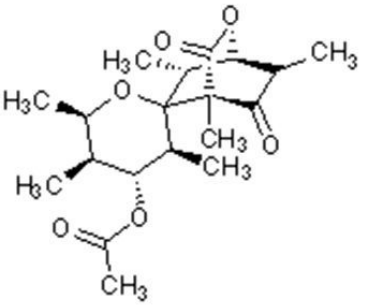

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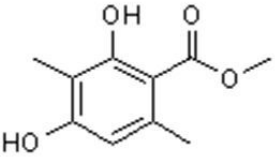
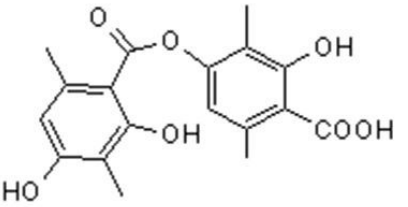
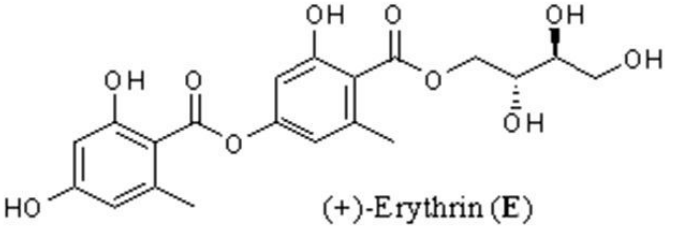


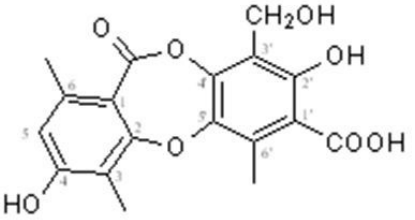
Methyl β-orcinol-carboxylate (**M**)



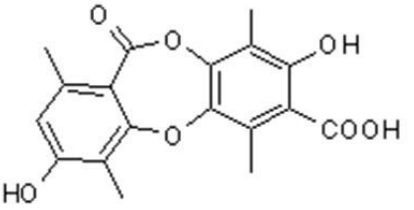
4-*O*-demethylbarbatic acid (**D**)



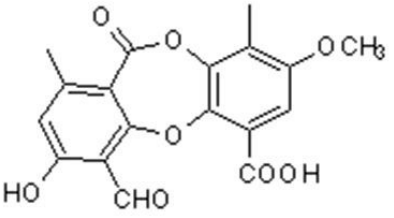
(+)-Erythrin (**E**)



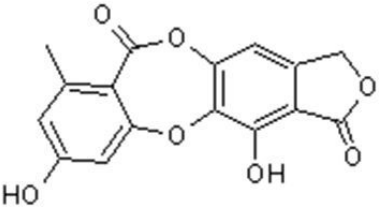
Conhypoprotocetraric acid (**C**)



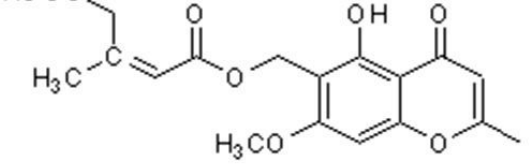
Hypoprotocetraric acid (**H**)



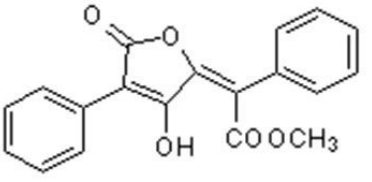
Psoromic acid (**P**)



Variolaric acid (**Var**)



Lepraric acid (**L**)



Vulpinic acid (**Vul**)

Figure 1:

Table 1:
List of the natural lichen compounds with their lichen species source, along with the solvents used to prepare the solutions and their initial concentrations.

Lichen compounds and their derivations	Lichen Species	Solvents used (%)	Initial concentration prepared (µg/mL)
(+)-**A**cetylportentol (**A**)	*Roccella fuciformis* [18]	Methanol (100)	2000
Conhypoprotocetraric acid (**C**)	*Ramalina siliquosa var. x* [15]	Methanol (100)	1400
Demethylbarbatic acid (**D**)	*Ramalina siliquosa* var. *druidarum* [15]	Acetone (100)	1400
(+)-**E**rythrin (**E**)	*Roccella fuciformis*, *Roccella phycopsis* [18]	DMSO/Methanol (50/50)	3000
Hypoprotocetraric acid (**H**)	*Ramalina siliquosa* var. *zopfii* [15]	DMSO/Methanol (50/50)	1000
Lepraric acid (**L**)	*Roccella fuciformis* [18]	DMSO/Methanol (50/50)	2500
Methyl-beta-orcinocarboxylate (**M**)*	Various lichens [17]	Acetone/Methanol (50/50)	3000
Psoromic acid (**P**)*	*Squamarina cartilaginea* [17]	DMSO/Methanol (50/50)	3000
(+)-**R**occellic acid (**R**)	*Roccella phycopsis* [18]	Methanol (100)	3000
Variolaric acid (**Var**)	*Ochrolechia parella* [16]	DMSO/Methanol (50/50)	3000
Vulpinic acid (**Vul**)	*Letharia vulpina* [17]	Chloroform/Methanol (50/50)	3000

(*): Purchased

Table 2:
The antibacterial activity of the natural lichen compounds against *S. gordonii* and *P. gingivalis* and their calculated Log P.

Compound	MIC (µg/mL)		MBC (µg/mL)		Log P[#]
	S. gordonii	*P. gingivalis*	*S. gordonii*	*P. gingivalis*	
A	>i	1000	>i	2000	2.18 (± 0.66)
C	700	175	>i	700	2.19 (± 0.37)
D	21.8	10.94	700	175	3.55 (± 0.67)
E	750	375	3000	1500	1.43 (± 0.44)
H	250	62.5	1000	500	3.49 (± 0.47)
L	>i	625	>i	2500	1.95 (± 0.40)
M	375	93.75	750	375	2.07 (± 0.23)
P	11.72	5.86	3000	11.72	2.68 (± 0.47)
R	46.9	46.9	750	375	5.28 (± 0.64)
Var	375	375	1500	3000	2.18 (± 0.33)
Vul	187.5	375	1500	375	2.96 (± 0.72)

>i: greater than the initial concentration; (#): calculated by ALOGPS 2.1.

II- Butyrolactone derivatives; articles 2, 3, and 4

After obtaining the screening results, lichesterinic acid was the best, so, it has been taken alone from the panel to synthesize some derivatives trying thereby to enhance the antibacterial activity.

A series of butyrolactone analogues based on the parental compound, lichesterinic acid, was synthesized and tested against *S. gordonii* by broth microdilution method. Then, the best derivatives were evaluated for their cytotoxicity against gingival epithelial cells and macrophages by MTT and LDH assays. This is demonstrated in article 2.

Article 3 will continue further to test the antibacterial activity of the butyrolactone series on the second strain, *P. gingivalis*, by broth microdilution method. In addition, the best compounds were tested for their antibiofilm activity by crystal violet assay against *S. gordonii* and *P. gingivalis* monospecies biofilms. The antibiofilm activity was confirmed by confocal microscope which was used to visualize these biofilms treated with the butyrolactone analogues. Finally, some genes involved in the biofilm formation were quantified by qPCR.

The target of these derivatives is the objective of article 4 which utilized microscopical (transmission electron and confocal microscopes), chemical (HPLC), and molecular (qPCR) approaches trying to decipher the underpinning mechanism of action.

ARTICLE 2

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Design, synthesis and biological evaluation of potential antibacterial butyrolactones

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ABSTRACT

Novel butyrolactone analogues were designed and synthesized based on the known lichen antibacterial compounds, lichesterinic acids (**B-10** and **B-11**), by substituting different functional groups on the butyrolactone ring trying to enhance its activity. All synthesized butyrolactone analogues were evaluated for their in vitro antibacterial activity against *Streptococcus gordonii*. Among the derivatives, **B-12** and **B-13** had the lowest MIC of 9.38 μg/mL where they have shown to be stronger bactericidals, by 2–3 times, than the reference antibiotic, doxycycline. These two compounds were then checked for their cytotoxicity against human gingival epithelial cell lines, Ca9–22, and macrophages, THP-1, by MTT and LDH assays which confirmed their safety against the tested cell lines. A preliminary study of the structure–activity relationships unveiled that the functional groups at the C_4 position had an important influence on the antibacterial activity. An optimum length of the alkyl chain at the C_5 position registered the best antibacterial inhibitory activity however as its length increased the bactericidal effect increased as well. This efficiency was attained by a carboxyl group substitution at the C_4 position indicating the important dual role contributed by these two substituents which might be involved in their mechanism of action.

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1. Introduction

The usages of antibiotics on a large scale alongside their misapplication have lead to the emergence of resistant pathogenic bacteria.[1] Both, the infection of these re-emergent strains which has increased the global mortality rate to be a growing concern and the global reduction in antibiotics production open a new era where other potent candidates should be found to fight against bacteria.[2] Indeed, an infinite number of plant species have been tested against a huge number of bacterial strains in vitro. In addition, many phytochemicals found effective against a broad spectrum of microorganisms comprising fungi, yeast and bacteria were uncovered.[3] Throughout the last 2 decades, plants are becoming a famous rich source of antimicrobial substances.[4] Furthermore, many other promising drug sources still need to be explored.[5] Lichens which are symbiotic organisms comprising a fungus and a photosynthetic alga and/or cyanobacterium constitutes a potential source of over 1000 distinct secondary metabolites.[6] They comprise antitumor, antiviral and antimicrobial activities.[6–9] Sensitive as well as several multi-drug resistant bacterial strains were shown to be susceptible to these lichen compounds.[6]

Streptococcus gordonii (*S. gordonii*) is an eminent member of the viridans streptococci large category. Not only was this bacteria described as an agent of septic arthritis but also it can colonise damaged heart valves and represents the primary etiological agent of subacute bacterial endocarditis.[10] In the oral cavity, *S. gordonii* adhere to the salivary pellicle which coats the teeth, proliferate and excrete an extracellular polysaccharide matrix protecting their developing microcolony on which secondary colonizers will adhere.[11] The late colonizing strains such as *Porphyromonas gingivalis* bind the sites provided by *S. gordonii* and form a highly pathogenic complex microbial community.[12,13] *S. gordonii* as a pioneer initial colonizer initiates the formation of dental plaques contributing in turn to the onset of dental caries and periodontal diseases as well as their progression.[14,15] Inhibiting *S. gordonii* might block the successive steps leading to acute oral diseases and this may constitute prevention rather than a risky cure after biofilm formation.

To address this oral issue, we synthesized a natural butyrolactone, L-lichesterinic acid. Cavalito et al. have extracted it from the lichen, *Cetraria islandica*, and shown to have an activity against

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Streptococcus hemolyticus and *Staphylococcus aureus*.[16] Our goal in this study is to evaluate its antibacterial activity against *S. gordonii* in solid and liquid media under anaerobic conditions. Trying to enhance its activity, some derivatives were synthesized and tested (Fig. 1). Finally, the cytotoxic effect of the most active compounds was evaluated on two human cell lines, gingival epithelial cells, Ca9–22, and macrophages, THP-1. To the best of our knowledge, it is the first study to describe some of these synthetic derivatives, their antibacterial activity against *S. gordonii* and their cytotoxic effects.

2. Results and discussion

2.1. Chemistry

Scheme 1 illustrates the synthesis of enantiopure (−)-lichesterinic acid **B-10** and its derivatives. This straightforward asymmetric synthesis has already been described by Braukmüller and Brückner in 2006 for the preparation of paraconic acids.[17] To the best of our knowledge, this strategy had been used only for the synthesis of naturally aliphatic α-methylene butyrolactone (+)-methylenolactocin (R = C_5H_{11}) and (+)-protolichesterinic acid (R = $C_{13}H_{27}$) and their (−) enantiomers (Fig. 2). Based on a six steps method, one additional step is required to obtain a series of lichesterinic acid derivatives by isomerization of the double bond. Moreover, this lactone strategy has been extended to include different alkyl chain lengths R (C_7H_{15}, C_9H_{19}, $C_{15}H_{31}$, and $C_{16}H_{33}$). Briefly, it began with the preparation of hydroxyl lactones **1a–e** where the enantiocontrol was imposed by the asymmetric dihydroxylation of *trans*-configured β,γ-unsaturated carboxylic ester with AD mix-α® or AD mix-β®. The resulting lactones were dehydrated giving butenolides **2a–e**. For the two next steps we modified the approach according to Perepogu et al.[18] A Gilman addition of a vinyl group was added *trans*-selectively to the C=C bond giving vinyl lactones **3a–e,** followed by an oxidation of the double bond allowing access to HO_2C-substituted lactones **4a–e**. α-Activation by Stiles' reagent, followed by amino-methylation in situ fragmentation provided the α-methylene butyrolactones **5a–e**. Then, the target enantiopure lichesterinic acid derivatives **6a–e** were obtained by isomerization of the double bond using NEt_3 in DMF. This synthesis is achieved in seven steps and around 10% overall yield with good enantioselective excess determined by chiral HPLC.

2.2. Biological activity

2.2.1. Antibacterial activity

2.2.1.1. Agar dilution. Seven out of the thirteen butyrolactones screened (Fig. 1) showed an activity with the concentrations tested against *S. gordonii* under anaerobic conditions (Table 1). Compounds **B-2**, **B-4**, **B-5, B-6**, **B-7**, and **B-13** didn't exhibit any activity. The least active compounds were **B-1** and **B-3** showing the highest Minimal Inhibitory Concentration (MIC) of 300 μg/mL. Then, the MIC decreased to be 200 μg/mL for **B-12** and continued decreasing to pass by 150 μg/mL for **B-8** and **B-9** and reaches the lowest value with **B-10** and **B-11** registering 90 μg/mL (Fig. 3A, Table 1). Alongside, doxycycline displayed an MIC of 0.41 μg/mL which was fixed and used always as a positive control (Fig. 3B, Table 1). In addition, the mixture of the solvents (DMSO + methanol) used to dissolute our compounds was found inactive at the highest concentration tested. These results were taken into the liquid medium to confirm and compare.

2.2.1.2. Broth microdilution. Compared to the solid medium, all butyrolactones were found active except **B-7**. At this step, **B-2**, **B-4**, **B-5** and **B-6** joined the antibacterial panel (Table 2).

According to the efficiency of the compounds, they can be distributed into 3 groups. The least efficient were **B-1**, **B-3**, **B-8**, and **B-9**. The most effective were **B-10**, **B-11**, **B-12**, and **B-13**.

The highest inhibitory activity was for **B-10** and **B-11** which registered the same results with MIC = 4.69 μg/mL and Minimal Bactericidal Concentration (MBC) = 18.75 μg/mL. While MIC

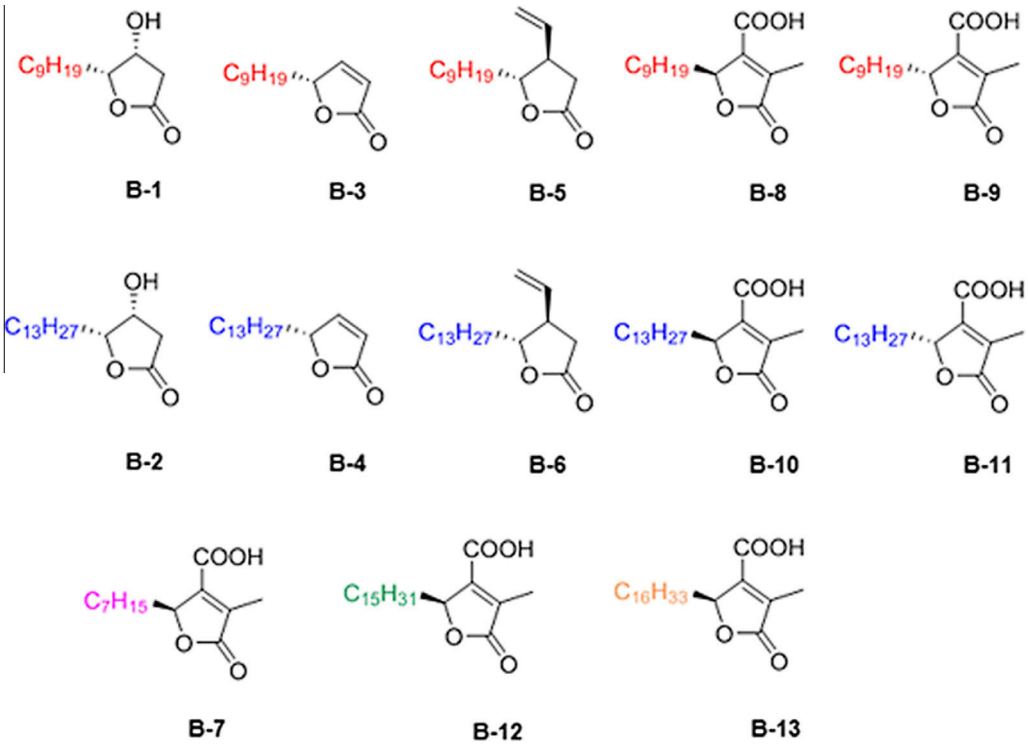
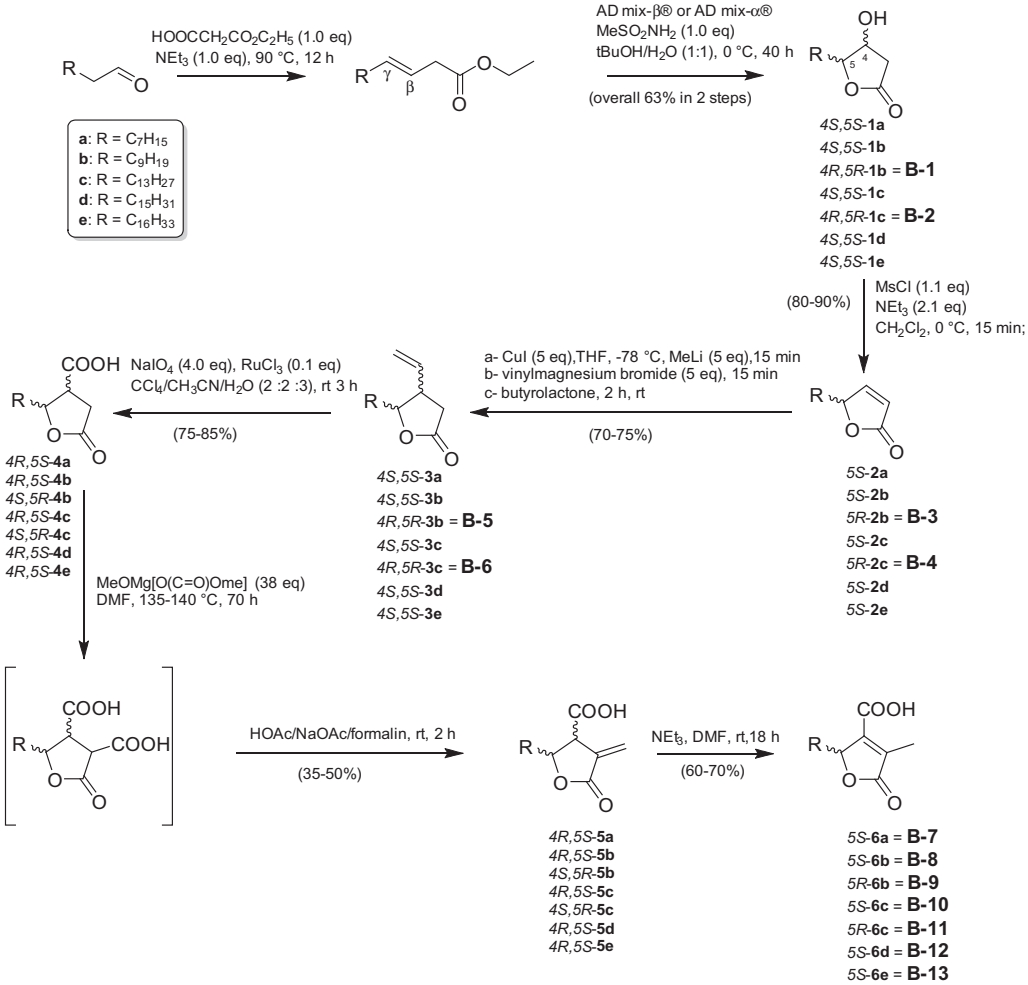


Figure 1. Chemical structures of butyrolactones.



Scheme 1. Enantioselective synthesis route of butyrolactones.

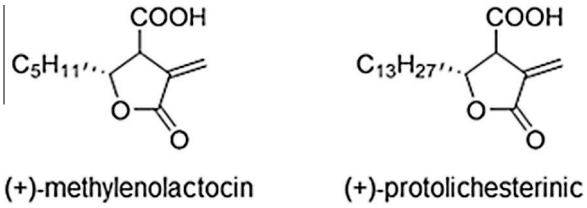


Figure 2. Chemical structures of two aliphatic α-methylene butyrolactones.

Table 1
Minimal Inhibitory Concentration of butyrolactones against *S. gordonii* by agar dilution

Compound	MIC (μg/mL)
B-1	300
B-2	>300
B-3	300
B-4	>300
B-5	>300
B-6	>300
B-7	>300
B-8	150
B-9	150
B-10	90
B-11	90
B-12	200
B-13	>300
Doxycycline	0.41

increased to be 9.38 μg/mL for **B-12**, its MBC remained at the same value. **B-13** showed the same MIC as **B-12** and it was also its MBC exhibiting the strongest killing effect.

Doxycycline activity decreased here to have MIC = 0.51 μg/mL and MBC = 32.8 μg/mL which were fixed and used always as positive control. If we compare butyrolactones to doxycycline antibiotic, we can notice that the latter's MBC was higher than that of **B-12** and **B-13** by 2 or 3 times, respectively (Table 2). For the next experiments we have selected **B-12** and **B-13** because **B-10** and **B-11** are already known natural compounds.

2.2.2. Cytotoxicity

B-10 and **B-11** were already described so we have chosen **B-12** and **B-13** to check their cytotoxicity. The viability of gingival epithelial cells, Ca9-22, and macrophage-like cells, THP-1, was evaluated by LDH and MTT assays (Fig. 4A and B).

2.2.2.1. LDH assay. The positive control, Triton 1%, was considered as the maximum with 100 percent cytotoxicity (Fig. 4A). Cells alone displayed 9% and 18% of LDH release from Ca9-22 and THP-1, respectively. No significant difference was displayed between the cells treated with the compounds and the cells alone showing around the same percentages of cell death. Regarding Ca9-22, 9.6% and 12% were found for **B-12**, **B-13**, respectively. With the same order, 20% and 10% of cell death were registered for THP-1 (Fig. 4A).

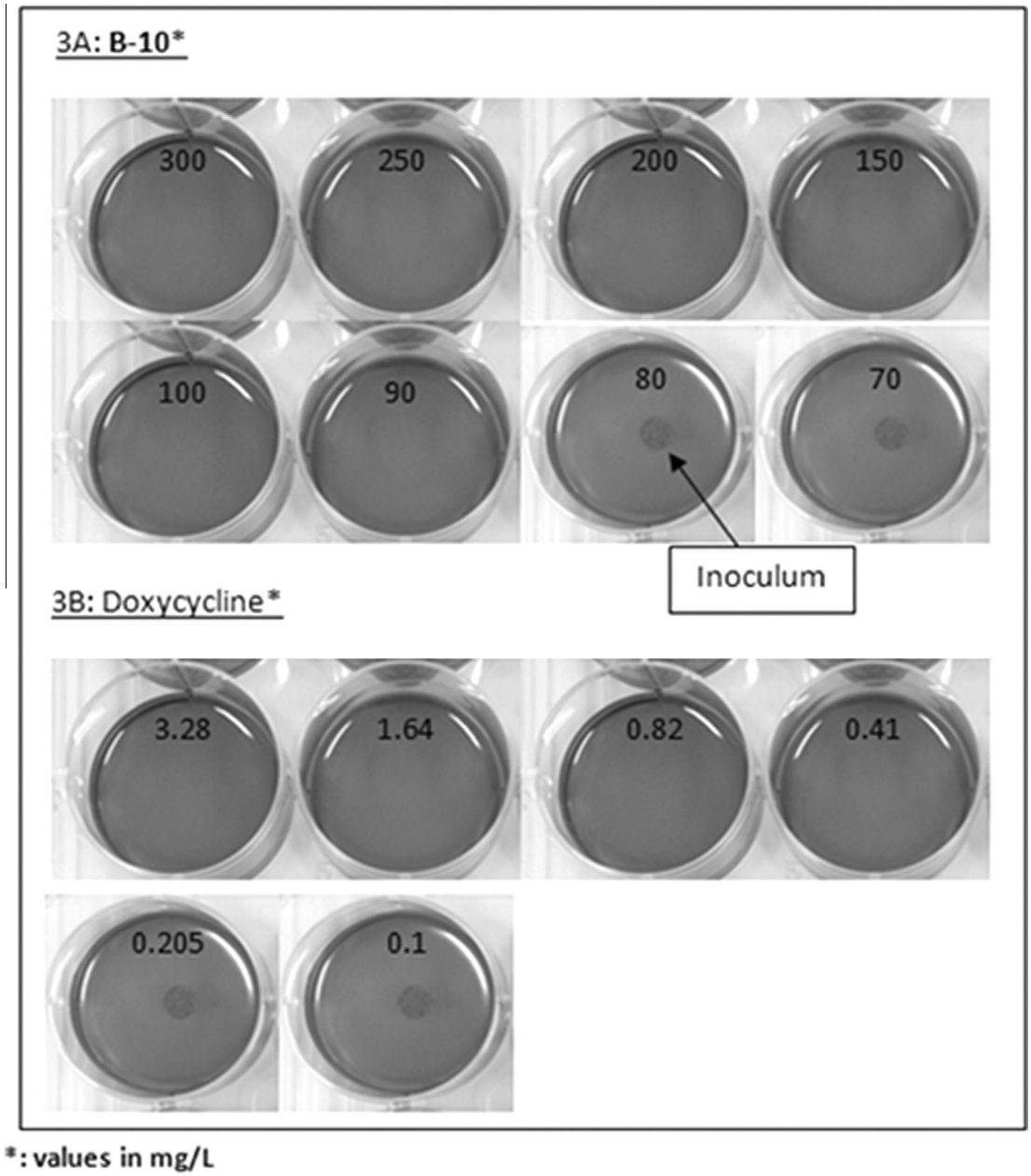


Figure 3. Effect of butyrolactones on *S. gordonii* in agar dilution.

Table 2
Minimal inhibitory and bactericidal concentrations of butyrolactones against *S. gordonii* by broth microdilution

Compound	MIC (μg/mL)	MBC (μg/mL)
B-1	150	300
B-2	75	150
B-3	150	300
B-4	75	75
B-5	75	150
B-6	75	75
B-7	>300	>300
B-8	150	>300
B-9	150	>300
B-10	4.69	18.75
B-11	4.69	18.75
B-12	9.38	18.75
B-13	9.38	9.38
Doxycycline	0.51	32.80

2.2.2.2. MTT assay. Cells alone were considered the maximum with 100% cell viability (Fig. 4B). Triton 1% was the positive control which showed a significant different result decreasing the cell population into around 3% for both types of cells. The compounds were fluctuating in a very close range around 100% and their activities were not significantly different from those against the cells alone. With respect to Ca9-22, 100% and 96% were displayed by **B-12**, **B-13**, respectively. Following the same pattern, 107% and 106% were found for THP-1 (Fig. 4B).

3. Structure–activity relationships

To analyze structure–activity relationships, four structural components were considered: the saturation of the C_3–C_4 bond, the nature of the substituent (X) and (Y) at the C_3 and C_4 position respectively, and the length of the alkyl chain (R) (Fig. 5).

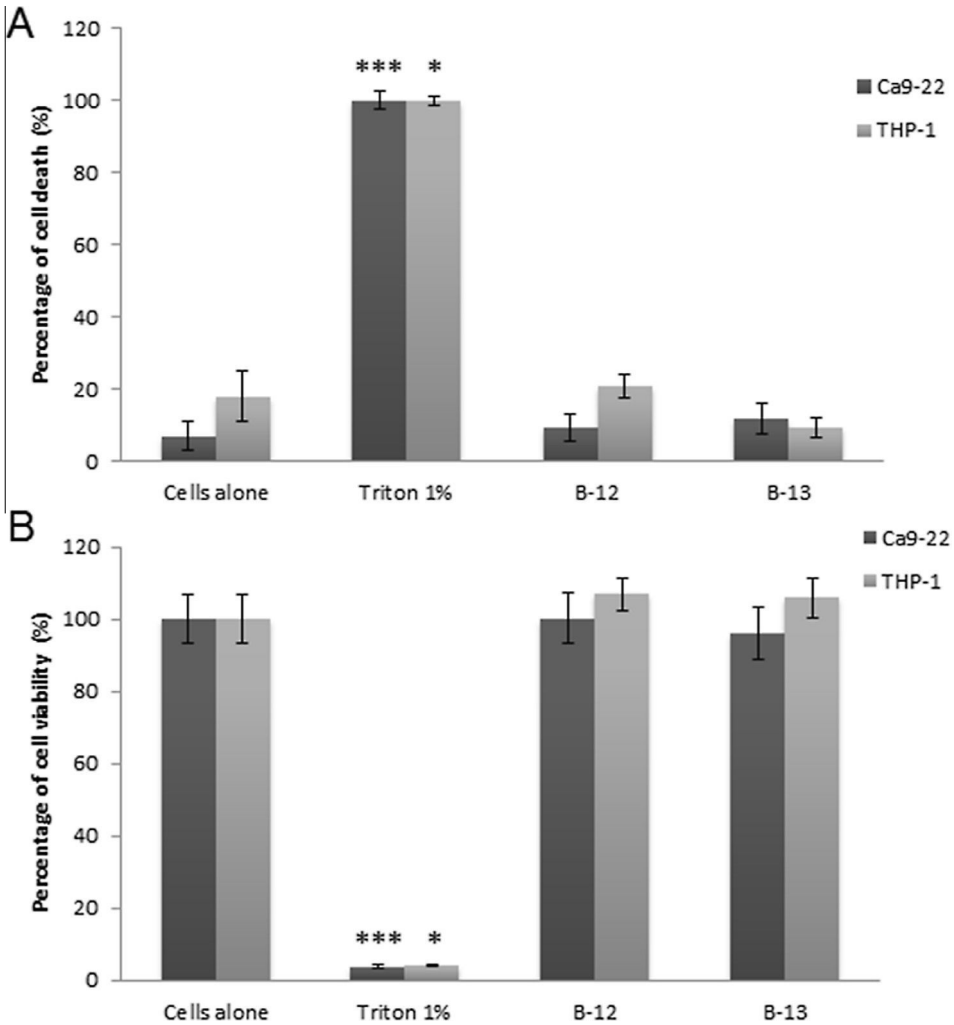


Figure 4. Evaluation of butyrolactones cytotoxicity on Ca9–22 and THP-1 cells by LDH (A) and MTT (B) assays. $^{*}P < 0.05$; $^{***}P < 0.001$.

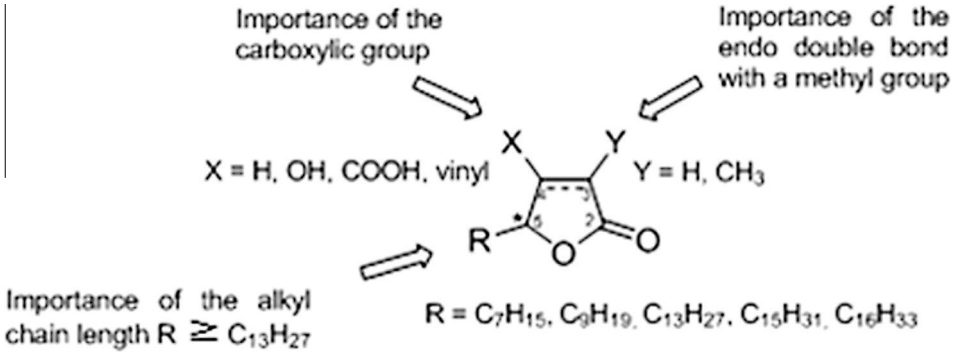


Figure 5. Pharmacomodulation.

Starting with the Agar dilution assay, the MIC was controlled by the length of the R alkyl chain. As the length of R increased, the activity increased. This was observed between C_9H_{19} chain compounds **B-8** and **B-9**, which had MIC = 150 μg/mL and $C_{13}H_{27}$ chain counterparts or lichesterinic acids (**B-10** and **B-11**), which had MIC = 90 μg/mL (Table 1). Hence, we decided to test shorter and longer chains to confirm our hypothesis. The C_7H_{15} chain compound **B-7,** as well as **B-13,** with the longest chain $C_{16}H_{33}$, didn't show any activity at the highest concentration tested. But, **B-12** which was shorter than **B-13** by only 1 carbon atom, showed an MIC = 200 μg/mL. This was interpreted as that the length of the chain plays an important role in its activity where there is an optimum length of 13 carbon atoms which has the highest potential. As the chain length increases or decreases, the activity decreases as well (Table 1).

In broth microdilution assay, all of the compounds were found active except **B-7** and exhibited better activity than in solid medium assay (Table 2). This discrepancy between the two media

was also shown by Guzman et al. They screened natural products from Columbian plants against *Mycobacterium tuberculosis* and they obtained different activity between the two media.[19] Therefore, we can propose that butyrolactones can move more freely due to their lipophilicity and inhibit more efficiently in liquid medium compared to the other different physiological solid state, where the bacteria are confined to the surface. The liquid results confirm the hypothesis of being $C_{13}H_{27}$ chain is the optimum length. **B-7** with a C_7H_{15} alkyl group did not show any activity and the effect decreased when the chain length increased. Comparing to **B-10**, MIC increased to be 9.38 μg/mL for **B-12** but the MBC remains the same. Then, when the chain length increases more to be 16 carbon atoms, **B-13** showed the same MIC as **B-12** but its MBC was the strongest to exhibit a surprising effect that the liquid medium owns. The latter elects an optimum chain length of 13 carbon atoms for the best inhibition but the killing effect increases as the R chain increases in length since their lipophilicity increases as well. This chain length contribution was discussed by Yang et al. where they tested the derivatives of 8-alkylberberine against Gram-positive and Gram-negative strains to find an optimum length of 8 carbon atoms. Shorter or longer chains showed lowering in the antibacterial activity. They also mentioned that Gram-positive strains were more susceptible to these derivatives.[20] It can be proposed that the saturated chain, R, may be involved in the butyrolactones mechanism of action. The optimum length hypothesis was also supported by comparing other compounds which can be grouped into 3 couples having the same main structure differing only in the length of the R chain, **B-1**/**B-2**, **B-3**/**B-4** and **B-5**/**B-6**, where when the length increased to 13 carbon atoms the activity increased. MIC and MBC decreased by half comparing **B-1** to **B-2**, from 150 and 300 μg/mL for **B-1** into 75 and 150 μg/mL for **B-2,** respectively. Also, the other couples were displaying the same effect. We can see clearly that MIC and MBC decreased from 150 and 300 μg/mL for **B-3** and **B-5** into 75 μg/mL for **B-4** and **B-6**, respectively. So, the activity is better regarding the couples, **B-3**/**B-4** and **B-5**/**B-6**, than the first couple, **B-1**/**B-2,** highlighting the drawback of the introduction of a hydrophilic group for the antibacterial activity of these butyrolactones. We can also notice that the enantiomers showed the same antibacterial effect comparing the value between **B-8** and **B-9** and **B-10** and **B-11**.

In addition to the R chain, substitution of different functional groups at C_4 position constitutes a second factor affecting the antibacterial activity. These groups divided our compounds into four classes. The first class, **B-1** and **B-2,** comprised a hydroxyl group, OH, the second class, **B-3** and **B-4,** comprised a double bond in the ring with no substitutions, the third class, **B-5** and **B-6,** possessed a vinyl group with a saturated ring and finally, the fourth class, **B-8**, **B-9**, **B-10**, **B-11**, **B-12**, and **B-13,** contained an unsaturated ring with two substituents, carboxyl and methyl groups. Since the highest activity was demonstrated for the last class, this suggests that the carboxyl group may stand behind this potency. The importance of this functional group was mentioned by Sebastianes et al. who tested the antibacterial activity of a fungal compound, 3-hydroxy propionic acid, 3-HPA, against *Staphylococcus aureus* and *Salmonella typhi*. Indeed, 3-HPA showed relevant antibacterial activity against the tested strains. When it was esterified to produce 3-hydroxypropanoic ethyl ester, no antimicrobial activity was registered.[21] This gives a complementary idea for the probable underpinning mechanism of action in which the carboxylic group and the R chain could be implicated (Fig. 5). Moreover, these butyrolactones have a similar structure to the γ-butyrolactone autoregulators described formerly. The latter are produced by the Gram-positive *Streptomyces* genus and they regulate the DNA binding activity of cognate receptor proteins triggering antibiotic production as mentioned by Kitani et al.[22] Hence, lichesterinic acid and its analogues may modulate the DNA binding activity of some proteins.

4. Conclusions

To conclude, all butyrolactone derivatives were synthesized in good yield with an efficient enantioselective strategy. All compounds were then screened for their antibacterial activity against *S. gordonii* in solid and liquid media using agar dilution and broth microdilution methods, respectively. The compounds have shown a stronger activity in the liquid medium than in the solid one where only **B-7** was found not active. The alkyl chain of 13 carbons showed the best inhibitory activity with an MIC of 4.69 μg/mL. Among the derivatives, **B-12** and **B-13** were the best promising compounds registering a better bactericidal activity than the reference antibiotic used, doxycycline, by 2 or 3 times, respectively. This chain alongside the carboxyl functional group may be involved in their mechanism of action. Finally, **B-12** and **B-13** were evaluated for their cytotoxicity against human gingival epithelial cells, Ca9-22, and macrophages, THP-1, and found not toxic. This gives a bright hope to continue with these two butyrolactones into their antibiofilm activity for their graduation as new oral antibiotic agents. These new compounds are capable to inhibit *S. gordonii* which may block the successive steps leading to oral complications, thus, a safe prevention rather than a risky late treatment after biofilm formation.

5. Experimental

5.1. Chemistry

All reagents of high quality were purchased from commercial suppliers and used without further purification. Melting points were recorded on a Kofler Leica VMHB melting point apparatus and are uncorrected. IR spectra were obtained with PerkinElmer UATR Two infrared spectrophotometer. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were performed on a Bruker DMX 300 spectrometer. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: $\delta_H = 7.26$, $\delta_C = 77.0$). The δ values are given in parts per million (ppm), and the coupling constants (J values) are given in Hertz (Hz). The multiplicity of the signals is reported as s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet). ESI-HRMS were carried out on a MICROMASS ZabspecTOF spectrometer for electrospray ionization at the CRMPO (Centre Régional de Mesures Physiques de l'Ouest), University of Rennes 1. Elemental analyses were performed on a microanalysor Flash EA1112 CHNS/O Thermo Electron at the CRMPO. Optical rotations were measured on a Perkin Elmer Model 341 polarimeter at 20 °C using thermostable optical glass cell (1 dm path length and c in g/100 mL). The ee values were determined by chiral LC with a TSP Spectra System UV2000 and P1000 XR apparatus with a CHIRALPACK® IC or IA column. Reactions were monitored by TLC on Merk 60 F254 (0.25 mm) plates which were visualized by UV detection or sprayed with vanillin or $KMNO_4$ solutions, then heated.

5.1.1. General procedures for compounds 1a–e

A mixture of the appropriate aldehyde (28.1 mmol), monoethyl malonate (3.32 mL, 3.72 g, 28.1 mmol, 1.0 equiv) and NEt_3 (3.92 mL, 2.85 g, 28.1 mmol, 1.0 equiv) was heated at 90–95 °C under argon atmosphere. After stirring overnight, the reaction mixture was cooled at room temperature and poured at 0 °C into an aq H_2SO_4 solution (20%, 100 mL). The organic phase was separated and the aqueous phase was extracted with diethyl ether (3 × 100 mL). The combined organic phases were dried with

$MgSO_4$. After filtration, the solution was concentrated under vacuum to give quantitatively the β,γ-unsaturated carboxylic ester. Then a mixture of β,γ-unsaturated carboxylic ester (28.1 mmol), AD mix-α® (39.34 g), methanesulfonamide (2.67 g, 28.1 mmol, 1.0 equiv) was added to a 1:1 mixture of *t*BuOH and H_2O (180 mL) at 0 °C. After 40 h the reaction was quenched by adding a satd aqueous solution of Na_2SO_3 (100 mL), this solution was stirred for 1 h before extraction with diethyl ether (3 × 50 mL). The combined organic layers were dried with $MgSO_4$ and concentrated under vacuum. The residue was purified by chromatography on silicagel using ethyl acetate/petroleum ether 2:8 and then 4:6 as eluents.

5.1.1.1. (4*S*,5*S*)-5-Heptyl-4-hydroxy-dihydrofuran-2(3*H*)-one (4*S*,5*S*-1a). Yield: 64%; white solid; mp 75 °C. R_f = 0.26 (petroleum ether/ethyl acetate 6:4). $[\alpha]_D$ = −47.6 (*c* 1.07, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.89 (t, *J* = 6.2 Hz, 3H), 1.28–1.93 (m, 12H), 2.55 (d, *J* = 17.7 Hz, 1H), 2.80 (dd, *J* = 5.4 Hz and *J* = 17.7 Hz, 1H), 4.34–4.40 (m, 1H), 4.46–4.48 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2, 22.7, 25.7, 28.4, 29.2, 29.5, 31.9, 39.6, 69.1, 85.3, 176.4 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{11}H_{20}O_3Na$: 223.13101, found $[M+Na]^+$: 223.1309.

5.1.1.2. (4*S*,5*S*)-4-Hydroxy-5-nonyl-dihydrofuran-2(3*H*)-one (4*S*,5*S*-1b). This compound was prepared as published.[23] Yield = 63%. $[\alpha]_D$ = −38.9 (*c* 1.15, $CHCl_3$). HRMS (ESI, *m*/*z*): calcd for $C_{13}H_{24}O_3Na$: 251.16231, found $[M+Na]^+$: 251.1618.

5.1.1.3. (4*R*,5*R*)-4-Hydroxy-5-nonyl-dihydrofuran-2(3*H*)-one (4*R*,5*R*-1b) (B-1). Yield = 63%; white solid; mp 69 °C. R_f (petroleum ether/ethyl acetate 8:2) = 0.10. $[\alpha]_D$ = +44.2 (*c* 1.07, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.27–1.95 (m, 16H), 2.55 (dd, *J* = 0.9 Hz and *J* = 17.7 Hz, 1H), 2.80 (dd, *J* = 5.4 Hz and *J* = 17.7 Hz, 1H), 4.33–4.39 (m, 1H), 4.46–4.51 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 13.4, 22.0, 24.9, 27.6, 28.6, 28.7, 28.8, 31.2, 38.8, 68.3, 84.4, 175.4 ppm. IR (ATR) 3466, 2952, 2922, 2850, 1740. HRMS (ESI, *m*/*z*): calcd for $C_{13}H_{24}O_3Na$: 251.1623, found $[M+Na]^+$: 251.1626.

5.1.1.4. (4*S*,5*S*)-4-Hydroxy-5-tridecyl-dihydrofuran-2(3*H*)-one (4*S*,5*S*-1c). This compound was prepared as published.[17] Yield = 61%. $[\alpha]_D$ = −37.1 (*c* 1.07, $CHCl_3$). HRMS (ESI, *m*/*z*): calcd for $C_{17}H_{32}O_3Na$: 307.2249, found $[M+Na]^+$: 307.2244.

5.1.1.5. (4*R*,5*R*)-4-Hydroxy-5-tridecyl-dihydrofuran-2(3*H*)-one (4*R*,5*R*-1c) (B-2). This compound was prepared as published.[17] Yield = 61%. $[\alpha]_D$ = +18.5 (*c* 1.04, $CHCl_3$). HRMS (ESI, *m*/*z*): calcd for $C_{17}H_{32}O_3Na$: 307.2249, found $[M+Na]^+$: 307.2249.

5.1.1.6. (4*S*,5*S*)-4-Hydroxy-5-pentadecyl-dihydrofuran-2(3*H*)-one (4*S*,5*S*-1d). Yield = 53%; white solid; mp 96 °C. R_f (petroleum ether/ethyl acetate 8:2) = 0.11. $[\alpha]_D$ = −32.9 (*c* 0.93, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.93 (m, 29H), 2.56 (dd, *J* = 0.8 Hz and *J* = 17.7 Hz, 1H), 2.80 (dd, *J* = 5.4 Hz and *J* = 17.5 Hz, 1H), 4.34–4.40 (m, 1H), 4.46–4.49 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.3, 22.8, 25.7, 28.4, 29.5, 29.6, 29.7, 29.8, 29.9 32.1, 39.6, 69.2, 85.1, 176.05 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{19}H_{36}O_3Na$: 335.25622, found $[M+Na]^+$: 335.2559.

5.1.1.7. (4*S*,5*S*)-5-Hexadecyl-4-hydroxy-dihydrofuran-2(3*H*)-one (4*S*,5*S*-1e). Yield = 52%; white solid; mp 99 °C. R_f (petroleum ether/ethyl acetate 8:2) = 0.10. $[\alpha]_D$ = −34.6 (*c* 1.04, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.6 Hz, 3H), 1.25–1.93 (m, 30H), 2.56 (d, *J* = 17.7 Hz, 1H), 2.80 (dd, *J* = 5.4 Hz and *J* = 17.7 Hz, 1H), 4.34–4.39 (m, 1H), 4.46–4.49 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.3, 22.8, 25.7, 28.4, 29.5, 29.6, 29.7, 29.8, 32.1, 39.6, 43.6, 69.2, 85.0, 175.8; HRMS (ESI, *m*/*z*): calcd for $C_{20}H_{38}O_3Na$: 349.2719, found $[M+Na]^+$: 349.2719.

5.1.2. General procedure for compounds 2a–e

At 0 °C under argon atmosphere NEt_3 (5.33 mL, 3.87 g, 38.2 mmol, 2.2 equiv) and methanesulfonyl chloride (1.48 mL, 2.19 g, 19.1 mmol, 1.1 equiv) were added dropwise to a solution of hydroxylactone **1** (17.4 mmol) in anhydrous CH_2Cl_2 (85 mL). After stirring for 1 h the reaction was quenched by adding a satd. aqueous solution of NH_4Cl (150 mL). The organic phase was separated and the aqueous phase was extracted with diethyl ether (3 × 50 mL). The combined organic layers were dried with $MgSO_4$ and concentrated under vacuum. The residue was purified by chromatography on silicagel using diethyl ether/petroleum ether 1:4 as eluents.

5.1.2.1. (*S*)-5-Heptylfuran-2(5*H*)-one (5*S*-2a). Yield = 94%; colorless oil. R_f (petroleum ether/diethyl ether 8:2) = 0.17. $[\alpha]_D$ = +77.8 (*c* 1.21, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.28–1.83 (m, 12H), 5.02–5.07 (m, 1H), 6.11 (dd, *J* = 2.0 Hz and *J* = 5.7 Hz, 1H), 7.46 (dd, *J* = 1.45 Hz and *J* = 5.7 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2, 22.7, 25.1, 29.1, 29.3, 31.8, 33.3, 83.6, 121.6, 156.5, 173.3 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{11}H_{18}O_2Na$: 205.1205, found $[M+Na]^+$: 205.1203.

5.1.2.2. (*S*)-5-Nonylfuran-2(5*H*)-one (5*S*-2b). This compound was prepared as published.[23] Yield = 94%. $[\alpha]_D$ = +63.5 (*c* 0.94, $CHCl_3$). HRMS (ESI, *m*/*z*): calcd for $C_{13}H_{22}O_2Na$: 233.1518, found $[M+Na]^+$: 233.1517.

5.1.2.3. (*R*)-5-Nonylfuran-2(5*H*)-one (5*R*-2b) (B-3). Yield = 91%; colorless oil. R_f (petroleum ether/diethyl ether 8:2) = 0.16. $[\alpha]_D$ = −62.7 (*c* 1.17, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.82 (m, 16H), 5.01–5.07 (m, 1H), 6.11 (dd, *J* = 2.0 Hz and *J* = 5.7 Hz, 1H), 7.46 (dd, *J* = 1.5 Hz and *J* = 5.7 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.4, 23.0, 25.3, 29.6, 29.62, 29.7, 29.8, 32.2, 33.5, 83.8, 121.8, 156.7, 173.5 ppm. IR (ATR) 2923, 2853, 1744. HRMS (ESI, *m*/*z*): calcd for $C_{13}H_{22}O_2Na$: 233.1518, found $[M+Na]^+$: 233.1519.

5.1.2.4. (*S*)-5-Tridecylfuran-2(5*H*)-one (5*S*-2c). This compound was prepared as published.[17] Yield = 77%. $[\alpha]_D$ = +54.2 (*c* 1.01, $CHCl_3$). HRMS (ESI, *m*/*z*): calcd for $C_{17}H_{30}O_2Na$: 289.2144, found $[M+Na]^+$: 289.2138.

5.1.2.5. (*R*)-5-Tridecylfuran-2(5*H*)-one (5*R*-2c) (B-4). This compound was prepared as published.[17] Yield = 81%. $[\alpha]_D$ = −57.2 (*c* 1.09, $CHCl_3$), HRMS (ESI, *m*/*z*): calcd for $C_{17}H_{30}O_2Na$: 289.2144, found $[M+Na]^+$: 289.2144.

5.1.2.6. (*S*)-5-Pentadecylfuran-2(5*H*)-one (5*S*-2d). Yield = 75%; white solid; mp 67 °C. R_f (petroleum ether/ethyl acetate 7:3) = 0.78. $[\alpha]_D$ = +48.9 (*c* 1.17, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.83 (m, 28H), 5.01–5.07 (m, 1H), 6.10 (dd, *J* = 1.9 Hz and *J* = 5.7 Hz, 1H), 7.46 (dd, *J* = 1.4 Hz and *J* = 5.7 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2, 22.8, 25.1, 29.4, 29.5, 29.6, 29.7, 29.8, 32.0, 33.3, 83.6, 121.6, 156.5, 173.3 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{19}H_{34}O_2Na$: 317.2457, found $[M+Na]^+$: 317.2456.

5.1.2.7. (*S*)-5-Hexadecylfuran-2(5*H*)-one (5*S*-2e). Yield = 91%; white solid; mp 74 °C. R_f (petroleum ether/diethyl ether 8:2) = 0.26. $[\alpha]_D$ = +50.7 (*c* 1.04, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.5 Hz, 3H), 1.25–1.83 (m, 30H), 5.01–5.06 (m, 1H), 6.11 (dd, *J* = 2.0 Hz and *J* = 5.7 Hz, 1H), 7.45 (dd, *J* = 1.4 Hz and *J* = 5.7 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.3, 22.8, 25.1,

29.4, 29.5, 29.6, 29.7, 29.8, 32.1, 33.3, 83.6, 121.6, 156.4, 173.3 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{20}H_{36}O_2Na$: 331.2613, found $[M+Na]^+$: 331.2613.

5.1.3. General procedure for compounds 3a–e

To a −78 °C solution of CuI (15.41 g, 80.9 mmol, 5.0 equiv) in anhydrous tetrahydrofuran (THF, 120 mL) was slowly added a solution of methyl lithium (50.6 mL, 80.9 mmol of 1.6 M, 5.0 equiv). The suspension was allowed to reach rt during 15 min and then cooled to −78 °C and vinyl magnesium bromide (80.9 mL, 80.9 mmol of 1 M, 5.0 equiv) was added. The mixture was stirred for 15 min at −78 °C and then butenolide **2** (16.2 mmol) in THF (20 mL) was added dropwise. The resulting black solution was allowed to warm at rt and was stirred for 2 h. Then the mixture was poured into 500 mL of vigorously stirred satd aqueous NH_4Cl solution. The pH of solution was adjusted to 8–10 by addition of conc NH_4OH. The mixture was stirred at rt for 1.5 h until all the copper salts had dissolved. The blue solution was extracted with diethyl ether, the organic phase was filtered through celite and then washed with a solution of ethylenediamine (50 mL with 450 mL H_2O). The organic layer was dried with $MgSO_4$ and concentrated under vacuum. The residue was purified by chromatography on silicagel using petroleum ether/ethyl acetate 9.5:0.5 as eluents.

5.1.3.1. (4*S*,5*S*)-5-Heptyl-4-vinyl-dihydrofuran-2(3*H*)-one (4*S*,5*S*-3a). Yield = 81%; colorless oil. R_f (petroleum ether/ethyl acetate 9.5:0.5) = 0.27. $[\alpha]_D = -63.1$ (*c* 1.13, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.6 Hz, 3H), 1.28–1.76 (m, 12H), 2.45 (dd, *J* = 10.5 Hz and *J* = 17.6 Hz, 1H), 2.68 (dd, *J* = 8.2 Hz and *J* = 17.0 Hz, 1H), 2.73–2.85 (m, 1H), 4.11–4.18 (m, 1H), 5.14–5.21 (m, 2H), 5.67–5.79 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.1, 22.6, 25.8, 29.1, 29.3, 31.7, 33.6, 35.5, 46.4, 84.8, 118.0, 135.8, 175.8 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{13}H_{22}O_2Na$: 233.1518, found $[M+Na]^+$: 233.1517.

5.1.3.2. (4*S*,5*S*)-5-Nonyl-4-vinyl-dihydrofuran-2(3*H*)-one (4*S*,5*S*-3b). Yield = 62%; colorless oil. R_f (petroleum ether/ethyl acetate 9.5:0.5) = 0.14. $[\alpha]_D = -54.5$ (*c* 1.19, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.78 (m, 16H), 2.45 (dd, *J* = 10.3 Hz and *J* = 16.9 Hz, 1H), 2.68 (dd, *J* = 8.2 Hz and *J* = 16.9 Hz, 1H), 2.73–2.84 (m, 1H), 4.11–4.17 (m, 1H), 5.15–5.21 (m, 2H), 5.67–5.78 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.1, 22.6, 25.7, 29.2, 29.3, 29.4, 29.45, 31.8, 33.6, 35.4, 46.3, 84.8, 117.9, 135.8, 175.8 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{15}H_{26}O_2Na$: 261.1831, found $[M+Na]^+$: 261.1832.

5.1.3.3. (4*R*,5*R*)-5-Nonyl-4-vinyl-dihydrofuran-2(3*H*)-one (4*R*,5*R*-3b) (B-5). Yield = 70%; colorless oil. R_f (petroleum ether/ethyl acetate 9.5:0.5) = 0.14. $[\alpha]_D = +55.6$ (*c* 1.27, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.78 (m, 16H), 2.45 (dd, *J* = 10.3 Hz and *J* = 16.9 Hz, 1H), 2.68 (dd, *J* = 8.2 Hz and *J* = 16.9 Hz, 1H), 2.73–2.84 (m, 1H), 4.11–4.17 (m, 1H), 5.15–5.21 (m, 2H), 5.67–5.78 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.1, 22.6, 25.7, 29.2, 29.3, 29.4, 29.5, 31.8, 33.6, 35.4, 46.3, 84.8, 117.9, 135.8, 175.8 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{15}H_{26}O_2Na$: 261.1831, found $[M+Na]^+$: 261.1832.

5.1.3.4. (4*S*,5*S*)-5-Tridecyl-4-vinyl-dihydrofuran-2(3*H*)-one (4*S*,5*S*-3c). Yield = 77%; white solid; mp 48 °C. R_f (petroleum ether/ethyl acetate 9:1) = 0.51. $[\alpha]_D = -42.9$ (*c* 1.20, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.5 Hz, 3H), 1.26–1.80 (m, 24H), 2.44 (dd, *J* = 10.3 Hz and *J* = 16.9 Hz, 1H), 2.68 (dd, *J* = 8.2 Hz and *J* = 16.9 Hz, 1H), 2.72–2.81 (m, 1H), 4.11–4.17 (m, 1H), 5.15–5.21 (m, 2H), 5.66–5.78 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2, 22.8, 25.8, 29.5, 29.6, 29.7, 29.8, 32.0, 33.8, 35.6, 46.5, 85.0, 118.1, 135.95, 175.9 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{19}H_{34}O_2Na$: 317.2457, found $[M+Na]^+$: 317.2456.

5.1.3.5. (4*R*,5*R*)-5-Tridecyl-4-vinyl-dihydrofuran-2(3*H*)-one (4*R*,5*R*-3c) (B-6). This compound was prepared as published.[24] Yield = 71%. $[\alpha]_D = +45.0$ (*c* 1.11, $CHCl_3$). HRMS (ESI, *m*/*z*): calcd for $C_{19}H_{34}O_2Na$: 317.2457, found $[M+Na]^+$: 317.2456.

5.1.3.6. (4*S*,5*S*)-5-Pentadecyl-4-vinyl-dihydrofuran-2(3*H*)-one (4*S*,5*S*-3d). Yield = 80%; white solid; mp 61 °C. R_f (petroleum ether/ethyl acetate 9:1) = 0.43. $[\alpha]_D = -34.6$ (*c* 1.33, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.71 (m, 28H), 2.45 (dd, *J* = 10.4 Hz and *J* = 17.0 Hz, 1H), 2.69 (dd, *J* = 8.2 Hz and *J* = 16.9 Hz, 1H), 2.75–2.81 (m, 1H), 4.11–4.17 (m, 1H), 5.15–5.21 (m, 2H), 5.66–5.78 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2, 22.8, 25.8, 29.5, 29.6, 29.7, 29.8, 32.0, 33.7, 35.6, 46.5, 84.9, 118.1, 135.9, 175.9. HRMS (ESI, *m*/*z*): calcd for $C_{21}H_{38}O_2Na$: 345.2770, found $[M+Na]^+$: 345.2767.

5.1.3.7. (4*S*,5*S*)-5-Hexadecyl-4-vinyl-dihydrofuran-2(3*H*)-one (4*S*,5*S*-3e). Yield = 65%; white solid; mp 65 °C. R_f (petroleum ether/ethyl acetate 9:1) = 0.38. $[\alpha]_D = -41.3$ (c 1.04, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.76 (m, 30H), 2.45 (dd, *J* = 10.3 Hz and *J* = 16.9 Hz, 1H), 2.68 (dd, *J* = 8.2 Hz and *J* = 17.0 Hz, 1H), 2.75–2.84 (m, 1H), 4.11–4.17 (m, 1H), 5.15–5.21 (m, 2H), 5.67–5.78 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2, 22.8, 25.8, 29.5, 29.6, 29.7, 29.8, 32.0, 33.8, 35.6, 46.5, 84.9, 118.1, 135.9, 175.9 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{22}H_{40}O_2Na$: 359.2926, found $[M+Na]^+$: 359.2925.

5.1.4. General procedure for compounds 4a–e

To a stirred solution at rt of vinyl lactone **3** (13.1 mmol) in a solvent mixture of $CH_3CN/CCl_4/H_2O$ (14:14:21 mL) were added $NaIO_4$ (11.19 g, 52.3 mmol, 4.0 equiv) and $RuCl_3$ (0.27 g, 1.31 mmol, 0.1 equiv). After 3 h at rt, CH_2Cl_2 was added and the aq phase was separated and extracted with CH_2Cl_2. The combined organic layers were filtered once through celite and then through silicagel + celite. The filtrate was concentrated under vacuum and the residue was diluted with diethyl ether (200 mL) and satd $NaHCO_3$ solution (200 mL) was added. After separation of the two phases, the aq. phase was acidified with HCl 1 M until pH = 2. The product was extracted with CH_2Cl_2, dried with $MgSO_4$ and concentrated under vacuum to yield the desired compounds in pure form.

5.1.4.1. (2*S*,3*R*)-2-Heptyl-5-oxo-tetrahydrofuran-3-carboxylic acid (4*R*,5*S*-4a). Yield = 80%; white solid; mp 110 °C. R_f (CH_2Cl_2/MeOH 9:1) = 0.48. $[\alpha]_D = -29.2$ (*c* 1.13; $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.4 Hz, 3H), 1.28–1.83 (m, 12H), 2.78–3.00 (m, 2H), 3.07–3.15 (m, 1H), 4.60–4.69 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2, 22.7, 25.3, 29.2, 29.3, 31.8, 32.0, 35.5, 45.5, 82.0, 174.5, 176.4 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{12}H_{19}O_4$: 227.1289, found $[M-H]^-$: 227.1290.

5.1.4.2. (2*S*,3*R*)-2-Nonyl-5-oxo-tetrahydrofuran-3-carboxylic acid (4*R*,5*S*-4b). Yield = 81%; white solid; mp 115 °C. R_f (CH_2Cl_2/MeOH 9:1) = 0.45. $[\alpha]_D = -44.7$ (*c* 1.05, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.27–1.60 (m, 14H), 1.72–1.82 (m,2H), 2.82 (dd, *J* = 9.7 Hz and *J* = 17.8 Hz, 1H), 2.95 (dd, *J* = 8.3 Hz and *J* = 17.8 Hz, 1H), 3.06–3.15 (m, 1H), 4.59–4.65 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.1, 22.6, 25.1, 29.1, 29.2, 29.3, 29.4, 31.8, 31.9, 35.3, 45.3, 81.8, 174.4, 175.5 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{14}H_{23}O_4$: 255.1596, found $[M-H]^-$: 255.1602.

5.1.4.3. (2*R*,3*S*)-2-Nonyl-5-oxo-tetrahydrofuran-3-carboxylic acid (4*S*,5*R*-4b). Yield = 78%; white solid; mp 115 °C. R_f (CH_2Cl_2/

MeOH 9:1) = 0.45. $[\alpha]_D$ = +44.7 (*c* 1.05, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.27–1.60 (m, 14H), 1.72–1.82 (m,2H), 2.82 (dd, *J* = 9.7 Hz and *J* = 17.8 Hz, 1H), 2.95 (dd, *J* = 8.3 Hz and *J* = 17.8 Hz, 1H), 3.06–3.15 (m, 1H), 4.59–4.65 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.1, 22.6, 25.1, 29.1, 29.2, 29.3, 29.4, 31.8, 31.9, 35.3, 45.3, 81.8, 174.4, 175.5 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{14}H_{23}O_4$: 255.1596, found $[M-H]^-$: 255.1604.

5.1.4.4. (2*S*,3*R*)-5-Oxo-2-tridecyl-tetrahydrofuran-3-carboxylic acid (4*R*,5*S*-4c). Yield = 71%; white solid; mp 114 °C; Ref. 17: 112 °C. $[\alpha]_D$ = −25.3 (*c* 1.01, $CHCl_3$); Ref. 17: $[\alpha]_D$ = −42.8 (*c* 1.76, $CHCl_3$). HRMS (ESI, *m*/*z*): calcd for $C_{18}H_{31}O_4$: 311.2222, found $[M-H]^-$: 311.2225.

5.1.4.5. (2*R*,3*S*)-5-Oxo-2-tridecyl-tetrahydrofuran-3-carboxylic acid (4*S*,5*R*-4c). Yield = 66%; white solid; mp 114 °C; Ref. 17: 110 °C. $[\alpha]_D$ = +34.4 (*c* 0.81, $CHCl_3$). HRMS (ESI, *m*/*z*): calcd for $C_{18}H_{31}O_4$: 311.2222, found $[M-H]^-$: 311.2228.

5.1.4.6. (2*S*,3*R*)-5-Oxo-2-pentadecyl-tetrahydrofuran-3-carboxylic acid (4*R*,5*S*-4d). Yield = 92%; white solid; mp 108 °C. R_f (CH_2Cl_2/MeOH 9:1) = 0.33. $[\alpha]_D$ = −29.9 (*c* 1.12, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.82 (m, 26H), 1.72–1.82 (m, 2H), 2.77–2.99 (m, 2H), 3.06–3.14 (m,1H), 4.59–4.65 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.3, 22.8, 25.3, 29.3, 29.5, 29.6, 29.8, 32.0, 32.1, 35.5, 45.4, 81.9, 174.4, 176.0 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{20}H_{35}O_4$: 339.2541, found $[M-H]^-$: 339.2543.

5.1.4.7. (2*S*,3*R*)-5-Oxo-2-hexadecyl-tetrahydrofuran-3-carboxylic acid (4*S*,5*R*-4e). Yield = 95%; brownish solid; mp 114 °C. R_f (CH_2Cl_2/MeOH 9:1) = 0.35. $[\alpha]_D$ = −29.0 (*c* 0.98, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.6 Hz, 3H), 1.27–1.56 (m, 28H), 1.72–1.81 (m, 2H), 2.79 (dd, *J* = 9.8 Hz and *J* = 17.8 Hz, 1H), 2.90 (dd, *J* = 8.3 Hz and *J* = 17.8 Hz, 1H), 3.04–3.12 (m, 1H), 4.58–4.64 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.1, 22.8, 25.3, 29.4, 29.5, 29.6, 29.7, 29.8, 32.1, 35.6, 35.7, 45.5, 52.7, 82.0, 174.3, 175.4; HRMS (ESI, *m*/*z*): calcd for $C_{21}H_{37}O_4$: 353.2697, found $[M-H]^-$: 353.2701.

5.1.5. General procedure for compounds 5a–e

A mixture of carboxy lactone **4** (7.88 mmol) in a solution of methoxymagnesium monomethylcarbonate MMC (150 mL, 299.6 mmol of 2 M in DMF, 38 equiv) was heated at 135–140 °C for 70 h under argon atmosphere. After the system had cooled to rt, the reaction was quenched by adding a solution of HCl 10% (150 mL), this solution was then extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were dried with $MgSO_4$ and concentrated under vacuum. The resulting brown oil was dissolved in a solvent mixture of acetic acid (38 mL), formaldehyde (28 mL), *N*-methylaniline (9.8 mL) and NaOAc (1.13 g). After stirring for 2 h at rt, the mixture was poured into a solution of HCl 10%. The solution was extracted with *tert*-butyl methyl ether (3 × 100 mL), the combined organic layers were washed once with brine and then three times with H_2O. The organic layer was dried with $MgSO_4$ and concentrated under vacuum. The residue was purified over silicagel Geduran®Si 60 (diethyl ether/petroleum ether/acetic acid 3:7:0.2) to yield compounds **5**.

5.1.5.1. (2*S*,3*R*)-2-Heptyl-4-methylene-5-oxo-tetrahydrofuran-3-carboxylic acid (4*R*,5*S*-5a). Yield = 30%; white solid; mp 71 °C. R_f (petroleum ether/diethyl ether/acetic acid 8:2:0.2) = 0.13. $[\alpha]_D$ = −11.4 (*c* 1.10, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.4 Hz, 3H), 1.28–1.79 (m, 12H), 3.62–3.66 (m, 1H), 4.82 (d, *J* = 5.3 Hz, 1H), 6.03 (d, *J* = 2.4 Hz, 1H), 6.46 (d, *J* = 2.7 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.2, 22.7, 24.8, 29.1, 29.2, 31.8, 35.8, 49.6, 79.1, 126.2, 132.5, 168.6, 174.7 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{13}H_{19}O_4$: 239.12888, found $[M-H]^-$: 239.1292.

5.1.5.2. (2*R*,3*S*)-4-Methylene-2-nonyl-5-oxo-tetrahydrofuran-3-carboxylic acid (4*S*,5*R*-5b). Yield = 41%; white solid; mp 93 °C.[25] R_f (petroleum ether/diethyl ether/acetic acid 8:2:0.2) = 0.20. $[\alpha]_D$ = +12.82 (*c* 0.975, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.87 (t, *J* = 6.6 Hz, 3H), 1.26–1.50 (m,14H), 1.71–1.76 (m, 2H), 3.61–3.63 (m, 1H), 4.81 (dt, *J* = 5.8 Hz and *J* = 7 Hz, 1H), 6.02 (d, *J* = 2.6 Hz, 1H), 6.46 (d, *J* = 3.0 Hz, 1H), 10.58 (s, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.0, 22.6, 24.7, 29.1, 29.2, 29.4, 31.8, 35.7, 49.5, 79.0, 126.2, 132.5, 168.4, 174.9 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{15}H_{23}O_4$: 267.1596, found $[M-H]^-$: 267.1603.

5.1.5.3. (2*S*,3*R*)-4-Methylene-2-nonyl-5-oxo-tetrahydrofuran-3-carboxylic acid (4*R*,5*S*-5b). Yield = 54%; white solid; mp 93 °C. R_f (petroleum ether/diethyl ether/acetic acid 8:2:0.2) = 0.20. $[\alpha]_D$ = −6.3 (c 0.98, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.87 (t, *J* = 6.6 Hz, 3H), 1.26–1.50 (m, 14H), 1.71–1.76 (m, 2H), 3.61–3.63 (m, 1H), 4.81 (dt, *J* = 5.8 Hz and *J* = 7 Hz, 1H), 6.02 (d, *J* = 2.6 Hz, 1H), 6.46 (d, *J* = 3.0 Hz, 1H), 10.58 (s, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.0, 22.6, 24.7, 29.1, 29.2, 29.4, 31.8, 35.7, 49.5, 79.0, 126.2, 132.5, 168.4, 174.9 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{15}H_{23}O_4$: 267.1596, found $[M-H]^-$: 267.1603.

5.1.5.4. (2*R*,3*S*)-4-Methylene-5-oxo-2-tridecyl-tetrahydrofuran-3-carboxylic acid (4*S*,5*R*-5c). This compound was prepared as published.[17] Yield = 36%. Mp 108 °C, Ref. 17: 104–105 °C. $[\alpha]_D$ = +18.3 (*c* 0.24, CH_2Cl_2), Ref. 17: $[\alpha]_D$ = +13.6 (*c* 1.72, $CHCl_3$). HRMS (ESI, *m*/*z*): calcd for $C_{19}H_{31}O_4$: 323.22223, found $[M-H]^-$: 324.2228.

5.1.5.5. (2*S*,3*R*)-4-Methylene-5-oxo-2-tridecyl-tetrahydrofuran-3-carboxylic acid (4*R*,5*S*-5c). This compound was prepared as published.[17] Yield = 28%. Mp 108 °C, Ref. 17: 104–105 °C. $[\alpha]_D$ = −7.9 (*c* 0.99, $CHCl_3$), Ref. 17: $[\alpha]_D$ = −13.2 (*c* 1.52, $CHCl_3$). HRMS (ESI, *m*/*z*): calcd for $C_{19}H_{32}O_4$: 324.2301, found $[M]^+$: 324.2291.

5.1.5.6. (2*S*,3*R*)-4-Methylene-5-oxo-2-pentadecyl-tetrahydrofuran-3-carboxylic acid (4*R*,5*S*-5d). Yield = 56%; white solid; mp 108 °C. R_f (petroleum ether/diethyl ether/acetic acid 6:3.8:0.2) = 0.33. $[\alpha]_D$ = −9.4 (*c* 1.13, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.5 Hz, 3H), 1.26–1.75 (m, 28H), 3.62–3.64 (m, 1H), 4.81 (dt, *J* = 6.0 Hz and *J* = 12.3 Hz, 1H), 6.02 (d, *J* = 2.4 Hz, 1H), 6.47 (d, *J* = 2.8 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.3, 22.8, 24.9, 29.3, 29.5, 29.6, 29.8, 32.1, 35.9, 49.6, 79.0, 126.2, 132.5, 168.4, 174.8 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{21}H_{35}O_4Na$: 375.2111, found $[M+Na]^+$: 375.2518.

5.1.5.7. (2*S*,3*R*)-2-Hexadecyl-4-methylene-5-oxo-tetrahydrofuran-3-carboxylic acid (4*R*,5*S*-5e). Yield = 45%; white solid; mp 107 °C. R_f (petroleum ether/diethyl ether/acetic acid 6:3.8:0.2) = 0.33. $[\alpha]_D$ = −2.3 (*c* 1.01, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.55 (m, 28H), 1.68–1.81 (m, 2H), 3.63 (ddd, *J* = 5.6 Hz, *J* = 2.5 Hz and *J* = 2.8 Hz, 1H), 4.81 (dt, *J* = 5.8 Hz and *J* = 7 Hz, 1H), 6.03 (d, *J* = 2.7 Hz, 1H), 6.47 (d, *J* = 3.0 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 14.3, 22.8, 24.9, 29.3, 29.5, 29.6, 29.7, 29.8, 32.1, 35.9, 49.6, 79.0, 126.0, 132.6, 168.3, 174.2 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{22}H_{37}O_4$: 365.2697, found $[M-H]^-$: 365.2695.

5.1.6. General procedure for compounds 6a–e

To a solution of compound **5** (1.99 mmol) in anhydrous DMF (17 mL) under argon atmosphere, was added NEt_3 (279 µL, 1.99 mmol, 1 equiv). After stirring overnight at rt, the reaction

was quenched by adding a solution of HCl 1 M, this solution was then extracted with diethyl ether (3 × 50 mL). The combined organic layers were washed with H_2O, dried with $MgSO_4$ and concentrated under vacuum. The residue was purified over silicagel Geduran®Si 60 (diethyl ether/petroleum ether/acetic acid 3:7:0.2) to yield the desired compound **6**.

5.1.6.1. (*S*)-2-Heptyl-4-methyl-5-oxo-2,5-dihydrofuran-3-carboxylic acid (5*S*-6a) (B-7). Yield = 71%; white solid; mp 120 °C. R_f (petroleum ether/diethyl ether/acetic acid 8:2:0.2) = 0.21. $[\alpha]_D = -11.4$ (*c* 1.10, $CHCl_3$), ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.27–1.67 (m, 12H), 2.08–2.19 (m, 1H), 2.25 (d, *J* = 2.1 Hz, 3H), 5.11–5.15 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 11.2, 14.2, 22.7, 24.9, 29.2, 29.3, 31.8, 32.9, 81.5, 140.3, 146.7, 166.7, 172.7 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{13}H_{19}O_4$: 239.1289, found $[M-H]^-$: 239.1291. Anal. Calcd for $C_{13}H_{20}O_4$: C, 64.98; H, 8.39. Found: C, 64.17; H, 8.25. HPLC: Chiralpak IC, *n*-heptane/MtBE/TFA 80:20:0.1, 250 nm, 1 mL/min, 0.5 mg/mL. T_R = 15.09 min, ee = 91%.

5.1.6.2. (*S*)-4-Methyl-2-nonyl-5-oxo-2,5-dihydrofuran-3-carboxylic acid (5*S*-6b) (B-8). Yield = 65%; white solid; mp 120 °C. R_f (petroleum ether/diethyl ether/acetic acid 8:2:0.2) = 0.29. $[\alpha]_D = -36.3$ (*c* 1.30, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.67 (m, 16H), 2.09–2.16 (m, 1H), 2.24 (d, *J* = 2.1 Hz, 3H), 5.12–5.13 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 11.0, 14.1, 22.6, 24.7, 29.1, 29.2, 29.3, 29.4, 31.8, 32.7, 81.4, 139.7, 146.7, 165.5, 172.6 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{15}H_{24}O_4Na$: 291.15723, found $[M+Na]^+$: 291.1572. Anal. Calcd for $C_{15}H_{24}O_4$: C, 67.14; H, 9.01. Found: C, 67.97; H, 9.07. HPLC: Chiralpak IC, *n*-heptane/MtBE/TFA 80:20:0.1, 250 nm, 1 mL/min, 0.5 mg/mL. T_R = 16.92 min, ee = 97%.

5.1.6.3. (*R*)-4-Methyl-2-nonyl-5-oxo-2,5-dihydrofuran-3-carboxylic acid (5*R*-6b) (B-9)[26]. Yield = 67%; white solid; mp 117 °C. R_f (petroleum ether/diethyl ether/acetic acid 8:2:0.2) = 0.29. $[\alpha]_D = +37.3$ (*c* 1.04, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.67 (m, 16H), 2.09–2.16 (m, 1H), 2.24 (d, *J* = 2.1 Hz, 3H), 5.12–5.13 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 11.0, 14.1, 22.6, 24.7, 29.1, 29.2, 29.3, 29.4, 31.8, 32.7, 81.4, 139.7, 146.7, 165.5, 172.6 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{15}H_{23}O_4$: 267.15963, found $[M-H]^-$: 267.1602. Anal. Calcd for $C_{15}H_{24}O_4$: C, 67.14; H, 9.01. Found: C, 66.90; H, 8.98. HPLC: Chiralpak IC, *n*-heptane/MtBE/TFA 80:20:0.1, 250 nm, 1 mL/min, 0.5 mg/mL. T_R = 18.92 min, ee = 99%.

5.1.6.4. (−)-Lichesterinic acid (5*S*-6c) (B-10). Yield = 69%; white solid; mp 122 °C; Ref. 27: 120–121 °C. $[\alpha]_D = -23.5$ (*c* 1.055, $CHCl_3$); Ref. 27 $[\alpha]_D = -35$ (*c* 0.6, $CHCl_3$). HRMS (ESI, *m*/*z*): calcd for $C_{19}H_{32}O_4$: 324.2301, found $[M]^+$: 324.2288. Anal. Calcd for $C_{19}H_{32}O_4$: C, 70.33; H, 9.94. Found: C, 71.48; H, 10.03. HPLC: Chiralpak IA, 100% ACN + 0.1% HCOOH, 210 nm, 1 mL/min, 0.5 mg/mL. T_R = 6.16 min, ee = 98%.

5.1.6.5. (+)-Lichesterinic acid (5*R*-6c) (B-11). Yield = 71%; white solid; mp 122 °C; Ref. 28: 120–122 °C. $[\alpha]_D$ +24.9 (*c* 1.03, $CHCl_3$); Ref. 28: $[\alpha]_D = +31.9$. HRMS (ESI, *m*/*z*): calcd for $C_{19}H_{32}O_4$: 324.2301, found $[M]^+$: 324.2288. Anal. Calcd for $C_{19}H_{32}O_4$: C, 70.33; H, 9.94. Found: C, 71.20; H, 9.95. HPLC: Chiralpak IA, 100% ACN + 0.1% HCOOH, 210 nm, 1 mL/min, 0.5 mg/mL. T_R = 6.91 min, ee = 91%.

5.1.6.6. (*S*)-4-Methyl-5-oxo-2-pentadecyl-2,5-dihydrofuran-3-carboxylic acid (5*S*-6d) (B-12). Yield = 89%; white solid; mp 120 °C. R_f (petroleum ether/diethyl ether/acetic acid 6:3.8:0.2) = 0.38. $[\alpha]_D = -21.8$ (*c* 0.98, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.5 Hz, 3H), 1.25–1.64 (m, 28H), 2.09–2.20 (m, 1H), 2.25 (d, *J* = 1.8 Hz, 3H), 5.12–5.14 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 11.2, 14.3, 22.8, 24.9, 29.4, 29.5, 29.7, 29.8, 32.1, 32.9, 81.5, 140.3, 146.8, 167.0, 172.8 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{21}H_{35}O_4$: 351.25408, found $[M-H]^-$: 351.2545. Anal. Calcd for $C_{21}H_{36}O_4$: C, 71.55; H, 10.29. Found: C, 71.80; H, 10.37. HPLC: Chiralpak IA, 100% ACN + 0.1% HCOOH, 210 nm, 1 mL/min, 0.5 mg/mL. T_R = 7.58 min, ee = 86%.

5.1.6.7. (*S*)-2-Hexadecyl-4-methyl-5-oxo-2,5-dihydrofuran-3-carboxylic acid (5*S*-6e) (B-13). Yield = 62%; white solid; mp 125 °C. R_f (petroleum ether/diethyl ether/acetic acid 6:3.8:0.2) = 0.38. $[\alpha]_D = -25.9$ (*c* 1.015, $CHCl_3$). ^1H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.26–1.67 (m, 30H), 2.07–2.18 (m, 1H), 2.24 (d, *J* = 2.1 Hz, 3H), 5.09–5.13 (m, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 11.2, 14.3, 22.8, 24.9, 29.4, 29.5, 29.7, 29.8, 32.1, 32.9, 81.5, 140.3, 146.8, 166.7, 172.8 ppm. HRMS (ESI, *m*/*z*): calcd for $C_{22}H_{37}O_4$: 365.26974, found $[M-H]^-$: 365.2699. Anal. Calcd for $C_{22}H_{38}O_4$: C, 72.09; H, 10.45. Found: C, 72.24; H, 10.39. HPLC: Chiralpak IA, 100% ACN + 0.1% HCOOH, 210 nm, 1 mL/min, 0.5 mg/mL. T_R = 8.09 min, ee > 99%.

5.1.7. Chemical compounds

All butyrolactones were dissolved in pure DMSO and then diluted with pure methanol to get 3 μg/mL and to reach a final concentration of DMSO less than 25%. After that, they were filter sterilized through a 0.22-μm-pore-size filter.

5.2. Biological activity

5.2.1. Bacterial culture

Streptococcus gordonii DL1 was used in this study.[29] Brain–heart infusion broth (BHI) (DIFCO, France) and/or blood Columbia agar plates (AES Chemunex, France) supplemented with hemin (5 μg/mL) and menadione (1 μg/mL) (Sigma Aldrich, France) were used for its growth. *S. gordonii* was grown under anaerobic conditions (N_2–H_2–CO_2 [80:10:10]) at 37 °C to mimic the conditions created by the microorganisms colonizing the tooth surface rendering it rapidly anaerobic.[30]

5.2.2. Cell lines

Two different human cell lines were chosen: a gingival epithelial carcinoma cell line, Ca9-22 (Health Science Research Resources Bank, Osaka, Japan) and a macrophage-like monocytic leukemia cell line, THP-1. Ca9-22 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, France) whereas RPMI 1640 medium with sodium pyruvate (1 mM) and Hepes buffer (1 M) (Sigma Aldrich) was used for THP-1 growth. Both lines were grown in a 5% CO_2 atmosphere at 37 °C and their media were supplemented with L-Glutamine (2 mM), 10% heat-inactivated fetal bovine serum (FBS, Lonza, France) and antibiotics (penicillin 100 mg/mL and streptomycin 50 mg/mL) (Sigma Aldrich). For THP-1 differentiation into macrophages, Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) was used at 10 ng/mL for 72 h.

5.2.3. Antibacterial assay

5.2.3.1. Agar dilution. Agar dilution assay was chosen to test the antibacterial activity against *S. gordonii* strain under anaerobic conditions as recommended by the Clinical and Laboratory Standards Institute (CLSI).[31] Briefly, Columbia agar is mixed with hoarse blood and different concentrations of butyrolactones (300, 250, 200, 150, 100, 90, 80 and 70 μg/mL), or doxycycline (1:2 serial dilutions from 1.31 to 2×10^{-5} μg/mL) as a positive control or the mixture of solvents used to dissolute the compounds (DMSO + methanol) or distilled water as negative controls and left to solidify. The agar mixture is then inoculated with 2 μl spot containing

10^5 CFU/mL before its incubation for 24 h under anaerobic conditions. The lowest concentration of the mixture that prevented the growth of the bacteria was then determined and the corresponding concentration was defined as the Minimal Inhibitory Concentration (MIC). This was repeated three times.

5.2.3.2. Broth microdilution. Broth microdilution test was done to confirm the results in broth as described by CLSI.[31] In brief, starting with 300 or 32.8 μg/mL as an initial concentration for the compounds to be tested or the positive control, doxycycline, respectively, 1:2 serial dilutions were made in BHI in a 96-well microtiter plate (Sterile, Flat bottom, with lid, Greiner Bio-one, Germany). Each well was then inoculated by 3×10^7 CFU/mL of *S. gordonii*. In addition, the mixture of the solvents (DMSO + methanol) used to dissolute the compounds was 1:2 serially diluted to check their activity. Then, the plate is incubated for 24 h under anaerobic conditions after which the clear wells will be spreaded on Columbia Petri plates to be incubated for another 24 h. The clear well with the lowest concentration represents the MIC which has inhibited the visible bacterial growth and the Petri plate showing no colonial growth will be the MBC defined as the lowest concentration that killed ⩾99% of the initial inoculum.

5.2.4. Cytotoxicity

Each well of a 96-well plate (Sterile, Flat bottom, with lid, Greiner Bio-one, Germany) was seeded with 70,000 cells after their trypsination and counting in case of Ca9-22 cells or only counting for THP-1. Ca9-22 cells were incubated for 24 h whereas THP-1 cells were incubated with PMA for 72 h. After that and for the two cell lines, the contents of the wells were removed and the compounds or only media as negative controls were added to be incubated for 24 h. The compounds best inhibitory concentrations, MICs, were chosen to test whether they have a cytotoxic effect or not and Triton 1% was used as positive control. Finally, LDH and MTT assays were done to investigate the cytotoxicity. The experiments were done three times in triplicate.

5.2.4.1. LDH. According to Promega protocol, 50 μL of the supernatant from each well was transferred into a new 96-well plate. Then, 50 μL of the CytoTox Reagent was added to each well and the plate was incubated for 30 min in the dark at room temperature. Finally, 50 μL of the stop solution was added and the OD was then read at 490 nm.

5.2.4.2. MTT. Ten microliters of 5 mg/mL MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma Aldrich, France), prepared in PBS and filter sterilized through a 0.22 μm filter, was added to the wells containing 100 μL of medium. The 96-well plate was then incubated for 4 h at 37 °C under 5% CO_2. After that, 100 μL of acid-isopropanol, 0.04 N HCL in isopropanol, was added to the wells and mixed very well to dissolve the formazan crystals. Finally, the O.D was read after a few minutes at 595 nm and at 655 nm (measurement and reference, respectively).[32] The results were presented as percent MTT activity where the readings for the untreated control cells were considered as 100%.

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ARTICLE 3

Antibiofilm activity of butyrolactone derivatives against oral bacteria

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Abstract A symbiotic association between fungus and algae and/or cyanobacterium called lichen is a rich source of biologically active metabolites. Based on one of its antibacterial compounds, lichesterinic acid, a series of butyrolactones have been synthesized to fight the oral bacteria. The latter form dental biofilms confering to these bacteria increased resistance and virulence to the host. In our previous study, the synthesized butyrolactones antibacterial evaluation against one of the primary colonizers, *Streptococcus gordonii*, was shown. Our preliminary aim here was to test these butyrolactones antibacterial activity against the causative agent of periodontitis, *Porphyromonas gingivalis (P. gingivalis).* **B-12** and **B-13** were the most active derivatives on *P. gingivalis* exhibiting minimal inhibitory concentrations (MIC) of 0.037 and 0.293 µg/mL and minimal bactericidal concentrations (MBC) of 1.17 and 0.586 µg/mL respectively. They were even stronger than the reference antibiotic, doxycycline, with MIC of 0.13 µg/mL and no MBC. This was followed by the bigger objective which is to evaluate the most active butyrolactone derivatives (**B-12** and **B-13**) for their antibiofilm activity against both oral strains. By using crystal violet assay, we highlighted the antibiofilm activity of **B-12** and **B-13** which was confirmed by confocal microscopy. Both derivatives displayed a lowest concentration with maximal biofilm inhibition, LCMI, of 9.38 µg/mL against *S. gordonii* and 1.17 µg/mL against *P. gingivalis*. In the present study, we have also demonstrated that the two investigated strains were able to form biofilms *in vitro* when sub-inhibitory concentrations of **B-12** and **B-13** were used. Indeed, when MIC/2 was used, this antibiofilm activity decreased as indicated by the expression of the genes implicated in adhesion and biofilm formation such as streptococcal surface protein (*sspA*).

Keywords Lichens · Butyrolactones · Antibacterial · Antibiofilm · Surface adhesion proteins

Introduction

One of the oldest symbiotic relationships comprising an ecological significance is lichen (Ramanan et al. 2015). It's a self-supporting and stable mutualistic association encompassing a filamentous fungus and a photosynthetic partner, eukaryotic algae and/or a cyanobacterium, and in some cases non-photosynthetic bacteria (Shrestha and St. Clair 2013). Their distinct genera have been utilized throughout the ages in curing many ailments in folk medicine and for other various aims such as dyes and perfumes (Shukla et al. 2010). Lichens which may grow under extreme ecological conditions, in tropical rainforest habitats or even on the surface of living leaves produce a wide range of secondary metabolites. These metabolites exhibit antibiotic, antitumor, antimutagenic and antiviral potentials to control their inhabitants (Boustie and Grube 2005). One of these metabolites is lichesterinic acid which was extracted from the lichen, *Cetraria islandica*, and shown to have an activity against *Streptococcus hemolyticus* and *Staphylococcus aureus* (Cavallito et al. 1948).

This antibacterial reputation has urged us to evaluate the effect of lichesterinic acid and some of its derivatives trying to get the best possible activity on *Streptococcus gordonii* which is an early colonizer in the oral cavity (Sweidan et al. 2016). This bacterial strain binds to the receptors provided by the salivary pellicle, a film that coats the teeth. Then, they expose sites for late colonizers attachment leading to coaggregation of oral bacteria thereby forming a complex biofilm (Kreth et al. 2009). Co-adhesion of the periodontal pathogen, *Porphyromonas gingivalis,* with *S. gordonii* is one of the best identified interspecies binding combinations (Kuboniwa and Lamont 2010a). *P. gingivalis* which is a Gram-negative anaerobic bacterial strain causes inflammation in the teeth-supporting soft and hard tissues, periodontium. A case called periodontitis which can lead to teeth loss if infections were left untreated (Mysak et al. 2014; How et al. 2016).

However, teeth loss is not the end, several facts could occur after these infections due to cytokine and inflammatory, immune and autoimmune responses. They include endothelial dysfunction, lipid deposition, monocyte migration, smooth muscle proliferation and release of platelets and reactant plasma proteins. These pave the way into atherosclerosis, thrombosis and cardiovascular disease (Bartold and Narayanan 2006). Furthermore, periodontal diseases lead to other systemic complications including bacteremia, endotoxemia, adverse pregnancy outcomes, nonalcoholic liver diseases, rheumatoid arthritis, osteoporosis, respiratory lung infections, pancreatic and oral cancers, obesity, type 2 diabetes, and Alzheimer's disease (Bartold and Narayanan 2006; Singhrao et al. 2015).

The early streptococcal plaque formation depends on several gene products. *S. gordonii* attaches primarily via Ssp surface adhesion proteins, SspA and SspB (Jenkinson and Demuth 1997; D. Dû and E. Kolenbrander 2000). This attachment depends also on the enzyme, α-amylase, which exists in abundant proportion in the human saliva. *S. gordonii* binds this protein with high affinity through surface receptors called α-amylase binding protein, *abpA* (Rogers et al. 1998). After binding, *S. gordonii* can sense their environment and population density by the quorum sensing regulation system composed of the *com* regulon. The latter

contains several genes and operons (Andreas Podbielski and Bernd Kreikemeyer 2004a). A biofilm-defective *S. gordonii* mutant had been shown to have an insertion within the *comD* gene that encodes for histidine kinase acting as an environmental sensor (Lunsford and London 1996; Loo et al. 2000). In addition, it has been suggested that *S. gordonii* produces an autoinducer-2 signaling molecule or LuxS serving as an intercellular communicator essential for biofilm formation between non-growing cells of *P. gingivalis* and *S. gordonii* (McNab et al. 2003).

On the other side, for the monospecies *P. gingivalis* biofilm to form, Mfa and FimA fimbriae were suggested to be required for autoaggregation where the expression of the long fimbriae, FimA, is controlled by the FimS-FimR two-component system (Kuboniwa and Lamont 2010b). UspA, the universal stress protein, is also involved in its development as shown before in microtiter plate assays and in flow cells (Chen et al. 2006). Alongside, some gene products were found to be inhibitors of this homotypic biofilm accumulation such as GalE, UDP-galactose 4-epimerase, and their loss enhanced its growth (R. Nakao, H. Senpuku, and H. Watanabe 2006a; Capestany et al. 2008).

Around 90% of bacteria live in biofilms which were reported to be responsible for about 80% of human infections in the United States. Not only do biofilms resist the antibiotics, but also they escape the host defense system (O'Toole et al. 2000; Bueno 2011). Hence, a promising hypothesis we found worth to test is the ability of our formerly uncovered efficient butyrolactones to inhibit the biofilm formation of the oral bacteria. In a previous study, out of a wide variety of butyrolactones synthesized based on the natural compounds, lichesterinic acids (**B-10** and **B-11)**, compounds **B-12** and **B-13** (Figure 1) were shown to be non-cytotoxic against gingival epithelial cells, Ca9-22, and macrophage-like cells, THP-1, and the most effective against *S. gordonii* (Sweidan et al. 2016). The present study leads to the evaluation of the antibacterial activity of all the butyrolactone derivatives against *P. gingivalis* to go further and evaluate, for the first time, the antibiofilm activity of the most active compounds (**B-12** and **B-13**) against *S. gordonii* and *P. gingivalis*.

Materials and methods

Chemical compounds

The butyrolactone derivatives were previously described by Sweidan et al. (Figure 1) (Sweidan et al. 2016).

Bacterial strains, growth media and conditions

We used in this study the oral bacteria, *Streptococcus gordonii* DL1 and *Porphyromonas gingivalis* ATCC 33277. We have grown them in an anaerobic environment (N_2-H_2-CO_2 [80:10:10]) at 37°C utilizing brain-heart infusion (BHI) medium (DIFCO, France) and blood

Columbia agar plates (AES Chemunex, France) supplemented with hemin (5 µg/mL) and menadione (1 µg/mL) (Sigma Aldrich, France) (Sweidan et al. 2016).

Finding the minimum inhibitory concentration and minimum bactericidal concentration

Broth microdilution test was done as described formerly (Sweidan et al. 2016). In nutshell, 1:2 serial dilutions of the butyrolactone derivatives were done in BHI medium in 96-well plate (Sterile, Flat bottom, with lid, Greiner Bio-one, Germany). Doxycycline antibiotic was used as the positive control (Park et al. 2014). The wells were inoculated with $3x10^7$ CFU/mL of *P. gingivalis*. After that, the plate is incubated anaerobically for 48 hours at 37°C. The clear well corresponding to the lowest concentration was defined as the minimal inhibitory concentration or MIC and this well was spread on Columbia agar plates to be incubated for 5 days to uncover the minimal bactericidal concentration or MBC which is the lowest concentration plate with no colonial growth.

Assessment of the antibiofilm activity using crystal violet assay

The ability of butyrolactones to inhibit *S. gordonii* or *P. gingivalis* monospecies biofilm formation was evaluated by a modified version of crystal violet assay as described previously (Christensen et al. 1985).

The biofilm was formed in a 96-well plate (untreated, flat bottom, with lid, Evergreen Scientific), where each well was inoculated with 200 µL from the bacterial suspension prepared in BHI containing $3x10^7$ CFU/mL. Wells containing only BHI or BHI mixed with butyrolactones served as negative controls. The plate was then incubated under anaerobic conditions for 24 h.

To quantify the biofilm, the wells were washed 3 times with sterile water to eliminate the non-adherent bacterial cells from the biofilm formed at their bottoms. The attached bacteria were then colored by 0.4% crystal violet solution for 15 min at room temperature. After that, the wells were again washed 3 times with sterile water to remove the excess of the colorant to be dried for 2 h at 37°C. Finally, 100 µL of 95% ethanol were added and the O.D was measured at 595 nm.

Visualization of *S. gordonii* and *P. gingivalis* monospecies biofilms by Confocal Laser Scanning Microscope (CLSM)

Monospecies biofilms of *S. gordonii* or *P. gingivalis* were formed in Ludin Chambers® (Life Imaging Services, Switzerland) (750 µL volume) (Nicolle et al. 2010). After assembly of the mounted flow-cell chambers with glass cover slips, they were connected to a peristaltic pump with a flow rate of 7 mL/h. The pump draws fresh medium driving it to the chambers and evacuates the liquid into a waste container through silicone tubing. First of all, the tubules

used were sterilized by flowing hydrochloric acid (0.1 M) for 30 min to be washed with distilled water after that. Then, the chambers were connected and the whole system was washed with distilled water and then with ethanol. The flow system was left one night with the ethanol filling its tubules and chambers under sterile conditions. In the following day, the system was washed with distilled water for 15 min to be followed with a 25% saliva flow for 15 min also. After that, an enough volume of BHI containing $3x10^7$ CFU/mL with or without our compounds were flew for 15 min to be incubated under anaerobic conditions for 24 h for *S. gordonii* or 48 h for *P. gingivalis*.

CLSM analyses were obtained as follows: The chambers were first washed with PBS for 30 min and then a solution of Syto 9/Propidium iodide (PI) (5 µM/40 µM) dyes (Molecular Probes, France) prepared in PBS were used to stain the biofilms for 15 min. After that, the chambers were visualized *in situ* utilizing a Leica TCS-SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with an inverted microscope (Fluorescence Microscopy Platform, IFR 140 GFAS, Université de Rennes I). The images were captured by the oil immersion objective lens (HC PL Apo 63X, 1.4 NA) applying 1.52 as a numerical zoom. For an entire bacterial detection with the dyes, specific excitation lasers and emission filters were used. The 488-nm excitation laser and 506-539 nm band-pass emission filter was utilized for Syto 9 and the 561-nm excitation laser and 600-700 nm band-pass emission filter in case of PI. Image acquisition and microscope piloting were done by the Leica software (LAS AF V.2.2.1), and Comstat 2 plugin in ImageJ software V1.48m (National Institute of Health) was used for images recovery.

Extraction of RNA from *S. gordonii* and *P. gingivalis* monospecies biofilms cells and quantification of some genes by qPCR

Performing three independent experiments and conserving the same conditions for biofilm formation, the contents of the wells corresponding to the MIC/2 were transferred into eppendorfs to be treated as required by the Gram type to extract the RNA.

Briefly, RNA from *S. gordonii*, a Gram-positive strain with a more rigid cell wall, was extracted using a rapid method for RNA preparation as described by (Mauro et al. 2016). Concerning *P. gingivalis*, their RNA were extracted using mirVana™ miRNA Isolation Kit (Ambion, France).

The samples of both strains were then treated by TURBO DNA-free (Ambion, France), according to the manufacturer's instructions, to digest the contaminating DNA. Before and after TURBO DNA-free experiment, the RNA was quantified using a Nanodrop ND1000 (NanoDrop Technologies). After that, 12µM of random primers (New England *Biolabs*, France) with 1 mM of dNTP (New England *Biolabs*, France) were added to 1µg of RNA. To denature sample RNA/primer, the mix was incubated for 5 minutes at 70°C. After that, 1X ProtoScript II Buffer (New England *Biolabs*, France), 10 mM of dithiothreitol (DTT), 0.4 UI of RNase Inhibitor and 10 UI of ProtoScript II reverse transcriptase (New England *Biolabs*,

France) were used to reverse-transcribe the RNA into cDNA. The RT-PCR procedure started with 5 min at 25°C, then, 60 min at 42°C, and finally, 5 min at 80°C. To determine if genomic DNA contamination was present or not, we have included a R*everse transcription* negative controls ("-RT") in real-time RT-PCR experiments. R*everse transcription* negative control is a mock reverse transcription containing all the RT-PCR reagents, except the reverse transcriptase. Then, the genes described in the primers list in table 2 were relatively quantified using StepOnePlus (Applied Biosystems) with the SYBR Green PCR Master Mix (Applied Biosystems) (Le Bars et al. 2012). Each gene was normalized to the 16S rRNA internal control to calculate the fold-change values. At least, 2-fold change relative to the control was considered as a relevant gene expression change.

Results

Effect of butyrolactone analogues on the growth of *P. gingivalis* planktonic cells

The synthetized butyrolactones were shown to be potentially active against *P. gingivalis* where the COOH-containing compounds carrying an aliphatic chain with a minimal number of 13 carbon atoms were the best (Table 1). **B-7** was the least active with MIC = 75 µg/mL and no MBC. It was followed by the unsaturated compounds (**B-3** and **B-4**) or saturated compounds substituted with a hydroxyl (**B-1** and **B-2**), or a vinyl group (**B-5** and **B-6**) which exhibited moderate activity. The activity increased to reach MICs = 0.037 and 0.293 µg/mL and MBCs = 1.17 and 0.586 µg/mL for **B-12** and **B-13**, respectively. These latter butyrolactones possess a conjugated carboxylic acid group and the longer aliphatic chains. They were even stronger than the reference antibiotic, doxycycline which presented a MIC = 0.13 µg/mL and no MBC. It is worth noting that **B-12** was more efficient than doxycycline by about 3 times comparing their MICs and much more effective regarding their MBCs.

Effect of the most active butyrolactone derivatives (B-12 and B13) on the formation of *S. gordonii* and *P. gingivalis* monospecies biofilms

The ability of the most active butyrolactones to prevent the monospecies biofilm formation (BF) by *S. gordonii* or *P. gingivalis* was evaluated by crystal violet assay. As a Gram-negative strain, *P.* gingivalis was more sensitive than the Gram-positive strain, *S. gordonii* (Figure 2).
Starting with *S. gordonii* (Figure 2A), as the butyrolactones concentration decreased, BF remained approximately constant with a small fluctuation ($0.4<O.D_{595nm}<0.5$) until reaching 9.38 µg/mL where the BF was 5-fold lower than the positive growth control ($O.D_{595nm}$ =2.25) which is equivalent to 80% of inhibition for both butyrolactones. This was the lowest concentration with the maximal biofilm inhibition, called LCMI. Beyond this value, BF started a dramatic increase to register finally an $O.D_{595nm}$ of 1.95 at 1.17 µg/mL for both compounds. However, the reference antibiotic, doxycycline, was stronger registering $O.D_{595nm}$

= 0.3 at 2.34 µg/mL as the LCMI after which BF increased by about two fold ($O.D_{595nm}$ = 0.72) at 1.17 µg/mL (Figure 2A).
Regarding *P. gingivalis* (Figure 2B), butyrolactones were more active but have the same initial constant pattern in comparison to the first strain. The $O.D_{595nm}$ of butyrolactones and doxycycline (about 0.45) was alternating in a range of $O.D_{595nm}$ =0.1 until reaching 18.75 µg/mL for doxycycline and 1.17 µg/mL for butyrolactones. These concentrations were the corresponding LCMIs for doxycycline and butyrolactones. After that and with respect to doxycycline, BF started a slight gradual increase to reach $O.D_{595nm}$ =1 at 0.15 µg/mL after which a dramatic increase was observed to register finally $O.D_{595nm}$ = 3 at 0.037 µg/mL. Concerning the butyrolactones, the LCMI, 1.17 µg/mL, has registered an $O.D_{595nm}$ 6-fold lower than that of the positive growth control ($O.D_{595nm}$ =3). In equivalence, **B-12** and **B-13** have inhibited the initial biofilm by about 83%. Doxycycline was less active than the butyrolactones by having a higher LCMI, 18.75 µg/mL, compared to 1.17 µg/mL for butyrolactones. To confirm this fact, at the LCMI of butyrolactones, 1.17 µg/mL, the latter registered $O.D_{595nm}$ =0.5 compared to $O.D_{595nm}$ =0.8 for doxycycline. However, doxycycline was more able to maintain its activity where at the concentration of 0.073 µg/mL, the butyrolactones registered an $O.D_{595nm}$ equal to that of the positive growth control reflecting no activity, but doxycycline was still active at this concentration with an $O.D_{595nm}$ = 2.1. At the final concentration, 0.037 µg/mL, doxycycline also has lost its activity to be as the untreated control (Figure 2B).

Effect of the butyrolactone derivatives (B-12 and B-13) on *S. gordonii* and *P. gingivalis* monospecies biofilms

The ability of the butyrolactones to inhibit *S. gordonii* or *P. gingivalis* monospecies biofilm formation was confirmed by the CLSM images of biofilms formed in ludin chambers with or without the compounds (Figure 3).
In case of *S. gordonii*, a highly condensed agglomerations of viable cells shown by the green fluoresence in addition to very few dead cells fluorescing in red were shown when the bacteria were incubated alone (Figure 3A). However, when they were treated with **B-12** (Figure 3B) and **B-13** (Figure 3C), the number of adhered bacterial cells was diminished dramatically and the attached cells were dead. A few green viable cells appeared when **B-13** was used. However, doxycycline has generated large zones without bacteria as if it has highly interfered with their adhesion, where the green labeling was prevailing in the remaining cells (Figure 3D). Hence, doxycycline was more efective than butyrolactones regarding the inhibition of adhesion whereas, **B-12** and **B-13** were stronger killers.
In contrast, with respect to *P. gingivalis*, the initial cellular mass was less diminished than that of *S. gordonii* relative to the positive growth control (Figure 3E). Also, the effects of butyrolactones (Figures 3F and 3G) and the antibiotic doxyxycline (Figure 3H) against *P. gingivalis* biofilms is different than that against *S. gordonii* biofilms. In this case, doxycycline was like the butyrolactones in preventing the adhesion of the cells except for **B-13** which had left small agglomeration zones formed of living and dead populations.

Effect of the butyrolactone derivatives (B-12 and B13) on the expression of some genes involved in the formation of *S. gordonii* and *P. gingivalis* monospecies biofilms

B-12 and **B-13** at their MIC/2 caused the same pattern of gene expression profile in strains (Figure 4). Concerning *S. gordonii* (Figure 4A), *luxS* and *comD* genes related to the quorum sensing were expressed in the same way in the presence of both butyrolactones registering about 11 fold change compared to the control. *SspB* comes in the second place with 10 fold change and this histidine kinase was near to *comD* histidine kinase as if the butyrolactones were acting in a kinase pathway. Then, *abpA* and *sspA* were then impacted with about 4 fold change. However, in case of doxycycline, *luxS* and *sspB* were almost totally downregulated. *SspA* registered a 4 fold change whereas *comD* and *abpA* were the most upregulated with 15 and 20 fold changes, respectively.

Concerning *P.gingivalis* (Figure 4B), in presence of doxycycline, only *galE* expression was decreased. When *P. gingivalis* was treated with **B-13**, *fimR* was increased and *galE* decreased. In contrary to ***B-13, B-12*** increased the expression of *Mfa1, fimS/fimR.* For *P.gingivalis*, the role of the FimS/FimR in expression of the *fimA* gene is well defined. A comparison of the transcriptional level of the *mfa1* in *P. gingivalis* wild-type strain and in the *fimR* mutant indicates that the FimS/FimR system is a positive regulator for the *mfa1* gene, although the system controls two fimbrial genes (Wu et al. 2007) explaining the results obtained in presence of **B-12.** Here we can speculate that *fimS/fimR* regulated *Mfa* gene expression whereas *fimA* was down regulated by other mechanism. Nakao et al. suggested that *galE* plays an important role in both the synthesis of O antigen and the formation of biofilms (2006b).

Discussion

Due to the prevalence and high impact of periodontal diseases caused by the oral biofilms on individuals and society as well as the high cost to treat these infections (Batchelor 2014), we attempted to evaluate the antibiofilm effect of new uncovered antibacterial butyrolactones against *S. gordonii* and *P. gingivalis* monospecies biofilms. The bacterial strains used here were highly studied before due to their importance not only in the oral cavity but also on the whole body (Yombi et al. 2012; Mysak et al. 2014; How et al. 2016).

With respect to the antibacterial activity of the butyrolactone analogues against *P. gingivalis*, the latter were shown more sensitive than *S. gordonii*. Their pattern of efficiency related to their structures was a little different. A clear and logical explanation has been given to their activity on *S. gordonii* concluding the importance of the aliphatic chain length. As the chain length increased the activity increased but the best activity was reached when the COOH group joined. However, there was an optimum length for providing the best inhibitory activity where beyond it the latter started to decrease whereas the killing effect continued enhancing (Sweidan et al. 2016). However, with respect to *P. gingivalis* in this study, the chain length role hasn't been clearly established (Table 1). Indeed, when the COOH was the functional group, both, the inhibitory and killing activities, were enhanced when the length increased

from **B-7** into **B-8/B-9**, then, **B-10/B-11**, and finally, **B-12** and **B-13**. Surprisingly, the stereochemical configuration has interfered here registering different activities as for **B-8/B-9** and **B-10/B-11**. It was realized that the inhibitory activity was better for the 5S enantiomers. However, the MBC was the same for **B-8** and **B-9**, but, **B-11** was stronger killer than **B-10** (Table 1). Several authors have previously described the role of stereochemistry in having different activities for the stereoisomers. Gerster et al. has mentioned that the S isomer of 6,7-dihydro-5,8-dimethyl-9-fluoro-1-oxo-1*H*,5*H*-benzo[ij]quinolizine -2-carboxylic acid was much more active than its R counterpart against several Gram-positive and Gram-negative bacteria (1987). Wakiyama et al have also demonstrated that the 7(S)-configuration of lincomycin derivatives was necessary for enhancing the antibacterial activity against respiratory infection-related Gram-positive bacteria (2016).

Concerning the antibiofilm activity of **B-12** and **B-13**, *P. gingivalis* was more sensitive than *S. gordonii* coinciding with the antibacterial activity. However, the sessile cells were more resistant than the planktonic cells and this agrees with previous studies (Wilson 1996; Song et al. 2013). Regardless of some discrepancies, **B-12** and **B-13** had the same effect on the biofilms tested. These little variations were translated in the CLSM images. A few more viable *S. gordonii* cells and small assembly zones of dead and living *P. gingivalis* cells appear in case of **B-13**. But, the CLSM has confirmed vividly the antibiofilm effect of the compounds which inhibited efficiently the biofilm formation of both strains. Hence, this antibiofilm activity confers the butyrolactones a considerable importance since biofilm inhibitors don't cause resistance immediately as mentioned by Stadler et al. (2016). Moreover, it is worth noting that the buytrolactones can act as inhibitors of quorum sensing systems since they share a structural similarity with the communicating molecules used among the Gram-negative bacteria. The butyrolactones can act as anatagonists competing acyl-homoserine-lactones for their binding sites leading to quorum sensing perturbation and inhibition of its consequent virulence and biofilm formation (Andreas Podbielski and Bernd Kreikemeyer 2004b; Swem et al. 2009).

At the lethal dose, the compounds kill or inhibit the bacterial populations; however, sub-inhibitory concentrations can act as selectors of resistance, generators of genetic and phenotypic variations, and signaling molecules modulating several physiological activities such as virulence, biofilm formation and gene expression (Andersson and Hughes 2014). The importance of this issue in the medical field has pushed us to use MIC/2 of the butyrolactones and doxycycline to quantify the selected biofilm genes by qPCR. A surprising result has been obtained after doing three independent experiments. The antibacterial compounds have up-regulated the expression of the chosen genes and consequently, promoted the biofilm formation. This can be predicted as the crystal violet assay has shown a weak antibiofilm effect at sub-MIC concentrations of the compounds. Alongside, other previous studies have reported this issue where the biofilm formation has been favored at sub-MIC of several antibiotics including tetracyclines where one of which, doxycycline, has displayed this effect in our present study. Ahmed et al. has mentioned that the sub-MICs of three antibiotics used in their study, ampicillin, ciprofloxacin, and tetracycline, have increased the biofilm formation of *Streptococcus intermedius* WT due to the role of autoinducer-2/LuxS (2009). This proposes

that these actors could be the reason behind the increased biofilm formation in our case. This is supported by the fact that *luxS* was one of the genes quantified and showed to be highly expressed in the presence of MIC/2 of the compounds. Also, Aka and Haji have shown that incubating *Pseudomonas aeruginosa* isolates with sub-MICs of antibiotics in the presence of chlorhexidine has stimulated biofilm formation (2015). Nevertheless, the induction of biofilm formation in the presence of MIC/2 of the highly efficient antibacterial butyrolactones and the involvement of AI-2/LuxS in the intercellular signaling as a bacterial survival strategy need further investigation.

As a conclusion, **B-12** and **B-13** derivatives had a promising antibiofilm activity shown by crystal violet and confirmed by CLSM. They should be used at concentrations higher than MIC/2 to induce the desired antibacterial effect.

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Conflict of interest We declare that we have no conflict of interest

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Table 1 MIC and MBC of butyrolactones against *P. gingivalis* by broth microdilution

Compound	MIC (μg/mL)	MBC (μg/mL)
B-1	9.38	37.5
B-2	37.5	150
B-3	4.69	37.5
B-4	4.69	75
B-5	37.5	75
B-6	2.34	75
B-7	75	>i
B-8	9.38	150
B-9	37.5	150
B-10	0.073	9.38
B-11	0.586	4.69
B-12	0.037	1.17
B-13	0.293	0.586
Doxycycline	0.13	>i

>*i*: greater than the initial concentration

Table 2 List of the genes used in this study along with their primers for each strain

Bacterial strain	Gene	Left primer	Right primer
S. gordonii	16S ribosomal RNA	AGCGTTGTCCGGATTTATTG	CATTTCACCGCTACACATGG
	Histidine kinase (*comD*)	CTACTCCACACTCTCGAGCC	TTGCAAACCGGAATTAACTCAG
	Autoinducer-2 production protein (*luxS*)	TGACGTCCCCACATGATCAT	AGCGGACCAAAAGGAGATGT
	Streptococcal surface protein A (*sspA*)	CAGCACCAGTAGTACCGACA	TTGATGGCTCCGGTTGATCT
	Streptococcal surface protein B (s*spB*)	TCGCCATTCCAAGCTGAAAC	GGATCCTTTGGTTTTGGCGT
	Amylase-binding protein (*abpA*)	CTTTGTCTTCTGCAGCTGGG	GAAGCAGCATTCAACAACGC
P. gingivalis	16S ribosomal RNA	TGGGTTTAAAGGGTGCGTAG	CAATCGGAGTTCCTCGTGAT
	fimA type I fimbrillin	GCCGAAAATGCGACTAAGGT	TGGCTCTGCTGTCATGATCA
	Two-component system response regulator (*fimR*)	GTTCGGCTGCATTGGAGAAT	AAACAGCAACAGCAGCAGAA
	Two-component system sensor histidine kinase (*fimS*)	GTCGCCATGGTTGCATACTT	CTTTTCCAAATAGCGGCCGA
	Mfa1 fimbrillin	ATTATGCCGGTCTGTGGGAA	AGTCTGACGAGGCAGCATTA
	UDP-glucose 4-epimerase (*galE*)	TAGCCTTGTACTCTGCTCCG	CGAACTGATGGAGCGATTCG
	Putative universal stress protein (*uspA*)	CTCGGATTGAAGAAGCGAGC	CGGAATGGAACCAAGTGCAA

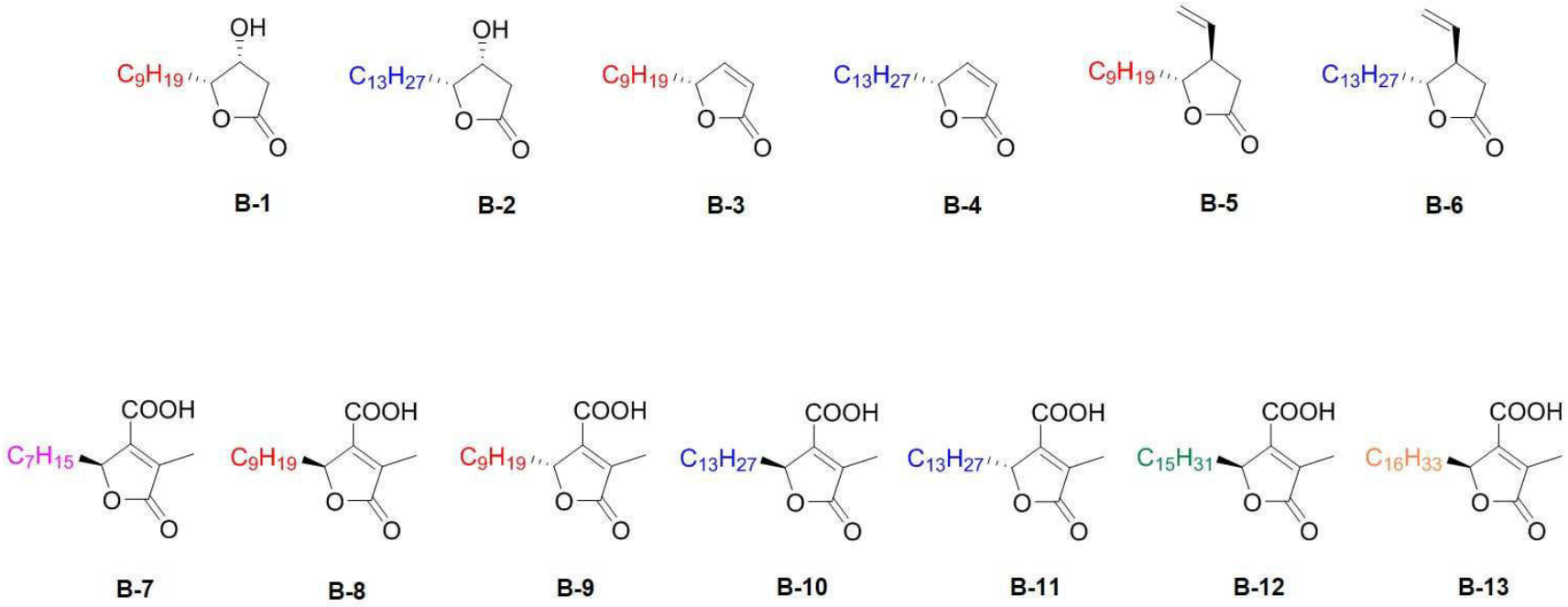

Fig. 1 Chemical structures of the butyrolactones

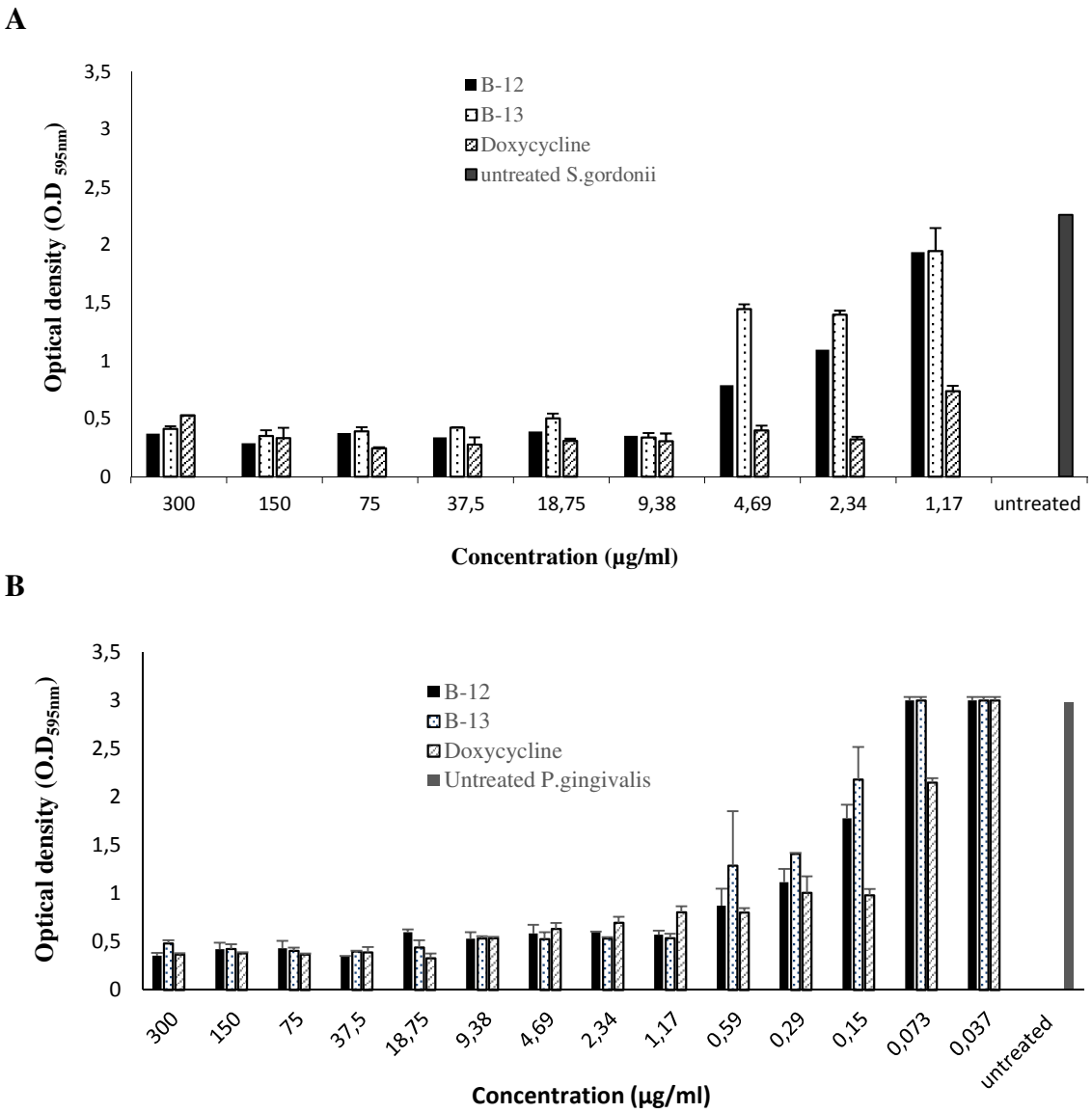

Fig. 2 Antibiofilm activity of butyrolactones (**B-12** and **B-13**) against *S. gordonii* (A) and *P. gingivalis* (B) monospecies biofilms by crystal violet assay

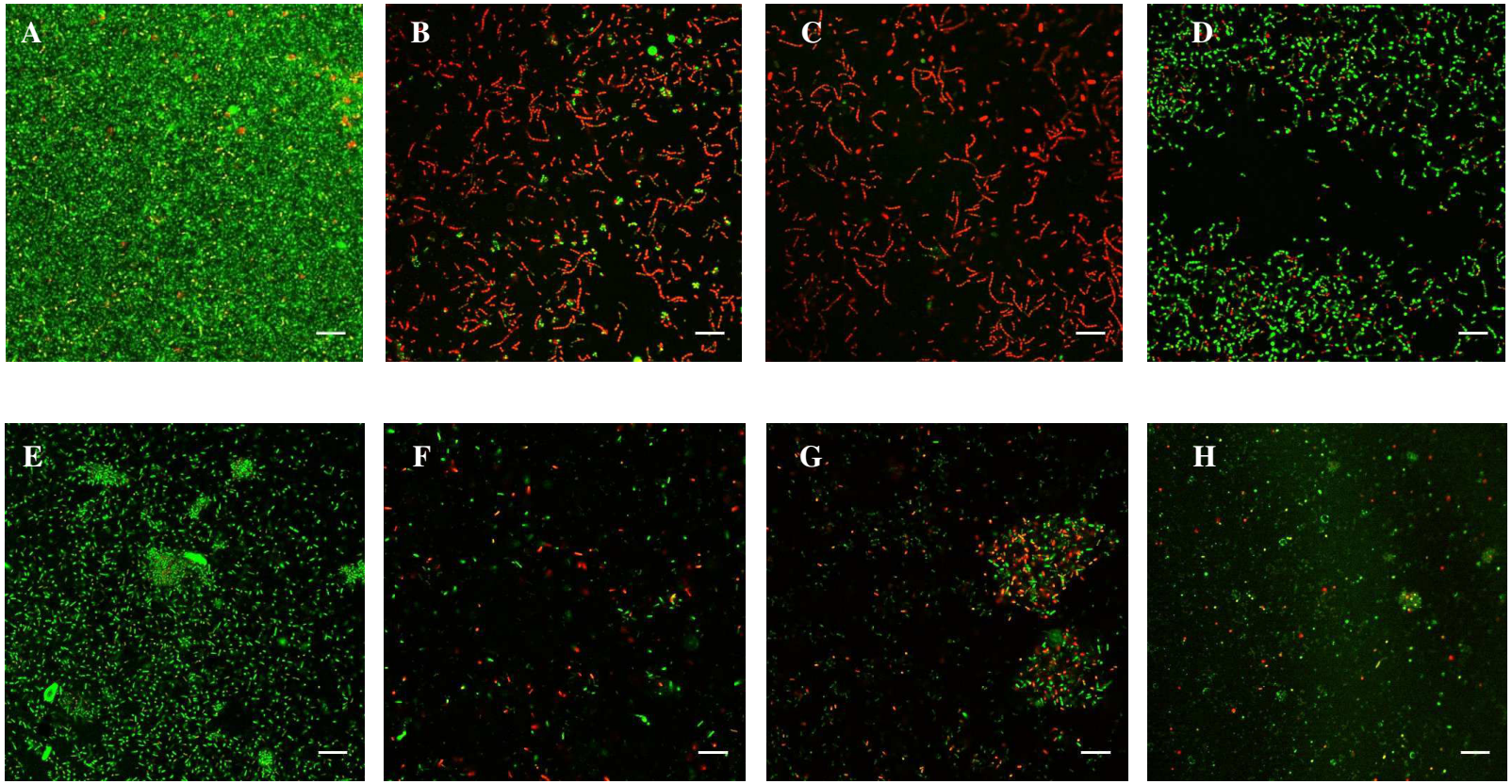

Fig. 3 CLSM images of *S. gordonii* (A, B, C, D) and *P. gingivalis* (E,F,G,H) monospecies biofilms using Live/Dead BacLight viability staining (Syto9/PI). (A) untreated *S. gordonii,* (B) *S. gordonii* incubated with **B-12**, (C) *S. gordonii* incubated with **B-13**, (D) *S. gordonii* incubated with doxycycline, (E) Untreated *P. gingivalis,* (F) *P. gingivalis* incubated with **B-12**,(G) *P. gingivalis* incubated with **B-13** and (H) *P. gingivalis* incubated with doxycycline .Viable cells are stained with green fluorescence (Syto9) and dead cells are stained with red fluorescence (PI). Scale bar= 20 µm.

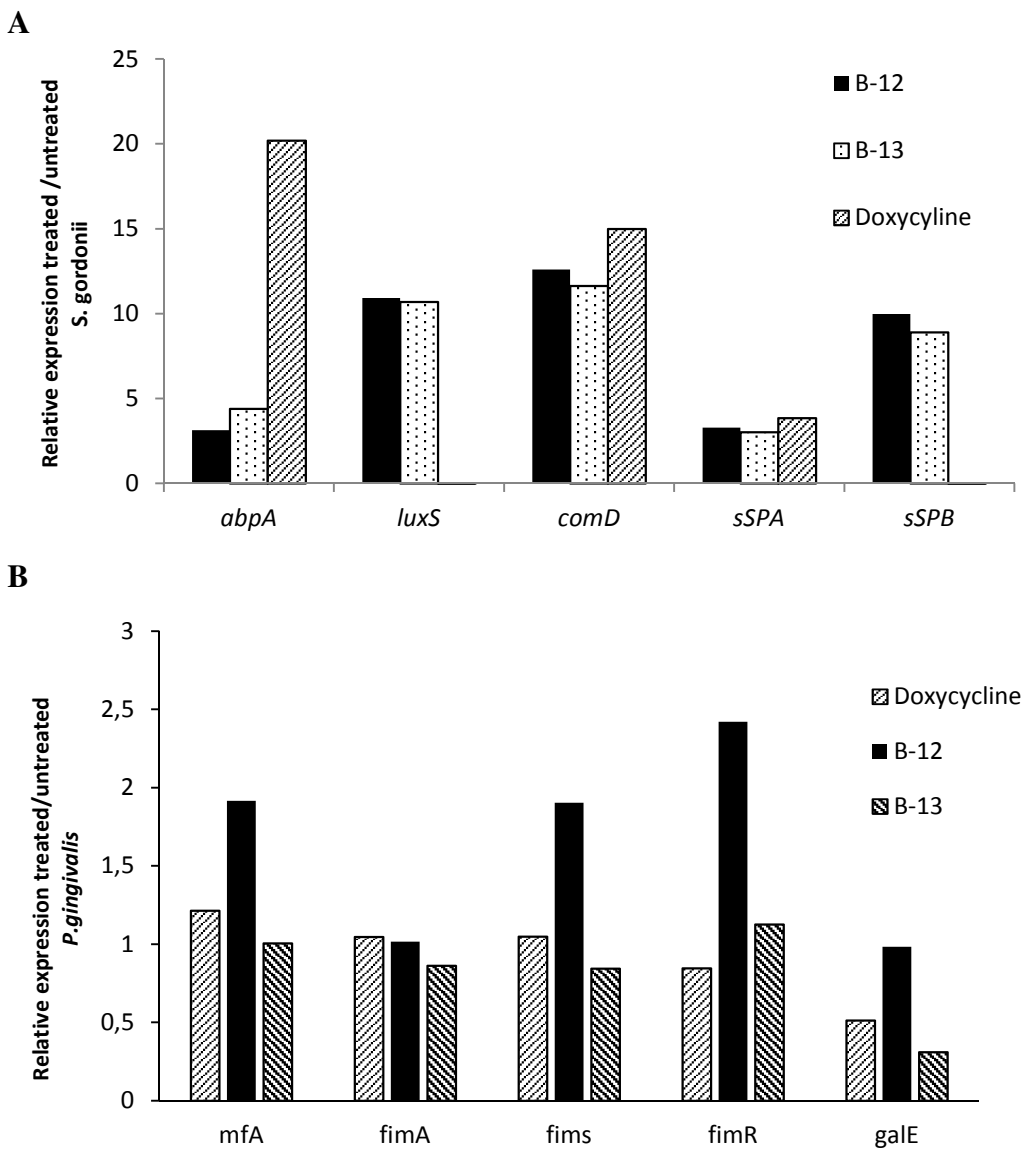

Fig. 4 Analysis of selected genes expression profile by qRT-PCR in presence of butyrolactones (**B-12** and **B-13**) and control antibiotics (Ampicillin and doxycycline). For *S. gordonii*, selected genes were: genes related to quorum sensing (*luxS* and c*omD*), α-amylase binding protein (*abpA*) and adhesion surface genes (*sspA* and *sspB*). For *P. gingivalis,* fimbriae genes *(Mfa* and *fimA),* their regulators *fimS* and *fimR,* and *galE* were studied.

ARTICLE 4

Lichen butyrolactone derivatives disrupted the cell wall of oral bacteria

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Abstract

Previously, we have demonstrated that out of the butyrolactones series synthesized based on the natural lichen metabolite, lichesterinic acid, compound (**B-13**) was the most effective against oral bacteria. However, its antibacterial mechanisms are still unknown. In this study, we have investigated its bacterial localization by synthesizing a fluorescently labeled **B-13** with NBD (4-nitro-benzo[1,2,5]oxadiazole) without modifying its antibacterial activity. We showed that this compound binds to *Streptococcus gordonii* cell surface, as demonstrated by HPLC analysis where compound **B-13** was found in the cell wall and membrane fraction after 1h of incubation. This compound was not detected in the cytoplasm even after 18h of incubation. By adhering to cell surface, **B-13** induced cell wall disruption leading to the release of bacterial constituents and consequently, the death of *S. gordonii*, a Gram-positive bacteria. The expression of two genes, *murA* and *alr,* implicated in cell wall synthesis, was modified in the presence of this butyrolactone. Gram-negative bacteria such as *Porphyromanas gingivalis* showed also cracked and ruptured cells in the presence of **B-13**, suggesting that this butyrolactone acts on Gram-positive and Gram-negative strains. However, it showed greater efficacy against the Gram-negative strains in comparison to the Gram-positive counterpart. Besides, we also demonstrated that the analogue of **B-13**, **B-12**, has also induced disruption of *P. gingivalis* and *S. gordonii.* This study demonstrated that butyrolactone derived from a lichen metabolite, disrupted the cell wall of bacteria introducing them as potent antibacterial compounds against oral pathogens causing serious medical complications.

INTRODUCTION

Treatment of infections with antibiotics reduces morbidity; however, the erroneous or unsuitable antibiotic can lead to the emergence of resistant pathogenic bacteria [1]. Facing this worldwide concern, alternative antimicrobial candidates against multidrug-resistant bacteria were developed. The new drugs, which are of natural origin, are capable of surpassing the bacterial resistance mechanisms [2]. Among the natural sources is the association of fungus and alga and/or cyanobacterium forming a symbiotic organism named lichen which produces more than 1000 distinct secondary metabolites. They were shown to be effective against sensitive and several multi-drug resistant bacterial strains [3–6]. Among the bacteria sensitive to lichen secondary metabolites, we have previously described that synthesized butyrolactone analogues, **B-13** and **B-12**, can inhibit the growth of *Streptococcus gordonii* [7] and *Porphyromonas gingivalis* (in submission). *S. gordonii* is a member of the viridans streptococci large category. In the oral cavity, *S. gordonii* adhere to the salivary pellicle which coats the teeth, proliferate and excrete an extracellular polysaccharide matrix protecting their developing microcolony on which secondary colonizers will adhere. The late colonizing strains such as *P. gingivalis* bind the sites provided by *S. gordonii* and form a highly pathogenic complex microbial community [8], [9]. *S. gordonii*, as a pioneer initial colonizer, initiates the formation of dental plaques contributing in turn to the onset of dental caries and periodontal diseases as well as their progression [10]. Inhibiting *S. gordonii* might block the successive steps leading to acute oral diseases and introduce new antibiotics that might be able to prevent and treat the periodontal diseases.

The most common way of antimicrobial killing is triggered by disruption of the cytoplasmic membrane. Bacterial cell membrane is responsible for many essential functions: transport, osmoregulation and respiration processes, biosynthesis and cross-linking of peptidoglycan, and synthesis of lipids. It is doubtless that for all these functions membrane integrity is absolutely necessary and its disturbance can directly or indirectly cause metabolic dysfunction and cell death. Alternative mechanisms of action include antimicrobial translocation into the cytoplasm where they interfere with metabolic processes, such as protein synthesis or DNA

replication [11]. All of these modes of action have in common that the bacterial membrane will be severely damaged in the end leading to cell death. One of the most validated targets for antibacterial therapy is the enzymes of peptidoglycan biosynthesis. This biosynthesis is a complex process, which involves numerous steps in the cytoplasm and the inner and outer leaflets of the cytoplasmic membrane [12], [13]. The process commences in the cytoplasm where the nucleotide precursors uridine-5'-diphosphate-GlcNAc (UDP-GlcNAc) and UDP-MurNAc-pentapeptide are synthesized. The first reaction is the transfer of enolpyruvate to the position 3 of UDP-GlcNAc by MurA [14]. D-amino acids are important components of the peptidoglycan layer in bacterial cell walls, where their presence is thought to protect the bacterial cell from the action of proteases. A small group of stem peptides consisting of L-alanine, D-glutamate (D-Glu), D-alanine, covalently attach to MurNAc to form a complex cellular skeleton [15]. Intracellular D-Glu is derived from the racemization of L-glutamate (L-Glu) by glutamate racemase (MurI) [16]. The D-Ala dipeptide is synthesized by two enzymes: alanine racemase (Alr), which converts L-enantiomers of alanine to the D-counterparts, and D-Ala-D-Ala ligase, which generates the D-Ala dipeptide [17]. Antimicrobials acting at the cell wall level are the most selective compared to other antibiotics, possess a bactericidal activity since inhibition of peptidoglycan synthesis leads to cell lysis.

In this study, we investigated mechanisms of action of butyrolactone analogue **B-13** on two oral bacteria implicated in periodontal diseases: *S. gordonii* and *P. gingivalis*. We have also compared its mechanism to another butyrolactone analogue (**B-12**).

RESULTS AND DISCUSSION

Fluorescent B-13 butyrolactone adheres to S. gordonii membrane

Previously, we have demonstrated that lichen butyrolactones derivatives such as **B-13** inhibits two oral bacteria *S. gordonii* [7] and *P. gingivalis* (in submission).

However, further studies are needed to determine the putative mechanisms for their antimicrobial activity. At first, we have generated in this study a NBD-labeled **B-13** (Scheme 1, Figure 1). The synthesis of the NBD spacer arm has already been described [18]. Fluorescence is provided by NBD, which is a compound widely used in the synthesis of fluorescent probes. The molecule corresponding to the arm is a diamine mono-protected by a Boc group (*N*-BOC-2, 2′-(ethylenedioxy)bis(ethylamine)). The reaction between the arm and

NBD chloride was performed in the presence of cesium carbonate in acetonitrile in order to obtain the fluorescent arm (step 1). The Boc group was then cleaved under anhydrous conditions, in the presence of TFA and sodium sulfate in CH_2Cl_2 (step 2). The last step consists in a peptide coupling between the probe and **B-13** (step 3). This coupling is carried out in the presence of TBTU and DIEA in anhydrous DMF. This last step gives the final **B-13 NBD** compound with a yield of 63%.

B-13 NBD biological activity was evaluated and compared to **B-13**. The conjugation of NBD to **B-13** did not impair their biological activity as their antimicrobial activity against *S. gordonii* was seen (data not shown). Zhao et al. 2016 [19] also showed that NBD is a highly tolerated fluorescence label and Matijašić et al. in 2012 [20] demonstrated that 9a-NBD-azithromycin has antimicrobial properties comparable to azithromycin.

After that we have used **B-13 NBD** to investigate cellular localization of **B-13** in *S. gordonii* by confocal laser scanning microscopy (CLSM). Confocal microscopic images of *S. gordonii* following the treatment of **B-13 NBD** showed green fluorescence (Figure 2) which indicated considerable binding of this compound onto the bacteria, as seen by the presence of green fluorescence on cell membranes. The green fluorescence has surrounded the bacterial DNA stained in blue with Syto 40, suggesting a strong localization in the cell wall.

Cellular dysfunctions can result from the interaction of an antimicrobial with the microbial cell membrane [21]. The antimicrobial can only attach to the cell membrane and alter its structure, permeability and transport activity. Another pathway exist, after binding the cell membrane, the antimicrobial may also penetrate inside the cells and affect vital cellular functions. To determine the antibacterial mechanisms of **B-13** in *S. gordonii,* localization of **B-13** in the bacteria was performed after incubating the latter with the compound for 1h and 18h. Lysed bacteria were centrifuged and their cell wall and membrane pellet (Figure 3a) and cytoplasmic (Figure 3b) fractions were analyzed using HPLC. Chromatograms in Figure 3 indicated that the compound was clearly visualized only in the cell wall and membrane fraction of the treated cells with the same manner than the control **B-13** after 1h of incubation. After 18h of treatment, a low level of **B-13** was detected in the cell membrane. This signal decrease of the compound may be caused by the degradation or the release by bacteria of this butyrolactone. In contrast, **B-13** was not detected in the cytoplasm fraction. This kinetic study suggests that **B-13** binds the membrane and does not cross to the cytoplasm.

B-13 Butyrolactone induced cell wall disruption of S. gordonii

To obtain deeper insight about the mode of action of **B-13**, morphology of *S. gordonii* was visualized by transmission electron microscopy (TEM) after treatment with this compound (Figure 4). Untreated bacteria appeared intact with a cell wall typically delimited and well-defined membrane, and a cytoplasmic content with few electron dense areas. Treatment with doxycycline, the reference antibiotic [22], caused cytoplasmic alterations as suggested by the presence of round bodies in the cytoplasm with a similar electron density like clear vesicles. However, in presence of **B-13**, bacteria showed lysed cells with broken walls and membranes, and heterogeneous electron density zones in the cytoplasm (Figure 4). The cells showed aberrant morphology; they were cracked and ruptured leading to the death. In some cells, the externalization of cytoplasmic material could be seen by the way of vesicles and rupture of the cell. Altogether, the results suggested that **B-13** inflicted considerable damage to *S. gordonii* membrane and this membrane disruption property could be the basis of the antibacterial activity of this compound.

CLSM analysis was used to provide further confirmation of our hypothesis on this membrane disruption. In order to follow permeability modification due to **B-13** exposure, we monitored the fluorescent intensity of bacterial culture mixed with propidium iodide (PI) and Syto 9 [23]. The green fluorescence caused by Syto 9 labels the living cells and the red fluorescence emitted by PI labels the dead ones. As shown in figure 5, untreated control *S. gordonii* cells appeared predominantly green (demonstrating live cells); whereas the **B-13** treated cells appeared substantially red, indicating the highly permeability of PI dye and that most of the cells were dead. This result confirmed that **B-13** had a destructive effect on the cell membranes where the majority of bacteria were dead.

B-13 Butyrolactone induced cell wall genes inhibition of S. gordonii

In this study, we have investigated the effect of **B-13** on peptidoglycan biosynthesis in *S. gordonii* by conducting qRT-PCR analysis of selected genes implicated in peptidoglycan synthesis: *murA*, *alr* and *murI* and *luxS* as a non-specific gene of peptidoglycan. The data

showed that **B-13**, significantly upregulated *murA* (4.2 fold), *murI* (4.4 fold) and *alr* (1.7 fold) expressions but lower than doxycycline (Figure 6A). Interestingly, in presence of doxycycline, *murA* was overexpressed in the treated group compared to that in the control one. For *S. gordonii*, D-alanine and D-glutamate are catalyzed by alanine racemase (Alr) and glutamate racemase (MurI), respectively. These results reveal that **B-13** increased the expression of murA, *murI* and *alr* of *S.gordonii* peptidoglycan biosynthesis. In summary, **B-13** increased the expression of peptidoglycan biosynthesis-related genes of *S. gordonii* at the transcriptional level as another antibiotic control which is ampicillin (Figure 6A), acting as an irreversible inhibitor of trans-peptidases, enzymes responsible for the formation of the bacterial cell wall[13].

The induction of *murA*, *murI and alr* genes can be interpreted as an attempt by *S. gordonii* cells to boost the rate of peptidoglycan synthesis and murein remodeling to restore stress-induced damaged or missing cell wall material. In *S. aureus*, [24] reported that bacitracin-challenged cells induced *pbpB*, *sgtB*, *murA*, and *bacA* gene expression to increase the rate of peptidoglycan synthesis. Maintaining cell envelope integrity in the face of environmental insults by responding to cell envelope stress is critical for bacterial survival. This response to environmental stress is regulated by two-component signal transduction systems (TCSTSs) are among the primary sensory-regulatory mechanisms that mediate bacterial adaptation processes (e.g., countering envelope stress) in response to environmental perturbations [25;26]. These systems modulate the expression of genes, encoding products crucial to cell survival, via a cytoplasmic response regulator (RR), upon receipt of an external stimulus detected by a membrane-bound histidine kinase (HK) sensor. This TCSTS was also identified in *S. mutans* UA159 genome, playing a prominent role in regulating environmental stress tolerance and other diverse phenotypes conducive to persistence [27; 28].

B-13 Butyrolactone has also disrupted the Gram-negative bacterial membrane as with P. gingivalis

In order to determine if its antimicrobial potential is influenced by composition of the cell wall of the microorganisms, we have investigated the effect of **B-13** on *Porphyromonas gingivalis*, a Gram-negative strain. When we used **B-13 NBD** on *P. gingivalis* (Figure 2), we have observed a prominent green fluorescence on cell membranes. Figure 7 showed TEM

micrograph of *P. gingivalis* control with a typical cell wall, outer and cytoplasmic membrane, periplasmic space, cytoplasmic content with few electron-dense areas. When *P. gingivalis* was treated with **B-13**, cells were damaged and showed either localized or complete separation of the cell membrane from the cell wall. The cellular degradation was also accompanied by electron-translucent cytoplasm and cellular disruption in the damaged cells. These differences in structure, thickness and composition of the cell can explain why Gram-positive *S. gordonii* were less inhibited and Gram-negative *P. gingivalis* showed substantial inhibition even at low antibiotic concentration. Besides, peptidoglycan genes expression by qRT-PCR showed that in *P. gingivalis* treated with butyrolactones*, alr, murA* and *murI* were downregulated as with the treatment with ampicillin (Figure 6B). These results also showed that butyrolactones are highly efficient on Gram-negative. Todorovic et al. 2017 [29]
indicated that antimicrobial activity of their compound was equal for Gram-positive bacteria or even significantly enhanced for Gram-negative bacteria [29], Mandal et al. 2016 [30] showed greater efficacy against Gram-positive and comparatively less efficacy against Gram-negative bacteria. May be these differences result from their peptidoglycan, which is a polymer of alternating N-acetylglucosamine and N-acetylmuramic acid and exists substantially more highly in Gram-positive than in Gram-negative bacteria. Lipoteichoic acids (LTA) are linked to the cytoplasmic membrane of most Gram-positive bacteria and vary between different species.

B-13 Butyrolactone analogue, B-12, has also disrupted the membrane of oral bacteria

In order to determine if this membrane disruption is specific to the synthesized buryrolactone, we have used an analogue of **B-13**, **B-12** (Figure 1), which we have already described in Sweidan et al. 2016 [7]. Electron Micrographs of *S. gordonii* or *P. gingivalis* treated with **B-12** showed broken membrane and dead bacteria (Figure 4 and figure 7). However, as for **B-13**, we found that the effect of **B-12** are also more active against Gram-negative as seen by the formation of unwinded structures on the cell wall, irregularly shaped cells, and scratched cell surface on *P. gingivalis* (Figure 7). Besides, peptidoglycan genes expression by qRT-PCR showed that in *P. gingivalis, alr, murA* and *murI* were downregulated as with the treatment with ampicillin whereas with *S. gordonii* these genes were upregulated (Figure 6B).

The finding that **B-12** has the ability to break and damage the bacterial membrane leads to think that the bactericidal effect of our synthesized butyrolactones is based on destroying the cell surface of bacteria.

Conclusion:

S. gordonii and *P. gingivalis* are oral bacteria initiating the formation of dental plaques contributing in turn to the onset of dental caries and periodontal diseases as well as their progression (Ng et al., 2016). We have previously demonstrated that these bacteria are sensitive to new drugs which are of natural origin, derived from lichen secondary metabolites [7] confirming that latter [31]. However, its antibacterial mechanisms are still unknown. By using compound (**B-13**) which is one of the most active butyrolactones series synthesized based on the natural compound, lichesterinic acid, we have shown that this compound has bound to the bacterial surface and induced membrane modification with the break of cell wall and the release of cytoplasm constituents leading to bacterial death. Our study showed that this effect is associated with an overexpression of genes implicated in peptidoglycan synthesis for Gram-positive such as *S. gordonii*, suggesting that this is the strain response to the stress generated by this antiobiotic [28]; whereas, in Gram-negative such as *P. gingivalis*, where the effect is more important, these genes were downregulated. These results suggested that its antimicrobial potential is influenced by the composition of the cell wall of the microorganisms [32].

This study shows for the first time the mechanism of action of synthesized butyrolactones, analogues of lichesterinic acid. To our knowledge, there are only at least two studies on the mechanism of action of lichen-derived compounds [33,3]. Gupta et al. (2012) demonstrated that usnic acid, a commonly occurring polyphenolic compound in many species of lichens, can destabilize the membrane integrity of MRSA[33]. Shrestha et al. 2016 demonstrated also that *L. vulpina* extracts disrupted the integrity of MRSA membranes and targeted the cell division processes in MRSA[3]. These studies have only investigated Gram-positive strains.

Our study opens the door for future mechanistic research on lichen secondary metabolites which will give a better understanding of lichen as an association of fungus and alga and/or cyanobacterium forming a symbiotic organism and how to use its secondary metabolites as

antibiotics. However, to better develop a new antibiotic, it is necessary to make further investigations on lichen secondary metabolites such as **B-13**.

EXPERIMENTAL PROCEDURES

Chemistry

All reagents of high quality were purchased from commercial suppliers and used without further purification. IR spectra were obtained with PerkinElmer UATR Two infrared spectrophotometer. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were performed on a Bruker GMX 300 spectrometer. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$:δ_H = 7.26, δ_C = 77.0). The δ values are given in parts per million (ppm), and the coupling constants (*J* values) are given in Hertz (Hz). The multiplicity of the signals is reported as s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet). ESI-HRMS were carried out on a Bruker MicroTof QII spectrometer for electrospray ionization at the CRMPO (Centre Régional de Mesures Physiques de l'Ouest), University of Rennes 1. Elemental analyses were performed on a microanalysor Flash EA1112 CHNS/O Thermo Electron at the CRMPO. Reactions were monitored by TLC on Merk 60 F254 (0.25 mm) plates which were visualized by UV detection or sprayed with $KMNO_4$ solutions, then heated.

(S)-4-methyl-5-oxo-2-pentadecyl-2,5-dihydrofuran-3-carboxylic acid (B-12) and (S)-2-hexadecyl-4-methyl-5-oxo-2,5-dihydrofuran-3-carboxylic acid (B-13):

B-13 and **B-12** derivatives described previously were used in this study [34].

(2-{2-[2-(7-Nitro-benzo[1,2,5]oxadiazol-4-ylamino)-ethoxy]-ethoxy}-ethyl)-carbamic acid *tert*-butyl ester:

This compound was prepared as published [18]. Yield: 70%; red viscous solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.42 (s, 9H), 3.35 (q, J=6,1 Hz, 2H), 3.59 (t, J=6 Hz, 2H), 3.68 (m, 4H), 3.85 (m, 4H), 4.98 (s, 1H), 6.19 (d, J=8 Hz, 1H), 6.87 (s, 1H), 8.49 (d, J=8,1 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 28.6, 41.1, 44.4, 69.5, 70.8, 71.2, 71.3, 78.8, 100.2, 137.9,

145.2, 145.6, 146.1, 156.1.

N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine:
This compound was prepared as published (Noël et al., 2011) and was used as it in step (3) without any further purification.

Probe B-13 NBD:

To a solution of butyrolactone **B-13** (44.6 mg, 0,122 mmol) and 1.28 eq. of TBTU (50.1 mg, 0,156 mmol) in anhydrous DMF (10 mL) under argon atmosphere, was added the NBD spacer (40 mg, 0.122 mmol, 1 eq.) and anhydrous DIEA (42.5 µL, 0.244 mmol, 2eq.). After stirring overnight at rt, the reaction was quenched by adding H_2O, this solution was then extracted with diethyl ether (3 x 50 mL) and once with ethyl acetate. The combined organic layers were washed with brine, dried with $MgSO_4$ and concentrated under vacuum. The residue was purified over preparative TLC (CH_2Cl_2/EtOH 95:5) to yield the desired compound.Yield = 63%; dark red solid. Rf (SiO_2, CH_2Cl_2/EtOH (95:5)) = 0,6. IR (ATR): ν_{C-O} (ester) = 1133 cm^{-1} ; ν_{C-O} (lactone) = 1213 cm^{-1} ; $\nu_{C=C}$ (ar) = 1586 cm^{-1} ; $\nu_{C=C}$ = 1630 cm^{-1} ; $\nu_{C=O}$ (ester) = 1698 cm^{-1} ; $\nu_{C=O}$ (lactone) = 1734 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.87 (t, J=6,7 Hz, 3H), 1.24 (m, 28H), 1.38 (m, 2H), 2.05 (s, 3H), 3.71 (m, 10H), 3.86 (t, J=5,4 Hz, 2H), 5.15 (t, J=5,8 Hz, 1H), 6.18 (d, J=8,7 Hz, 1H), 6.42 (s, 1H), 6.82 (s, 1H), 8.46 (d, J=8,7 Hz, 1H) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz) δ 10.8, 14.3, 21.2, 22.8, 25.1, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8, 29.8, 29.8, 30.2, 32.1, 32.9, 39.4, 43.6, 68.2, 69.7, 70.4, 70.6, 81.8, 99.1, 124.3, 129.1, 136.5, 143.8, 144.1, 153.8, 162.5, 171.3, 173.4 ppm. HRMS (ESI, m/z) : Calcd. for $C_{34}H_{53}N_5O_8Na$ 682,37918, found $[M+Na]^+$: 682.37920. Anal. Calcd. For $C_{34}H_{53}N_5O_8$: C, 61,89; H, 8.10 ; N, 10.61. Found : C, 62.40 ; H, 8.06 ; N, 9.70.

Bacterial strains

We used in this study the oral bacteria, *Streptococcus gordonii* DL1 and *Porphyromonas gingivalis* ATCC 33277. We have grown them in an anaerobic environment (N_2-H_2-CO_2 [80:10:10]) at 37°C utilizing brain-heart infusion (BHI) medium (DIFCO, France) and blood

Columbia agar plates (AES Chemunex, France) supplemented with hemin (5 µg/mL) and menadione (1 µg/mL) (Sigma Aldrich, France) [34].

Cellular localization of butyrolactone derivatives at the surface of oral bacteria surface.

Confocal microscopy (CLSM)

After inoculating 1 mL of BHI medium to have finally 3 x 10^7 CFU/mL of *S. gordonii* or *P. gingivalis*, they were incubated anaerobically for 18 or 48 hours, respectively. Then, the samples were centrifuged and the pellets were resuspended in PBS to be labeled by Syto 40 and/or B-13-NBD for 15 minutes. After that, 6 µL from each condition was transferred into a microscopic glass slide and visualized under CLSM.

For CLSM images, Leica-SP8 scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with an inverted microscope (Fluorescence Microscopy Platform, IFR 140 GFAS, Université de Rennes I) was used to visualize the slides *in situ*. The resonant option with 8000 Hertz was selected to capture the images utilizing the oil immersion objective lens (HC PL Apo 63X, 1.4 NA) where the numerical zoom was set at 8. The 405-nm excitation laser and 430-440 nm band-pass emission filter were used for Syto 40 and the 488-nm excitation laser and 506-535 nm band-pass emission filter in case of **B-13 NBD**. Leica software (LAS AF V.2.2.1) was utilised for image acquisition and microscope piloting and ImageJ software V1.48m (National Institute of Health) was used for images recovery.

CLSM was also used to evaluate bacterial viability after their exposure to compound **B-13**. This viability is investigated by using two nucleic acid-specific dyes: Syto 9 is membrane-permeable, stains all cells and can be detected by green fluorescence, and propidium iodide (PI) which is membrane impermeable, stains cells with damaged membranes and gives red fluorescence. Bacteria treated with **B-13** were visualized *in situ* utilizing a Leica TCS-SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with an inverted microscope (Fluorescence Microscopy Platform, IFR 140 GFAS, Université de Rennes I). The images were captured by the oil immersion objective lens (HC PL Apo 63X, 1.4 NA) applying 1.52 as a numerical zoom. For an entire bacterial detection with the dyes, specific excitation lasers and emission filters were used. The 488-nm excitation laser and 506-

539 nm band-pass emission filter was utilized for Syto 9 and the 561-nm excitation laser and 600-700 nm band-pass emission filter in case of PI. Image acquisition and microscope piloting were done by the Leica software (LAS AF V.2.2.1), and Comstat 2 plugin in ImageJ software V1.48m (National Institute of Health) was used to analyse the images.

HPLC-DAD analysis

In a 96-well plate (Sterile, Flat bottom, with lid, Biolite, Thermo Fisher Scientific, Korea) containing 100 µL of BHI, B-12 and **B-13**, 100 µL of *S. gordonii* were added into each well to have a final concentration of 3 x 10^7 CFU/mL. A replicate for each condition was prepared. After incubation under anaerobic condition at 37°C for 18 hours, the contents of the MIC/2 wells were collected in an ependorf and extracted using an optimized protocol of that described by Leejae et al. 2013 [35]. Briefly, the samples were centrifuged at 5000 rpm for 10 minutes at 4 °C. The pellets obtained were washed 2 times with a buffer containing (10 mM Tris.HCl of pH = 8, 0.1 mM EDTA, 1 mM DTT, 5% ethanol, and 50 mM NaCl). Then, the pellets were lysed by sonication on ice for 5-10 min to be centrifuged at 5000 rpm for 10 min at 4°C. Supernatants comprising the cytoplasm and the pellets containing the cell wall and cell membrane were separated to be extracted by ethyl acetate. The organic upper layer was transferred into a new tube to be washed with distilled water. Again, the upper layer was transferred into another tube in which anhydrous sodium sulfate was added. The remaining liquid was finally transferred into a new tube and evaporated to dryness for the powder to be dissolved in THF. HPLC analysis were performed on a Prominence Shimadzu HPLC system (Marne La Vallée, France) equipped with a C18 hypersil Gold aQ column (250 x 4.6 mm, 5 µm, Thermo Scientific) and consisting on a binary pump (LC-20ADSP), a surveyor autosampler (SIL-20AHT) and a diode array detector (SPD-M20A). The mobile phase consisted of (H_2O + 0.1% HCOOH) as solvent A and (ACN + 0.1% HCOOH) as solvent B with gradient: 0% of B during 5 min, 0%–100% of B during 5 min, 100% of B during 5 min, 100%–0% of B during 5 min, 0% of B during 10 min. The flow rate was 1 mL/min and 20 µL of each sample were injected. DAD data were recorded at 228 and 254 nm and absorption spectra (210–400 nm) were recorded each second. The two samples, pellet and supernatant of each condition, had their own HPLC chromatograms allowing us to determine in which one (cell wall/cytoplasm) the butyrolactones were detected. Identification of **B-13** in the samples was done by comparison of its retention time and UV-spectra with the standard under the same chromatographic conditions.

Transmission electron microscopy (TEM):

Following the overnight incubation, treated or untreated bacteria were collected, transferred to Eppendorf tubes and washed three times in Cacodilic buffer (0.15 M, pH 7.4). Fractions of bacterial suspension were fixed at 4°C for 60 min with 2% glutaraldehyde in 0.15 M Cacodilic buffer then washed three times in the same buffer. Cells were post-fixed in 1% OsO4 for 60 min at 4°C, rinsed in Cacodilic buffer and embedded in 2 % Low melting agar (Sigma). After dehydration series in acetone, the cells samples were embedded in conventional EPON (EMS 1420) and then polymerized at 60°C for 48h. Resins blocks were sectioned into 80 nm ultrathin sections using ultramicrotome LEICA UC7. These sections were mounted on copper grids and stained. Grids were observed using a TEM JEOL-JEM 1400 (Jeol Ltd, Tokyo, Japan) at an accelerating voltage of 120 kV and equipped with a Gatan Inc. Orius 1000 camera.

RNA extraction

A replicate of **B-12**, **B-13**, doxycycline or ampicillin was done in 100 µL of BHI in 96-well plate (Sterile, Flat bottom, with lid, Biolite, Thermo Fisher Scientific, Korea). Then, 100 µL of *S. gordonii* or *P. gingivalis* were added into each well to have a final concentration of 3 x 10^7 CFU/mL. After anaerobic incubation at 37 °C for 18 or 48 hours in case of *S. gordonii* or *P. gingivalis*, respectively, the contents of the MIC/2 wells of each condition were collected in an eppendorf to be centrifuged and pellets were treated for RNA extraction. Briefly, *S. gordonii*, a Gram-positive strain with a more rigid cell wall, needs FastPrep pre-treatment to lyse the cells. RNAs were isolated by using water-saturated phenol (pH 5.0), precipitated and washed with ethanol. Concerning *P. gingivalis*, their RNA were extracted using mirVana™ miRNA Isolation Kit (Ambion) 4°C.

The samples of both strains were then treated by TURBO DNA-free (Ambion), according to the manufacturer's instructions, to digest the contaminating DNA. Before and after TURBO DNA-free experiment, the RNA was quantified using a Nanodrop ND1000 (NanoDrop Technologies). After that, 12 µM of random primers (New England *Biolabs*) with 1 mM of dNTP (New England *Biolabs*) were added to 1µg of RNA. To denature sample RNA/primer,

the mix was incubated for 5 min at 70°C. After that, 1X ProtoScript II Buffer (New England *Biolabs*), 10 mM of dithiothreitol (DTT), 0,4 UI of RNase Inhibitor and 10 UI of ProtoScript II reverse transcriptase (New England *Biolabs*) were used to reverse-transcribe the RNA into cDNA. The RT-PCR procedure started with 5 min at 25°C, then, 60 min at 42°C, and finally, 5 min at 80°C. To determine if genomic DNA contamination was present or not, we have included a R*everse transcription* negative controls ("-RT") in real-time RT-PCR experiments. R*everse transcription* negative control is a mock reverse transcription containing all the RT-PCR reagents, except the reverse transcriptase. Then, the genes described in the primers list in table 2 were relatively quantified using StepOnePlus (Applied Biosystems) with the SYBR Green PCR Master Mix (Applied Biosystems) [36]. Each gene was normalized to the 16S rRNA internal control to calculate the fold-change values. At least, 1.5 fold change relative to the control was considered as a relevant gene expression change.

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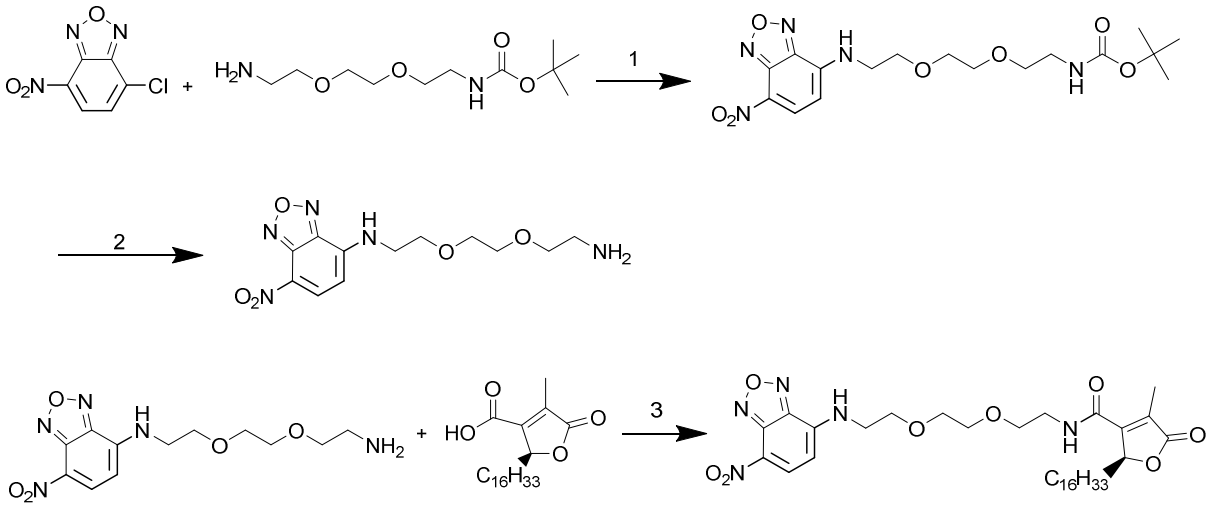
Table 1: Primers used in this study:

Strains	Genes	Right primers (5'→3')	Left primers (5'→3')
S.gordonii	Alanine racemase (*alr*)	AATCCGGCCCATACCAGAAT	GAGAGGGTGGCCTGGATAAA
	Glutamate racemase (*murI*)	ATGTGATGGGACCGGATGTT	CCACGCTTACCTTCTGTCCT
	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (murA)	ATCTCTGGTTCTCTAGCGGC	AAAGGCCTTGAAGCAATGGG
P. gingivalis	Alanine racemase (*alr)*	CGCTCTATCTCACCGGATGT	TGTCCGACATCTTGCAAAGC
	Glutamate racemase (*murI*)	TTCGGGGAACAATTTTGCGA	AAGCCTTGCGAACCATTCAG
	D-alanyl-D-alanine carboxypeptidase (*murA)*	GTGCCGTGCCTCATATCAAG	ACAGACCACGCTCTGTTGTA

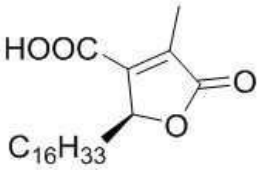
Scheme 1: Synthesis of fluorescent probe **B-13 NBD**



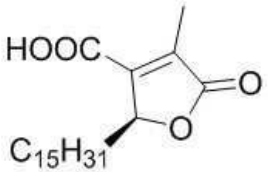
1 : CH_3CN, Cs_2CO_3 2 eq, 40°C, 30 min.

2 : CH_2Cl_2, TFA, Na_2SO_4 2 eq, rt, 1 h

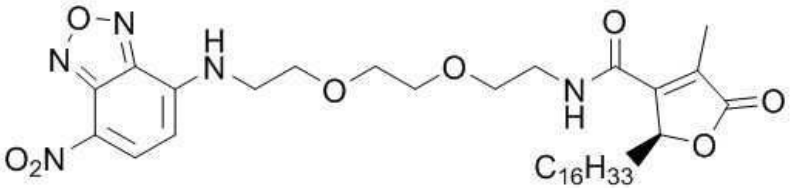
3 : DMF anhydrous, DIEA anhydrous 2 eq, TBTU 1.28 eq, Ar, rt, 18 h.



B-13



B-12



B-13 NBD

Figure 1: Chemical structures of **B-12**, **B-13** and **B-13 NBD**

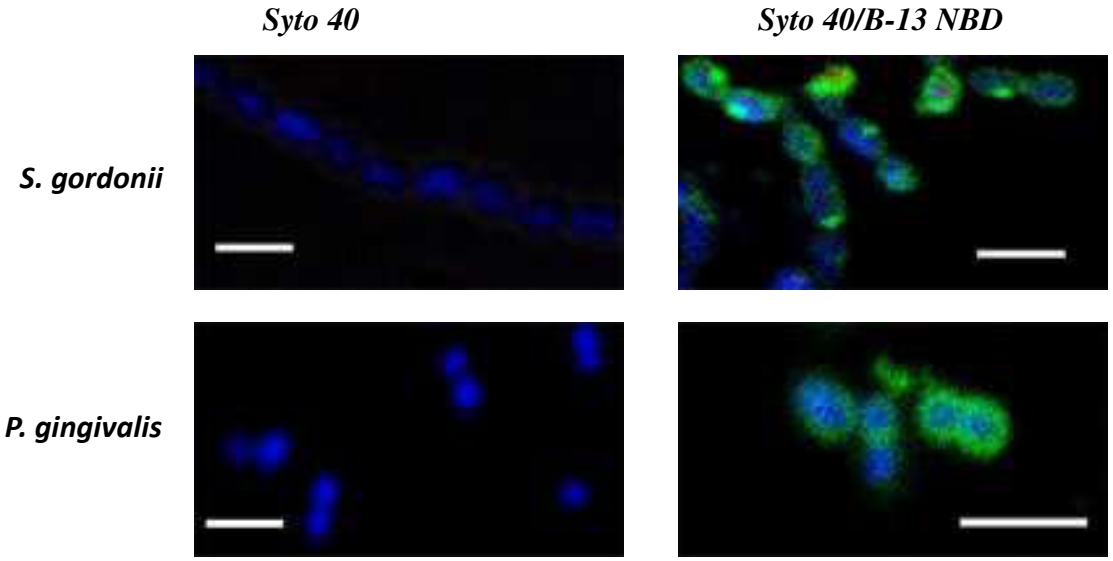
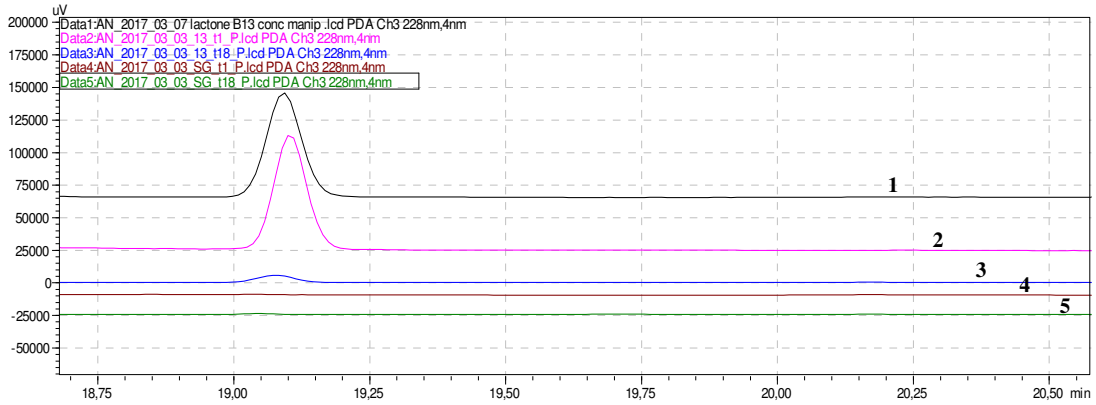


Figure 2: Localization of fluorescently labeled **B-13 NBD** on *S. gordonii* and *P. gingivalis* by confocal microscopy. **B-13 NBD** adhered to bacteria surface. Bacteria DNA is stained by Syto 40 in blue and **B-13 NBD** is seen in green. Bars represent 2µm

a



b

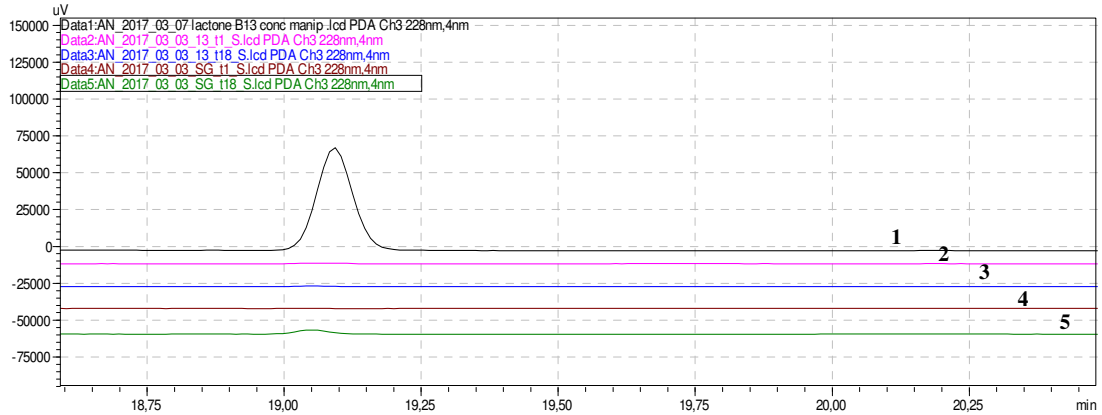


Figure 3: Localization of **B-13** in *S,gordonii* as determined by HPLC chromatogram. *S. gordonii* after incubation with **B-13** was lysed, and the cell wall and cell membrane were separated from the cytoplasm by centrifugation. (a) represents HPLC elution patterns of cell wall and membrane and (b) corresponds to cytoplasm. **B-13** are found in cell wall and membrane fraction of lysed *S. gordonii*. (1) **B-13** as reference, (2) **B-13** incubated with *S. gordonii* for 1h, (3) *S. gordonii* for18h, (4) *S. gordonii* without compound for 1h, (5) *S. gordonii* without compound for 18h.

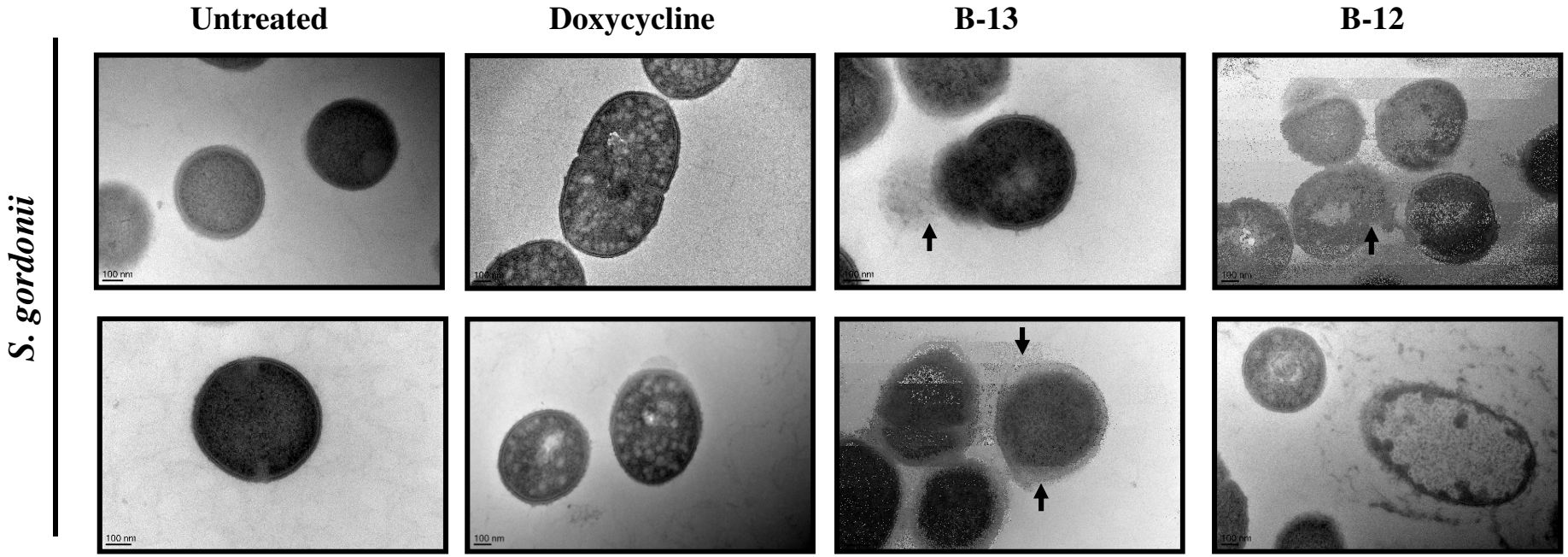


Figure 4:Transmission electron micrographs of *S. gordonii*. **B-13** as **B-12** disrupted *S.gordonii* cell wall (as indicated by black arrows).

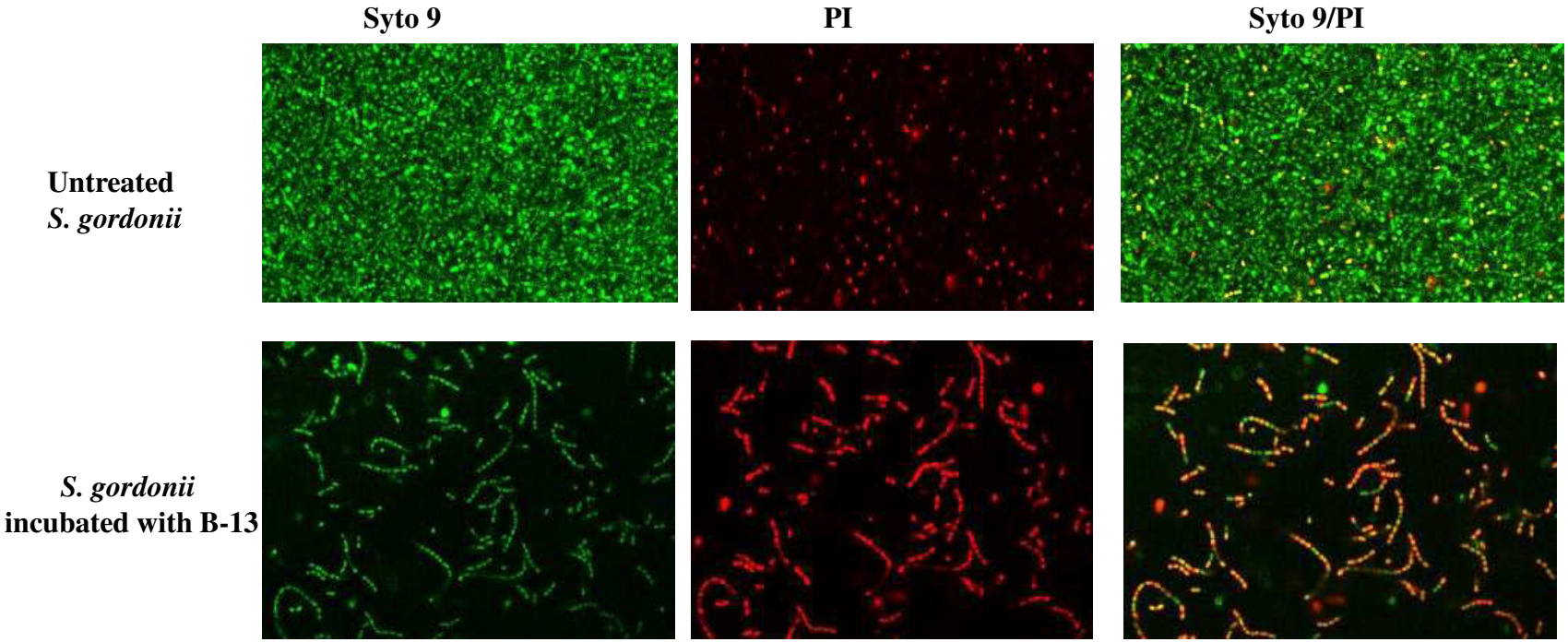


Figure 5: *S. gordonii* cell suspension, untreated or treated with **B-13**, stained with Syto 9 and PI, analysed by CLSM. Cells with membrane undisturbed stained by green fluorescence whereas cells with ruptured surface stained by red fluorescence. The overlap of the green and the red appears as orange.

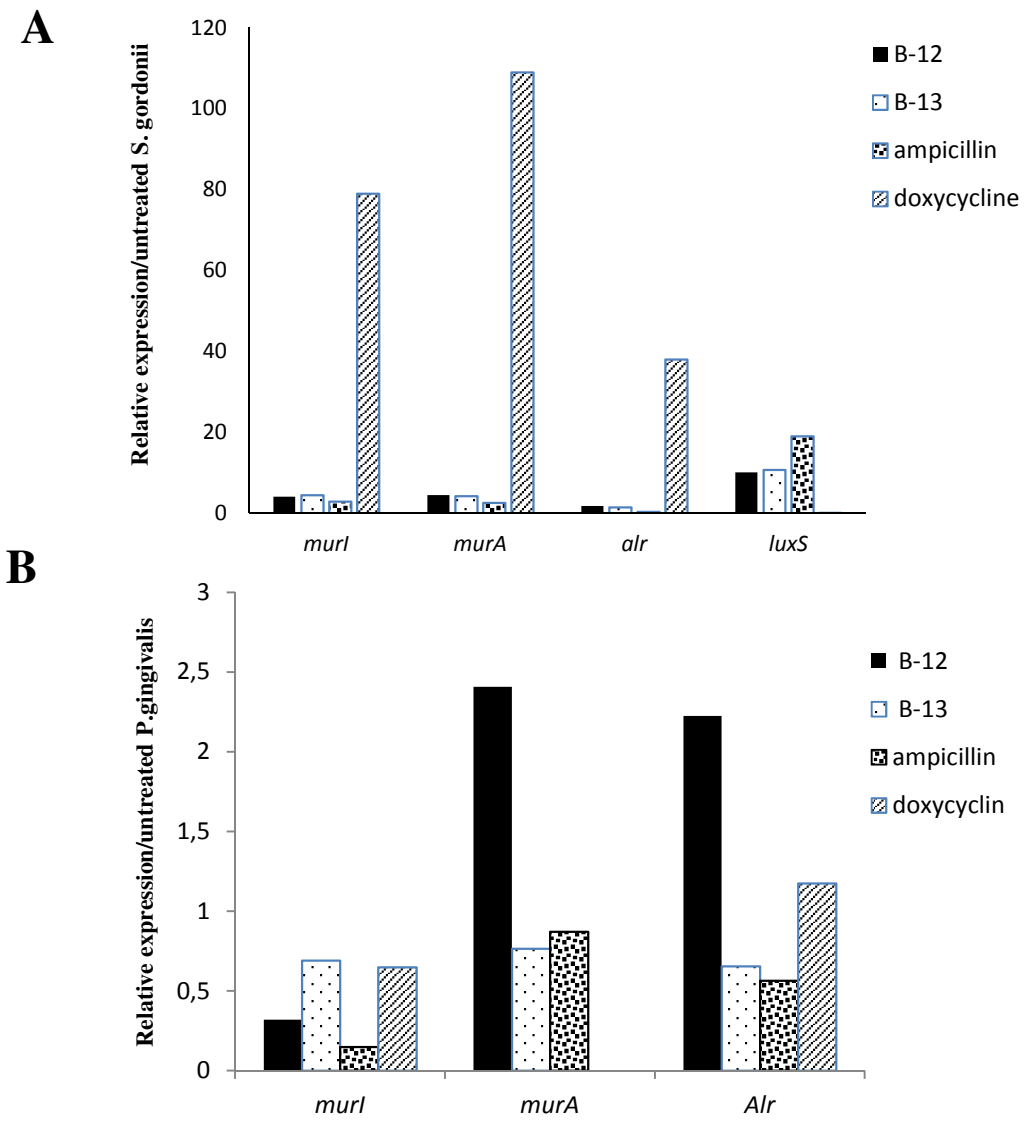


Figure 6: Analysis of selected peptidoglycan genes by qRT-PCR after treatment of *S. gordonii* (A) or *P. gingivalis* (B) with **B-13** and **B-12** or antibiotic controls (doxycycline, ampicillin).

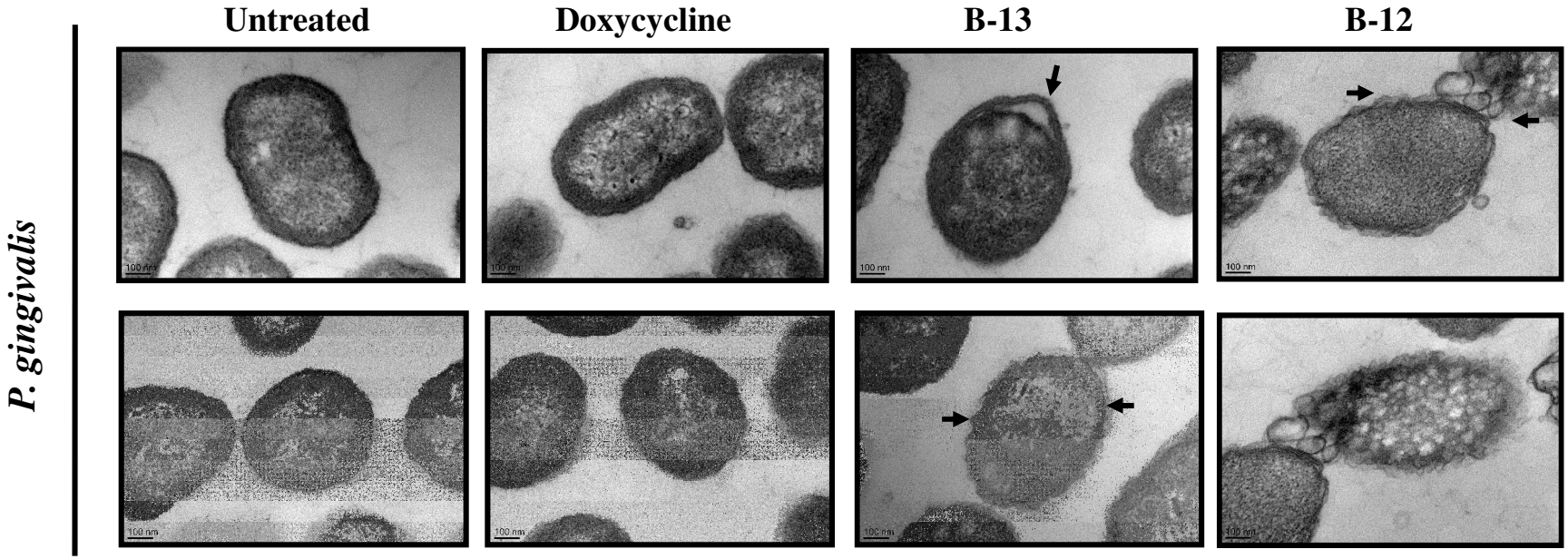


Figure 7:Transmission electron micrographs of *S. gordonii* et *P. gingivalis.* **B-13** as **B-12** disrupted *S.gordonii* and *P. gingivalis* cell wall (as indicated by black arrows).

GENERAL DISCUSSION AND CONCLUSIONS

E- General discussion and conclusions

In a purpose to support the scientific community with a modest contribution concerning the periodontal disease complications and against the universal bacterial resistance invading our patients, the present thesis was launched trying to find a new generation of efficient antibiotics from the medicinal natural sources which were shown to possess many biological potentials including strong antibacterial activity.

One of the most important ecological niches of microorganisms in the human body is the oral cavity which represented our battle field [1]. The oral bacteria form a dental biofilm which can lead to gingivitis due to bad oral hygiene. Several factors discussed in the introduction such as systemic diseases can contribute to the probability of gingivitis progression to a more advanced stage called periodontitis [2]. Not only will the teeth be lost but also several systemic complications as cancers can occur as a result of this stage [3], [4]. To combat these oral and the consequent beyond-oral complications, in addition to the universal bacterial resistance crisis, we tried to uncover new antibiotics of natural origin as the latter have been reported to be very efficient against bacterial infections [5]–[7]. Lichens were chosen being the promising natural source known for their biological potencies and especially their antibacterial activity via the secondary metabolites they secrete [8]–[12].

A panel of natural lichen compounds belonging to different classes of structures and spanning from linear into cyclic and aromatic features were screened by broth microdilution method against the oral infection-implicated bacteria, *Streptococcus gordonii* and *Porphyromonas gingivalis*. The results showed that (+)-roccellic, demethylbarbatic, psoromic, and lichesterinic acids were the best, with lichesterinic acid being the most active and *P. gingivalis* was shown to be more sensible than *S. gordonii* (Article 1, under revision).

Starting with article 1, the natural lichen compounds showed differential activities according to their structures (Figure 1 and table 2, article 1). We can find among them 5 compounds that possess close structure, **C**, **D**, **H**, **P** and **Var**. Compounds **C**, **H** and **Var** were less active than **D** and **P**. Regarding **C** and **Var**, they showed different activity regarding the Gram type of the bacteria. **C** was more effective against *P. gingivalis* (Gram-negative) whereas **Var** was more active to kill *S. gordonii* (Gram-positive). This result is in accordance with those of protocetraric and lobaric acids which showed a good activity against *Salmonella typhi* [13] and *P. gingivalis* [14], respectively. Then, we can conclude that some functional groups have a selective antibacterial activity that will target a certain type. Summarizing the structural differences, we can conclude the importance of the following groups in depsidone core to obtain the best

antibacterial activity: a) An aldehyde group at carbon 3, b) A methyl group at carbon 3' instead of CH_2OH, c) A hydroxyl or methoxy group at carbon 2' and d) presence of a carboxyl group.

The lipophilicity of compounds can play an important role in their antibacterial properties since the bacterial lipid membrane is lipophilic. Nevertheless, other physicochemical properties such as pKa could be an important parameter to determine the partition coefficient of these lichen compounds as already mentioned by Honda et al [15]. All the active compounds possess a carboxylic group indicating that these compounds are mostly ionized at pH 7. Our results are in agreement with those reported previously [15].

After that, we have focused on the forgotten antibiotic, lichesterinic acid, where we have synthesized a butyrolactone series trying to obtain the best possible activity. After testing their antibacterial activity against the Gram-positive strain, *S. gordonii*, by agar dilution and broth microdilution methods, the best compounds were checked for their cytotoxicity against gingival epithelial cells, Ca9-22, and macrophage-like cells, THP-1, by LDH and MTT assays (article 2). The complementary antibacterial activity of these derivatives against the second strain, *P. gingivalis*, is presented in article 3.

Several important points are worth to be addressed: i) which Gram-type was more sensitive to butyrolactones, ii) the differences between the two antibacterial testing methods, iii) the butyrolactones structure-activity relationships, and iv) the cytotoxic activity of the derivatives.

i) Regarding the butyrolactones efficiency, *S.gordonii* was shown to be less sensitive to these derivatives than *P.*gingivalis. Being *S. gordonii* more resistant to the antibacterial agents than *P. gingivalis* was not always the case as shown in other studies. Tsaousoglou et al. have reported 3 different responses of these 2 bacterial strains in their planktonic state against 3 different antibiotics. They respond similarly to ofloxacin, whereas, in the presence of moxifloxacin, *S. gordonii* was more resistant. In contrast, *P. gingivalis* was less sensitive to doxycycline [16].

ii) Comparing the testing methods, we have realized that the compounds were more efficient in the liquid medium (broth microdilution method) than in the solid medium (agar dilution) except for doxycycline which registered approximately the same activity in both media. This can be explained by the ability of the compounds to move more freely and inhibit the bacteria in the liquid medium compared to the solid counterpart where the bacteria are restricted to the inoculation zone at the middle of the agar surface. These differential results due to the medium utilized was previously mentioned by Guzman et al. who have tested natural compounds from Columbian plants against *Mycobacterium tuberculosis* and obtained discrepant results related to the medium used in the testing method [17]. This coincides also

with Hammer et al. who demonstrated differences in the essential oils and other plant extracts MICs obtained by these two methods reaching two serial dilutions against *Escherichia coli* and *Staphylococcus aureus* [18]. However, other studies have shown that the two methods can give similar results in certain conditions. For instance, Klancnik et al. have used several testing methods including agar dilution and broth microdilution to evaluate plant extracts, their mixtures and phenolic acids on three Gram-positive strains (*Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes*) and four Gram-negative bacteria (*Escherichia coli* O157:H7, *Salmonella infantis*, *Campylobacter jejuni*, and *Campylobacter coli*). They have reported comparable results and a good level of agreement only for Gram-positive bacteria [19]. This contradicts our results concerning the Gram-positive strain used in our study, *S. gordonii*, which displayed vividly distinct results obtained by the two methods.

iii) With respect to the chemical structure and the groups that stand behind the antibacterial activity, two sites were proposed to be involved: a) the aliphatic chain, and b) the carboxyl group. In addition, the stereochemical configuration has also played a role.

The aliphatic chain has clearly interfered in the activity of butyrolactones. In articles 2 and 3, this issue has been extensively discussed. The attribution of this chain to the activity which is affected by number of carbon atoms constituting this tail was more interpretable on case of *S. gordonii*. As the length increased the bactericidal activity increased, whereas there is an optimum length to get the best inhibitory activity and it was shown to be formed of 13 carbon atoms. This was not the case of *P. gingivalis* except when the COOH group was substituted on the butyrolactone ring. But, both activities, bacteriostatic and bactericidal, were improved as the length increased with no optimum length being realized. This chain length contribution was discussed by Yang et al. where they tested the derivatives of 8-alkylberberine against Gram-positive and Gram-negative strains to find an optimum length of 8 carbon atoms. Shorter or longer chains showed lowering in the antibacterial activity. They also mentioned that Gram-positive strains were more susceptible to these derivatives [20].

The second important site, the carboxyl group, was the second key to obtain the efficient activity as shown from the results obtained for both strains in articles 2 and 3. The importance of this functional group was mentioned by Sebastianes *et al* who tested the antibacterial activity of a fungal compound, 3-hydroxy propionic acid, 3-HPA, against *Staphylococcus aureus* and *Salmonella typhi*. Indeed, 3-HPA showed

relevant antibacterial activity against the tested strains. When it was esterified to produce 3-hydroxypropanoic ethyl ester, no antimicrobial activity was registered [21]. It is worth noting here that when we labeled the butyrolactone **B-13** by esterifying the carboxyl group and introducing NBD-chloride, the antibacterial activity remained but was lowered (Article 4).

The presence of both structures, the aliphatic chain and the carboxyl group, were indispensable for having the efficiency obtained, since the absence of one or both of them has led to the absence or lowering of the activity.

The stereochemical configuration didn't show any interference against *S. gordonii* in article 2. Both configurations had the same MICs and MBCs. But, in case of *P. gingivalis*, the two stereoisomers had different results where the 5S enantiomers were more active than the 5R counterparts. Several authors have previously described the role of stereochemistry in having different activities for the stereoisomers. Gerster et al. has mentioned that the S isomer of 6,7-dihydro-5,8-dimethyl-9-fluoro-1-oxo-1*H*,5*H*-benzo[ij]quinolizine -2-carboxylic acid was much more active than its R counterpart against several Gram-positive and Gram-negative bacteria [22]. Wakiyama et al have also demonstrated that the 7(S)-configuration of lincomycin derivatives was necessary for enhancing the antibacterial activity against respiratory infection-related Gram-positive bacteria [23].

iv) Concerning the cytotoxic activities of the selected butyrolactones, **B-12** and **B-13** were shown to be non-toxic against gingival epithelial cells and macrophages at their MICs. This provides a promising profile of butyrolactones to be used as an oral antibiotic safe on the gingival and immune cells of the host.

Finally, by comparing the antbacterial activity of the butyrolactones and the natural lichen compounds against the very strains, we can conclude that both of them have showed greater efficacy against the Gram-negative *P. gingivalis* in comparison to the Gram-positive *S. gordonii*. However, the buyrolactone derivatives were more active (Articles 1 and 2).

In the following step, and after screening the natural lichen compounds and lichesterinic acid derivatives against *S. gordonii* and *P. gingivalis*, the most active butyrolactone analogues, **B-12** and **B-13**, were evaluated for their antibiofilm activity against the same strains monospecies biofilms by crystal violet assay (article 3). *P. gingivalis* was more sensitive than *S. gordonii* coinciding with the antibacterial activity. However, the sessile cells were more resistant than the planktonic cells and this agrees with previous studies [24], [25]. This antibiofilm activity was confirmed by the confocal microscopy images which showed clearly the potency of these derivatives to interfere efficiently with the biofilm formation of the strains tested. Hence, this

antibiofilm activity confers the butyrolactones a considerable importance since biofilm inhibitors don't cause resistance immediately as mentioned by Stadler et al. [26].

After that, we tried, as shown in article 3, to get close to the mechanism of these derivatives by which they inhibit the biofilm formation. Several genes implicated in the monospecies biofilm formation of both strains were quantified by qPCR after treating these biofilms with sub-inhibitory concentrations of the derivatives. There is a universal importance of these concentrations because they act as selectors of resistance, generators of genetic and phenotypic variations, and signaling molecules modulating several physiological activities such as virulence, biofilm formation and gene expression [27]. The antibacterial compounds have up-regulated the expression of the chosen genes and consequently, promoted the biofilm formation. This can be predicted as the crystal violet assay has shown a weak antibiofilm effect at sub-MIC concentrations of the compounds. Alongside, other previous studies have reported this issue where the biofilm formation has been favored at sub-MIC of several antibiotics including tetracyclines where one of which, doxycycline, has displayed this effect in our present study. Ahmed et al. has mentioned that the sub-MICs of three antibiotics used in their study, ampicillin, ciprofloxacin, and tetracycline, have increased the biofilm formation of *Streptococcus intermedius* WT due to the role of autoinducer-2/LuxS [28]. This proposes that these actors could be the reason behind the increased biofilm formation in our case. This is supported by the fact that LuxS was one of the genes quantified and showed to be highly expressed in the presence of MIC/2 of the compounds. Also, Aka and Haji have shown that incubating *Pseudomonas aeruginosa* isolates with sub-MICs of antibiotics in the presence of chlorhexidine has stimulated biofilm formation [29]. Nevertheless, the induction of biofilm formation in the presence of MIC/2 of the highly efficient antibacterial butyrolactones and the involvement of AI-2/LuxS in the intercellular signaling as a bacterial survival strategy need further investigations.

Finally, article 4 went more deep in trying to understand the mode of action of butyrolactones and how they inhibit the strains used in this study. They were shown by transmission electron and confocal microscopy along with HPLC to target the cell wall which is one of the most efficient modes of actions including also targeting the ribosomes or DNA topoisomerases [30]. What makes the cell wall-targeting antibiotics more attractive than the others is that the eukaryotic cells comprise ribosomes and DNA topoisomerases which are the bacterial targets of these antibiotics suggesting that the eukaryotic cells can be more vulnerable to the latter.

The antibiotics targeting the cell wall as the butyrolactones may be favored over the others in treating the oral biofilms, since the bacteria will grow significantly more slower in its biofilm phase, thus, these metabolically reduced-activity bacteria regarding their biosynthesis of proteins, RNA, DNA, peptidoglycan, and folic acid, will be less inhibited by the antibiotics

targeting these processes such as ribosome and DNA topoisomerases inhibitors [39]. *Enterococcus faecalis* oral biofilms has been shown to require very high concentrations of antibiotics such as ampicillin (peptidoglycan synthesis inhibitor), vancomycin (peptidoglycan synthesis inhibitor), and linezolid (protein synthesis inhibitor) [30], [33].

Targeting the cell wall can be by targeting the synthesis of its components as peptidoglycan or it can be by binding directly to the bacterial membrane bilayer thereby disrupting physically its integrity and its functions. Hurdle et al. has mentioned also that the antimicrobials can target either the bacterial membrane organization or the functions of membrane-associated respiratory enzymes. Figure 20 summarizes these two pathways [39].

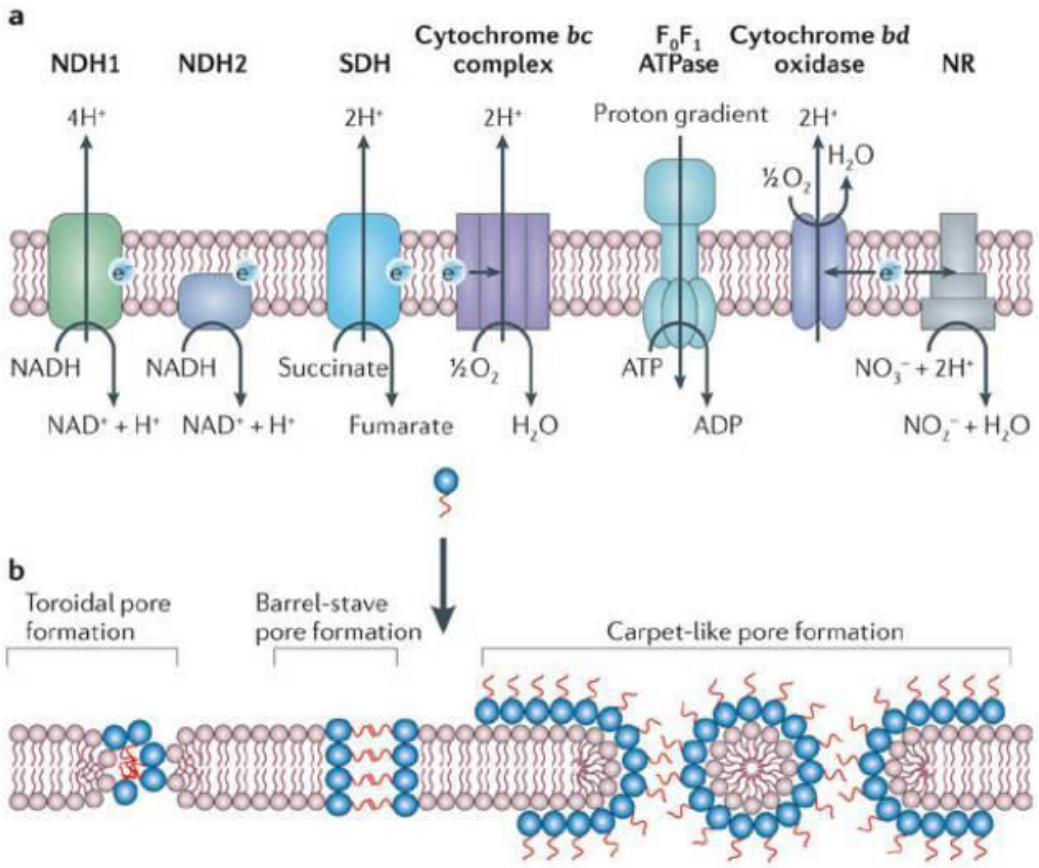


Figure 20: The antimicrobials can target the functions of membrane associated respiratory enzymes (a) or bind directly to the membrane and disrupts its physical integrity.

Daptomycin is a cyclic lipopeptide antibiotic which acts by inserting its lipophilic tail into the bacterial membrane leading to fast membrane depolarization and potassium ion efflux. This results in blocking the DNA, RNA, and protein synthesis and finally cell death. This antibiotic has a very efficient cidal activity rapidly killing more than 99.9% of methicillin-resistant and -susceptible *Staphylococcus aureus* (MRSA, MSSA) in less than one hour and remains bactericidal within 24 hours against the stationary phase cultures of these two strains having 10^9 CFU in a

simulated endocardial vegetation model. It has a broad-spectrum activity profile with the capacity to inhibit MRSA, MSSA, glycopeptide-intermediate *S. aureus* (GISA), methicillin-resistant coagulase-negative *Staphylococcus* spp. (CoNS), and vancomycin-resistant enterococci (VRE). Phase III clinical trials has confirmed the safety of this antibiotic and its efficacy against several pathogens including one of the oral biofilms forming strains, *Enterococcus faecalis* [40].

Butyrolactones have been shown to target the cell wall by several techniques, however, whether they bind and disrupt the membrane directly or inhibit the synthesis of some of its components couldn't be known by the transmission electron microscope which showed cells with disrupted membrane and this can be the reason of the cell death (binding directly and disrupting it) or it can be the result (inhibiting the synthesis of some of the cell wall components resulting in the disruption seen by electron micrographs). After adhering to the cell surface, **B-13** induced cell wall disruption leading to the release of bacterial constituents inducing the death of *S. gordonii.* The expression of the two genes, *murA* and *alr*, implicated in cell wall synthesis, were modified in presence of this butyrolactone. Gram-negative bacteria such as *P. gingivalis* showed also cracked and ruptured cells in presence of **B-13**, suggesting that this butyrolactone acts on Gram-positive and Gram-negative bacteria. However, it showed greater efficacy against the Gram-negative strain in comparison to the Gram-positive counterpart. Besides, we also demonstrated that the analogue of **B-13**, **B-12**, has also induced the disruption of *P. gingivalis* and *S. gordonii.* This study has demonstrated that the lichen butyrolactone derivatives have disrupted the cell wall of oral bacteria and that this effect was associated with an increase of genes implicated in peptidoglycan synthesis for the Gram-positive such as *S. gordonii*, suggesting that this strain response to the stress was generated by this antiobiotic [41]; whereas in the Gram-negative such as *P. gingivalis* where the effect was more important, these genes were downregulated. These results suggested that its antimicrobial potential is influenced by the composition of the cell wall of the microorganisms [42].

The doubt of being the butyrolactones bind the membrane directly and cause its disruption as shown in the transmission electron micrographs, or this effect is the consequence of the butyrolactones binding to something else inside the cell leading to membrane rupture was raised by the HPLC technique. The latter has proved the presence of butyrolactone derivatives in the cell wall fraction without being detected in the cytoplasmic one, proposing that they bind directly to the bacterial membrane and exert their effect. This has been supported by the confocal laser scanning microscope images where we have investigated the bacterial localization of the butyrolactones by synthesizing **B-13** labeled with NBD (**4-nitro-benzo[1,2,5]-oxadiazole)** keeping its antibacterial activity. This has shown vividly that the butyrolactone derivative (**B-13**) has bound the bacterial membrane as a ring, whereas the DNA labelings, Syto9 and Propidium Iodide (PI), were concentrated in the middle of the bacterial cells.

A known antibiotic, daptomycin, shares a close structural moiety with the butyrolactone derivatives and still big differences exist between the two. Possessing a lipophilic chain by butyrolactones as that of daptomycin also enforces the belief these derivatives bind directly to the bacterial membrane by inserting its aliphatic chain between the membrane entities. Also, the chain of butyrolactones is only a simple saturated carbon chain of 12 members in **B-12** or 13 in **B-13** compared to a shorter and highly more complicated counterpart in daptomycin. In addition, a giant head is found in daptomycin compared to a small 5-membered ring in butyrolactones. This suggests that they can share the binding step and the membrane perturbing result, but differ in the intermediate phase. Action of daptomycin is schematized in figure 21 which can propose a way by which the butyrolacones can bind and lead to membrane disruption [40]. However, since the butyrolactones skeleton is a bad metal chelator due to the absence of the strong chelating sites [36], binding of butyrolactones can't depend on the concentration of calcium as daptomycin or any other metal in the medium. Furthermore, butyrolactones can't oligomerise as their head structure suggests, but can interact with other chemical entities present on the surface of the bacterial membrane near their binding site. The CLSM images of fluorescently labeled butyrolactones in article 4 and the fluorescently labeled daptomycin in Pogliano et al. show different appearance on the staining. Butyrolactones formed a ring coating the entire bacterial surface with the same intensity, whereas daptomycin appeared as discrete foci and it stained intensely the active dividing site [37].

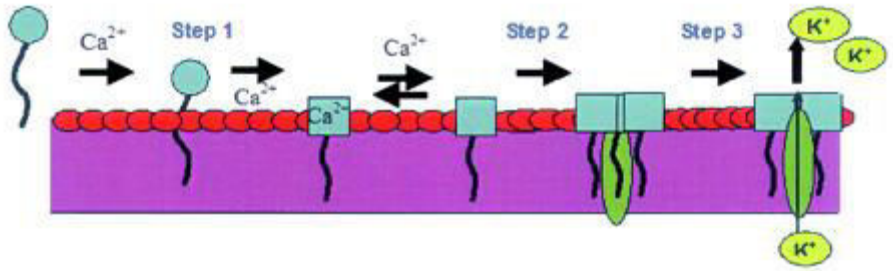


Figure 41: Daptomycin exerts its action in several steps starting by binding in step 1 to the membrane in a calcium-dependent manner. Then, in step2, daptomycin monomers oligomerise and disrupt the membrane. Finally, the intracellular ions are released in step 3 leading to cell death.

Another important probability can be proposed where the buytrolactones can act as inhibitors of quorum sensing systems since they share a structural similarity with the communicating molecules used among the Gram-negative bacteria. The butyrolactones can act as antagonists competing acyl-homoserine-lactones for their binding sites leading to quorum sensing perturbation and inhibition of its consequent virulence and biofilm formation [38], [39].

Moreover, these butyrolactones have a similar structure to the γ-butyrolactone autoregulators described formerly (Figure 22). The latter are produced by the Gram-positive Streptomyces

genus and they regulate the DNA binding activity of cognate receptor proteins triggering antibiotic production as mentioned by Kitani et al. [40]. Hence, butyrolactone analogues may modulate the DNA binding activity of some proteins.

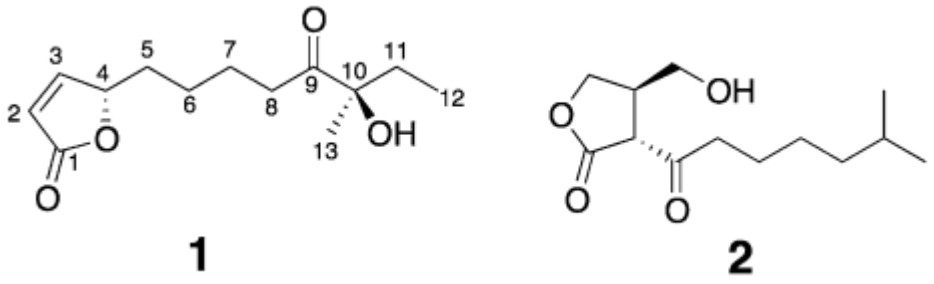


Figure 22: Example of the chemical structures of the γ-butyrolactone autoreguators, 1) Natural avenolide, and 2) A-factor.

To conclude, the butyrolactones were shown to be capable of efficiently inhibiting the Gram-positive early colonizer, *S. gordonii*, and the Gram-negative late colonizer, *P. gingivalis*, which is one of the most important pathogenic bacteria in the periodontal diseases where many authors have mentioned it as the etiological agent of this disease. When the healthy sites start to change into the diseased status, the microbial species present change gradually from mostly Gram-positive into mostly Gram-negative, respectively. Hence, these compounds can prevent or treat the oral infection as they can be used in the early, transit or advanced stages.

In addition, the butyrolactones were demonstrated to be stronger than the antibiotic doxycycline, safe on the gingival epithelial cells and macrophages, and efficient preventive antibiofilm agents; this introduces them to be used as prevention in early detection of the disease by the dentist to block its progression and reverse its pathway towards the healthy one, or as a treatment of periodontal disease. They can be provided as mouthrinses or adjunct therapy to mechanical debridement or after surgeries to kill the remnant pathogens more efficiently than doxycycline. Thus, the patient will require less time for his diseased sites to improve. Moreover, their usages can help reduce the need of surgeries for the patient.

PERSPECTIVES

F- Perspectives

1- After analyzing the structure-activity relationship of the efficient natural lichen compounds, and concluding the active sites involved in their antibacterial activity, it will be possible to synthesize some derivatives containing functional groups known in the literature for the antibacterial potency they provide to the hosting compound thereby improving the latter's efficiency, for instance, COOH group. By this way, the MIC will be lowered that is the concentration needed from this compound to treat the targeted infection in the host will be lowered and consequently, cytotoxicity will be avoided as much as possible.

2- The efficient natural lichen compounds and the butyrolactone derivatives of lichesterinic acid were shown to be effective against the Gram-positive, *S. gordonii*, and the Gram-negative, *P. gingivalis*, oral bacteria. It will be worth to test their efficiency against other sensitive or multi-drug resistant bacteria implicated in the oral, and in other systemic infections as well. This will also show if the greater sensitivity of the Gram-negative bacteria tested in this study in comparison to the Gram-positive counterpart is a universal fact that applies to other strains of the same Gram type as well. Furthermore, the difference in the efficiency of the stereoisomers seen against *P. gingivalis* is important to check its existence also against other bacterial strains of the same or distinct Gram type.

3- The dental biofilms are very resistant and require sometimes an antibiotic concentration that can reach to 500 times more than the systemic therapeutic dose as discussed earlier, hence, the mechanical methods are needed to disturb the biofilm physically and allow the antibiotics have access to the pathogenic bacteria. Since the butyrolactones have a strong preventive antibiofilm activity as shown in this project, it will be worth to test if they may have a strong curative antibiofilm activity as well. If butyrolactones were shown to eradicate the biofilm efficiently, they can be proposed as a standalone therapy without the need for mechanical debridement or surgeries.

4- The butyrolactone derivatives were shown to be non-toxic against gingival epithelial cells and macrophages at their MICs. However, a higher concentration of the antibacterial agent is sometimes needed as described in the previous point due to the biofilm resistance. Hence, testing a concentration gradient will be important to check the butyrolactones toxicity at a higher concentration. In addition, evaluating their toxicity against other cell types will be a must to check their systemic effects. Will they affect the red blood cells or immune cells other than macrophages if given through the blood for instance?

5- It is indispensable to test the ability of butyrolactone derivatives to inhibit bone resorption and promote periodontium reattachment which are very important

characteristics that an oral antibiotic is preferred to have. In addition, testing their anti-inflammatory capacity is highly considerable to be evaluated.

6- Sharing some structural and functional characteristics between butyrolactone derivatives and the known antibiotic, daptomycin, can predict a part of the butyrolactone analogues mode of action story. As a result, further investigations are needed to discover the actual mechanism of these derivatives.

7- The similarity between butyrolactone derivatives and acyl-homoserine-lactones (AHL) used in the quorum sensing systems between Gram-negative bacteria can suggest their interference in these systems as antagonists for the original communicating molecules leading to quorum sensing inhibition and consequently, virulence and biofilm formation. Testing their ability to interfere in the quorum sensing systems is a very important perspective to combat the biofilm infections that are very hard to be treated with the conventional antibiotics.

8- Similar structure to butyrolactone derivatives was also found in the γ-butyrolactone autoregulators. The latter regulate the DNA binding activity of cognate receptor proteins triggering antibiotic production. Hence, it will be important to check if butyrolactone analogues can modulate the DNA binding activity of some proteins and trigger antibiotic production.

9- Establishing *in vivo* studies of butyrolactone derivatives is important to be performed as they possess promising potencies to graduate into the clinical trials before introducing them into the market as a new generation of efficient antibiotics which differ in structure and mode of action from all the other antibiotics known to date. The butyrolactone derivatives can be a new resort for the patients in this post-antibiotic era.

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ANNEXE

Original articles

1- A. Sweidan, M. Chollet-Krugler, P. van de Weghe, A. Chokr, S. Tomasi, M. Bonnaure-Mallet, L. Bousarghin, Design, synthesis and biological evaluation of potential antibacterial butyrolactones, Bioorg. Med. Chem. 24 (2016) 5823–5833.

2- A. Sweidan, M. Chollet-Krugler, A. Sauvager, P. van de Weghe, A. Chokr, M. Bonnaure-Mallet, S. Tomasi, L. Bousarghin. Antibacterial activities of natural lichen compounds against *Streptococcus gordonii* and *Porphyromonas gingivalis*. Fitoterapia (Under revision).

3- A. Sweidan, Z. Tamanai-Shacoori, M. Chollet-Krugler, N. Oliviero, A. Chokr, M. Bonnaure-Mallet, P. van de Weghe, S. Tomasi, L. Bousarghin. Antibiofilm activity of butyrotactone derivatives against oral bacteria. Applied Microbiology and Biotechnology (Under preparation).

4- A. Sweidan, I. Smida, M. Chollet-Krugler, A. Sauvager, J. Vallet, N. Gouault, N. Oliviero, A. Burel, P. van de Weghe, A. Chokr, S. Tomasi, L. Bousarghin. Lichen butyrolactone derivatives disrupted the cell wall of oral bacteria. Journal of American Chemical Society (Under preparation).

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1- A. Sweidan, M. Chollet-Krugler, P. Van de Weghe, A. Chokr, S. Tomasi, L. Bousarghin. Antibacterial activity of some lichen compounds analogues on *Streptococcus gordonii*. 6ème Journée des doctorants, 20-01-2016, Rennes I University, Faculty of Pharmacy, poster and oral communication.

2- A. Sweidan, M. Chollet-Krugler, P. Van de Weghe, A. Chokr, S. Tomasi, L. Bousarghin. Treatment of *Streptococcus gordonii* infections by lichen butyrolactones. Young Research Fellow Meeting 2016, 15-17 February 2016, Lille University, Faculty of Pharmacy, flash poster presentation.

3- A. Sweidan, M. Chollet-Krugler, P. Van de Weghe, A. Chokr, S. Tomasi, L. Bousarghin. Antibacterial activity of butyrolactone analogues on *Streptococcus gordonii*. Conférence Internationale : Host-pathogen interactons : from bench to bedside, 2-3 Juin, 2016, Nantes, oral communication.

4- A. Sweidan, Z. Tamanai-Shacoori, M. Chollet-Krugler, N. Oliviero, A. Chokr, M. Bonnaure-Mallet, P. van de Weghe, S. Tomasi, L. Bousarghin. Antibacterial and antibiofilm activities of a butyrolactone series against oral bacteria. 7ème Journée des doctorants, 18-01-2017, Rennes I University, Faculty of Pharmacy, flash poster presentation.

Antibiofilm activity of lichen secondary metabolites

Alaa SWEIDAN, 2017

The oral bacteria do not only infect the mouth and reside there, but also travel through the blood and reach distant body organs. If left untreated, the dental biofilm that can cause destructive inflammation in the oral cavity may result in serious systemic medical complications. In dental biofilm, *Streptococcus gordonii*, a primary oral colonizer, constitutes the platform on which late pathogenic colonizers like *Porphyromonas gingivalis*, the causative agent of periodontal diseases, will bind. The aim of the first study was to determine the antibacterial activity of eleven natural lichen compounds belonging to different chemical families and spanning from linear into cyclic and aromatic structures to uncover new antibiotics which can fight against the oral bacteria. Three compounds were shown to have promising antibacterial activities where the depsidone core with certain functional groups constituted the best active compound, psoromic acid, with MICs = 11.72 and 5.86 µg/mL against *S. gordonii* and *P. gingivalis*, respectively. The compounds screened had promising antibacterial activity which might be attributed to some important functional groups.

Novel butyrolactone analogues were then designed and synthesized based on the known lichen antibacterial compounds, lichesterinic acids (**B-10** and **B-11**), by substituting different functional groups on the butyrolactone ring trying to enhance its activity on *S. Gordonii* and *P. gingivalis*. The substituents were hydroxyl, vinyl or carboxyl groups and/or an alkyl chain of different lengths. Saturated analogues were also designed. Among the derivatives, **B-12** and **B-13** had the lowest MIC of 9.38 µg/mL where they have shown to be stronger bactericidals, by 2-3 times, than the reference antibiotic, doxycycline. **B-12** and **B-13** were also the most efficient on *P. gingivalis* exhibiting MIC of 0.037 and 0.293 µg/mL and MBC of 1.17 and 0.586 µg/mL, respectively. These 2 compounds were then checked for their cytotoxicity against human gingival epithelial cell lines, Ca9-22, and macrophages, THP-1, by MTT and LDH assays which confirmed their safety against the tested cell lines. A preliminary study of the structure-activity relationships unveiled that the functional groups at the C4 position had an important influence on the antibacterial activity of butyrolactone analogues. An optimum length of the alkyl chain at the C5 position registered the optimum antibacterial inhibitory activity however as its length increased the bactericidal effect increased as well. This efficiency was attained by a carboxyl group substitution at the C4 position indicating the important dual role contributed by these two substituents which might be involved in their mechanism of action.

This was followed by the investigation of **B-12** and **B-13** for their antibiofilm activity against both oral strains using crystal violet assay and confocal microscopy. Both derivatives displayed a lowest concentration with maximal biofilm inhibition, LCMI, of 9.38 µg/mL against *S. gordonii* and 1.17 µg/mL against *P. gingivalis*. However, when sub-inhibitory concentrations of **B-12** and **B-13** were used, we demonstrated that the two investigated strains were able to form biofilms *in vitro*. Indeed, this antibiofilm activity decreased as indicated by the expression of the genes implicated in adhesion and biofilm formation such as streptococcal surface protein (*sspA*).

To better understanding the mechanisms of action of butyrolactone derivatives, we have investigated **B-13** bacterial localization by synthesizing a fluorescently labeled **B-13** with NBD (4-nitro-benzo[1,2,5]oxadiazole) without modifying its antibacterial activity. We showed that this compound binds to *Streptococcus gordonii* cell surface, as demonstrated by HPLC analysis where compound **B-13** was found in the cell wall and membrane fraction after 1h of incubation. This compound was not detected in the cytoplasm even after 18h of incubation. By adhering to cell surface, **B-13** induced cell wall disruption leading to the release of bacterial constituents and consequently, the death of *S. gordonii*, a Gram-positive bacterium. The expression of two genes, *murA* and *alr,* implicated in cell wall synthesis, was modified in the presence of this butyrolactone. Gram-negative bacteria such as *Porphyromanas gingivalis* showed also cracked and ruptured cells in the presence of **B-13**, suggesting that this butyrolactone acts on Gram-positive and Gram-negative strains. However, it showed greater efficacy against the Gram-negative strains in comparison to the Gram-positive counterpart. Besides, we also demonstrated that the analogue of **B-13**, **B-12**, has also induced disruption of *P. gingivalis* and *S. gordonii.*

All these studies demonstrated that butyrolactones derived from a lichen metabolite can be proposed as potent antibacterial compounds against oral pathogens causing serious medical complications.

Résumé

Cette thèse de doctorat a été proposée pour pallier au manque de développement de nouveaux antibiotiques. En effet, l'abus et le mauvais usage des antibiotiques est l'une des principales raisons de la résistance bactérienne qui se développe globalement (Özgenç 2016). Face à ce problème de santé publique, des candidats antimicrobiens potentiellement plus efficaces que les antibiotiques actuels ont été étudiés. Les nouveaux médicaments, d'origine naturelle, sont capables de surpasser les mécanismes de résistance bactérienne et le plus important est qu'ils peuvent affecter les bactéries à l'intérieur de leurs biofilms (Borges et al. 2016). Parmi les sources naturelles, nous pouvons citer l'association de champignons et d'algues et / ou de cyanobactéries formant un organisme symbiotique appelé lichen. Ces organismes peuvent produit plus de 1000 métabolites secondaires distincts. Ils comprennent les depsones, les depsidones, les depsides, les dibenzofuranes, les composés phénoliques, les lactones, les quinones et les dérivés de l'acide pulvinique possédant des activités cytotoxiques, antivirales et antimicrobiennes non négligeables. Certains de ces composés se sont avérés efficaces contre des souches bactériennes sensibles et résistantes à plusieurs médicaments (Boustie & Grube 2005; Shrestha & St. Clair 2013).

Le coût des soins dentaires est élevé, il arrive en quatrième position parmi le coût de toutes les maladies et consomme entre 5 et 10% de toutes les ressources de soins de santé. Parmi les complications buccales définies cliniquement, les maladies parodontales occupent une place importante en raison de leur prévalence, de leurs effets notables sur les individus et la société ainsi que du coût élevé des traitements (Batchelor 2014). Elles peuvent être identifiées comme une inflammation infectieuse des tissus de soutien des dents causée par les pathogènes buccaux résidant dans les biofilms dentaires. Une couche streptococcique se forme au-dessus de la pellicule salivaire et constitue un site de recrutement sur lequel les colonisateurs tardifs peuvent se lier. Ces derniers incluent l'agent étiologique de cette maladie, *Porphyromonas gingivalis*. L'inflammation commence lentement et peut s'aggraver si les infections ne sont pas traitées, détruisant les tissus avec le temps et entraînant une perte de dents (How et al. 2016).

Deux bactéries buccales sont utilisées dans le cadre de notre thèse. La première est *Streptococcus gordonii* qui est un Coque à Gram positif et un colonisateur précoce comparé au second qui est *P. gingivalis*, un Bacille à Gram négatif et un colonisateur tardif. Cette diversité confère à ce projet un objectif multidimensionnel concernant divers champs d'application. Tout d'abord, le projet a pour objectif de tester la capacité des nouveaux agents antibactériens d'origine lichénique à interférer positivement dans l'état, précoce ou avancé, d'infection buccale du patient. Ensuite, l'étude a suivi une stratégie multi-route pour combattre les infections buccales en testant la capacité des composés les plus actifs (dérivés butyrolactones) à empêcher la formation du biofilm et empêcher ainsi le déclenchement de l'infection ou cibler

le pathogène tardif, *P. gingivalis*, après le début de l'infection, et troisièmement, l'étude a consisté à évaluer ces composés lichéniques sur deux souches bactériennes Gram positif ou négatif et possédant des morphologies différentes et provoquant des infections systémiques différentielles.

Comme la plaque précoce constitue une base sur laquelle d'autres colonisateurs tardifs tels que *P. gingivalis* peuvent se lier et mener des actions inflammatoires, deux stratégies ont été utilisées dans ce projet. La première était de cibler et d'inhiber la souche bactérienne prédominante, *S. gordonii*, empêchant de former la plaque précoce. Ce serait un effort proactif pour prévenir les complications futures plutôt que de traiter un biofilm déjà existant. La deuxième stratégie a été d'utiliser les composés pour attaquer l'agent étiologique parodontite, *P. gingivalis*.

Afin de découvrir un nouvel agent antibactérien issu de lichens pour lutter contre ces bactéries buccales, nous avons sélectionné une série de composés lichéniques appartenant à différentes classes de structures allant des composés aliphatique à des composés cycliques ou aromatiques. Certains d'entre eux possèdent des structures proches de celles des composés de lichens antibactériens déjà connus, par ex. l'acide roccellique, une forme ouverte de l'acide lichestérinique (Sweidan et al. 2016), quatre depsidones et deux depsides proches du protocétrarique (Nishanth et al. 2015) et / ou physodique (Xu et al. 2016) ou lobarique (Carpentier et al. 2017) et l'acide évernique (Gökalsın & Sesal 2016). À notre connaissance, cette étude (article 1) présente pour la première fois les activités de ces composés licheniques contre les souches bactériennes ciblées.

Les composés lichéniques naturels criblés avaient une activité antibactérienne prometteuse contre les bactéries buccales. Les composés (+) - acide Roccellique (R), acide Demethylbarbatique (D) et acide Psoromique (P) avaient l'activité la plus élevée. Chimiquement, certains changements structuraux parmi les composés ont montré certains sites importants qui pourraient être impliqués dans l'activité antibactérienne. Cependant, cette activité ne semble pas être attribuée à leurs valeurs de log P. Ces résultats mettent en évidence de nouveaux composés ayant des activités antibactériennes puissantes contre des pathogènes buccaux pouvant entraîner de graves complications médicales.

Puisque l'acide lichestérinique était le composé le plus actif, ses résultats n'ont pas été inclus dans le premier article, mais ont été mis de côté pour être présenter dans un second. Une pharmacomodulation a été faite sur cette antibactérien dans le but d'augmenter son activité. Les composés les plus actifs ont été évalués pour leur cytotoxicité contre les cellules épithéliales gingivales et les macrophages et pour leur activité antibiofilmique. La conception et la synthèse des dérivés, leur évaluation biologique contre *S. gordonii*, ainsi que l'effet cytotoxique des meilleurs composés ont été publiées dans l'article 2.

Tous les dérivés de butyrolactone ont été synthétisés avec un bon rendement grâce à une stratégie énantiosélective efficace. Tous les composés ont ensuite été criblés pour leur activité antibactérienne contre *S. gordonii* en milieu solide et liquide en utilisant respectivement des méthodes de dilution sur gélose et de microdilution en bouillon. Les composés (**B1** à **B13**) ont montré une activité plus forte en milieu liquide que sur un milieu solide où seul **B-7** n'était pas actif. La chaîne alkyle de 13 carbones a montré la meilleure activité inhibitrice avec une CMI de 4,69 µg/mL. Parmi les dérivés, **B-12** et **B-13** étaient les composés les plus prometteurs enregistrant une meilleure activité bactéricide que l'antibiotique de référence utilisé, la doxycycline, par 2 ou 3 fois, respectivement. La chaîne à côté du groupe fonctionnel carboxyle peut être impliquée dans leur mécanisme d'action. Enfin, **B-12** et **B-13** ont été évalués pour leur cytotoxicité contre les cellules épithéliales gingivales humaines, Ca9-22, et les macrophages, THP-1, et trouvé non toxique. Cela offre de nouvelles perspectives de continuer avec ces deux butyrolactones pour la mesure de leur activité antibiofilmique. Ces nouveaux composés sont capables d'inhiber *S. gordonii*, ce qui peut bloquer les étapes successives conduisant à des complications buccales, donc une prévention sûre plutôt qu'un traitement tardif risqué après la formation du biofilm.

Ensuite, nous avons étudier l'activité de l'antibiotique comme démontré dans l'article 3. Environ 90% des bactéries vivent dans des biofilms qui seraient responsables d'environ 80% des infections humaines aux Etats-Unis. Non seulement les biofilms résistent aux antibiotiques, mais ils échappent aussi au système de défense de l'hôte (O'Toole et al. 2000; Bueno 2011). Par conséquent, une hypothèse prometteuse valant la peine d'être testée était la capacité des butyrolactones à inhiber la formation de biofilm des bactéries buccales. Dans une étude précédente, sur une grande variété de butyrolactones synthétisées sur la base du composé naturel, l'acide lichestérinique, les composés **B-12** et **B-13** se sont révélés non cytotoxiques contre les cellules eucaryotes utilisées, et les plus efficaces contre *S. gordonii* (Sweidan et al. 2016). La présente étude conduit à l'évaluation de l'activité antibactérienne de tous les dérivés de butyrolactones contre *P. gingivalis* pour aller plus loin et évaluer, pour la première fois, l'activité antibiofilmique des composés les plus actifs (**B-12** et **B-13**) contre *S. gordonii* et *P. gingivalis*.

En conclusion, les butyrolactones synthétisées ont démontré une activité antibactérienne efficace contre *P. gingivalis*. De plus, les dérivés **B-12** et **B-13** présentaient une activité antibiofilmique prometteuse, révélée par le cristal violet et confirmée par CLSM. Ils peuvent être utilisés comme revêtements antimicrobiens pour empêcher la formation de biofilm comme mentionné par Dror et al. (2009). Cependant, ils doivent être utilisés à des concentrations supérieures à la CMI/2 pour induire l'effet antibactérien souhaité, et inférieures à la CMB pour bloquer l'étape d'adhésion dans la formation de biofilm sans tuer les cellules

bactériennes, ce qui constitue une nouvelle stratégie prometteuse et efficace pour inhiber la formation de biofilm (Kostakioti et al. 2013).

Enfin, le mécanisme d'action de l'analogue de butyrolactone le plus actif, **B-13**, sur les deux bactéries buccales a été analysé pour trouver la cible bactérienne. Nous avons également comparé son mécanisme à un autre analogue de butyrolactone (**B-12**), ce qui est discuté à l'article 4.

Nous avons montré que ce composé se lie à la surface bactérienne et induit une modification membranaire avec la rupture de la paroi cellulaire et la libération de constituants cytoplasmiques conduisant à la mort bactérienne. Ces résultats suggèrent que son potentiel antimicrobien est influencé par la composition de la paroi cellulaire des micro-organismes (Malanovic & Lohner 2016).

Cette étude montre pour la première fois le mécanisme d'action des butyrolactones synthétisées, analogues de l'acide lichestérinique. Il ouvre la voie à de futures recherches mécanistiques sur les métabolites secondaires de lichens, qui permettront de mieux comprendre le lichen et d'utiliser ses métabolites secondaires comme antibiotiques. De plus, les structures des analogues de butyrolactones sont différentes de celles de tous les antibiotiques découverts à ce jour, y compris ceux ciblant les parois cellulaires. Ce fait appuyé par la façon dont ces composés ciblent les bactéries comme décrit dans notre étude, peut introduire une nouvelle génération d'antibiotiques avec un nouveau mode d'action. Cependant, pour mieux développer un nouvel antibiotique, il est nécessaire de poursuivre les investigations sur les métabolites secondaires lichéniques en général et **B-13** en particulier.