



Universidade do Minho
Escola de Engenharia

Maria Helena Rodrigues Macedo

**The role of L-arginine in the modulation
of oral biofilms in periodontal disease**





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of oral biofilms in periodontal disease**

Dissertation for the MSc in Biomedical Engineering
Area of Clinical Engineering

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ABSTRACT

Periodontitis is an infectious oral disease and remains one of the most prevalent chronic diseases worldwide. Although the exact microbial aetiology is unknown, oral biofilms, also known as dental plaque, seem to play an important role in the development of this disease. Studies already shown that bacterial composition of dental biofilms differs between healthy and diseased sites. To date, the occurrence of this disease is associated with three main factors: (1) the presence of pathogenic bacteria, (2) the absence of beneficial bacteria and (3) the susceptibility of the host.

This research project focussed on evaluating the interaction with the outgrowth of beneficial bacteria, where selective nutritional stimulation, here referred to as prebiotics, aimed to modulate oral biofilms by stimulating the growth of beneficial bacteria and, thereby, suppress the outgrowth of pathogens, shifting a complex microbiota towards a more healthy-associated composition.

The effect of the amino acid L-arginine on selective stimulation of beneficial oral bacteria was tested in single-, dual- and multi-species assays. Further, it was investigated if L-arginine may prevent pathogen incorporation into established biofilms.

It was demonstrated that L-arginine treatment could selectively trigger the outgrowth of beneficial bacteria throughout the experiments, leading to a shift in multi-species biofilms *in vitro*. Although a direct preventive effect of L-arginine could not be observed, continuous treatment showed a reduction in the pathogenic proportion in the complex microbial biofilms. Also, it was demonstrated that predictions about multi-species assays cannot be made based on the results obtained with single and dual-species assays.

In conclusion, this study showed that L-arginine has a promising potential to be used as a prebiotic compound in oral health, especially to treat periodontitis, as it is able to modulate oral biofilms towards a more beneficial state. However, *in vivo* studies are necessary to confirm the observed *in vitro* prebiotic effect.

Keywords: Oral health, periodontal disease, biofilm, periodontopathogens, prebiotics, L-arginine

RESUMO

A periodontite é uma doença oral infecciosa e é uma das doenças crônicas mais prevalentes a nível mundial. Apesar da etiologia exata da doença ser desconhecida, os biofilmes orais, também conhecidos como placa bacteriana, parecem desempenhar um papel importante no seu desenvolvimento. Alguns estudos já demonstraram que a composição dos biofilmes orais de pacientes saudáveis são diferentes dos de pacientes doentes. Até hoje a ocorrência desta doença está associada com três fatores principais: (1) a presença de bactérias patogênicas, (2) a ausência de bactérias benéficas e (3) a suscetibilidade do hospedeiro.

Este projeto de investigação focou-se em avaliar a interação com o crescimento de bactérias benéficas, onde uma estimulação nutricional seletiva, aqui referido como prebióticos, visou modular os biofilmes orais, estimulando o crescimento das bactérias benéficas e, desse modo, suprimindo o crescimento dos patogênicos, levando a uma mudança do microbioma oral em direção a um estado mais saudável.

O efeito da estimulação seletiva das bactérias benéficas foi testado usando o aminoácido L-arginina em ensaios de mono-, dual- e multi-espécies. Foi ainda investigado se a L-arginina é capaz evitar a incorporação de bactérias patogênicas em biofilmes já estabelecidos na cavidade oral.

O tratamento com L-arginina revelou que esta consegue estimular o crescimento das bactérias benéficas em detrimento dos patogênicos, levando a uma mudança nos biofilmes multi-espécie *in vitro*. Apesar de não se ter observado um efeito preventivo direto com o uso da L-arginina, o tratamento contínuo com este aminoácido demonstrou uma redução na proporção de espécies patogênicas no biofilme. Percebeu-se ainda que os resultados obtidos com ensaios mono-, dual- e multi-espécies diferem entre si, o que significa que previsões sobre o comportamento das espécies em biofilmes complexos não podem ser feitas através de ensaios onde são apenas testadas uma ou duas espécies.

Concluindo, este estudo demonstrou que a L-arginina tem um enorme potencial para ser usada como um prebiótico na saúde oral, com um grande potencial para tratar a periodontite, visto que é capaz de modular os biofilmes orais. No entanto, estudos *in vivo* são necessários para confirmar os efeitos observados *in vitro*.

Palavras-chave: Saúde oral, doença periodontal, biofilme, prebióticos, L-arginina

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CHAPTER 1 – MOTIVATION AND OBJECTIVES

1.1. MOTIVATION

Periodontitis is an infectious disease that affect millions of people worldwide and, today, oral healthcare professionals are still lacking an effective and durable treatment to fight this disease.

Studies have been showing that the development of this periodontal disease is probably related to oral biofilms and its composition. The presence of pathogenic bacteria, the absence of the so-called beneficial bacteria and the susceptibility of the host seem to be the three main factors that lead to the development of the disease.

As so, modulating oral biofilms towards a more healthy-associated microbiota might be a promising way to treat or even prevent the development of the disease.

Probiotics, also known as “beneficial bacteria”, have been studied in the oral field and have shown promising results in maintaining oral health. Besides probiotics, prebiotics have also been studied, as they are substances that are able to selectively stimulate the growth of beneficial bacteria, and thus, improve host health. However, their effect on the oral cavity has not yet been clarified since studies are still scarce.

Prebiotics might be a promising mechanism to modulate the oral microbiota by selectively stimulating the growth of beneficial bacteria. Moreover, promoting the outgrowth of beneficial bacteria, and as so, preventing the growth of pathogens in the biofilm might be important to prevent the establishment of periodontal diseases.

Therefore, this work was conducted in order to test if the amino-acid L-arginine could act as a prebiotic and trigger the growth of beneficial bacteria in the oral biofilm, thus reducing the proportion of pathogenic bacteria. If L-arginine is able to modulate the oral biofilm, it may be seen as a promising substrate to be used in oral healthcare.

1.2. OBJECTIVES

The main goal of this project was to selectively trigger the outgrowth of beneficial oral bacteria in complex biofilms by means of nutritional stimulation using the amino-acid L-arginine. To achieve this goal, the project was divided into following parts:

- Evaluating which specific bacteria are stimulated by L-arginine (growth and biofilm formation)
- Evaluating the selective stimulation of beneficial species by L-arginine and subsequent outgrowth over a pathogenic species in dual-species competition assays
- Inducing a shift towards a beneficial-dominated microbial composition in multispecies biofilms by L-arginine treatment
- Evaluating if L-arginine treatment causes a preventive effect in the incorporation of pathogenic species into established beneficial biofilms

CHAPTER 2 – LITERATURE REVIEW

2.1. THE ORAL CAVITY AND ITS MICROBIOTA

The oral cavity is of major importance in the human body. Although some physicians tend to believe it is resumed to a confined compartment within the human organism, from an anatomic point of view, the oral cavity is connected to the nasopharynx, the larynx, the tonsils, the middle ear and the gastrointestinal tract. As it has such a strong connection with different parts of the human body, it influences general health and it is also influenced by the state of the body [1]. It is known, for example, that inflammations of the mouth seem to weaken the ability of the body to control blood sugar, further complicating diabetes since the inflammation impairs the body's ability to use insulin. On the other hand, patients with diabetes tend to have a greater amount of pathogenic bacteria, which can lead to periodontal diseases [2]. Besides, epidemiological studies focusing on periodontitis have shown that this disease can increase the risk of systemic infections, coronary heart diseases, myocardial infarction, osteoporosis, among others [3].

The oral microbiota is a biological system of extremely high complexity [4]. More than 700 species have been detected in the human mouth being that over 400 have been identified in the periodontal pocket [5]. Thus, the oral microbiota is at least as complex as the gastrointestinal or vaginal microbiota.

In nature, as well as in the human body, the vast majority of microorganisms are not found in a planktonic state, but attached to surfaces where they form biofilms. Biofilms can be defined as matrix-enclosed bacterial populations that adhere to each other and/or to surfaces or interfaces [6]. The biofilm matrix is produced by the bacteria and is generally composed of water and microbial macromolecules [7]. Different bacterial species specifically attach to different surfaces or co-aggregate with specific partners in the mouth [8,9]. Often one species can co-aggregate with more than one partner and the partners, in their turn, can co-aggregate with other bacteria to form a dense biofilm composed of different species [9]. This strategy allows bacteria to survive and grow in hostiles environments because they are able to protect each other and try to create the perfect conditions for each species to thrive [9]. Bacteria in biofilms exhibit 'emergent properties' such as metabolic co-dependence and cooperation, facilitated horizontal gene transfer, and increased resistance to antimicrobial agents which differ from bacteria in the planktonic state [8,10]. These emergent properties may lead to alterations in their gene expression, which results in bacteria having a different phenotype [8]. These changes can make biofilms at least 500 times more resistant to antibacterial agents [6].

In the oral cavity biofilm formation is referred to as dental plaque [11]. Kolenbrander et al. [12] developed a model of bacterial adhesion to tooth surface and co-aggregation among bacterial species. Based on this model, bacteria can be considered as either early or late colonizers (Figure 1). Early colonizers, usually streptococci, bind to salivary receptors. Their ability to bind to other early colonizers and host molecules may confer an advantage in establishing early dental plaque [12]. *Fusobacterium nucleatum*, however, acts as a bridging organism between early and late colonizers, playing a special role in the development of dental plaque. *F. nucleatum* is the most numerous gram-negative species in healthy sites, with increasing abundance in oral diseased sites. It has also been noticed that this species is always present whenever *Treponema denticola* and *Porphyromonas gingivalis* are also present, which suggests that its presence may be required for the colonization of these two species [13]. Interestingly, *F. nucleatum* coaggregates with several late colonizers, although no coaggregation among the late colonizers have been observed [12,14,15]. As such, *F. nucleatum* may play a key role in the establishment of dental plaque and, consequently, the development of dental diseases.

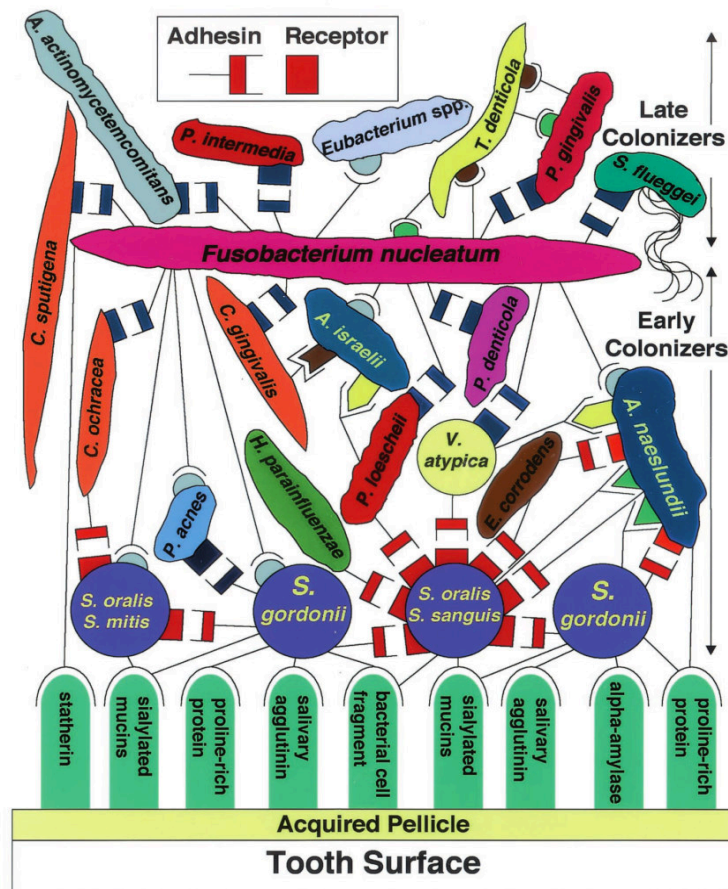


Figure 1 - Model of bacterial colonization showing early and late colonizers and *F. nucleatum* acting as a bridge between them [12].

Taken the complexity and important interspecies interactions into account, oral biofilms display difficult therapeutic targets [16]. Further, bacterial diversity is rather subject specific, and prone to changes during the lifetime of each individual [5]. As such, treatment of oral diseases requires a microbial community-based approach instead of targeting one specific oral pathogen, as this oral pathogen may be causing disease in some individuals but not in others [11,17]. Hence, the exact microbial aetiology of oral diseases remains unknown, although a different bacterial community composition was found between health and diseased sites.

In a healthy patient, the resident microflora acts as an important component of the host defences forming a barrier against exogenous and potentially pathogenic populations – preventing their establishment in the mouth – and by regulating the inflammatory host response to oral commensal bacteria [18]. Further, oral commensal bacteria have been shown to maintain healthy oral tissues by influencing the expression of mediators such as intracellular adhesion molecule 1 (ICAM-1), E-selectin and Interleukin 8 (IL-8), and modulating immune responses and enhancing cellular homeostatic mechanisms [19–22].

The resident microbiota also contributes to the normal development of the physiology, nutrition and defence systems of the organism, protecting the host from disease. Thus, beneficial organisms that colonize the oral cavity live in a stable symbiotic balance with the host [23]. Only when this balance is disturbed, an outbreak of disease may occur [18,24]. According to Devine and Marsh [25], a disturbance of the balance occurs “if the host becomes immunocompromised, if the resident microbial populations are suppressed or if microorganisms reach sites they normally do not have access to (e.g. through trauma)”.

2.2. ORAL DISEASES

There are several types of diseases that can affect the human cavity, like caries, halitosis, gingivitis, periodontitis, herpes, and oral candidiasis.

Dental caries is one of the most common disease in the oral cavity and one of the most prevalent infectious disease affecting humans worldwide [26]. This disease appears because of disturbances that occur in the bacterial biofilm on the tooth surface, disturbing the ecological balance and leading to loss of tooth mineral [27]. Some bacteria present in the mouth are classified as cariogenic, such as *Streptococcus mutans* and *Streptococcus sobrinus*, meaning that they can

induce the formation of caries [28]. These microorganisms are present in the dental plaque and are known to produce acids, due to the periodic ingestion of dietary carbohydrates, that dissolve the mineral matrix of the teeth [26,29]. Bacterial glycolysis leads to the production of acid, which induce repeated cycles of demineralization of the tooth enamel [29]. The demineralization phases are followed by periods of alkalisation, which restore the integrity of enamel. Caries occur when the acidification phases outweigh the alkalisation phases, translating in the establishment of a more acidogenic microbiota, which results in lower plaque pH values leading to enhanced enamel demineralization [29]. The production of acids, and consequently demineralization of the enamel is influenced by three factors, the host, the presence of bacteria and nutrients. Only the interplay of all three factors lead to the occurrence of caries [30]. Nowadays, the preventive strategies applied to dental caries focus on targeting the host and dietary factors and removing the dental plaque [31].

Although any disorder of the periodontium – tissue surrounding and supporting the teeth – can be defined as a periodontal disease, this term is usually used when referring to gingivitis and periodontitis, which are inflammatory disorders caused by a pathogenic microflora [32]. Basically, periodontal diseases develop when dental plaque accumulates at the gingival margin triggering an inflammatory response of the host [33]. The increase of plaque mass is followed by an increase of obligatory anaerobic and proteolytic bacteria, many of which are Gram-negative pathogens [25].

Gingivitis is the mildest form of periodontal disease and is reversible when the correct oral hygiene habits are adapted [32]. At this stage, the infection is limited to the gums. The chronic form of the disease results in mild bleeding from the gums during tooth brushing which is not considered a major inconvenience unless the patient suffers from a bleeding disorder [32].

Periodontitis appears when gingivitis is not treated and the disease progresses to a more severe state (Figure 2). The inflammatory process extends deep in to the tissues and leads to the loss of supporting connective tissue and alveolar bone [32]. Soft tissue pockets or deepened crevices are formed between the gingiva and the root of the tooth and can cause pain and discomfort to the patient. The mastication process may also be impaired and eventually the tooth can be lost. Further, severe periodontitis can be associated with periodontal abscesses and halitosis. Moreover, if the biofilm is not thoroughly removed the flow of the gingival crevicular fluid is increased which also introduces molecules such as haemoglobin, haptoglobin and transferrin which select for the proteolytic bacteria that can degrade host molecules that regulates inflammation, resulting in an exaggerated and inappropriate inflammatory response [23]. Loss of

connective tissue attachment and loss of alveolar bone are the two main diagnostic criteria used for the detection of periodontitis [34]. Periodontitis is highly prevalent, affecting billions of people around the world [34].

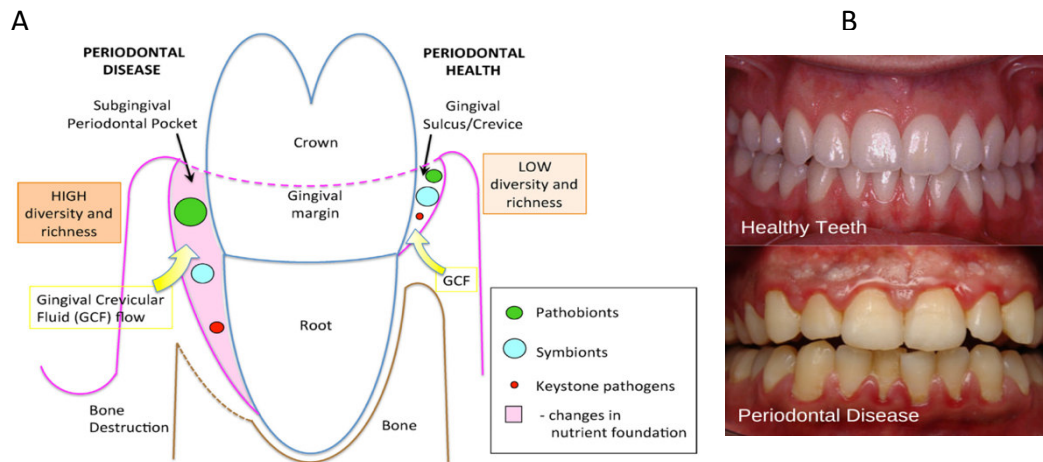


Figure 2 - Difference between periodontal health and periodontitis. A – Schematic representation of the difference between periodontal health and periodontitis [101]. B – Difference in the appearance of the mouth of a patient with healthy teeth and a patient with periodontitis (<http://www.esthetixdentalspa.com/uncategorized/5-things-you-should-know-about-gum-disease-periodontitis/>).

2.3. PERIODONTITIS

2.3.1. WHY DOES PERIODONTITIS OCCUR?

To date it is assumed that the occurrence of periodontitis is linked to several factors (Figure 3).

There are three main factors that, when combined, lead to disease [35]:

- The presence of pathogenic bacteria
- The absence of beneficial bacteria
- The susceptibility of the host

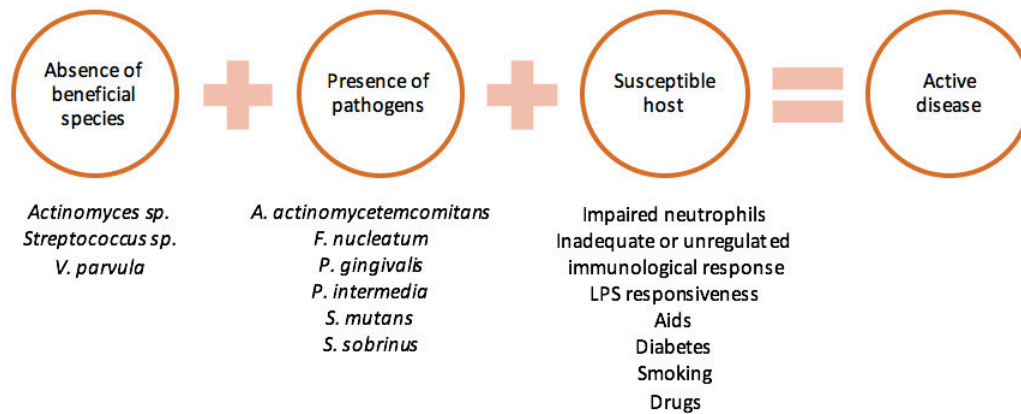


Figure 3 - Combination of factors that lead to periodontitis (adapted from [35]).

This is in accordance with the “Ecological Plaque Hypothesis” introduced by Marsh [36], trying to describe and explain the dynamic relationship between the oral microflora and the host in health and disease. In the past, the occurrence of periodontitis was explained by varied hypothesis. First, the “Specific Plaque Hypothesis” was proposed. This hypothesis claimed that some specific pathogenic bacteria are solely responsible for the formation and aggravation of oral diseases [37]. However, the fact that disease sometimes occurred even in the absence of these pathogenic species lead to the rejection of this theory. So, the “Non-specific Plaque Hypothesis” arose. This theory claimed that bacteria present in the oral biofilm produce specific factors and substances and the accumulation of these products are responsible for inflammation and damage to the gingival tissues and lead to oral disease [38]. Here, the quantity of plaque, rather than its composition was considered to be the rationale for oral diseases. However, with the aid of technological advances, it was possible to observe that, in fact, there are some bacteria that are more associated with the occurrence of disease than others. As so, the “Ecological Plaque Hypothesis” arose as a combination of the key points of the previously two mentioned hypotheses. It links the local environment changes with the activity and composition of the biofilm, where any change in the environment induces a response in the microflora (Figure 4) [18].

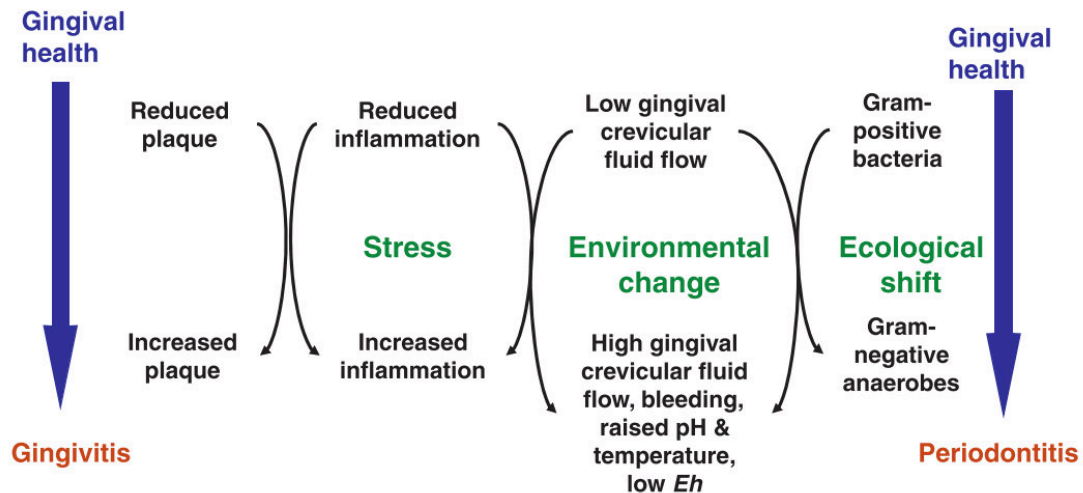


Figure 4 - Schematic representation of the "Ecological Plaque Hypothesis" in relation to gingivitis and periodontitis (Eh = redox potential) [18]

This hypothesis combines environmental factors of the host with the biofilm itself. The accumulation of plaque might trigger an inflammatory response by the host, which in turn leads to changes in the local environment and induces the production of new proteins which might favour the growth of mostly anaerobic Gram-negative bacteria – the pathogens [18].

The shift that occurs from Gram-positive bacteria dominated biofilms to mostly Gram-negative anaerobic biofilms might trigger the occurrence of periodontal diseases [39]. The presence of these periodontopathogens is clearly linked to periodontitis, as microbiota from diseased sites was shown to be markedly different from the one seen in healthy patients [40].

Although a specific microbial aetiology is not known, there are several strains that could be related to the disease and play an important role in its development. When these bacteria are present in the oral cavity of a susceptible host, and abundant in sufficient amounts to cause a shift in the oral microbiota, disease might occur. Each species in the pathogenic biofilm has a different role and some are more involved in the disease and are more virulent than others. For example, it is considered that by the time *P. gingivalis* or *Aggregatibacter actinomycetemcomitans* are prevalent, the disease is well established and the site is already affected [25].

The same way some species are defined as pathogens, others are considered to be commensal or beneficial and they are considered to play a positive role in the community. Beneficial/commensal bacteria may have an effect in protecting the host from pathogens. Therefore, some authors have started to focus on the fact that the reduction or absence of these bacteria is another factor for plaque-related periodontal inflammation [12]. Thus, the presence of

these species in the oral cavity might prevent the shift from a health towards a disease associated biofilm composition [1]. Kilian et al [41] listed *Streptococcus mitis*, *Streptococcus oralis*, *Actinomyces naeslundii*, *F. nucleatum* and some species of *Prevotella* as being oral commensal/beneficial bacteria.

2.3.3. PATHOGENS INVOLVED IN PERIODONTITIS

- ***Aggregatibacter actinomycetemcomitans***

A. actinomycetemcomitans (formerly known as *Actinobacillus actinomycetemcomitans*) is an exogenous Gram-negative microorganism. It has been considered, for many years, the most likely aetiological agent in aggressive periodontitis (AgP) and has also been implicated in chronic periodontitis and other severe non-oral infections [34,42–44]. The term aggressive periodontitis was suggested in order to differentiate certain rapidly progression forms of periodontitis from the more common form of chronic periodontitis [45]. AgP was formerly known as juvenile periodontitis as it generally affects systemically healthy individuals aged less than 30 years old [46]. Two forms of AgP have been distinguished, based on the number of teeth affected and the distribution of lesions: the localized form (LAgP) and the generalized form (GAgP) [34]. Some scientists believe that the main organism implicated in localized AgP is *A. actinomycetemcomitans*, colonizing the subgingival plaque biofilm, whereas generalized AgP usually affects more teeth and is caused by *P. gingivalis* [46]. *A. actinomycetemcomitans* is present in 90% of localized AgP and in 30 to 50% of severe adult periodontitis [43].

A. actinomycetemcomitans is considered to be an opportunist, with varied virulence factors and mechanisms. Its ability to produce virulence factors leads to serious infections, transmissible among exposed individuals [43]. This bacterium moves from the initial colonization site to the gingival crevices where it competes with other bacteria [34]. The production of leukotoxin is considered a major virulence factor of *A. actinomycetemcomitans*, since it enables the bacterium to evade the host response by killing leukocytes [47].

A study in West African individuals showed that there was a significantly increased risk of attachment loss among young individuals when *A. actinomycetemcomitans* was present in the oral cavity compared with the ones who did not harbour this bacterium [48]. Also, several studies on

various adolescent's populations have shown that the presence of this species in the subgingival plaque increases the risk of attachment loss [34].

Although *A. actinomycetemcomitans* has been considered by many as the major pathogen in AgP, this is a controversial concept. Different studies showed different results and there is one study that shows no significant association between this bacterium and periodontal diseases, whereas the prevalence and levels of *P. gingivalis*, *T. denticola* and *Prevotella intermedia* were significantly associated with the disease, in descending order [42,49]. This shows that more evidence is needed to fully understand the role of bacteria and their interactions in dental biofilms.

- ***Porphyromonas gingivalis***

P. gingivalis is a black pigmented, non-motile, Gram-negative bacterium that requires anaerobic conditions and that has been associated with periodontitis for a long time [50]. It is considered a natural member of the human microbiota, that under perturbations to the host or microflora can cause pathology [51]. It was found in samples of epithelial cells obtained from periodontal pockets, gingival crevices, and buccal mucosa specimens collected from patients with periodontitis but also from subjects with healthy gingivae [52]. Its metabolic energy is obtained through fermentation of amino acids, which is decisive for survival in deep periodontal pockets, where sugars are extremely scarce [53]. It is the species most associated with the chronic form of periodontitis, leading to chronic inflammation and destruction of the soft and hard tissues that support the teeth and can be detected in up to 85% of diseased sites [54–57]. It is considered a late colonizer of the periodontal biofilm as can be observed in Figure 1.

According to Sakanaka et al, *P. gingivalis* can elevate the virulence of the periodontal biofilm, even in low numbers, by subverting host responses, altering biofilm community structures and facilitating an increase in overall bacteria load leading to its designation as a keystone pathogen [54]. Inter-bacterial interactions between *P. gingivalis* and other species of the bacterial community are considered to play a critical role in promoting the pathogenicity of this bacterium in the biofilm [54]. *P. gingivalis* possesses a great number of virulence factors such as cysteine proteinases, lipopolysaccharide (LPS), capsule and fimbriae, which enable the bacterium to colonise and invade periodontal pockets [57,58].

P. gingivalis invades cells and tissues in the host as a strategy to survive, avoiding, this way, immune surveillance. It can invade gingival epithelial cells, maintaining their viability and replication [53,59,60].

- ***Fusobacterium nucleatum***

F. nucleatum can be associated with a great number of diseases that affect the human body, but on the other hand, can also be isolated from healthy subjects, being the most numerous Gram-negative species present in healthy sites [61]. Thus *F. nucleatum* can be considered either as an oral commensal or as a periodontal pathogen that is associated with a variety of human diseases. It is one of the first Gram-negative species to become established in plaque biofilms acting as a bridge between early and late colonizers [12]. *F. nucleatum* is a central species in oral biofilms since it enables the aggregation between Gram-positive and Gram-negative species [12,62].

This bacterium is ubiquitous in the oral cavity and is dominant within the periodontium and dental plaque biofilms and hence, plays an important role in biofilm ecology and infectious diseases in humans. It has been demonstrated that it can be invasive and pro-inflammatory in human oral epithelial cells, eliciting the secretion of IL-8. [39,61–63].

In a study performed with several species to evaluate the adherence and invasion of different bacteria to epithelial cells (using *P. gingivalis* as a positive control since it is considered a periodontal pathogen), results showed that *F. nucleatum* was different from the rest of the group of bacterial species in the invasion assays. This organism showed a high capacity to invade cells, with its activity being comparable to *P. gingivalis* [39].

The presence of this species is affected by environmental factors such as smoking. Smoking increases the number of these bacteria in the oral cavity in both healthy and diseased patients [64].

- ***Prevotella intermedia***

P. intermedia is an obligated anaerobic gram-negative, black-pigmented, rod-shape bacterium that was previously classified in the genus *Bacteroides*. *P. intermedia* has long been known to be associated with periodontal disease [65]. It has been implicated as a putative periodontal pathogen due to its isolation from lesions of subjects with early and advanced periodontitis [66]. *P. intermedia* has also been shown to invade human coronary artery endothelial and smooth muscle cells *in vitro* [66]. It is the most commonly isolated species of the genus *Prevotella* from dental plaque [67].

Although *P. intermedia* is considered less virulent than *P. gingivalis*, it has several surface properties which may be regarded as potential virulence factors [67].

Information about this pathogen and its role in disease is still limited and more studies need to be conducted in order to fully understand its role in diseases like periodontitis.

- ***Streptococcus mutans* and *Streptococcus sobrinus***

Oral streptococci are early colonizers of the oral cavity and are abundantly found in dental plaque, considering the oral cavity as their natural habitat [68].

S. mutans and *S. sobrinus* are the two most important bacteria of the group *mutans streptococci*, the major pathogenic bacteria in caries process [69]. These bacteria are Gram-positive, catalase-negative, non-motile, aerobic members of the genus *Streptococcus*. These two species are closely related and they are both pathogenic within humans, enhancing the formation of dental caries, as they produce organic acids that demineralize hard tissues [70,71]. They are considered the two most significant human pathogens regarding dental caries [72].

S. mutans is a major matrix producer and when dietary sucrose and starch are present, it can rapidly modulate the formation of cariogenic biofilms [11,73]. *S. mutans* thrives in cariogenic plaque because of its ability to tolerate, to grow and to metabolize carbohydrates in an environment with a low pH [74–76]. It is considered a key aetiological agent in dental caries, being predominant in carious lesions microflora [68].

Studies performed on subjects with caries do not always show the same results. While some studies showed that *S. sobrinus* is more acidogenic and more cariogenic than *S. mutans*, others did not find this difference [72]. However, it is possible to understand that both species have a great influence in the development of caries and, as such, are considered pathogenic.

2.3.3. CURRENT APPROACHES TO TREAT PERIODONTITIS

In the treatment of periodontitis, initial therapy focuses on reducing dental plaque [77]. It is based on a mechanical approach, namely scaling and root planning (SRP) and eventually can include surgery in order to reduce the depth of the periodontal pocket, with instructions for an improved correct oral hygiene by tooth brushing and interdental cleaning [33,78,79]. Although SRP can reduce the number of pathogens, they rapidly re-colonize the treated sites [80]. The establishment of a more aggressive microbiota occurs within weeks or months [81]. Treatment can be combined with the use of antimicrobials, however the shift of the oral microbiota is only

temporary, since the use of antibiotics have not been proven to improve the long term effect of periodontal therapy [78]. This may be due to the failure of the antimicrobial agent in penetrating the full depth of the biofilm [9].

Like periodontitis, most of the infectious diseases that affect the human population have been treated with antibiotics. However, infectious diseases remain one of the most important health problems affecting the world population nowadays. In fact, hospital infection rates are not declining and multi-drug-resistant bacteria continue to emerge [1]. This widespread use of antibiotics also reflects on the level of resistance in the subgingival microbiota of adults with periodontitis [82]. Also, antibiotics can also lead to undesired side effects such as antibiotic-associated diarrhoea [83].

Taking this into account, there is a great necessity to develop novel non-antibiotic treatment therapies. New emerging concepts for treatment or prevention of periodontal diseases might be the use of probiotics and prebiotics in dental health care [25].

2.4. PROBIOTICS

Over the last decades, an increasing interest in the use of probiotics, also known as beneficial bacteria, and their use to modulate the microbiota and maintain health has been reported [1].

Probiotics are defined by the World Health Organization (WHO) as living microorganism that confer a health benefit to the host when administered in sufficient amounts (http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf). They are referred to as “health-promoting” bacteria and might be used for therapeutic purposes in humans [81].

In order for periodontitis to develop a combination of three factors is needed: a susceptible host, the presence of pathogens and the reduction or absence of beneficial species [35]. Since it is difficult to modulate the host immune response, researchers are focusing on the third aetiological factor, namely restoring the number of beneficial bacteria [1]. Probiotics are seen as a promising strategy to treat periodontitis since they might be able to increase the number of beneficial bacteria in dental plaque, and thus, leading to a decrease of pathogenic bacteria.

Healthcare professionals are focusing more and more on probiotics as their oral intake, to promote general health, has already shown promising effects [81]. For example, probiotics have been shown to reduce *mutans streptococci* counts in saliva and plaque, which is promising since

they are key aetiological agents in caries [84]. Moreover, studies using *Lactobacillus* species show significant decreases in gingivitis when compared to the control [83]. Researchers have also observed that a strain of *Lactobacillus salivarius* named *L. salivarius* TI 2711 starts to kill *P. gingivalis*, *P. intermedia* and *P. nigrescens* after 6-12h in co-culture *in vitro* [85]. The same researchers also tested *L. salivarius in vivo*, letting tablets containing this bacterium to dissolve in subject's mouth 5 times a day for a period of 8 weeks. Results showed significant reductions of salivary black pigmented anaerobic species for subjects treated with probiotics compared to the control [85]. A study by Krasse et al. [86] using *Lactobacillus reuteri* in patients with gingivitis showed a significant reduction of gingivitis and plaque compared to the control. Also, a study performed in a beagle dog model showed that beneficial oral bacteria such as *Streptococcus sanguinis*, *Streptococcus salivarius* and *Streptococcus mitis* delayed the recolonization of the oral cavity by periodontal pathogens, reduced inflammation and improved density and bone levels [87].

In addition, positive effects have been shown on the gastrointestinal tract for diarrhoea, inflammatory bowel disease and irritable bowel syndrome, being that probiotics also showed promising effects for atopic diseases and cancer [88].

For now, it is known that probiotics have at least the following modes of action [1,89]:

- Modulation of the host defences (innate and acquired immune system) – probiotics can modulate the immune system towards an anti-inflammatory action, acting on different cells. Host cells such as immune cells and epithelial cells are able to recognize bacteria and their products. Also they can regulate the expression of phagocytosis receptors in the neutrophils and enhance natural killer cell activity [89–92]. They are capable of reducing the production of pro-inflammatory cytokines and increase the production of anti-inflammatory cytokines such as Interleukin 10 (IL-10) and host defence peptides such as beta-defensin 2 [25];
- Production of antimicrobial substances – probiotics can exert a direct effect against pathogens by producing several compounds such as lactic acid, hydrogen peroxide (H₂O₂), bacteriocins and bacteriocin-like inhibitory substances that act as microbial agents against periodontopathogens and that are capable of inhibiting their growth or kill them [25,83,89];
- Competitive exclusion mechanisms – probiotic can also exert an indirect effect against pathogenic bacteria as they can compete against pathogens for the same nutrients or hamper their adhesion. If beneficial strains are better adapted to the respective niche, the

adhesion of pathogens to the surfaces may be prevented by them, impairing pathogens growth and vitality [13,83]. This indirect effect is very important as the adhesion of pathogens to host surfaces is the first step of infection [33].

However, it is likely that one single probiotic strain does not exhibit all three mechanisms, so probiotic strains are used in combination with each other to increase their beneficial effects [1,93]. It is also likely that these mechanisms vary according to the combinations of probiotics used, the condition that is being treated and its stage and also the presence of prebiotics [94].

Studies concerning probiotics have already shown that certain streptococci, such as *S. salivarius*, *S. mitis* and *S. sanguinis* can attenuate the IL-8 response on epithelial cells induced by pathogens such as *F. nucleatum* and *A. actinomycetemcomitans* and that these streptococci are also capable of producing hydrogen peroxide and inhibiting the outgrowth of periodontopathogens [22,95,96].

Teughels et al. [80] showed that the application of probiotics in a beagle dog model after SRP delayed and reduced the recolonization of the site by periodonpathogens, when compared to the control treatment.

Probiotics can be used as a method to prevent oral diseases, supplementing the oral cavity with beneficial bacteria, creating a healthy oral biofilm, preventing pathogens and consequently disease to become established in the mouth [97]. When disease is already established at periodontal sites, probiotics might be used as a method of restoring beneficial bacteria at those sites and shifting the microflora to a healthy state and preventing the growth of pathogens, treating the disease [97].

It is also important to understand that probiotics are more likely to have a durable effect on young individuals since their resident microbiota seems to be less stable and more subject to flux than the microbiota of adults, so perhaps it is in childhood that long-term changes on the oral microbiota can be achieved. In adults, shifting the oral microbiota through probiotics may be difficult as a definitive shift may be hard to accomplish [1,25]. It has been shown that the probiotic effect might disappear if the patient discontinues its use [1].

Besides age of the subject, a careful selection of the probiotic must be performed according to the disease, also the mode and time of administration can be crucial, as well as the general health state of the patient. All of these are determinant factors for the success of probiotics [25].

2.5. PREBIOTICS

Similar to probiotics, the objective of prebiotic use can be defined as to improve host health through modulation of the microbiota [81].

The definition of prebiotics is adapted to the gastrointestinal tract, since studies of prebiotics have been mainly focused on the gastrointestinal microbiota [25]. Prebiotics are defined as “non digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already established in the colon and thus in effect improve host health” [98] or more recently as “selectively fermented ingredients that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health” [99]. However, beneficial effects of prebiotics might be extended to other parts of the body, in this case to the oral cavity.

To put simply, prebiotics might be seen as selective triggers to stimulate beneficial bacteria, as they aim to enhance their growth and proliferation [25]. The combination of probiotics and prebiotics is defined as a symbioses, beneficially affecting the host [81].

The main mechanism of action of prebiotics is thought to be indirect since they facilitate the proliferation of beneficial bacteria of the microflora, exerting probiotic effects. However, they may also have direct effects on the host, which include the stimulation of expression of the anti-inflammatory cytokine IL-10 and interferon gamma, the enhancement of secretion of IgA and the modulation of inflammatory responses [25].

Prebiotics might be a promising mechanism to modulate the human resident microbiota without disturbing the health associated homeostasis. However, to date, very few studies on prebiotics in oral health have been conducted.

CHAPTER 3 – MATERIALS AND METHODS

3.1. STRAINS AND CULTURE CONDITIONS

In total 15 bacterial species known from the oral cavity were used as model organisms. *Aggregatibacter actinomycetemcomitans* ATCC 43718, *Fusobacterium nucleatum* ATCC 10953, *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611, *Streptococcus mutans* ATCC 25175 and *Streptococcus sobrinus* ATCC 33478 were considered as pathogenic species. Commensal, albeit potentially beneficial species included *Actinomyces naeslundii* ATCC 51655, *Capnocytophaga sputigena* ATCC 33612, *Streptococcus gordonii* ATCC 49818, *Actinomyces viscosus* ATCC 15987, *Streptococcus mitis* ATCC 49456, *Streptococcus oralis* clinical isolate, *Streptococcus salivarius* TOVE-R, *Streptococcus sanguinis* LMG 14657 and *Veillonella parvula* DSM 2008.

All bacterial strains were cultured on blood agar plates (Blood Agar Base No 2, Oxoid Ltd, Basingstoke, UK) supplemented with 5 µg/ml hemin (Sigma-Aldrich Co, St. Louis, MO, USA), 1 µg/ml menadione (Sigma-Aldrich Co, St. Louis, MO, USA) and 5% sterile horse blood (Defibrinated Horse Blood – E&O Laboratories Ltd, Burnhouse, Bonnybridge, Scotland). *A. actinomycetemcomitans*, *S. mutans*, *S. sobrinus*, *S. gordonii*, *S. mitis*, *S. oralis*, *S. salivarius* and *S. sanguinis* were incubated at 37°C in a 5% CO₂ environment. *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *A. naeslundii*, *C. sputigena*, *A. viscosus* and *V. parvula* were incubated at 37°C under anaerobic conditions (10% CO₂, 10% H₂, 80% N₂).

3.2. GROWTH AND BIOFILM FORMATION IN THE PRESENCE OF L-ARGININE

In order to observe if L-arginine promotes the growth and biofilm formation of the different oral bacteria, single-species growth curves and biofilm formation assays were established. Bacteria were collected from the blood agar plates and transferred to 10 ml of brain heart infusion broth (BHI) (Difco Laboratories, Detroit, MI, USA). The cultures were incubated overnight at 37°C either under anaerobic conditions or in a 5% CO₂ environment. The bacterial concentration of the overnight cultures was adjusted with fresh BHI to 1x10⁷ Colony Forming Units (CFU)/ml by measuring the optical density (OD) at 600 nm.

Stock solutions of L-arginine (Sigma-Aldrich Co, St. Louis, MO, USA) were prepared in demineralized water with concentrations of 250, 200, 100 and 50 $\mu\text{mol/ml}$ and filter sterilized through a 0.2 μm filter (Pall Corporation, Ann Arbor, MI, USA).

In 96-well plates (Greiner Bio-One, Wemmel, Belgium), 200 μl of bacteria were added to either 20 μl of 0.03% chlorhexidine (CHX) (Sigma-Aldrich Co, St. Louis, MO, USA) (negative control), or 20 μl of L-arginine. Also, in each assay there was one well with just 200 μl BHI, without bacteria or substrate (white), and one well with just 200 μl of bacteria (positive control). Final concentrations of L-arginine were 22.73, 18.18, 9.09 and 4.55 $\mu\text{mol/ml}$. Four technical replicates for each condition were used. The plates were incubated at 37°C under anaerobic conditions or in a 5% CO_2 environment. Experiments were repeated three times at different days.

The growth was monitored by measuring the OD at 630 nm. For aerobic species the measurements were made every hour from 0h to 9h, and at 24h and 48h. For anaerobic species measurements were made at 0h, 24h and 48h.

At 24h and 48h, biofilm formation was monitored. The supernatant was removed and the wells were washed 2 times with 100 μl of sterilized 1 x phosphate buffered saline (PBS). Afterwards, 100 μl of ethanol (EtOH) were added to each well to fix the biofilms for 20 min. EtOH was removed and the plates were dried. Biofilms were stained by adding 100 $\mu\text{l/well}$ of a crystal violet solution (Sigma-Aldrich Co, St. Louis, MO, USA). After 15 min, the plates were rinsed with demineralized water to remove the remaining crystal violet and subsequently left to dry. The bound dye was solubilized by adding 75 $\mu\text{l/well}$ of 5% acetic acid for about 30-45 min. Finally, OD was measured at 630 nm.

3.3. DUAL-SPECIES COMPETITION ASSAYS

Overnight cultures of the pathogenic species *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *S. mutans*, *S. sobrinus* and the commensal species *S. gordonii*, *A. viscosus*, *S. mitis*, *S. oralis*, *S. salivarius* and *S. sanguinis* were prepared. Both *C. sputigena* and *V. parvula* were not used in this assay since previous experiences performed by the research team shown that these species were not able to grow. Since it is not known if *Actinomyces* are truly beneficial bacteria or just commensals, only *A. viscosus* was used in the assay since it showed significant results in the first assay, unlike *A. naeslundii*. Bacteria were collected from the blood

agar plates and transferred to 10 ml of BHI. The cultures were incubated overnight at 37°C either under anaerobic conditions or in a 5% CO₂ environment and concentration was adjusted to 1x10⁷ CFU/ml.

A mixture of 1 ml of the beneficial culture and 1 ml of the pathogenic culture were added to 24-well plates (Greiner Bio-One, Wemmel, Belgium). 200 µl of L-arginine (250 µmol/ml) or BHI as a negative control were added to the cultures and the plates were incubated for 24 h under anaerobic conditions at 37°C.

Biofilms at the bottom of the plates were gently washed with 1xPBS to detach the non-adhered cells and were disrupted by trypsinization (Trypsin-EDTA (0.05%), Life Technologies, Paisley, UK) for 15 min under anaerobic conditions at 37°C. Bacterial cells were harvested by centrifugation (5 min, 6,000 x g) and were resuspended in 400 µl of 1xPBS. Aliquots of biofilm samples were immediately incubated with propidium monoazide (PMA) (Biotium, Hayward, CA) at a final concentration of 100 µg/ml as described by Loozen et al [100]. Briefly, this process consisted of adding 10 µl of PMA to 90 µl of each sample. The samples were incubated for 5 min in the dark, and subsequently exposed for 10 min to a 400 W halogen light source placed 20 cm above the samples to induce the cross-linking of PMA. During this step samples were kept on ice to avoid excess heating. Samples were centrifuged at 20,000 x g for 10 min and DNA extraction was performed using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) in accordance to the manufacturer's instructions.

A quantitative PCR (qPCR) assay was performed using a CFX96 Real-Time System (Biorad, Hercules, CA, USA). The Taqman 5' nuclease assay PCR method was used for detection and quantification of bacterial DNA. Specific primers and Taqman probes for each species are shown in Table 1. Taqman reaction contained 12.5 µl Mastermix (Eurogentec, Seraing, Belgium), 4.5 µl sterile distilled water, 1 µl of each primer and probe and 5 µl template DNA. Assay conditions for all primer/probe sets consisted of an initial 2 min at 50°, followed by a denaturation step for 10 min at 95°C, followed by a denaturation step for 10 min at 95°C, followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. Quantification was based on a plasmid standard curve.

Table 1 - Primers/probes used in qPCR

STRAIN		Primer/Probe (5'-3')	Fragment length
<i>Aggregatibacter actinomycetemcomitans</i>	Forward Reverse Probe	GAA CCT TAC CTA CTC TTG ACA TCC GAA TGC AGC ACC TGT CTC AAA GC AGA ACT CAG AGA TGG GTT TGT GCC TTA GGG	80 bp
<i>Fusobacterium nucleatum</i>	Forward Reverse Probe	GGA TTT ATT GGG CGT AAA GC GGC ATT CCT ACA AAT ATC TAC GAA CTC TAC ACT TGT AGT TCC G	162 bp
<i>Porphyromonas gingivalis</i>	Forward Reverse Probe	GCG CTC AAC GTT CAG CC CAC GAA TTC CGC CTG C CAC TGA ACT CAA GCC CGG CAG TTT CAA	68 bp
<i>Prevotella intermedia</i>	Forward Reverse Probe	CGG TCT GTT AAG CGT GTT GTG CAC CAT GAA TTC CGC ATA CG TGG CGG ACT TGA GTG CAC GC	99 bp
<i>Streptococcus mutans</i>	Forward Reverse Probe	GCC TAC AGC TCA GAG ATG CTA TTC T GCC ATA CAC CAC TCA TGA ATT GA TGG AAA TGA CGG TCG CCG TTA TGA A	114 bp
<i>Streptococcus sobrinus</i>	Forward Reverse Probe	TTC AAA GCC AAG ACC AAG CTA GT CCA GCC TGA GAT TCA GCT TGT CCT GCT CCA GCG ACA AAG GCA GC	88 bp
<i>Actinomyces naeslundii</i>	Forward Reverse Probe	TCG AAA CTC AGC AAG TAG CCG AGA GGA GGG CCA CAA AAG AAA GGG TAC TCT AGT CCA AAC TGG CGG ATA GCG	96 bp
<i>Streptococcus gordonii</i>	Forward Reverse Probe	CGG ATG ATG CTA ATC AAG TGA CC GTT AGC TGT TGG ATT GGT TGC C AGA ACA GTC CGC TGT TCA GAG CAA	177 bp
<i>Actinomyces viscosus</i>	Forward Reverse Probe	GTG AAG GAG CCA GCT TGC TGG TTC TG CGG AAC AAA CCT TTC CCA GGC ATG AGT GGC GAA CGG GTG AGT AAC	155 bp
<i>Streptococcus salivarius</i>	Forward Reverse Probe	AAC GTT GAC CTT ACG CTA GC ACC GTA ACG TGG GAA AAC TG GTA GCG TCA GAG TGG TTG AC	192 bp
<i>Streptococcus oralis</i>	Forward Reverse Probe	ACC AGC AGA TAC GAA AGA AGC AT AGG TTC GGG CAA GCG ATC TTT CT AAG GCT GCT GTT GCT GAA GAA GT	229 bp
<i>Streptococcus mitis</i>	Forward Reverse Probe	GGC TCG TAG TCT GGA GAT GG TAG GTC GTC GTC CCA AGG AA CGA AGA GCA CCA ATA GCA CCT CCC	133 bp
<i>Streptococcus sanguinis</i>	Forward Reverse Probe	CAA AAT TGT TGC AAA TCC AAA GG GCT ATC GCT CCC TGT CTT TGA AAA GAA AGA TCG CTT GCC AGA ACC GG	75 bp
<i>Veillonella parvula</i>	Forward Reverse Probe	GAC GAA AGT CTG ACG GAG CA TGC CAC CTA CGT ATT ACC GC AGC TCT GTT AAT CGG GAC GAA AGG C	171 bp

3.4. MULTI-SPECIES ASSAYS

3.4.1. SET-UP BIOREACTOR

To establish multi-species communities, a Biostat B Twin 1L bioreactor (Sartorius Stedim Biotech GmbH, Goettingen, Germany) was used. Each of the culture vessels contained 750 ml BHI (37g/L) (Difco Laboratories, Detroit, MI, USA) supplemented with 2.5 g/L mucin from porcine stomach type III (Sigma-Aldrich Co, St. Louis, MO, USA), 1.0 g/L yeast extract (Oxoid Ltd, Basingstoke, Hampshire, England), 0.1 g/L L-cysteine (Calbiochem, San Diego, CA, USA), 2.0 g/L sodium bicarbonate, 5 mg/L hemin, 1 mg/L menadione and 0.25% (w/v) glutamic acid (all Sigma-Aldrich Co, St. Louis, MO, USA) (from now on referred as BHI 2). Growth conditions were set to 37°C, 0% O₂, 5% CO₂, pH 6.7 ± 0.1, stirring at 300 rpm.

The chemostat culture was prepared, containing 14 bacterial species including beneficial as well as pathogenic species. Overnight cultures of the pathogenic species *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *S. mutans*, *S. sobrinus* and the commensal/beneficial species *A. naeslundii*, *S. gordonii*, *A. viscosus*, *S. mitis*, *S. oralis*, *S. salivarius*, *S. sanguinis* and *V. parvula* were prepared. *C. sputigena* was not used for the reason described under 3.3. First, *S. mitis* was inoculated into the chemostat and grown until late exponential phase. The OD_{600nm} of each of the remaining species was adjusted to 1.4 and a volume of 750 µl of each species was inoculated to the chemostat culture. To stabilize the composition of the community the culture was kept undiluted for 48h and subsequently kept in continuous culture with an exchange of medium of 200 ml within 24h.

3.4.2. BIOFILM TREATMENT WITH L-ARGININE

A sample of the chemostat culture was taken and diluted 1:10 in fresh BHI 2 medium. Respectively, 2 ml of the culture were added to two wells of a 24-well plate. The plates were incubated for 24h under anaerobic conditions at 37°C.

For the following 3 days, biofilms were treated three times a day. Supernatant was removed from the wells and 500 µl of L-arginine dissolved in PBS (250 µmol/ml) or PBS as a negative control were applied for 3 min, while shaking at 250 rpm. The substrate solutions were removed and 2 ml of BHI 2 were added to the wells. On the fourth day, biofilms were washed once with PBS, followed by trypsinization for 15 min under anaerobic conditions at 37°C. Bacterial cells were harvested by centrifugation (5 min, 6,000 × g) and re-suspended in 500 µl PBS. Vitality DNA extraction and qPCR assays were performed as described under 3.3. The experiment was repeated on three different days.

3.4.3. PREVENTIVE EFFECT OF L-ARGININE

In addition to the above mentioned 14-species chemostat culture, a 'beneficial'-chemostat culture was established containing the commensal/beneficial species *A. naeslundii*, *S. gordonii*, *A. viscosus*, *S. mitis*, *S. oralis*, *S. salivarius*, *S. sanguinis* and *V. parvula*. Inoculation of the bacteria into the chemostat was carried out as previously described.

A sample of this beneficial-community was taken and diluted 1:10 in fresh BHI 2 medium. Respectively, 2 ml of the culture were added to two wells of a 24-well plate, as well as to two well containing calcium deficient hydroxyapatite (HA) discs (Hitemco Medical, Old Bethpage, NY, USA). The plates were incubated for 24h under anaerobic conditions at 37°C.

For treatment of the biofilms, L-arginine (250 µmol/ml) was dissolved in a mouthwash base (pH 5.7) provided by Colgate-Palmolive Company, NJ, USA. For the following two days biofilms were rinsed twice a day as described above, however after treatment with the substrate a washing step with 500 µl of BHI 2 was included to remove any residues of the substrate. The mouthwash base (pH 5.7) was used as a control. After the second day of treatment, biofilms were challenged overnight with a 1:10 dilution of the 14-species community, containing beneficial as well as pathogenic species. To monitor if incorporation of pathogens into the biofilm is prevented or slowed down due to the previous L-arginine treatment, over the following 24 h biofilms were rinsed twice with BHI 2 to provide new nutrients.

On the fourth day, biofilms were washed once with PBS, followed by trypsinization for 45 min at 37°C, 350 rpm. Biofilms grown on HA discs were vortexed for 15 sec, and the supernatant was sonicated for 2 min (Branson 2510 Ultrasonic Cleaner, Branson Ultrasonics Corp., Danbury, CT, USA) to deposit remaining HA.

Bacterial cells were harvested by centrifugation (5 min, 6,000 × g) and re-suspended in 500 µl PBS. Vitality DNA extraction and qPCR assays were performed as described under 3.3. The experiment was repeated on three different days.

3.4.4. PREVENTIVE EFFECT OF L-ARGININE WITH CONTINUOUS TREATMENT

Assays were performed as described under 3.4.3., however rinsing was continuously carried out over 3 days with L-arginine, to monitor for a preventive and on-going effect of L-arginine. The assays were repeated on four different days.

3.5. DATA ANALYSIS

For single-species growth and biofilm formation assays the maximum OD value within 48 h was determined. A linear mixed model was used for each species separately using compound and time as crossed fixed factors and run as random factor. Inference for difference from the control between concentrations and the null concentration was calculated by means of contrasts. All p-values were corrected for simultaneous hypothesis testing according to Sidak [101].

Data of the dual species competition assays and multi species assays were analyzed by using a linear mixed model for each combination of species using compound as fixed factor and runs as random factor. Biofilm formation and composition in response to L-arginine was compared with the control. All p-values were corrected for simultaneous hypothesis testing according to Dunnett [102]. Analysis was performed on percent quantity of each bacterium for dual species competition assays, and on log(CFU/ml) values for multi species assays. The validity of each linear mixed model was assessed by means of a normal quantile plot and residual dot plot. Data of prevention assays were analyzed by means of Student's t-test. Significance level was set at $p < 0.05$.

CHAPTER 4 – RESULTS

4.1. GROWTH AND BIOFILM FORMATION IN THE PRESENCE OF L-ARGININE

To monitor the stimulatory effect of L-arginine on beneficial/commensal species, growth curves and biofilm formation assays were set up over 48 hours (Annex A - Growth and biofilm formation in the presence of L-arginine). Statistically significant values for growth stimulation and biofilm formation are shown in Figure 5 (A and B, respectively).

Regarding Figure 5A, it is possible to observe that growth of *A. viscosus* was stimulated by L-arginine in concentrations of 20 $\mu\text{mol/ml}$ and 10 $\mu\text{mol/ml}$ by a factor of $1,39 \pm 0,13$ and $1,19 \pm 0,21$, respectively, in comparison to the control ($p < 0,05$). *S. sanguinis* also showed significant growth stimulation by L-arginine concentrations of 20, 10 and 5 $\mu\text{mol/ml}$ by a factor of up to $1,31 \pm 0,07$. Moreover, growth stimulation of *S. gordonii* was observed for all concentrations of L-arginine, with the highest stimulation of a factor of $1,26 \pm 0,08$ (25 $\mu\text{mol/ml}$) ($p < 0,05$). However, none of the pathogenic strains was stimulated by L-arginine.

Observing Figure 5B, which regards biofilm formation, L-arginine significantly increased biofilm formation of *A. viscosus* at a concentration of 20 $\mu\text{mol/ml}$ and *S. oralis* at a concentration of 5 $\mu\text{mol/ml}$ by a factor of $1,55 \pm 0,31$ and $1,03 \pm 0,09$, respectively ($p < 0,05$). The pathogenic strains *A. actinomycetemcomitans* and *P. gingivalis* were stimulated by L-arginine by a factor of up to $2,53 \pm 0,46$ (5 $\mu\text{mol/ml}$) for *A. actinomycetemcomitans* and by a factor of $1,03 \pm 0,14$ (10 $\mu\text{mol/ml}$) for *P. gingivalis*.

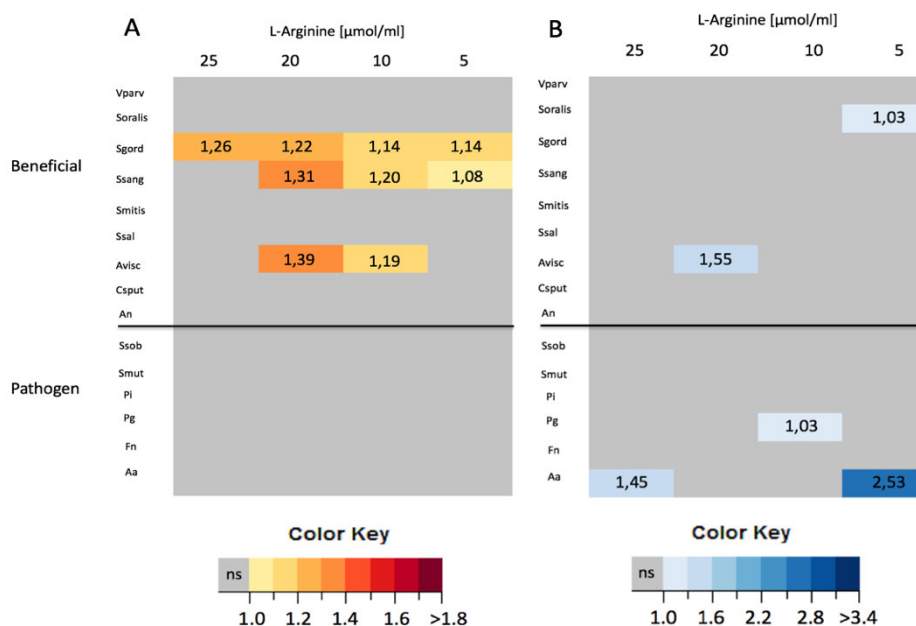


Figure 5 - Statistically significant values regarding the growth stimulation assays (A) and the biofilm formation assays (B) and the respective color key scale ($p < 0,05$), the reference control was set to a value of 1. Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Smut: *Streptococcus mutans*, Ssob: *Streptococcus sobrinus*, Avisc: *Actinomyces viscosus*, Ssal: *Streptococcus salivarius*, Smitis: *Streptococcus mitis*, Ssang: *Streptococcus sanguinis*, Sgord: *Streptococcus gordonii*, Soralis: *Streptococcus oralis*.

4.2. DUAL-SPECIES COMPETITION ASSAYS

Competitive assays were used to determine if in a dual species environment, the beneficial species can outgrow the pathogens by means of selective nutritional stimulation (Annex B – Dual species competition assays). The proportional decrease/increase of the pathogenic species was calculated for each combination (Figure 6).

The addition of L-arginine to the combination of *A. actinomycetemcomitans* and either *S. gordonii* or *S. mitis* led to an increase of the proportion of the pathogenic species ($p < 0,05$). On the contrary, L-arginine did stimulate the outgrowth of *A. viscosus* while reducing the pathogens *A. actinomycetemcomitans* and *P. gingivalis* by $-25,66\% \pm 5,93\%$ and $-97,31\% \pm 1,73\%$, respectively. Even more efficient was the addition of L-arginine to the assays of *S. sanguinis* in combination with *P. gingivalis*, *S. mutans* or *S. sobrinus*, resulting in a reduction of the pathogenic proportion in the biofilm of $-85,49\% \pm 19,69\%$ for *P. gingivalis*, $-19,30\% \pm 5,74\%$ for *S. mutans* and $-40,73\% \pm 4,21\%$ for *S. sobrinus* ($p < 0,05$).

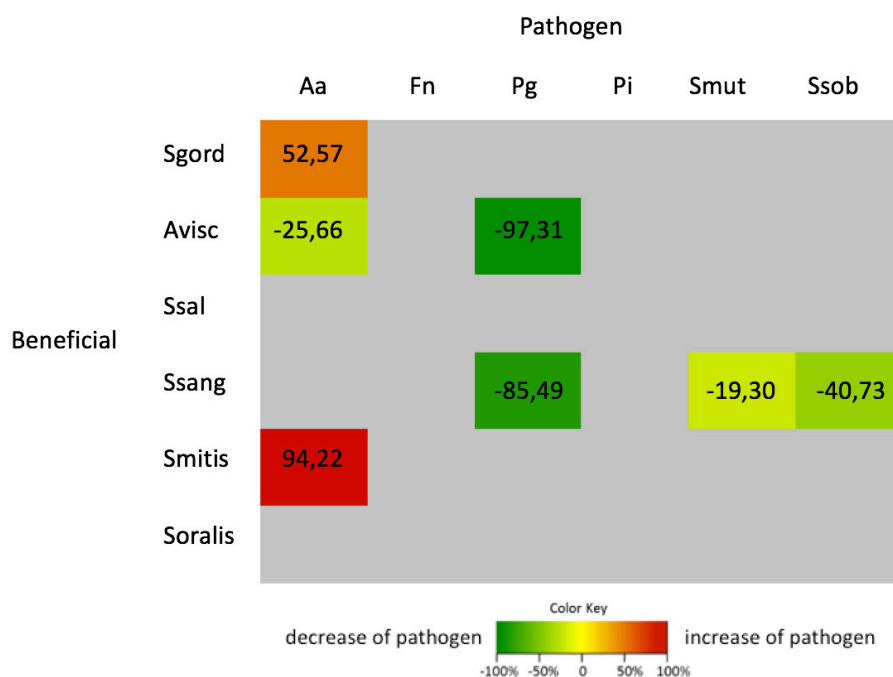


Figure 6 - Decrease/increase of pathogenic species in dual species biofilms in comparison to the no substrate control [%] ($p < 0.05$). Grey area shows non-significant data. Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Smut: *Streptococcus mutans*, Ssob: *Streptococcus sobrinus*, Avisc: *Actinomyces viscosus*, Ssal: *Streptococcus salivarius*, Smitis: *Streptococcus mitis*, Ssang: *Streptococcus sanguinis*, Sgord: *Streptococcus gordonii*, Soralis: *Streptococcus oralis*.

4.3. MULTI-SPECIES ASSAYS

Biofilm formation and composition in response to the treatment with L-arginine was compared to the negative control and proportional decrease or increase of the species was calculated. For pathogenic species a decrease, for beneficial species an increase compared to the negative control was highlighted (Tables 2, 3 and 4).

4.3.1. BIOFILM TREATMENT WITH L-ARGININE

Biofilm treatment with L-arginine showed a reduction of the pathogens *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *S. mutans* and *S. sobrinus*. Numbers of *P. gingivalis* and *P. intermedia* were reduced by more than a 1-log reduction, with the decrease of *P. gingivalis* being statistically significant ($p < 0,05$) (Table 2). Additionally, there was an increase of the beneficial species *A.*

naeslundii, *A. viscosus*, *S. sanguinis* and *S. oralis* due to the treatment with L-arginine (Table 2), however, data were not statistically significant ($p>0,05$).

Table 2 - Bacterial numbers shown as log(CFU/ml) comparing treatment of biofilms with L-arginine and the control (PBS). Reduction of pathogenic species and increase of beneficial species in comparison to the negative control are highlighted with green background. Bold numbers indicate a -1log reduction in comparison to the negative control. Significant reduction of pathogenic species are highlighted by black rectangles ($p<0.05$). Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Smut: *Streptococcus mutans*, Ssob: *Streptococcus sobrinus*, Avisc: *Actinomyces viscosus*, Ssal: *Streptococcus salivarius*, Smitis: *Streptococcus mitis*, Ssang: *Streptococcus sanguinis*, Sgord: *Streptococcus gordonii*, Soralis: *Streptococcus oralis*.

		Biofilm treatment [log(CFU/ml)]	
		No Substrate	L-arginine
Pathogens	Aa	6,04±(2,47)	6,42±(3,64)
	Fn	9,97±(0,68)	9,64±(1,03)
	Pg	7,93±(0,36)	5,93±(1,17)
	Pi	8,52±(0,28)	5,93±(1,75)
	Smut	7,45±(0,50)	7,24±(0,74)
	Ssob	7,6±(0,58)	7,13±(0,71)
Commensal/Beneficial	An	6,68±(1,63)	7,15±(1,47)
	Sgord	8,96±(0,49)	8,68±(0,40)
	Avisc	6,32±(0,08)	6,72±(0,97)
	Ssal	3,06±(2,68)	2,64±(2,31)
	Ssang	3,18±(2,84)	3,39±(2,93)
	Smitis	8,33±(0,13)	8,15±(0,79)
	Soralis	7,2±(0,25)	7,3±(0,21)
	Vparv	10,42±(0,58)	10,03±(0,41)

4.3.2. PREVENTIVE EFFECT OF L-ARGININE

Beneficial biofilms grown on the bottom of 24-well plates and treated with L-arginine did not show a prevention of the incorporation of pathogenic species into the biofilm when compared to the control (Table 3). On the other hand, for biofilms grown on HA discs and treated with L-arginine, a significant reduction of *P. intermedia* by -8% was observed ($p<0,05$) (Table 3).

The overall beneficial proportion of the biofilms did not change due to treatment with L-arginine when compared to the control. However, *A. viscosus* was significantly stimulated by

treatment with L-arginine when biofilms were grown on the bottom of 24-well plates ($p < 0,05$) (Table 3).

Table 3 - Bacterial numbers shown as log(CFU/ml) comparing preventive treatment of biofilms with L-arginine and the control (PBS). Biofilms were grown on the bottom of 24-well plates (polyethylene) or on hydroxyapatite discs. Reduction of pathogenic species and increase of beneficial species in comparison to the control are highlighted with green background. Significant reduction of pathogenic species or increase of beneficial species are highlighted by black rectangles ($p < 0.05$). Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Smut: *Streptococcus mutans*, Ssob: *Streptococcus sobrinus*, Avisc: *Actinomyces viscosus*, Ssal: *Streptococcus salivarius*, Smitis: *Streptococcus mitis*, Ssang: *Streptococcus sanguinis*, Sgord: *Streptococcus gordonii*, Soralis: *Streptococcus oralis*.

		Prevention [log(CFU/ml)]			
		Plastic		Hydroxyapatite	
		No substrate	L-arginine	No substrate	L-arginine
Pathogens	Aa	2,45±(2,15)	3,16±(0,80)	2,02±(1,75)	2,24±(1,94)
	Fn	9,10±(0,41)	9,59±(0,46)	8,94±(0,38)	8,93±(1,10)
	Pg	4,78±(0,56)	5,12±(0,25)	4,43±(1,34)	4,47±(0,83)
	Pi	5,89±(3,12)	5,90±(3,10)	5,95±(2,90)	5,48±(2,96)
	Smut	6,47±(0,35)	6,62±(0,36)	6,55±(0,68)	6,70±(0,64)
	Ssob	5,42±(0,94)	5,50±(0,78)	6,00±(0,95)	6,80±(0,25)
Commensal/Beneficial	An	7,57±(0,55)	7,64±(0,41)	6,83±(0,45)	7,02±(0,77)
	Sgord	9,49±(0,51)	9,59±(0,48)	9,21±(0,32)	8,99±(0,25)
	Avisc	6,13±(4,05)	6,49±(0,49)	5,63±(0,58)	5,51±(0,26)
	Ssal	4,05±(3,82)	4,05±(7,93)	7,92±(0,29)	7,11±(0,74)
	Ssang	7,92±(0,48)	7,93±(0,48)	7,96±(0,48)	7,85±(0,54)
	Smitis	2,60±(2,25)	2,62±(2,28)	2,79±(2,44)	2,76±(2,54)
	Soralis	7,78±(1,63)	7,76±(1,47)	8,58±(2,19)	9,00±(1,30)
	Vparv	9,90±(0,61)	9,99±(0,42)	10,15±(0,58)	10,00±(0,63)

4.3.3. PREVENTIVE EFFECT OF L-ARGININE WITH CONTINUOUS TREATMENT

For the prevention assays with continuous treatment of biofilms with L-arginine a decrease of pathogenic strains was observed for biofilms grown on the bottom of 24-well plates as well as for biofilms grown on HA discs (Table 4). Treatment with L-arginine of biofilms grown on HA discs significantly reduced the pathogen *S. mutans* by -10 % ($p < 0,05$). Further, a decrease of *A. actinomycetemcomitans* by more than -1 log reduction was observed (Table 4).

No significant effect of the treatment with L-arginine was observed for the beneficial bacteria.

Table 4 - Bacterial numbers shown as log(CFU/ml) comparing preventive, albeit continuous treatment of biofilms with L-arginine and the control (PBS). Biofilms were grown on the bottom of 24-well plates (polyethylene) or on hydroxyapatite discs. Reduction of pathogenic species and increase of beneficial species in comparison to the negative control are highlighted with green background. Bold numbers indicate a -1log reduction in comparison to the negative control. Significant reduction of pathogenic species are highlighted by black rectangles ($p < 0.05$). Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Smut: *Streptococcus mutans*, Ssob: *Streptococcus sobrinus*, Avisc: *Actinomyces viscosus*, Ssal: *Streptococcus salivarius*, Smitis: *Streptococcus mitis*, Ssang: *Streptococcus sanguinis*, Sgord: *Streptococcus gordonii*, Soralis: *Streptococcus oralis*.

		Prevention + Continuous Treatment [log(CFU/ml)]			
		Plastic		Hydroxyapatite	
		No substrate	L-arginine	No substrate	L-arginine
Pathogens	Aa	2,68±(1,83)	3,07±(0,62)	4,35±(2,12)	2,73±(0,74)
	Fn	8,37±(0,60)	8,84±(0,32)	7,79±(1,10)	7,00±(0,91)
	Pg	5,01±(0,44)	4,83±(0,24)	5,05±(0,91)	4,47±(0,50)
	Pi	6,33±(2,59)	5,96±(2,93)	6,03±(2,92)	5,19±(2,45)
	Smut	6,71±(0,33)	6,56±(0,48)	7,84±(0,77)	7,07±(0,80)
	Ssob	5,67±(0,55)	5,65±(0,37)	7,10±(0,80)	6,47±(0,84)
Commensal/Beneficial	An	8,03±(0,35)	7,74±(0,19)	8,88±(3,37)	6,56±(1,42)
	Sgord	7,34±(4,91)	9,54±(0,61)	9,29±(0,28)	8,94±(0,20)
	Avisc	6,08±(0,51)	5,82±(0,38)	5,40±(0,68)	4,98±(0,49)
	Ssal	4,95±(2,48)	4,22±(2,46)	7,87±(1,05)	6,38±(1,11)
	Ssang	8,40±(0,38)	8,15±(0,17)	8,37±(0,36)	8,15±(0,40)
	Smitis	2,40±(2,00)	2,39±(2,04)	2,30±(2,66)	2,48±(2,39)
	Soralis	7,33±(1,62)	6,95±(1,81)	8,38±(1,10)	7,84±(1,11)
	Vparv	10,17±(0,43)	9,99±(0,24)	10,09±(0,53)	9,42±(0,26)

CHAPTER 5 – DISCUSSION

5. DISCUSSION

Oral biofilms are complex biological systems, characterized by multiple interspecies interactions as well as being in a dynamic relationship with the surrounding environment. As such, several exo- and endogenous factors determine the beneficial or pathogenic effect of these biofilms towards the host. In the present study we demonstrated that prebiotic treatment of oral biofilms with L-arginine resulted in a modulation of multi-species biofilms composition towards a more healthy-associated microbiota in vitro.

Until nowadays the prebiotic definition is still limited to the gastrointestinal tract. This study reveals one of the first attempts to transfer the concept of selective outgrowth of endogenous beneficial bacteria by means of nutritional stimulation to oral health. Here, the amino acid L-arginine triggered the growth of beneficial/commensal oral bacteria and thereby leading to a suppression of pathogenic species. In fact, it is well documented in literature that arginine deiminase system (ADS)-positive bacteria are abundant members of the oral microbiota that colonize the teeth and soft tissues. Several streptococci, in particular *S. gordonii*, *S. sanguinis*, *S. mitis*, *S. salivarius* and certain *Actinomyces* species have been shown to metabolize arginine by the ADS [29,103,104]. The ADS is a three enzyme pathway that releases ornithine, ammonia and CO₂ and provides adenosine triphosphate (ATP) to bacteria. First, arginine is converted to citrulline via arginine deiminase (AD). Then, the citrulline generated in the previous phase is acted on by a catabolic ornithine transcarbamylase (cOTC) to produce ornithine and carbamyl phosphate in the presence of inorganic phosphate. Finally, the third enzyme in the pathway, carbamate kinase (CK), cleaves the carbamyl phosphate to ammonia and CO₂, simultaneously donating the phosphate to ADP, producing ATP [29,105]. The released ammonia can increase the environmental pH, and might prevent the outgrowth of cariogenic bacteria, that tend to prefer a more acidic environment, which in consequence might reduce the risk of oral diseases [104,106,107]. The capacity of oral bacteria to modulate the environmental pH can be a critical factor in the prevention of caries, since neutralization of acids might prevent the emergence of a cariogenic microbiota [29]. Studies show that subjects that are caries-resistant have a greater concentration of ammonia in their plaque and a higher resting pH, which controls the outgrowth of cariogenic bacteria that depend on low pH to gain an ecological advantage [104,108].

In this study L-arginine was shown to be used by *A. viscosus*, *S. gordonii* and *S. sanguinis* (Figure 5A), species that are known for being ADS-positive [29,103]. Also Huang et al [109] showed

that ADS-activity of *S. gordonii* and *S. sanguinis* was higher when arginine was present. A higher ADS activity may result in increased ATP production, favouring the growth of bacteria. Although significant results were obtained for these species, other beneficial species were not stimulated by the substrate. According to Burne et al [29], arginolysis can supply sufficient ATP for growth, however, this growth is normally modest. Additionally, growth stimulation of L-arginine was selective, as no increased growth of any of the six pathogenic species used in this study could be observed (Figure 5A).

On the other hand, biofilm formation of *P. gingivalis* and *A. actinomycetemcomitans* were stimulated by L-arginine (Figure 5B). This might be related to the fact that *A. actinomycetemcomitans* is able to form extremely tenacious biofilms in vitro, being that biofilm formation is an important virulence factor in this species [44,110]. As so, the presence of L-arginine by itself was not sufficient to prevent the biofilm formation by this bacterium. Cugini and co-workers [51] showed that *P. gingivalis* can use L-arginine to promote its biofilm formation, so we might predict that this specie used the present L-arginine to form the biofilm.

Although the presence of L-arginine and its utilization by the ADS affected and modulated bacterial growth and biofilm formation in this study, bacterial, as well as, metabolic interactions need to be taken into consideration. Several oral bacteria, mainly streptococci, are capable of producing antimicrobial compounds, such as hydrogen peroxide (H_2O_2), which was shown to inhibit the growth of pathogenic oral bacteria [4,96,111]. A study by Herrero et al [96] showed that *S. sanguinis*, *S. gordonii*, *S. mitis* and *S. oralis* significantly inhibited the growth of three periodontal pathogens, namely *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* using H_2O_2 . However, it was also observed that inhibition only occurred when the commensal species were inoculated 24 hours before adding the pathogen and that inhibition was more pronounced under aerobic conditions, indicating strong influence of environmental factors on pathogen inhibition. In accordance with the study of Herrero et al [96], our findings in dual species competition assays showed (Figure 6) that *A. actinomycetemcomitans* was rarely inhibited by beneficial species. Previous studies already claimed that *S. gordonii* might act as an accessory pathogen since it produces lactate as an end-product of glycolysis, which subsequently might be consumed by *A. actinomycetemcomitans* [54,112,113]. This metabolic interaction is also known as cross-feeding, in which metabolic by-products, in this case lactate, of one species are used by other species as an energy or nutrient source and supporting the growth of both organisms [114]. Similar metabolic

interactions have been reported for *P. gingivalis* and *S. gordonii*, where *S. gordonii* provides metabolic support for the growth of *P. gingivalis* [54].

A. viscosus and *S. sanguinis* were stimulated by L-arginine treatment, and by this actively reduced the proportion of *P. gingivalis* in dual-species competition assays (Figure 6). The expression of fimbriae is a key determinant that regulates colonization and virulence in *P. gingivalis*. The surface *P. gingivalis* cells present two distinct fimbrial structures: the long fimbriae, mainly consisting of FimA protein subunits, and the short fimbriae composed of Mfa1 subunit. Long fimbriae have been associated with adherence and invasion of *P. gingivalis* to gingival epithelial cells, to bone resorption, co-aggregation with other oral isolates, and biofilm formation [115]. A study showed that exposing *P. gingivalis* for 1h to arginine deiminase, translated in the down regulation of the transcription of both FimA and Mfa1 [116]. This down regulation was shown to be due to the conversion of arginine to citrulline by the ADS [51]. Citrulline accumulation has negative effects on FimA expression and biofilm formation by *P. gingivalis* [54]. The arginine deiminase showed inhibition of *P. gingivalis* biofilm formation [116]. Although *P. gingivalis* can use arginine for biofilm formation, in combination with ADS-positive strains, such as *A. viscosus* and *S. sanguinis*, *P. gingivalis* tends to be inhibited [51]. Also, *S. sanguinis* was capable of reducing counts of *S. mutans* and *S. sobrinus* (Figure 6) when in combination with these two pathogens. Studies have been showing that *S. sanguinis* is capable of antagonizing *S. mutans* [4,117]. Since *S. mutans* and *S. sobrinus* are both members of the group mutans streptococci and are closely related, it is plausible that *S. sanguinis* is also capable of antagonizing *S. sobrinus* [70,71].

In the present study, it was demonstrated that L-arginine is capable of modulating multi-species oral biofilms, as beneficial species were stimulated, likely to change the environmental pH and to produce ATP. As a result, the number of pathogenic oral species in the biofilm were reduced. However, it seems very likely that the production of H₂O₂ by beneficial strains, mainly streptococci, also plays a role in modulating the biofilm. The present study also showed that results vary in accordance to the complexity of the biofilm community, and predictions about multi-species biofilm could not be made based on the outcome of single or dual-species assays.

The treatment with L-arginine (Table 2) resulted in a reduction of *P. gingivalis*, which is a keystone in chronic adult periodontitis [118]. This reduction of *P. gingivalis* was expected, according to the explanation aforementioned.

Treatment of beneficial biofilms with L-arginine did not show a preventive effect on the incorporation of pathogenic species into the biofilm community (Table 3). It might be possible that

beneficial strains constantly need L-arginine in order to produce ammonia to maintain a higher environmental pH and also to produce ATP to favour their growth. However a statistically significant reduction in this assay (Table 3) and also a reduction of 1log in the treatment assay (Table 2) was observed for *P. intermedia*. It is possible that the beneficial species, mainly streptococci, were able to inhibit the growth of *P. intermedia*. In fact, a study by Herrero et al [96] has shown that *P. intermedia* was inhibited when incubated with *S. sanguinis*, *S. oralis*, *S. gordonii* and *S. mitis*.

Promising results were obtained when the combination of a preventive treatment effect plus a continuous treatment was applied, since a reduction of pathogenic species could be observed (Table 4). For *A. actinomycescomitans* a reduction of 1 log was detected which can be considered as being clinically relevant. A possible explanation might be that *S. sanguinis* produces H₂O₂ which is known to inhibit growth of *A. actinomycescomitans* [119]. Additionally, the number of *S. mutans* were significantly decreased (Table 4). *S. sanguinis* is capable of antagonizing *S. mutans* [4,117]. Epidemiological studies showed that when *S. sanguinis* was the early colonizer and high levels of this specie were present in an infant's oral cavity, colonization by *S. mutans* was delayed [120]. As all beneficial species were inoculated prior to the pathogens, inhibition by H₂O₂ seems plausible [96]. However, qualitative and quantitative analysis of H₂O₂ needs to be carried out to confirm this hypothesis.

In the present study, it was demonstrated that L-arginine is capable of modulating the oral biofilm, since beneficial species present in the oral cavity are capable of using this substrate in order to raise the pH and to produce ATP. This mechanism favours the growth of the beneficial species and, as a result, reduces the pathogenic oral species in the biofilm. However, it seems very likely that the production of H₂O₂ by beneficial strains, mainly streptococci, also plays a role in modulating the biofilm. This study also showed that results vary in accordance to the type of biofilm, and predictions about a multi-specie biofilm cannot be made based on single or dual-species assays.

Nonetheless, the findings point towards a potential preventive plus continuous treatment strategy, using L-arginine in young individuals in order to prevent the establishment of aggressive periodontitis, since this disease affects mainly young people and its primary etiologic agent is *A. actinomycescomitans*. Also the emergence of caries might be addressed by L-arginine treatment, since *S. mutans* is considered one of the major cariogenic bacteria [4,34,42].

Noteworthy, the decrease of pathogenic species was more pronounced when biofilms were grown on hydroxyapatite discs when compared to biofilms grown on polyethylene (PE) surfaces

(Tables 3 and 4). Hydroxyapatite is one of the most studied materials in dental practise because of its chemical similarity with the tooth enamel, its biocompatibility and bioactivity [121]. As such, salivary pellicle formation and primary colonization by bacteria might be similar on hydroxyapatite discs to natural teeth in the oral cavity. Further, hydroxyapatite shows a strong buffering capacity, which in consequence might influence the biofilm composition and efficacy of potential prebiotic solutions [122]. Using L-arginine to promote a more healthy-associated state of the oral microbiota might also be an important approach for people with dental implants, as dental implants are commonly coated with hydroxyapatite.

Also, one study showed that the age of the biofilm influences NH_3 production by the ADS [123]. The older the biofilm, the greater the ammonia production. It is possible that, if the experiment is extended in time, allowing biofilm to get older, better results may be accomplished. Also, the influence of various environmental factors, like oxygen concentration, nutrient availability etc., needs to be elucidated as the study by Herrero et al showed that these factors can play an important role in the inhibition of periodontal pathogens [96,124]. Additionally, alkali production from arginine depends on environmental factors like sucrose presence, pH and buffer capacity, which could further have an influence on the production of H_2O_2 [104,123].

In conclusion the present study showed a potential prebiotic effect of L-arginine on oral biofilms, shifting complex microbiota towards a more healthy-associated composition. Application of prebiotics in oral health might be a promising approach to prevent the shift from a health associated towards a dysfunctional microbiota. However, clinical studies need to prove and confirm the benefits of prebiotics as a concept in oral health *in vivo*.

CHAPTER 6 – CONCLUSIONS

6.1. CONCLUSIONS

It was demonstrated in this work that L-arginine could successfully trigger the selective outgrowth of beneficial oral bacteria in single-, dual- and multispecies biofilms, *in vitro*. By shifting and modulating a complex oral microbiota towards a more beneficial-dominated composition, L-arginine can be considered as a potential oral prebiotic compound.

Continuous application of L-arginine might reveal a novel approach to prevent the shift of a healthy microbiota to a pathogenic state.

In accordance with the definition of prebiotics, this study showed that interspecies interactions are of great importance as a prebiotic effect cannot be solely evaluated based on single-species experiments. On the other hand, the *in vitro* testing model used in this study based on a multi-species microbiota showed great potential to search for and identify promising oral prebiotic compounds.

6.2. FUTURE PERSPECTIVES

Studies about prebiotics in the oral field must continue to be performed since these compounds might be of great importance to treat oral diseases and to establish and maintain oral health.

Future studies on this field should take into account the influence of environmental factors such as oxygen concentration, nutrient availability and age of biofilms in order to ensure a prebiotic effect independent of varying conditions and elucidating the underlying mechanism of the prebiotic action.

Also, the prebiotic effect of L-arginine needs to be confirmed using *in vivo* assays and subsequent clinical trials.

Besides L-arginine, other prebiotic compounds should be tested in order to evaluate their effect in the oral environment.

CHAPTER 7 – BIBLIOGRAPHY

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ANNEXES

A. Growth and biofilm formation in the presence of L-arginine

A.1. Bacterial growth in the presence of L-arginine

The following Figures (7-11) show the growth curves for the species used in this study in the presence of different concentration of L-arginine compared to the control.

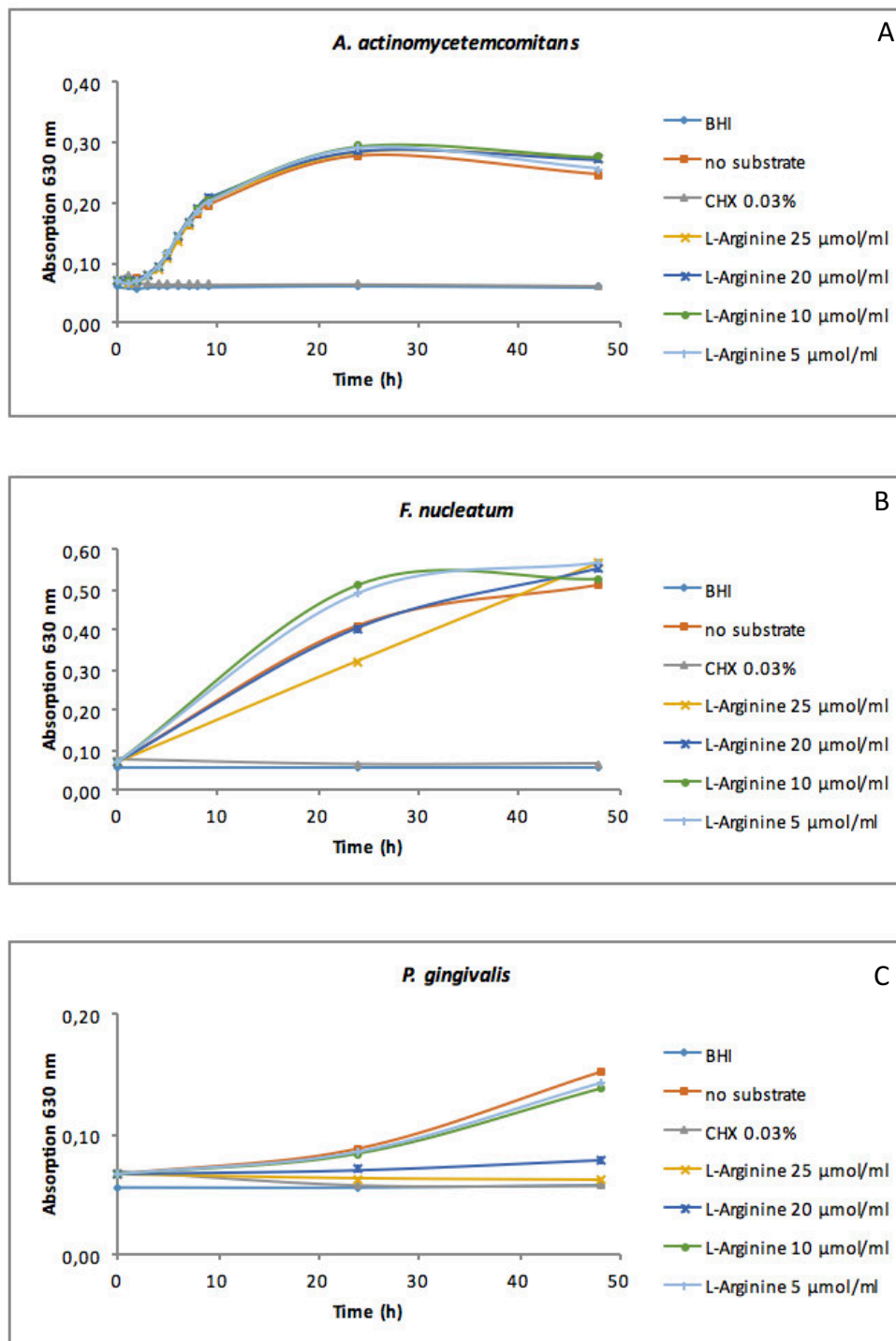


Figure 7 – Growth curves for *A. actinomycetemcomitans* (A), *F. nucleatum* (B) and *P. gingivalis* (C) in the presence of different concentrations of L-arginine.

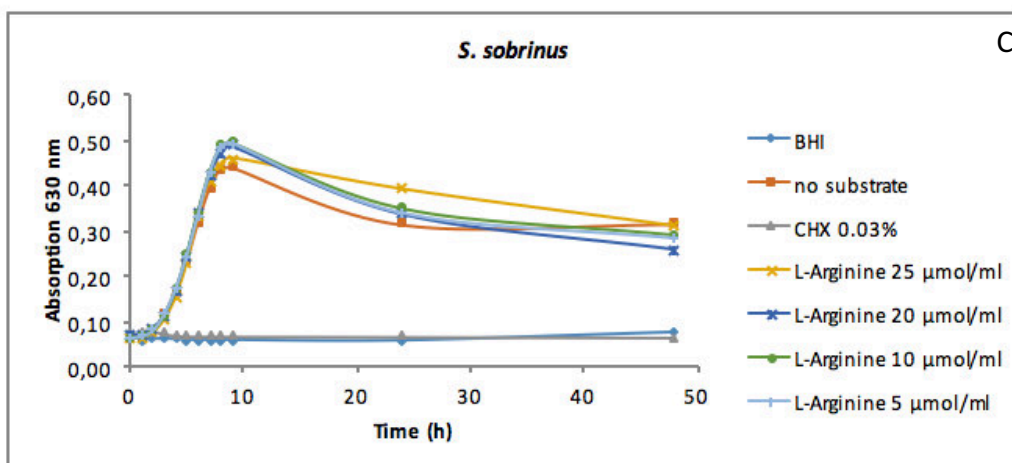
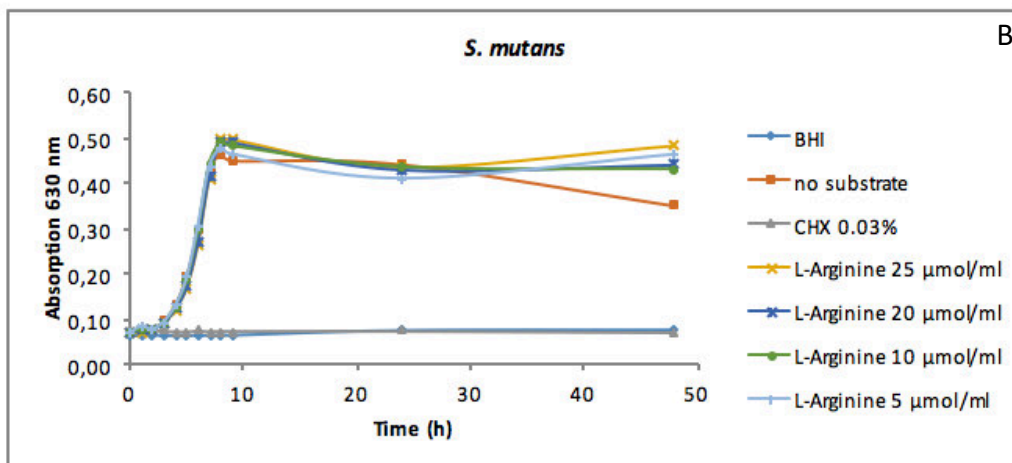
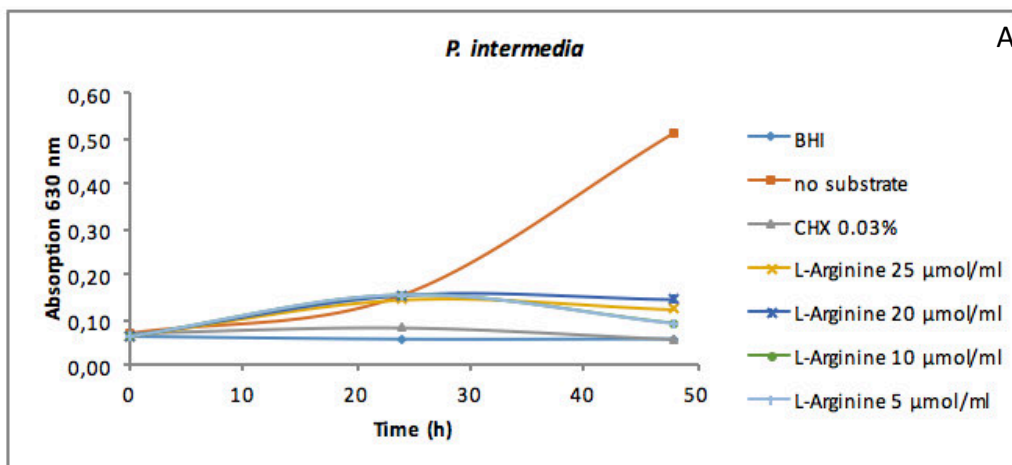


Figure 8 - Growth curves for *P. intermedia* (A), *S. mutans* (B) and *S. sobrinus* (C) in the presence of different concentrations of L-arginine.

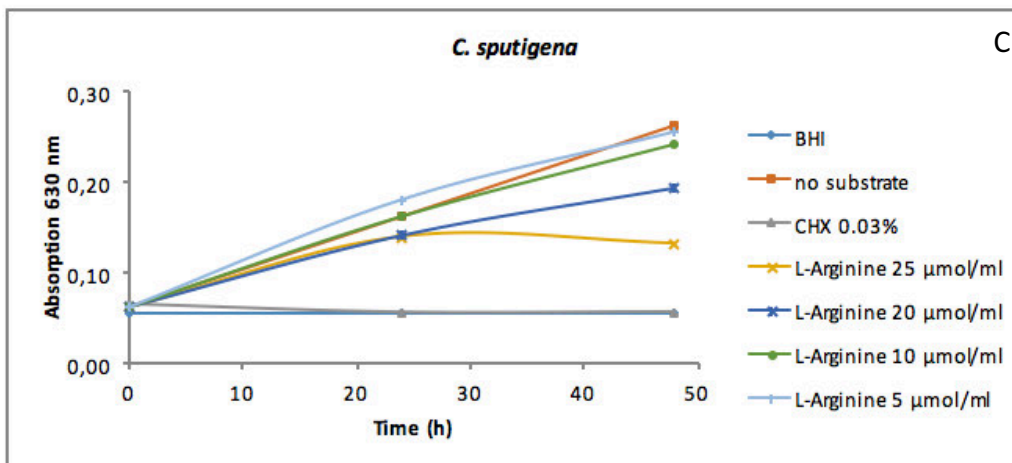
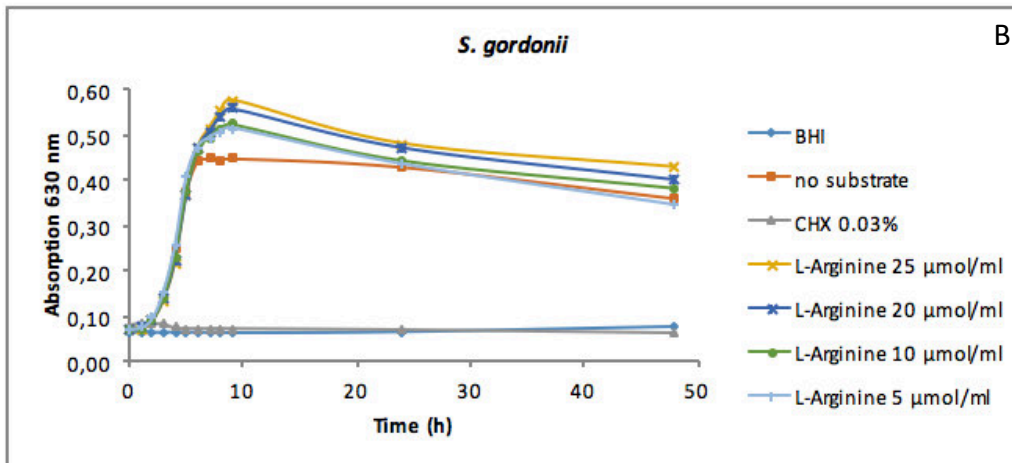
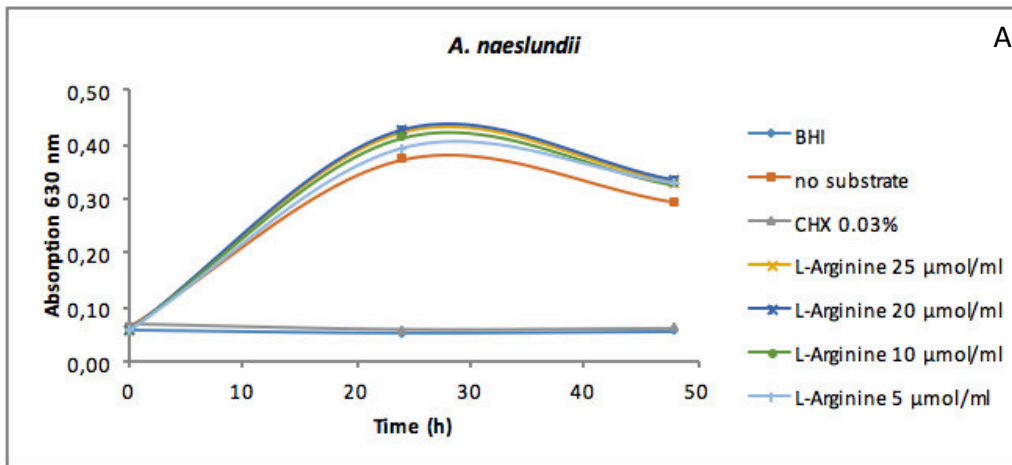


Figure 9 - Growth curves for *A. naeslundii* (A), *S. gordonii* (B) and *C. sputigena* (C) in the presence of different concentrations of L-arginine.

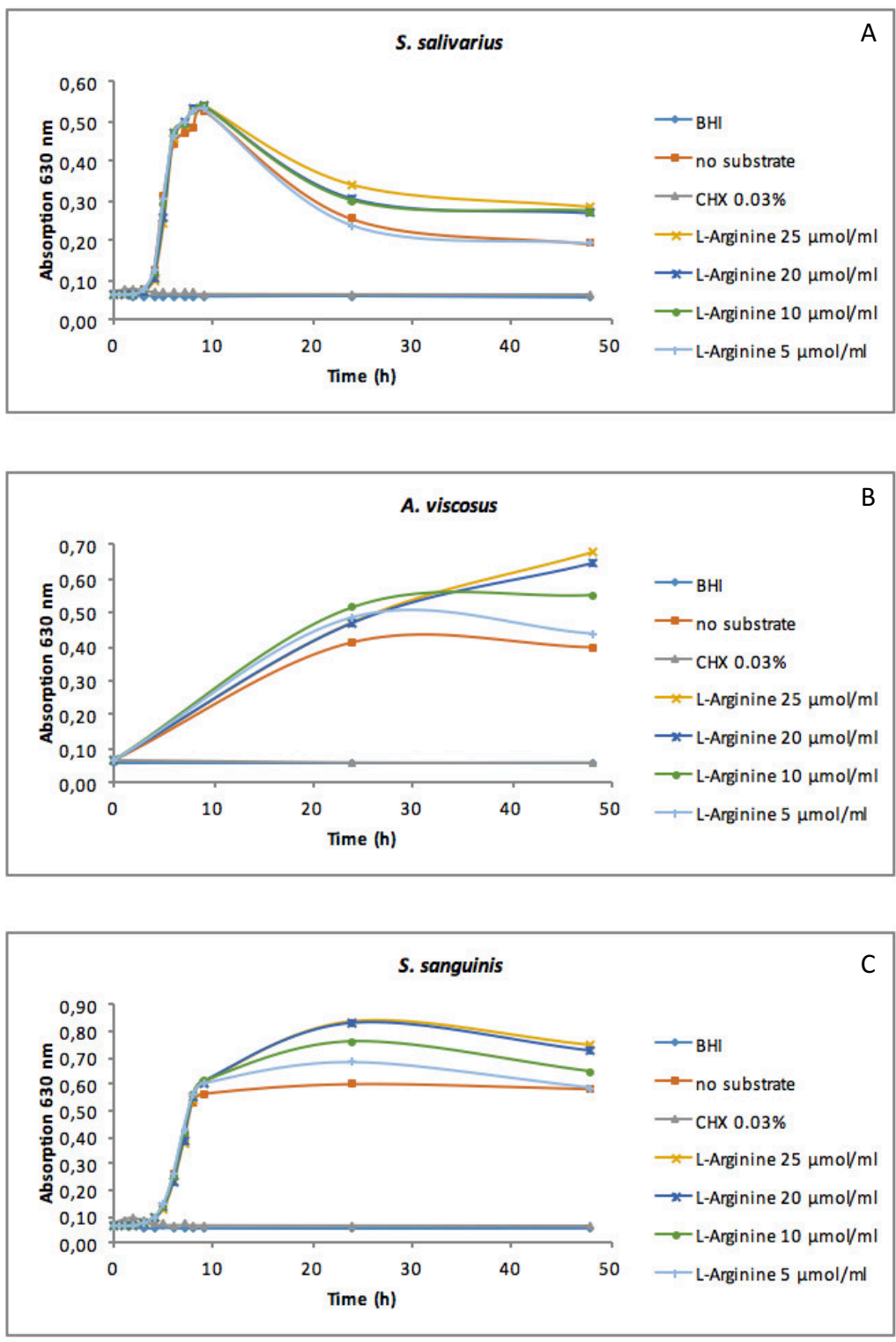


Figure 10 - Growth curves for *S. salivarius* (A), *A. viscosus* (B) and *S. sanguinis* (C) in the presence of different concentrations of L-arginine.

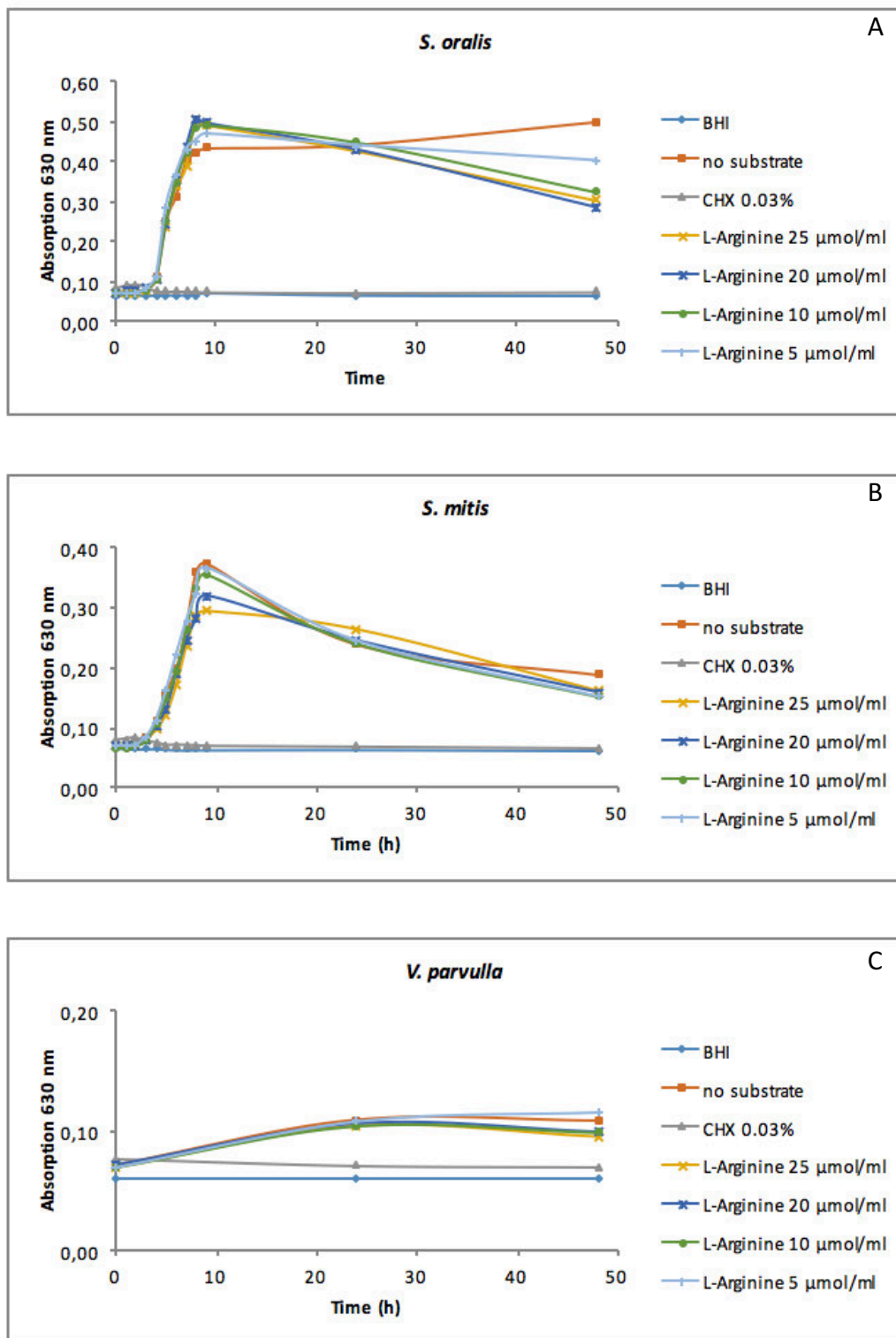


Figure 11 - Growth curves for *S. oralis* (A), *S. mitis* (B) and *V. parvulla* (C) in the presence of different concentrations of L-arginine.

A.2. Biofilm formation in the presence of L-arginine

The following Figures (12-16) show the biofilm formation of each species used in this study in the presence of different concentrations of L-arginine compared to the control.

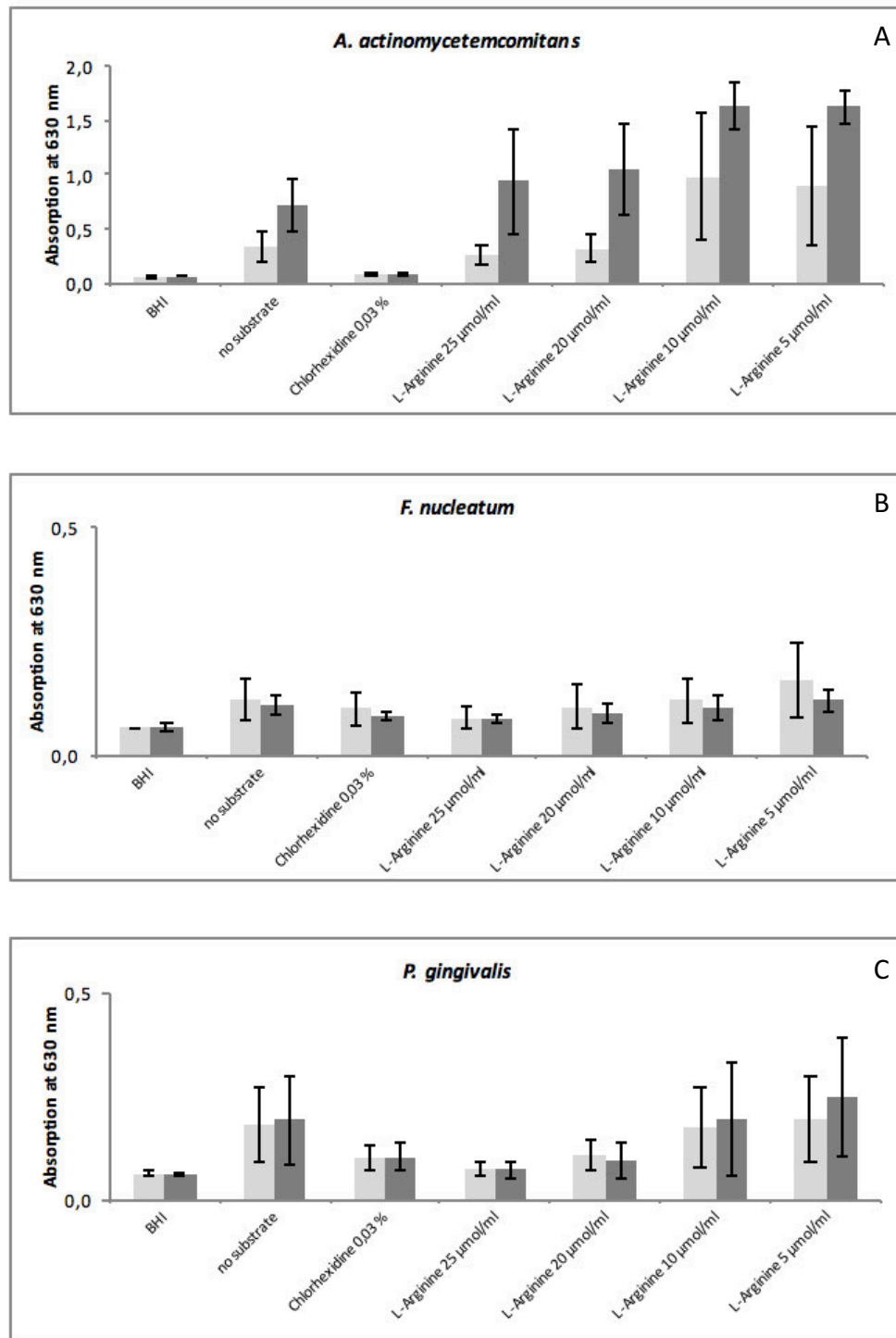


Figure 12 – Biofilm formation for *A. actinomycetemcomitans* (A), *F. nucleatum* (B) and *P. gingivalis* (C) in the presence of different concentrations of L-arginine. Light grey – 24h incubation; Dark grey – 48h incubation.

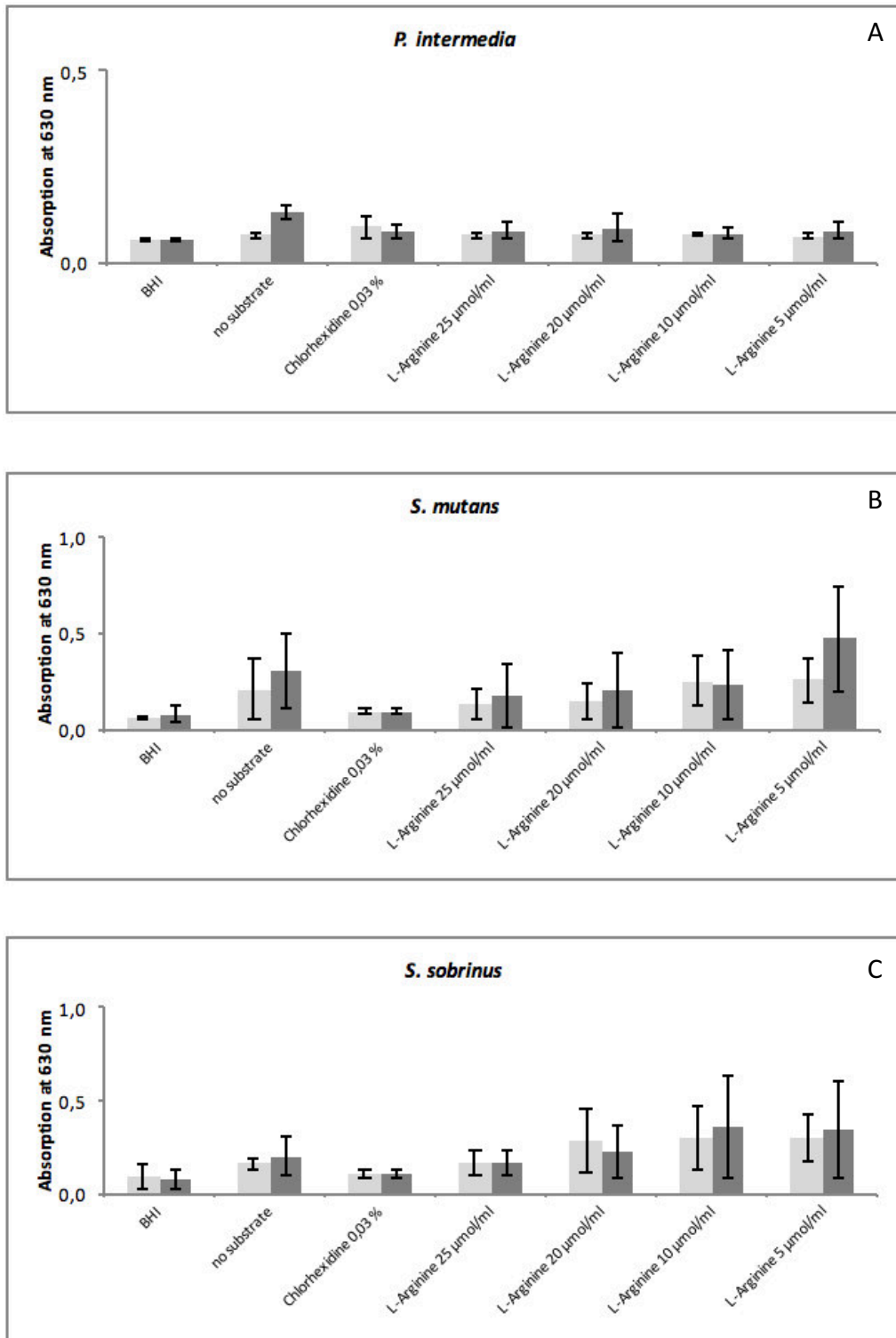


Figure 13 - Biofilm formation for *P. intermedia* (A), *S. mutans* (B) and *S. sobrinus* (C) in the presence of different concentrations of L-arginine. Light grey – 24h incubation; Dark grey – 48h incubation.

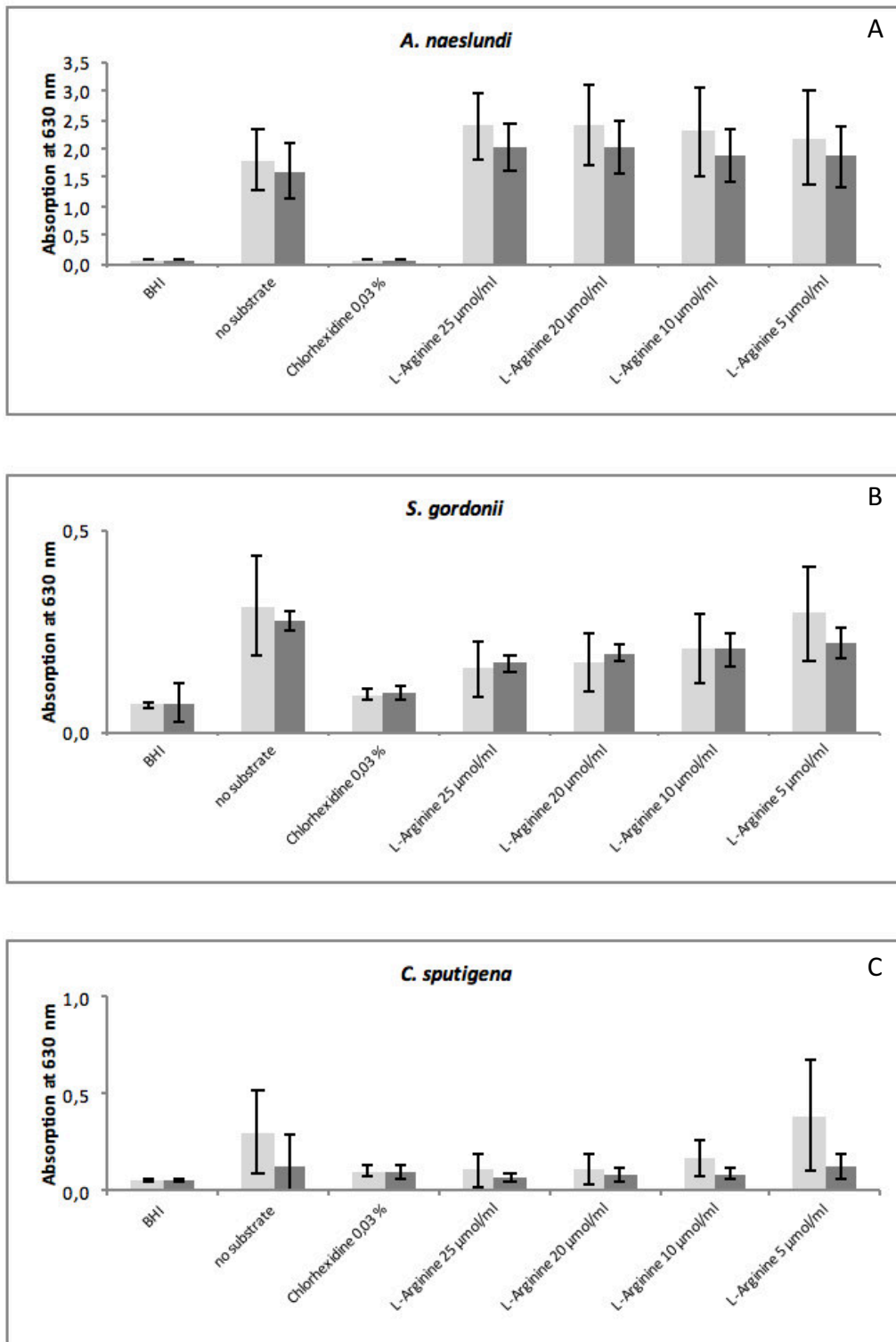


Figure 14 - Biofilm formation for *A. naeslundii* (A), *S. gordonii* (B) and *C. sputigena* (C) in the presence of different concentrations of L-arginine. Light grey – 24h incubation; Dark grey – 48h incubation.

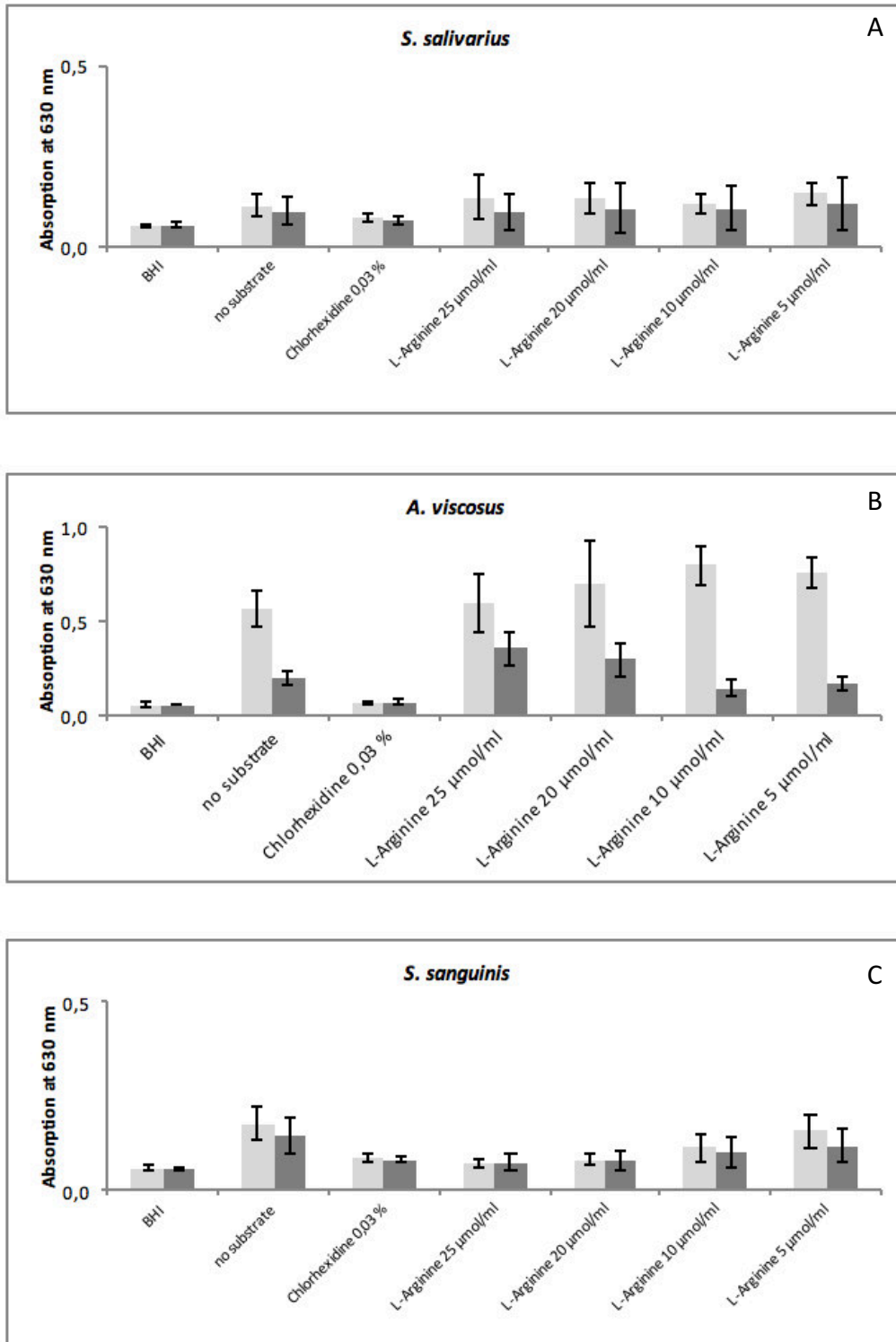


Figure 15 – Biofilm formation for *S. salivarius* (A), *A. viscosus* (B) and *S. sanguinis* (C) in the presence of different concentrations of L-arginine. Light grey – 24h incubation; Dark grey – 48h incubation.

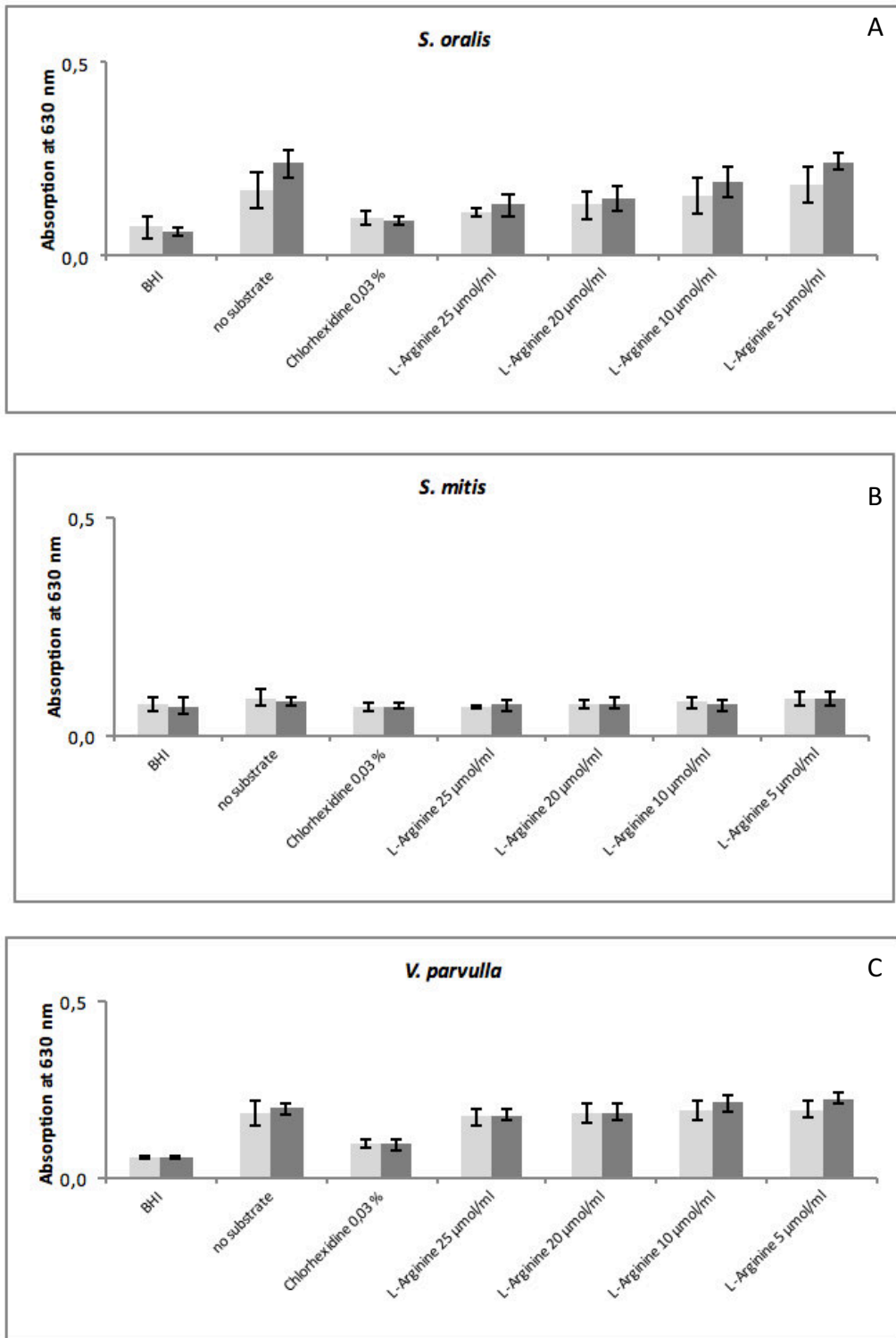


Figure 16 - Biofilm formation for *S. oralis* (A), *S. mitis* (B) and *V. parvulla* (C) in the presence of different concentrations of L-arginine. Light grey – 24h incubation; Dark grey – 48h incubation.

B. Dual-species competition assays

The following Figures (17-22) show the proportion in terms of percentage between the beneficial and pathogenic species in the dual-species competition assays.

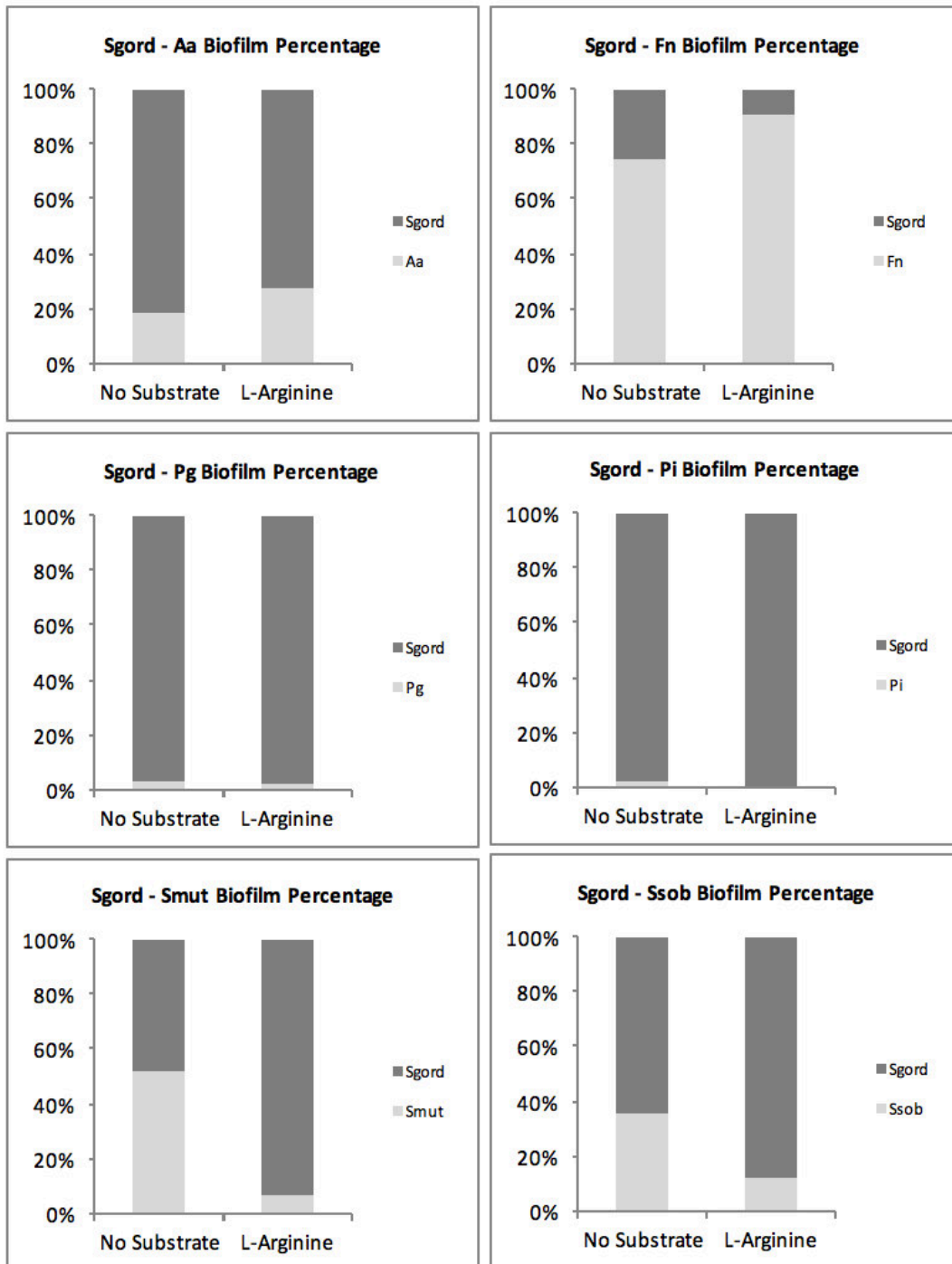


Figure 17 – Biofilm percentage when the beneficial species Sgord: *Streptococcus gordonii* is incubated with the pathogenic species Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Smut: *Streptococcus mutans* and Ssob: *Streptococcus sobrinus*.

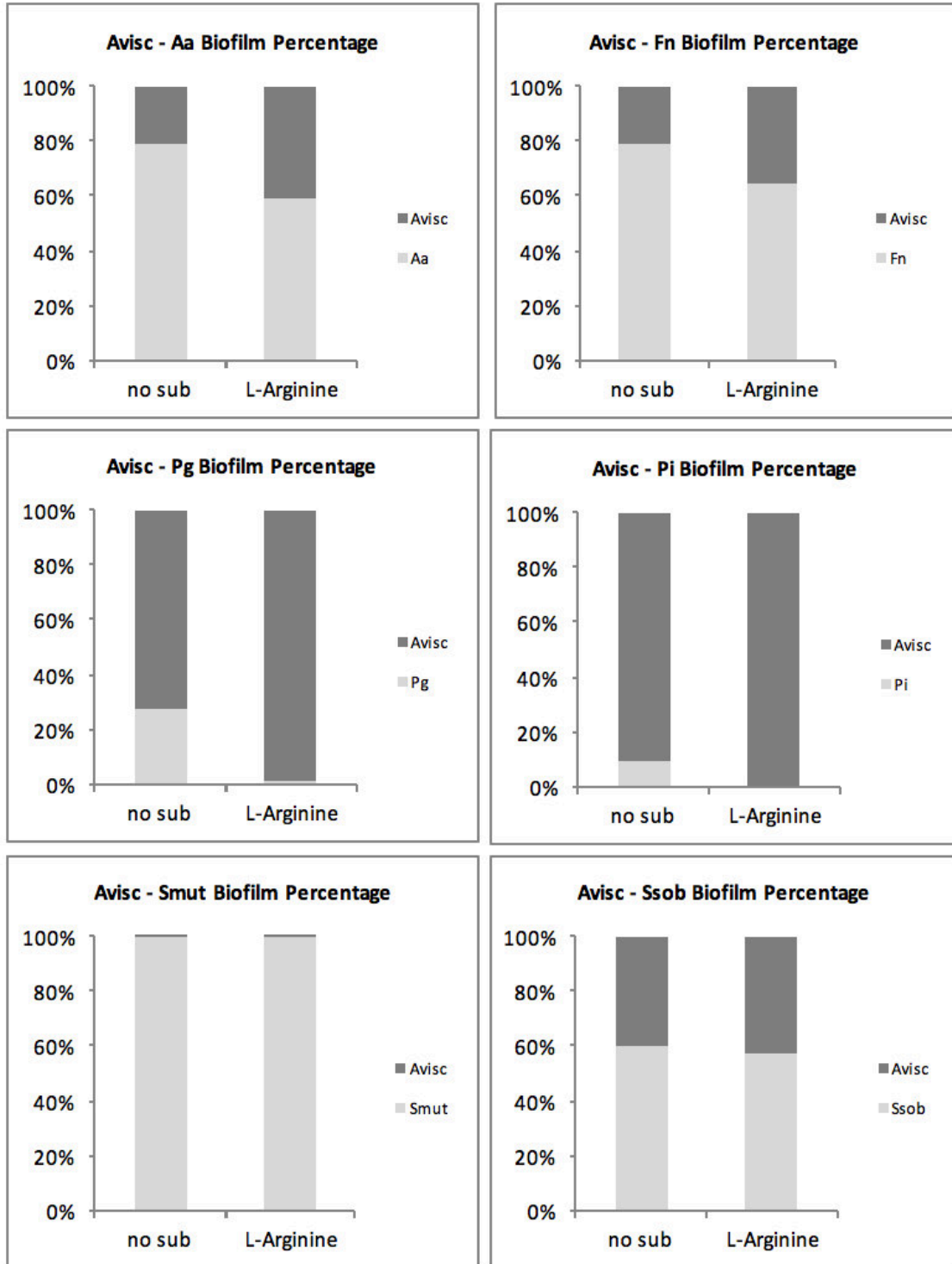


Figure 18 - Biofilm percentage when the beneficial species Avisc: *Actinomyces viscosus* is incubated with the pathogenic species Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Smut: *Streptococcus mutans* and Ssob: *Streptococcus sobrinus*.

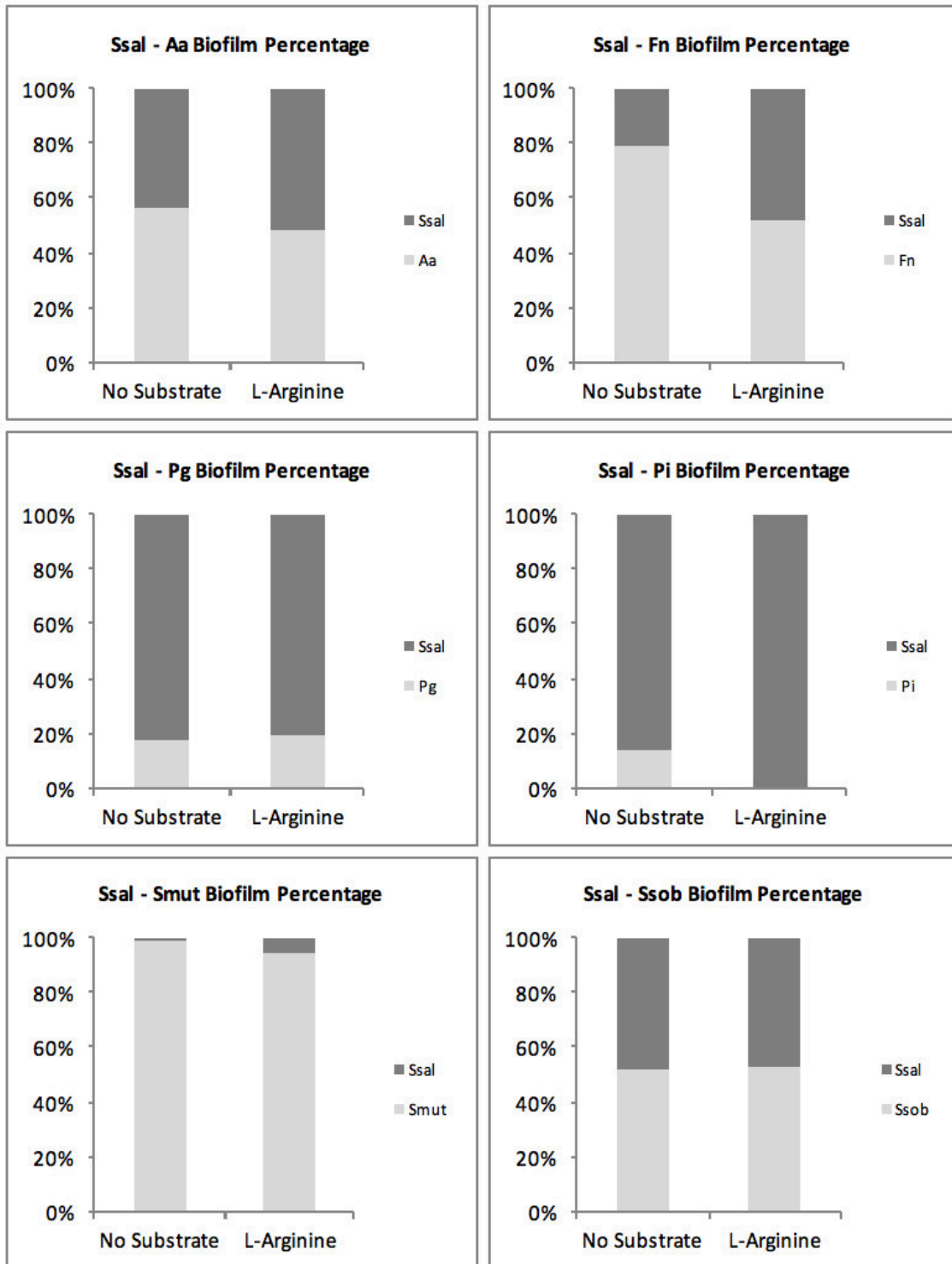


Figure 19 - Biofilm percentage when the beneficial species Ssal: *Streptococcus salivarius* is incubated with the pathogenic species Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Smut: *Streptococcus mutans* and Ssob: *Streptococcus sobrinus*.

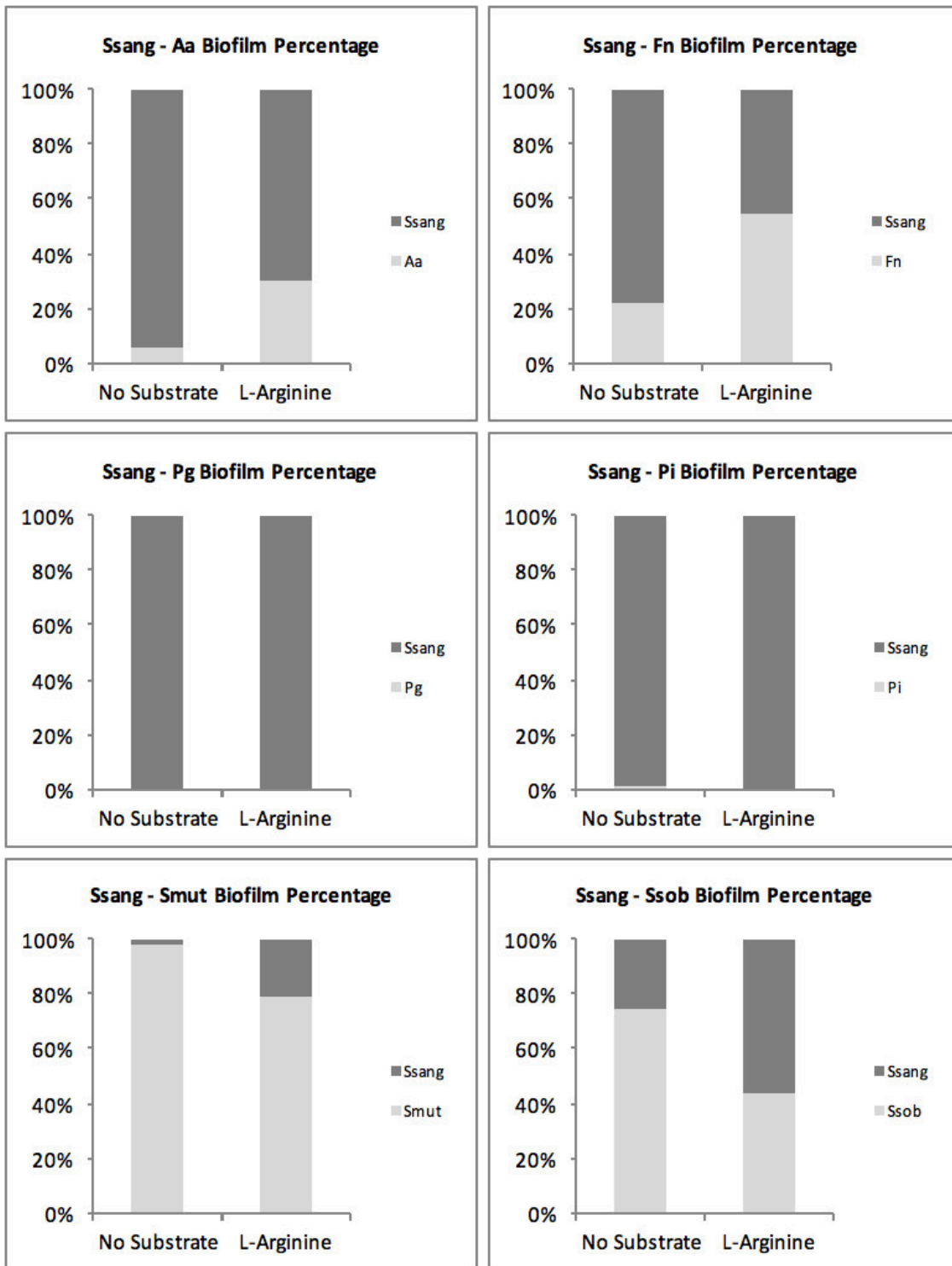


Figure 20 - Biofilm percentage when the beneficial species Ssang: *Streptococcus sanguinis* is incubated with the pathogenic species Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Smut: *Streptococcus mutans* and Ssob: *Streptococcus sobrinus*.



Figure 21 - Biofilm percentage when the beneficial species Smitis: *Streptococcus mitis* is incubated with the pathogenic species Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Smut: *Streptococcus mutans* and Ssob: *Streptococcus sobrinus*.

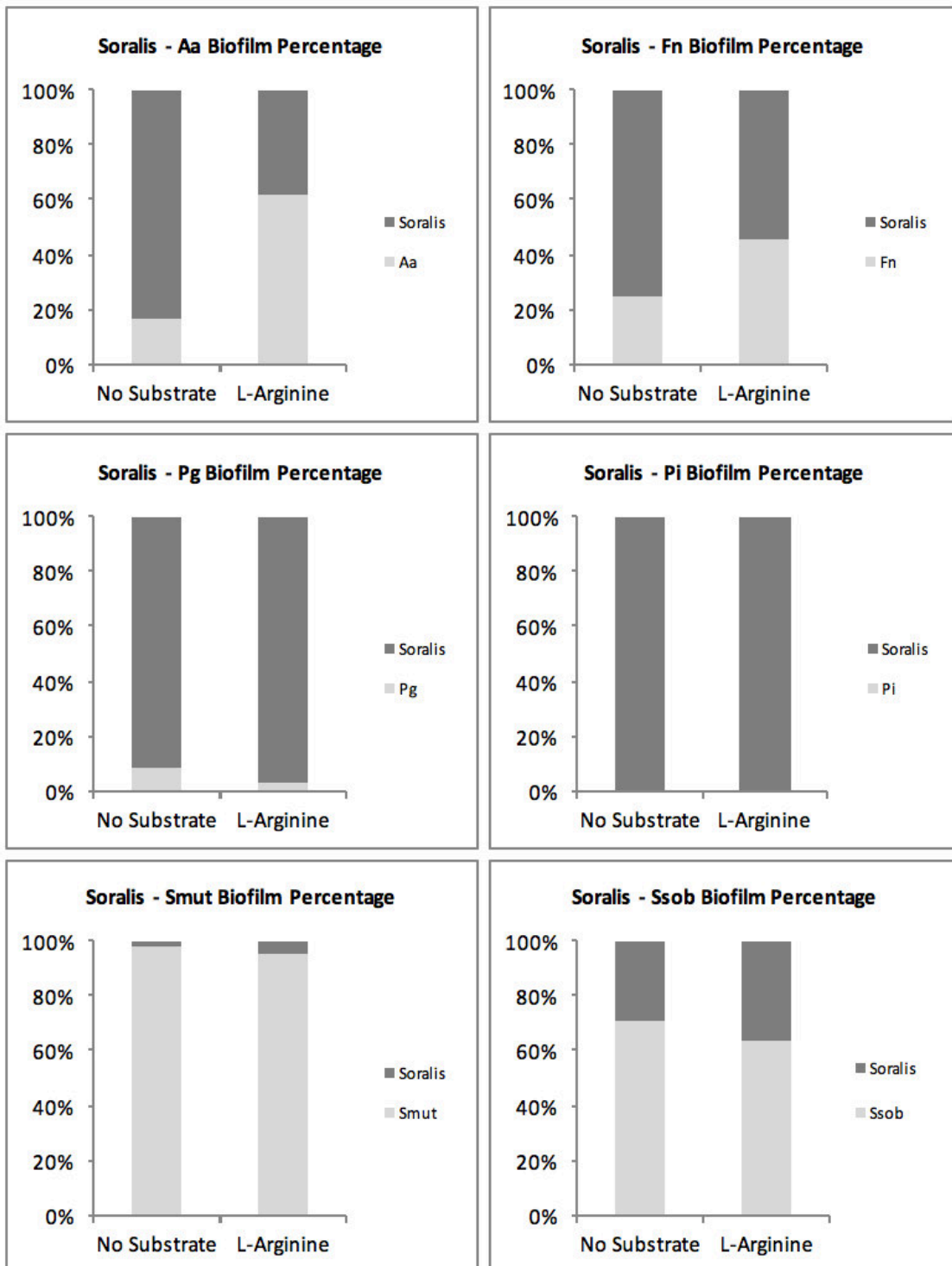


Figure 22 - Biofilm percentage when the beneficial species Soralis: *Streptococcus oralis* is incubated with the pathogenic species Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Smut: *Streptococcus mutans* and Ssob: *Streptococcus sobrinus*.