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DEVELOPMENT OF A POSTBIOTIC-BASED ORODISPERSIBLE FILM TO PREVENT DYSBIOSIS IN THE ORAL CAVITY

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of a Master of Science degree in Applied Microbiology

by
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Dedication

This thesis is dedicated to my son, Pedro Miguel, who taught me to see beauty in all the things around me; to my parents, Helena and Pedro, for always believing in me; to my husband, Sandro, for his unconditional support, and to my sister, Patrícia, for always keeping me company.

I would also like to dedicate my thesis to Professor Freni for being the best tutor I could ask for.

Last but not least, this thesis is dedicated to Dra. Andreia Montenegro, thank you for never giving up on me and helping me grow; and to Professora Sara, because “a little bit of everything I write will always be for her.”

Resumo

As doenças orais afetam mais de três mil milhões de pessoas, sendo uma das infeções mais comuns em todo o mundo. Estudos recentes demonstraram que, para reduzir o risco de cáries dentárias, a modulação da microbiota oral, ao invés da remoção de microrganismos benéficos e patogénicos, é mais eficaz. Isto baseia-se no facto de que as doenças orais são causadas por uma alteração da homeostase, intitulada disbiose, e não por um patogénico específico. Assim, a implementação de estratégias para prevenir e controlar a disbiose oral, é indubitavelmente importante. Os tratamentos convencionais incluem a utilização de antibióticos de largo espetro, que levam a uma maior disrupção deste equilíbrio, em conjunto com a limpeza/desbridamento mecânico da cavidade, após a formação da cárie.

Assim, é imperativa a implementação de estratégias alternativas para ultrapassar as desvantagens do uso de antibióticos de largo espectro. Neste sentido, probióticos e posbióticos têm recebido particular atenção, uma vez que são capazes de modular a microbiota oral e diminuir a taxa de disbiose. No entanto, os seus mecanismos de ação devem ser clarificados, por forma a que seja possível implementá-los como estratégias preventivas.

Neste trabalho, *Lactiplantibacillus plantarum* e *Lacticaseibacillus paracasei* foram cultivados em MRS líquido, centrifugados e filtrados após 48h. Os posbióticos foram diluídos em diferentes concentrações e co-incubados com *Streptococcus mutans*. A atividade antimicrobiana, o volume mínimo inibitório e o tempo necessário para a inibição do *S. mutans* foram avaliados. A capacidade antibiofilme foi determinada pelo método do cristal violeta. Finalmente, um filme orodispersível (ODF) à base de polímeros e plasticizantes foi desenvolvido como veículo de administração. Os posbióticos demonstraram capacidade antimicrobiana e antibiofilme contra *S. mutans* após 24h de co-incubação. A formulação do ODF impregnado com posbióticos foi optimizado.

Este estudo oferece uma visão geral do potencial da utilização dos posbióticos para prevenir a disbiose oral, focado na atividade antimicrobiana e antibiofilme. Dados os resultados obtidos, a utilização de um filme orodispersível impregnado com posbióticos deve ser considerado uma potencial alternativa para combater a disbiose oral.

Palavras-chave

Disbiose oral; Posbióticos; Saúde oral; *Streptococcus mutans*; Filme orodispersível

Abstract

Oral diseases affect over three billion people, being one of the most common infections worldwide. Recent studies show that to reduce the risk of caries, controlling the ecology of the oralome instead of the complete removal of both harmful and beneficial microorganisms, is more effective. This is based on the knowledge that oral diseases are not caused by a single pathogen but rather by a shift in homeostasis, called dysbiosis. Implementing strategies to prevent and control oral dysbiosis to avoid complications is of utmost importance. Conventional treatments include the use of antibiotics, which disrupt the equilibrium of the oral microbiota even further, together with the mechanical removal of the decayed area of the cavity once it is formed.

Therefore, it is imperative to implement alternative strategies to overcome the disadvantages of the current conventional therapies, namely the use of broad-spectrum antibiotics. In this sense, probiotics and postbiotics have received particular attention since they can modulate the oral microbiota and decrease the dysbiotic rate in the oral cavity. However, their mechanisms of action need to be addressed to clarify and drive their possible applications as preventive strategies.

In this work, *Lactiplantibacillus plantarum* and *Lacticaseibacillus paracasei* were grown in MRS broth, centrifuged, and filtered after 48h. The postbiotics were diluted to different concentrations and co-incubated with *Streptococcus mutans*. The antimicrobial activity was assessed. Additionally, the minimal inhibitory volume and the time needed for *S. mutans* inhibition were evaluated. Antibiofilm capacity was determined by the crystal violet method. Finally, an orodispersible film based on polymers and plasticizers was developed as an administration vehicle. Postbiotics demonstrated antimicrobial and antibiofilm activity against *S. mutans* after 24h in co-incubation. The formulation of a postbiotic-based orodispersible film based on polymers was optimized.

This study offered an overview of the potential of postbiotics to prevent oral dysbiosis, focusing on their antimicrobial and anti-biofilm activity. Given the obtained results, orodispersible films impregnated with postbiotics should be considered a potential alternative to target oral dysbiosis.

Keywords

Oral dysbiosis; Postbiotics; Oral health; *Streptococcus mutans*; Orodispersible films

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Introduction

Dental caries or tooth decay represent the most predominant infection worldwide among oral diseases, with more than 3.5 billion people experiencing it at least once in their lifetime (Hernández et al., 2022). Besides impacting the oral cavity, oral health is also highly related to general health (Thomas et al., 2021). Since as early as 1989, with the study of Mattila et al., it has been known that there is a link between insufficient oral health and heart problems (Mattila et al., 1989). Moreover, complications during pregnancy have also been linked with chronic periodontitis, as well as chronic obstructive pulmonary disease and bone resorption (Gao et al., 2022). Even gastric diseases, namely inflammatory bowel disease, are related to an unbalanced oral microbiota (Thomas et al., 2021; Gao et al., 2022). Additionally, there has been observed a potential link between oral diseases, namely periodontitis, and neurodegenerative diseases, such as Alzheimer's or Multiple Sclerosis, accompanied by a loss of bacterial diversity (Thomas et al., 2021; Nicholson & Landry, 2022). Obesity is also a risk factor for the unbalance of the oral microbiota; in fact, it represents an increased risk of developing oral diseases, namely periodontitis (Wu et al., 2018). In addition, periodontitis seems to be a risk factor for the aggravation of the health state of patients with SARS-CoV-2 and even for the progression of rheumatoid arthritis (Thomas et al., 2021).

In the work of Thomas and collaborators (2021), it was clear that diseases with an inflammatory base cannot be treated nor alleviated in the presence of oral dysbiosis, and it was particularly relevant in patients with diabetes. Another study by Lamont et al. (2018) showed that an unhealthy oral cavity is related to worsening a general illness state. This is partially caused by the presence of lipopolysaccharides (LPS), an endotoxin from Gram-negative bacteria, that can easily enter the blood circulation and reach different organs (Thomas et al., 2021).

Despite being related to different systems, like the cardiac and respiratory systems, oral health, specifically gum health, is also intrinsically connected to psychological well-being (EFSA, 2020). In fact, oral health is highly dependent on the equilibrium of the oral microbiota, which is also called oralbiota. Such microbiota, when balanced, has the function of protecting the oral cavity (Colombo et al., 2015; Urvashi et al., 2020). However, oral diseases, namely caries, appear when this community shifts and dysbiosis happens (Urvashi et al., 2020; Sánchez et al., 2021). In addition to caries, periodontitis is the second most prevalent dental problem, which is an inflammatory disease that can lead to the loss of teeth and support tissues (Butera et al., 2020; Yang et al., 2021).

The oral microbiota comprises more than seven hundred species of bacteria, being one of the most complex populations of the human body (Kado et al., 2020). For example, Loesche (1986) observed that the first colonizers, *Streptococcus mutans* and *Streptococcus sobrinus,* appeared as soon as the teeth started erupting (Mosaddad et al., 2019). However, these microorganisms are found in the saliva before other areas of the oral cavity (Kaan et al., 2021). Although they are highly associated with dental illness, they are colonizers of the oral microbiota (Mosaddad et al., 2019). These early colonizers are important in preventing oral diseases and modulating the host's immune system. (Kaan et al., 2021).

However, in dysbiosis, some microbiota members start an overgrowth process, which originates biofilm formation. The essential characteristic for biofilm formation is the bacterial ability to adhere to oral surfaces (Kaan et al., 2021). This is based on the ecological plaque hypothesis, which states there are no specific microorganisms responsible for the oral disease but rather an overgrowth of some species of the oralbiota. In this case, the treatment should focus on regulating and controlling the environment that causes the microbiome shift and not on the use of antimicrobial therapy (Bizzini et al., 2012; Cugini et al., 2021; Radaic et al., 2022).

The main bacteria found in dental biofilms are *Streptococcus*, *Actinomyces*, *Prevotella*, *Porphyromonas*, *Tannerella*, and *Fusobacterium* spp. (Colombo et al., 2015). However, only a few are associated with periodontitis, mainly Gram-negative and anaerobic (Bizzini et al., 2012), such as *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* (Jansen et al., 2021). The bacteria in biofilm synthesize a matrix rich in glucans and exopolysaccharides (EPS), allowing for better adherence to one another and surrounding tissues (Sánchez et al., 2021). Other important compounds of the biofilms are endotoxins, namely LPS, that trigger an inflammatory response in the host. The biofilm characteristics are also protective and do not allow chemical agents to penetrate the microbiome barrier, leading to higher antibiotic resistance (Yang et al., 2021). Another crucial factor in maintaining oral health is diet (Kaan et al., 2021). Cleaver et al. (2021) showed that the amount of carbohydrates ingested is related to acid production, providing an adequate environment for the growth of cariogenic microorganisms. The standard treatment for cavities and gingival inflammation is the mechanical removal of the lesion and the dental plaque. However, this means the removal of both beneficial and harmful microbiota and, consequently, increasing microbial unbalance. Furthermore, removing all of the microbiome offers dental pathogens more free colonization sites (He et al., 2009). In addition, bacteria in biofilm are usually more resistant than in the planktonic state, making antibiotherapy an inadequate choice (Sánchez et al., 2021). Furthermore, the consumption of antibiotics can lead to an even more notable

dysbiosis, to a point where it can become irreversible (Radaic et al., 2022). In fact, the excessive consumption of antibiotics can deplete entire taxons from the oral cavity, resulting in the loss of functional groups (Najmanová et al., 2022).

Recent studies have been focusing on preventive treatments capable of re-establishing the oralbiota equilibrium, modulating the microbiome, and presenting a positive effect on inflammation (Colombo et al., 2015; Nguyen et al., 2021). Taking this into consideration, probiotics, prebiotics, and postbiotics are promising strategies, considering consumer awareness of health as well (Bustamante et al., 2020; Lordello et al., 2021; Hernández et al., 2022).

The World Health Organization defines probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host" (FAO/WHO).

They can positively affect the microflora (Qiu et al., 2020), control the inflammatory response (R. Zhao et al., 2021), modulate the innate and adaptative immune response, and inhibit the production of cytokines by pathogens (Bustamante et al., 2020). It has also been shown that probiotics stimulate immunoglobulin production and alter the DNA of the host (Y. Zhao et al., 2021). Moreover, they do not cause any side effects felt with antimicrobial therapy and can be considered an alternative to this treatment (Lee et al., 2021). On this note, there has been increased attention towards probiotics as an adjunctive therapy since they are able to modulate both the inflammatory response (NĘdzi-GÓra et al., 2020; R. Zhao et al., 2021) and the microbiota, critical properties for the prevention of dental caries and other diseases (Coqueiro et al., 2018; Qiu et al., 2020).

Although it is also important to remember that probiotics cannot substitute primary oral care and hygiene practices, they are now an interesting choice for a preventive approach (NĘdzi-GÓra et al., 2020). This therapy will not substitute conventional treatment but shows better results than the current preventive strategies.

Besides probiotics, the knowledge of pre and postbiotics has been increasing in recent years, with more research focusing on the functions of cell-free supernatants and even non-living bacteria, especially to circumvent the disadvantages and limitations of probiotic use (Jastrząb et al., 2021; Scott et al., 2022).

1. Oral Microbiota

The oralbiota can be affected by several factors: dietary habits, type of dentition, medication, age, and general behaviors of the individual (Kaan et al., 2021; Thomas et al., 2021;

Babina et al., 2022; Zhang et al., 2022;). Regarding general behaviors, it was noted that dietary habits, namely carbohydrate intake, are of the utmost importance to define the type of bacteria that inhabit the oral cavity. The higher the ingestion of considerable amounts of carbohydrates, the more negatively affected the oral microbiota is (Hernández et al., 2022).

An interesting fact about the oral cavity is that it cannot be perceived as a whole but rather as a set of different microenvironments, with distinctive characteristics from one another, that reach equilibrium and allow different taxa to grow and multiply. According to recent research, it was observed that each specific niche has a separate set of microorganisms substantially fitted for environmental changes (Xu et al., 2015; Ren et al., 2017; Mason et al., 2018; Najmanová et al., 2022). This is presumably due to the different microenvironments that determine different conditions across the oral cavity, such as the temperature or the presence of oxygen, but also the type of tissue and the presence of nutrients. According to these differences, 4 different niches in the oral cavity can be perceived: the teeth, the tongue, the oral mucosa, and the gingiva (Najmanová et al., 2022). For example, the buccal mucosa presents minimal diversity, while the tongue and the dental surfaces show elevated levels of microbial communities (Kaan et al., 2021).

It should be noted that when daily oral hygiene is poor, there is an increase in the number and diversity of microorganisms present in the entire oral cavity (Mashima et al., 2017; Alves et al., 2020).

Despite presenting a high diversity of microorganisms, specific genera, such as *Streptococcus*, *Prevotella*, *Fusobacterium*, and *Veillonella*, are more commonly found in a healthy oral cavity. Other commensal microorganisms, such as viruses, archaea, fungi, and protozoa, have also been found among the oral biota; in fact, more than 85 distinct fungal genera can be linked to the oral cavity (Lin et al., 2022). Similarly to what happens with other commensal communities, all microorganisms have distinct roles when inhabiting a host, and they all contribute to the organization and survival of each other (Sampaio-Maia et al., 2016; Kaan et al., 2021).

In a healthy environment, the commensal bacteria of the mouth cohabit in symbiosis with the host. This means that an equilibrium is reached, not only between the bacteria and the individual's immune system but also between commensals themselves. This equilibrium is called homeostasis, an essential characteristic of a healthy oralome (Cugini et al., 2021; Thomas et al., 2021; Hernández et al., 2022). This healthy oralome is critical for oral health and general health since these commensals can determine as much as the susceptibility to disease (Cugini et al., 2021). It is essential to understand that the maintenance of oral homeostasis is

multifactorial. It mainly depends on the host and the microenvironments created in the different sites of the oral cavity, but also on the bacteria and other microorganisms present (Hernández et al., 2022).

The microorganisms in the oral cavity are acquired as soon as the individual is born, and they start changing over time. When the teeth erupt, new structures allow different microorganisms to inhabit the oral cavity: the enamel and the gingival sulcus. However, Kaan and collaborators (2021) demonstrated that a major microbiological shift also occurs with the alteration of food from liquid to solid.

The acquisition of specific bacteria seems highly dependent on the mother's oralome, which proves the importance of prenatal oral care to assure the children's health (Kaan et al., 2021). In fact, it was noted that if children's caretakers harbored certain species, namely *S. mutans*, *S. sobrinus*, *Fusobacterium nucleatum*, *Prevotella intermedia*, and *P. gingivalis*, there was a high chance that those species would be found in the children as well. This relation was particularly relevant at the time of the retrieval of anaerobes in the oral cavity (Kaan et al., 2021). When in homeostasis, the commensals of the oral cavity promote oral health and, as a result, general health. These microorganisms present a protective capacity against pathogens, either by competition for nutrients or binding sites (Cugini et al., 2021; Hernández et al., 2022).

Regarding the microorganisms that inhabit the oral cavity as commensals, it can be noted that Streptococci are the primary oral colonizers, namely *Streptococcus mitis, Streptococcus salivarius*, and *Streptococcus cristatus*, among other species. The emergence of these microorganisms in the oral cavity occurs as soon as the eruption of the first teeth, mainly due to the adherence ability of Streptococci to the tooth surface, tongue, and gingiva. They also allow other bacteria to bind to them, thus becoming part of the oral microbiota even if they do not present the capacity to adhere to dental surfaces and other tissues themselves (Hernández et al., 2022).

Due to the presence of the early colonizers, their metabolites, and excreted products, the environment becomes suitable for the growth of other bacteria. In this sense, new anaerobic sites emerge as the microorganisms start arranging themselves (Kaan et al., 2021).

As the early colonizers start changing the environment and allowing other bacteria to start inhabiting the oral cavity, significant changes occur regarding the present bacteria. This is called "permanent colonization" of the mouth and is responsible for creating the "core taxa" of the oral microbiota, mainly due to the adherence ability of Streptococci, which operates as a bridge between dental surfaces and other bacteria (Sampaio-Maia et al., 2016).

Similar to what happens in other areas of the human body, commensal microorganisms

present beneficial properties for the host: Streptococci species, namely *S. mitis*, *S. sanguinis*, and *S. cristatus* prevent the adhesion of pathogens, such as *P. gingivalis*, via an enzyme called arginine deaminase, that affects the production of binding proteins. They also have the ability to counteract acid production from cariogenic bacteria, like *S. mutans*, allowing the salivary pH to increase. In addition, they reduce cytokine expression, which helps lower inflammatory responses (Hernández et al., 2022).

It is important to understand that commensal microorganisms can also reduce nitrate, which prevents the occurrence of caries. Nitrate-reducing bacteria present the capacity to produce ammonia, which, in turn, similar to other commensals mentioned above, raises the pH of the oral cavity, functioning as a buffer for acid production from *S. mutans*. Furthermore, they can decrease the number of anaerobes, due to the presence of nitrate, nutrient competition, and binding sites competition. This is important since anaerobes are mostly responsible for periodontal disease and halitosis. Relevant nitrate-reducing bacteria belong to the genus *Actinomyces* and *Kingella*, among others, and are usually present in individuals who exhibit good oral health (Rosier, Moya-Gonzalvez, et al., 2020).

Besides homeostasis and the action of commensals in the equilibrium of the oral cavity, another critical factor for maintaining oral health is the presence of saliva. In fact, a deficient salivary flow has been related to a higher colonization of pathogens in the oral cavity (Hernández et al., 2022). The saliva presents several distinct functions, namely, the removal of microorganisms that could be potentially pathogenic, transferring microorganisms from one location to another, facilitating the colonization of the commensals, and its buffer capacity, which is critical for caries control (Cugini et al., 2021; Kaan et al., 2021; Thomas et al., 2021). In addition, its composition highly affects the types of microorganisms present in the biofilm's structure; for example, the presence of glycoproteins serves as a nutrient source for commensal bacteria (Thomas et al., 2021). Regarding the buffering ability, it should be mentioned that saliva influences the microorganisms that can survive in the biofilm since it can uphold a pH level of 6.5–7.5, which is neutral (Cugini et al., 2021; Lee et al., 2021; Sudhakaran et al., 2021). The most important consequence of pH maintenance is the remineralization of enamel, preventing the formation of cavities by acidogenic bacteria (Ferrer et al., 2020; Hernández et al., 2022). Saliva also possesses immunoglobulins in its composition, allowing it to serve as a limitation for bacterial growth since it presents antimicrobial capacity. However, this property is not visible when homeostasis is absent (Cugini et al., 2021; Kaan et al., 2021; Thomas et al., 2021).

Finally, it is essential to understand that, like other microbiotas of the human body, oral

bacteria can also act systemically in the individual. For this reason, some researchers noted that it serves as an indicator of overall health, an activator of the host's immune system, and, on the downside, a source of systemic inflammation when homeostasis is lost (Thomas et al., 2021).

2. Oral dysbiosis

As mentioned above, the oral microbiota is a community of microorganisms that inhabit the oral cavity. When they cohabit in homeostasis, they can prevent disease and maintain a certain environment suitable for the survival of the commensals (Hernández et al., 2022).

The oral microbiota presents a certain level of stability; nonetheless, certain circumstances, such as a modification in the dietary habits or the host's ability to interact with the commensals, disrupt the oral equilibrium, altering the environment and resulting in the loss of homeostasis, which is called dysbiosis. A relationship between oral dysbiosis and the appearance of oral diseases, such as caries, periodontitis, and gingivitis, is already established (Kaan et al., 2021). In addition, dysbiosis also impairs the host's general health, affecting the cardiovascular system (Radaic et al., 2022).

The loss of homeostasis is multifactorial: it can be host-related, and it can also occur due to the use of antibiotics. In this case, it is called chemically induced environmental dysbiosis (Cugini et al., 2021). Another underlying cause of dysbiosis is insufficient salivary flow, resulting in a deficient removal of microorganisms, contributing to biofilm maturation (Thomas et al., 2021).

This condition of dysbiosis allows some species to grow uncontrollably, causing profound alterations in the oral microbiota, ultimately resulting in a loss of oral and general health (Cugini et al., 2021; Thomas et al., 2021; Hernández et al., 2022). In general, dysbiosis can result in the loss of critical bacterial taxons, reduced metabolite production by commensals, uncontrolled immune system stimulation, and deficient response to pathogens (Najmanová et al., 2022). The systemic implications are mainly due to changes in the epithelial barriers in the oral cavity that occur in a dysbiotic environment due to the production of proinflammatory cytokines, such as tumor necrosis factor α and interleukin 6, by pathogens such as *P. gingivalis*, allowing the passage of LPS, which is produced by Gram-negative bacteria, into the bloodstream (Thomas et al., 2021).

This shift in the environment, i.e., the dysbiosis per se, usually improves the growth capacity of certain bacteria, namely species belonging to the genera *Streptococcus*, *Actinomyces*, and *Lactobacillus*, whose metabolic activity creates an anaerobic environment.

Eventually, this allows anaerobic bacteria, such as *P. gingivalis*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans,* to grow, which are usually pathogenic due to their ability to produce acids. This acid production results in an enhanced demineralization process of the dental enamel, resulting in a cavity (Hernández et al., 2022). Furthermore, it also creates conditions for the overgrowth of periodontopathogens since biofilm formation in the teeth can expand to subgingival areas (Gheisary et al., 2022).

An important aspect of dysbiosis in the oral cavity is that environmental alterations shape the type of microorganisms that can grow due to the creation of specific conditions (Thomas et al., 2021). One particular characteristic is the reduction of the levels of H_2O_2 produced by *Streptococcus gordonii,* which results in a loss of antagonistic activity against *S. mutans*, allowing it to overgrow. As *S. mutans* grows, it changes the EPS matrix with acidic metabolites, such as lactic acid, decreasing the oral pH (Hernández et al., 2022). Consequently, in the presence of oral dysbiosis, there is a less diverse oralome compared to a healthy oral microbiota (Giordani et al., 2021). Notably, the loss of commensal microorganisms accommodates new bonding sites for acidic pathogens to bind to teeth and gingiva.

The following segments give an overview of the main mechanisms that lead to dysbiosis, its correlation with oral diseases, as well as the major pathobionts and conventional treatment.

2.1 Biofilm formation

It is essential to understand that the bacteria in the oral cavity are organized in a multidimensional structure, usually called biofilm, composed primarily of EPS (Seminario-Amez et al., 2017; Barzegari et al., 2020; Wu et al., 2022). The excreted EPS has several functions, including adhesion facilitation and protection (Wasfi et al., 2019). Since the oral cavity presents so many challenging characteristics for bacteria to survive, their organization in biofilms is their most efficient survival mechanism (Chen et al., 2022; Gao et al., 2022; Hernández et al., 2022; Jung et al., 2022).

The biofilm allows the bacteria to interact with each other through quorum sensing (QS) and gene regulation, reaching an important level of organization and complexity by coaggregation, thus becoming less prone to removal (Sampaio-Maia et al., 2016; Kaan et al., 2021; Thomas et al., 2021; Chen et al., 2022). In addition, it has been noted that biofilm development increases the pathogenicity of specific microorganisms, like *S. mutans* (Wu et al., 2022). As the biofilm starts to form and the environment changes, there is a loss in diversity in the microbiota, which contributes to dysbiosis. This loss in diversity is not limited to bacteria

but also fungi and other microorganisms (Kaan et al., 2021).

One of the reasons bacteria in biofilms present a higher resistance to antibiotics may be the physical impossibility of the antibiotic molecule to reach the deeper levels of the biofilm (Barzegari et al., 2020). In addition, differences in the environment, such as the lower pH, can alter antibiotic action. Moreover, the capacity for horizontal transfer of genes allows microorganisms to acquire resistance genes quickly, rapidly becoming resistant to previously effective antibiotics (Barzegari et al., 2020; Giordani et al., 2021).

The ability to adhere is the main property of bacteria in the biofilm, depending on the presence of a receptor and a protein called adhesin (Kaan et al., 2021; Jung et al., 2022). The biofilm formation shows progressive development; it begins with reversible adherence, followed by irreversible adherence and consequent maturation. As the biofilm reaches its mature state, an equilibrium is formed between the microorganisms involved (Barzegari et al., 2020; Thomas et al., 2021; Hernández et al., 2022). When biofilm reaches its mature state, it is then called dental plaque (Thomas et al., 2021).

However, the biofilm must be removed before maturation to avoid deep dysbiosis in the oral cavity. Nonetheless, it should be noted that it is challenging to eliminate biofilm after the state of irreversible attachment (Jiang et al., 2020). Current preventive techniques include the use of fluoride, which has proven ineffective in removing biofilm and limiting its formation (Bijle et al., 2020).

After a couple of hours succeeding oral hygiene, the teeth become covered in a thin pellicle mostly composed of proteins, enzymes, and lipids, called the acquired enamel pellicle or acquired exogenous pellicle (AEP) (Cugini et al., 2021; Kaan et al., 2021). The AEP serves mainly as a protective barrier for the teeth, but it can also aid in the adherence of microorganisms to the dental surface, thus initiating biofilm formation. The first colonizers are mostly Gram-positive bacteria (Cugini et al., 2021; Thomas et al., 2021). They allow second and tertiary colonizers to adhere, forming the commensal ecosystem, including putative pathobionts (Cugini et al., 2021). In a healthy individual, the components present in the saliva can neutralize the plaque after 30 minutes of carbohydrate consumption. For this reason, a relationship between the salivary flow and the buffer capacity of an individual can be noted (Ferrer et al., 2020; Hernández et al., 2022).

With the ingestion of carbohydrates, more acid metabolites are produced and expelled to the matrix. The AEP becomes more acidic and allows the growth of tolerant microorganisms, favoring its growth instead of other commensals, thus contributing to the state of oral dysbiosis (Cugini et al., 2021). This represents a shift in plaque characteristics, ultimately resulting in the

growth of acidogenic and aciduric microorganisms (Hernández et al., 2022).

Specific microorganisms, such as *S. gordonii*, *Streptococcus oralis*, *Streptococcus mitis*, and those belonging to the genera of *Actinomycetes,* can bind to the proteins present in the AEP via GtfB protein (a glucosyltransferase) that have the ability to adhere to the tooth surface (Wu et al., 2022), serving as a bridge for other bacteria to bind, such as species belonging to the genera *Fusobacteria*, *Veillonella* and *Rothia* (Kaan et al., 2021; Chen et al., 2022; Wu et al., 2022). For this reason, these Gtfs are considered a key characteristic that allows cariogenic biofilm formation. In *S. mutans,* the most essential protein is considered to be GtfC, since it shows the maximum affinity for the tooth enamel (Wu et al., 2022).

To prevent this, the host's saliva is rich in antibodies, namely immunoglobulin A (IgA), which prevent bacterial adhesins from adhering to the AEP (Basir et al., 2022; Lin et al., 2022). However, some bacteria, both pathogens and commensals, have the ability to clive IgA through a specific protease production, which translates into a colonization advantage (Kaan et al., 2021).

Another important characteristic of the biofilm is the presence of an oxygen gradient, eventually becoming anaerobic, primarily due to the tridimensional organization of the bacteria, which allows the growth of anaerobic bacteria that, in other circumstances, would not be able to grow (Kang et al., 2020; Hernández et al., 2022). Of note, biofilm formation is not exclusive of a dysbiotic state; bacterial biofilms in a healthy oral cavity can occur when the alkaline compensation is balanced with the acid production (Hernández et al., 2022). As a matter of fact, dental plaque is considered by many authors an evolutive microbial community (Inchingolo et al., 2022; Wu et al., 2022). However, in an unbalanced oral cavity, it is observed that the biofilm promotes disease and facilitates dysbiosis since specific species exhibit the ability to overgrow in these new conditions (Cugini et al., 2021). Radaic and collaborators (2022) demonstrated the difference between healthy and pathogenic biofilm formation, and it was noted that the latter includes a significant change in certain present phyla, namely, an increase in *Proteobacteria* and *Fusobacteria*. It becomes clear that biofilm should be one of the first targets to act to prevent oral diseases, especially since the positive correlation with caries has been demonstrated several times. It was also noted that supragingival biofilm, rich in both Gram-positive and Gram-negative bacteria (Kaan et al., 2021), is highly correlated with the appearance of periodontitis (Hernández et al., 2022; Ma et al., 2023). Archael oral colonization of the subgingival plaque in periodontally healthy individuals was reported to increase the prevalence and abundance of periodontal disease (Kaan et al., 2021). In fact, *S. mutans* biofilm formation is a crucial step in the formation of oral caries and can even be related to the

appearance of oral candidiasis (Rossoni et al., 2020; Jang et al., 2021; Ebrahim et al., 2022).

It is important to consider that dysbiosis happens before the first symptoms of the oral disease appear (Najmanová et al., 2022), which proves the importance of controlling this condition as an effective technique to prevent oral diseases.

2.2 Oral diseases

In the last decade, our knowledge of oral diseases improved drastically, and the idea that specific pathogens were responsible for oral diseases was abandoned. Nowadays, a vast number of authors accept the ecological plaque hypothesis to explain the appearance of oral diseases. This hypothesis states that dysbiosis is the leading cause of pathobiont growth in the oral cavity, not a specific set of pathogens (Cugini et al., 2021).

Depending on the type of dysbiosis and, consequently, the type of plaque formed, different diseases can appear in the oral cavity (Seminario-Amez et al., 2017; Cugini et al., 2021; Thomas et al., 2021). The most common oral disease is caries, followed by gingivitis, periodontitis, peri-implantitis, halitosis, and oral cancer. The last one is the only oral disease that does not directly correlate with a dysbiotic state (Yang et al., 2021).

Regarding caries, a multifactorial infection, the biofilm formation tends to increase the development of Gram-positive bacteria, usually producers of acidic metabolites, which end up dropping the pH and demineralizing the tooth surface, which causes a cavity (Amargianitakis et al., 2021; García et al., 2021; Thomas et al., 2021; Hernández et al., 2022; Poorni et al., 2022; Staszczyk et al., 2022). The pH limit before the cavity starts to form is 5.5. For this reason, ingesting excessive amounts of sugar is considered a risk factor for caries development (Cugini et al., 2021; Jung et al., 2022). Curiously, one of the most important risk factors for developing caries is “caries re-experiencing,” demonstrating a tendency for the appearance of this disease in certain individuals with a predisposition for caries. (Poorni et al., 2022) Some authors consider dental caries a chronic infection because of this (Konde et al., 2021). This is particularly concerning since 61% of children from 6 to 12 years of age (Basir et al., 2022) and 50% of children between 3 and 6 years of age have developed caries in Europe (Inchingolo et al., 2022).

When caries appear under the age of six, they are named early childhood caries (ECC), and it is considered one of the most prevalent diseases worldwide at this age (Staszczyk et al., 2022). ECCs are closely related to a reduced quality of life and are also considered an expensive burden for families (Hasslöf et al., 2022). Additionally, when caries appear in young children,

it impacts their mastication technique and even their speaking ability (Basir et al., 2022). However, in the preliminary stages, caries is a reversible process of enamel loss, meaning that it can be prevented without invasive therapies (Hasslöf et al., 2022; Inchingolo et al., 2022).

Another common oral disease is gingivitis, which is called, in a more exacerbated state, periodontitis. Gingivitis is, similar to caries, a multifactorial disease that starts with dysbiosis (Cugini et al., 2021; Abikshyeet et al., 2022). It is thought that gingivitis worsens with the hormonal change that accompanies the start of puberty (Kaan et al., 2021). The primary symptom of gingivitis is bleeding, which, in turn, increases the number of blood components, like erythrocytes, hemin, and fibrin. These molecules represent key factors for specific groups of bacteria, usually existing in dwindling numbers but rapidly increasing when this imbalance is present (Cugini et al., 2021). Besides blood components, another factor contributing to this disease's progression is the presence of inflammatory cytokines produced by the individual immune system (Gheisary et al., 2022).

It is important to understand that gingivitis itself is a reversible condition and is not responsible for the destruction of gingival tissues (Volgenant et al., 2022). However, if untreated, it can progress to a more severe condition called periodontitis. This condition is a major cause of tooth loss in adults and can impair their general health (EFSA, 2020; Gheisary et al., 2022; Hardan et al., 2022; Zhang et al., 2022). In fact, the World Health Organization (WHO) states that up to 50% of the world's population can suffer from periodontitis (Lin et al., 2022). Periodontitis is then characterized by irreversible damage to the periodontium, the structure responsible for tooth attachment in the oral cavity (Thomas et al., 2021; Salinas-Azuceno et al., 2022; Zhang et al., 2022).

With the aggravation of dysbiosis and gingivitis, there is an accumulation of plaque at the supragingival level. Consequently, the environment becomes oxygen-deprived and allows Gram-negative anaerobic microorganisms to proliferate. As the pockets get deeper and the carbon source is depleted, the environment becomes more propitious for the growth of *Treponema denticola*, *P. gingivalis*, *Fusobacterium nucleatum*, and *Veillonella* sp., microorganisms usually found in periodontal dysbiosis (Cugini et al., 2021; Thomas et al., 2021; Butera et al., 2022; Salinas-Azuceno et al., 2022). Besides oxygen and carbon, the pH and temperature of the environment also change, resulting in new microorganisms having the possibility to grow, therefore increasing the diversity in the oral cavity, contrary to what happens in cariogenic dysbiosis (Thomas et al., 2021).

In addition to losing the teeth, periodontitis is also responsible for bone reabsorption, which leads to more tooth loss in the neighboring sites (Cugini et al., 2021).

Another important consequence of dysbiosis is oral malodor, also known as halitosis, which is deeply connected to the prevalence of oral diseases, even if it is not associated with any specific infection (Karbalaei et al., 2021). Halitosis affects almost 50% of the population, with significant effects on quality of life, specifically self-esteem and psychological factors (López-Valverde et al., 2022). The leading cause of halitosis is the plaque that forms on teeth surfaces and the tongue and the increase of Gram-negative anaerobic bacteria that produce volatile sulfur compounds, such as *P. gingivalis*, *Treponema denticola*, *Prevotella intermedia*, and *Fusobacterium nucleatum* (Yoo et al., 2020; Karbalaei et al., 2021; López-Valverde et al., 2022).

Furthermore, there is another group of oral diseases called oral mucosal diseases, which affect, as the name suggests, the mucosal tissues of the mouth. This group comprises oral infections such as oral candidiasis, oral lichen planus, and ulcerative lesions (Alves et al., 2020). Despite being multifactorial, it is well established that these diseases also result from oral dysbiosis (Thomas et al., 2021; Wang et al., 2021). However, oral mucositis is prevalent in individuals treated with radiotherapy and chemotherapy, which induces dysbiosis on its own (Wang et al., 2021).

Another common disease found in the oral cavity is oral candidiasis, an opportunistic infection caused by yeasts belonging to the genus *Candida*, which is incredibly challenging in immunocompromised individuals (Contaldo et al., 2023; Fusco et al., 2023). It appears as a consequence of oral dysbiosis since this state allows the growth of certain species, such as *Candida albicans*. However, infections caused by other species of *Candida* spp. are increasing worldwide since they are more resistant to treatment than *C. albicans* (Contaldo et al., 2023). In addition, fungal infections have been increasing recently, possibly due to the considerable use of broad-spectrum antibiotics, among other factors (Fusco et al., 2023).

Definitively, it is essential to indicate that certain types of cancer, such as oral squamous cell carcinoma, have been linked to the presence of an oral dysbiotic state, even though it was not demonstrated that it could initiate this disease (Radaic et al., 2022). In some oral cancers, an imbalanced number of streptococci can be found in the oral cavity, more precisely, they are decreased (Thomas et al., 2021).

Although the worldwide prevalence of oral diseases is increasing even with more access to general care, there is an urgent need to find new and alternative strategies to prevent this imbalance in the oral cavity, with many negative impacts on human health (Radaic et al., 2022; Thomas et al., 2021).

2.2.1 Major pathobionts

It has been demonstrated that there is not an oral pathogen responsible for causing oral diseases; instead, oral diseases usually initiate after a change in the environment, and this change is responsible for the modification of oral microbiota into a dysbiotic state (Thomas et al., 2021). For this reason, it is relevant to distinguish between pathogens and pathobionts. Pathogens are microorganisms that can cause disease in any environment they inhabit. By contrast, pathobionts are commensals that acquire pathogenic properties after specific environmental changes that benefit their growth (Cugini et al., 2021).

S. mutans, a Gram-positive bacteria (Jang et al., 2021), is the most studied member among oral pathogens. It is considered the primary colonizer and the microorganism responsible for increasing adherence of other bacteria, it can adhere to teeth surfaces, but it also has the ability to adhere to the oral mucosae (Kaan et al., 2021; Yang et al., 2021). Despite the difference between pathogenic microorganisms and pathobionts, *S. mutans* is considered a true pathogen of the oral cavity since its presence usually indicates a positive correlation to dental caries and even periodontitis (Kamble et al., 2022; Konde et al., 2022; Lin et al., 2022). In addition, the virulence factors that *S. mutans* present are essential for caries formation, such as the ability to produce acid from carbohydrate metabolization, survive in acidic environments, and produce EPS from sucrose (Wu et al., 2022). Despite that, its biofilm formation capacity has also been described, as well as its ability to facilitate the adherence of other microorganisms and form dental plaque (Abikshyeet et al., 2022).

Usually, in a state of oral disease, it is noticeable an increase in aciduric microorganisms, such as *Streptococcus* spp., *Lactobacillus* spp., *Bifidobacterium* spp., *Actinomyces* spp., and *Veillonella* spp. The complexity of the biofilm is not limited to bacteria. For instance, *C. albicans*, a well-known fungi member, is also more commonly found in a cariogenic biofilm (Thomas et al., 2021; Hernández et al., 2022).

However, there are other relevant pathobionts, such as *P. gingivalis*, *Tannarela forsythia*, and *Fusobacterium nucleatum*, Gram-negative anaerobes, and LPS producers, being characterized as major causes of oral inflammation in cases of periodontitis and halitosis (Yang et al., 2021; Giannini et al., 2022; Zhang et al., 2022;). *F. nucleatum* is also responsible for the co-aggregation of different colonizers (Karaca et al., 2022). The bacteria known as periodontopathogens, as are those mentioned above, and others, such as *Treponema denticola, Bacteroides* spp., *Aggregatibacter actinomycetemcomitans*, *Captnocytophaga* spp., and *Veillonella* spp., are also associated with oral lichen planus (Thomas et al., 2021; Zhang, Y et

al., 2022). *Veillonella* spp. has also been linked to halitosis and even dental caries (Lin et al., 2022). Regarding periodontitis and halitosis, the most studied pathogen is *P. gingivalis*, an anaerobic Gram-negative bacterium (Jung et al., 2021), since it is considered a key pathogen due to its ability to increase inflammation, worsen the dysbiotic state, and produce volatile sulfur compounds. Additionally, it also has the ability to evade the host's immune system (Ishikawa et al., 2020; Yoo et al., 2020; El-Bagoory et al., 2021; Jansen et al., 2021; Sudhakaran et al., 2021). Likewise, the presence of *P. gingivalis* and *A. actinomycetemcomitans* not only increases the inflammatory state but also promotes the virulence factors of the other species inhabiting the dental plaque (Bueno et al., 2022). *Prevotella* spp. can also be linked to halitosis and periodontal disease (Lin et al., 2022).

Another relevant pathobiont is *C. albicans* and other *Candida* species responsible for oral candidiasis. These microorganisms present specific adhesins capable of adhering to dental surfaces, mucosa, and even dental appliances, such as orthodontic braces or artificial crowns. Similar to other commonly found pathobionts, those from *Candida* species can organize in biofilms, resulting in augmented virulence and therapy resistance (Contaldo et al., 2023).

In immunocompromised individuals, a critical outcome of pathobionts are opportunistic infections (Rossoni et al., 2020).

2.2.2 Conventional treatment

Despite oral health being a general health problem and a burden for public health, there are no implemented preventive strategies for this matter. However, it has been noted that the best angle of preventive approaches should be the regulation of the commensal microorganisms to prevent oral dysbiosis (Salinas-Azuceno et al., 2022), especially since oral diseases like caries and periodontitis can be reversed when the contributing factors are eliminated (García et al., 2021).

Regarding conventional treatment for oral diseases, the most frequent guideline is to pay regular visits to the dentist, where the focus is removing dental plaque and implementing antibiotics in case of infection (Kaan et al., 2021).

The physical removal of dental plaque, either by brushing the teeth or with professional utensils such as a scaler, is one of the best traditional strategies to prevent the aggravation of plaque and, consequently, the emergence of caries and gingivitis. However, brushing the teeth is not always effective, and it is not feasible to depend solely on professional actions to prevent oral diseases (EFSA, 2020).

The most common treatment outside of the dentist's office for the alleviation of dental plaque is the use of chlorhexidine mouth rinses. Although its efficacy has been assessed towards different microorganisms, especially *S. mutans*, it has a few disadvantages, such as teeth staining, loss of salivary flow, and elimination of all microbiota, worsening the dysbiotic state eventually (Kamble et al., 2022).

In order to prevent caries, sealants are usually placed, forming a physical barrier on the fissures, thus not allowing the microorganisms to reach them. However, in the case of a cavity forming in a tooth with a sealant, what is noted is that it becomes much more severe than when the sealant is not implemented. In this case, the sealant can only be applied to molar teeth, the only ones that present deep sulcus and fissures; nonetheless, caries can affect all teeth in the oral cavity (Cugini et al., 2021). Another action to prevent caries formation is using fluoride due to its ability to remineralize the tooth surface and thus prevent cavity formation (Staszczyk et al., 2022). However, it does not prevent the adherence of microorganisms or biofilm formation, and it can still allow dysbiosis to occur in the oral cavity (Inchingolo et al., 2022).

Another significant disadvantage in the current treatment of oral caries and periodontitis is the use of antibiotics, particularly broad-spectrum antibiotics. The use of these drugs may not be effective due to the resistance of bacteria in the biofilm structure, and it may also aggravate the resistance to antibiotics while disrupting the oralome into a deeper state of dysbiosis (Chen et al., 2022).

Regarding periodontitis, the conventional treatment is based on physically removing infra-gingival plaque with a technique called scaling and root planning. Despite being effective in removing the plaque and lowering the levels of microorganisms in the sulcus, periodontopathogens can recolonize the subgingival pockets and rapidly enter a dysbiotic state again (Butera et al., 2022; Giannini et al., 2022).

Concerning oral candidiasis, the established treatment is the application of antifungal drugs, such as azoles. However, this therapy deeply affects the host since it is directed at synthesizing the eukaryotic cell wall. For this reason, common side effects, namely hepatotoxicity and nephrotoxicity, must be considered when treatment is prolonged (Contaldo et al., 2023). Moreover, these microorganisms have the potential to disseminate systemically, causing what is called candidemia. Besides, multi-resistant species belonging to the *Candida* genus, including *C. albicans*, have been found, translating into an ineffective biotherapy (Contaldo et al., 2023; Fusco et al., 2023).

Altogether, it is clear that new strategies for preventing oral dysbiosis need to be implemented to circumvent the problem of oral diseases. (Fig. 1)

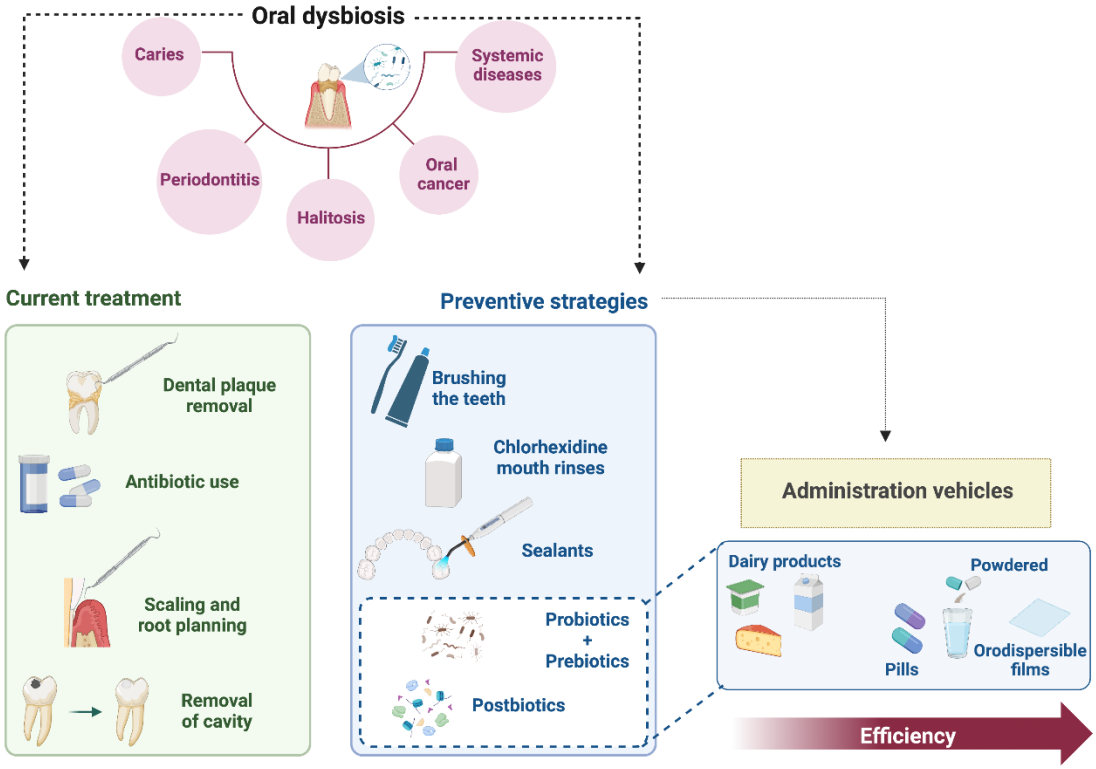

Fig. 1. Oral diseases, current treatments, and preventive strategies. Oral dysbiosis is the main factor contributing to the appearance of oral diseases, namely, caries, periodontitis, halitosis, and oral cancer. In addition, oral diseases are intimately correlated with systemic diseases, such as diabetes and heart and lung conditions. The current treatment is based on removing dental plaque, either physically or with antibiotics, scaling, root planning for periodontitis, and cavity removal for caries. Regarding preventive techniques, it is clear there is a lack of efficient strategies. The traditional prevention methods are teeth brushing and the use of chlorhexidine mouth rinses. Sealants can be used in molars, and they are more common in young patients. Recently, novel strategies, namely the use of probiotics, prebiotics, and postbiotics, have been gaining attention. Several studies correlated the efficiency of these methods with the administration vehicles. Notably, dairy products are the least effective vehicles regarding oral dysbiosis control, and orodispersible films, a promising approach, are the most effective. Created with BioRender.com

3. Novel strategies for the prevention of oral dysbiosis

Oral dysbiosis culminates in oral diseases if the balance is not restored. Interestingly, even though it is one of the most prevalent infectious diseases worldwide, they are still considered a "challenge to modern dentistry" (Kamble et al., 2022). For example, ECC is estimated to affect more than 6 hundred million children worldwide, even with educational strategies implemented worldwide (Staszczyk et al., 2022).

One of the most important strategies that must be implemented to prevent oral dysbiosis is controlling biofilm formation (Chen et al., 2022). For this reason, novel strategies should focus

on the removal of mature biofilm in order to maintain oral homeostasis, especially since it has been proved that oral dysbiosis is the leading cause of the emergence of a pathogenic environment (Cugini et al., 2021; Gao et al., 2022). Another important factor is that the microorganisms associated with the emergence of oral diseases and oral dysbiosis are mostly endogenous species, not pathogens from another environment. Consequently, the treatment and prevention strategies should focus on controlling the growth of these microorganisms instead of their complete removal (Abikshyeet et al., 2022).

Even with the implementation of novel techniques to prevent oral dysbiosis, oral hygiene should never be disregarded since it is one of the main factors influencing microbiota regulation (Kaan et al., 2021).

One aspect in which current strategies have been failing regarding preventing oral diseases is the maintenance of oral homeostasis or keeping the dysbiotic rate low. This rate is defined as the relation between pathobiont species present in the oral environment and species that are mostly associated with oral health (Ishikawa et al., 2020; Salinas-Azuceno et al., 2022). Current studies have focused on reducing the adhesion capacity of specific pathogens (Hernández et al., 2022). One approach that should be considered is using probiotics, prebiotics, and postbiotics to control the oral cavity, especially since they can modulate the oral microbiome and influence the dysbiotic rate by controlling the growth of disease-related microorganisms (Ishikawa et al., 2020; Thomas et al., 2021).

3.1 The use of probiotics

In order to fill the gap left by the lack of preventive strategies regarding oral diseases, a vast number of studies have been focusing on probiotics and their derivates to maintain homeostasis in the oral cavity (Ebrahim et al., 2022; Kamble et al., 2022; Salinas-Azuceno et al., 2022).

Probiotics have been used to reduce gut inflammation for over two decades and have shown the potential to modulate the normal microbiota in other parts of the human body (Giannini et al., 2022). Recently, it was noted that they are able to reduce the number of oral pathogens, such as *S. mutans,* when ingested daily for extended periods (Coqueiro et al., 2018; Qiu et al., 2020). Moreover, it was observed that probiotics may restore dysbiotic environments (Lordello et al., 2021; Gheisary et al., 2022; Hernández et al., 2022; Inchingolo et al., 2022). However, to obtain the full potential of probiotics for preventing dysbiosis, their use should be implemented before any signs of inflammation or disease appear (Seidel et al., 2022).

Since it is well-known that most oral diseases stem from dysbiosis, it is interesting to understand how probiotics can be of aid. Notably, only a reduced number of genera, such as *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp., and *Weissella* sp., have been considered adequate in the oral cavity because not all the bacteria that are usually administered as probiotics adhere to the oral mucosa (Zhang et al., 2022). Commonly, the species with potential probiotic effects are lactic acid bacteria (LAB), which can resist considerably low pH (Raheem et al., 2021). These species are "Generally Recognized as Safe" (GRAS) by the European Food Safety Authority (FDA, 2018). The *Lactobacillus* genus comprises a group of Gram-positive bacteria, facultative anaerobic, present in several areas of the human body (Zhang et al., 2022).

Several researchers have shown a positive correlation between oral health, or better yet, the reduction of pathobiont levels, using probiotic species (Gheisary et al., 2022; Giannini et al., 2022; Hardan et al., 2022; Seidel et al., 2022; Zhang et al., 2022). In fact, Kamble and collaborators (2022) concluded that using probiotics is as effective as chlorhexidine, a common disinfectant.

In addition, many studies have explored how probiotics can modulate the microbial composition of the oral cavity. These mechanisms have then addressed how the probiotics i) inhibit the adhesion of pathogens, ii) present antimicrobial activity, iii) affect biofilm formation, and iv) balance the pH change of the environment (Garcia et al., 2022; Hasslöf et al., 2022; Hernández et al., 2022; Kamble et al., 2022; Kijima et al., 2022; Konde et al., 2022; Wu et al., 2022). In this regard, probiotics have the capacity to compete directly for nutrients with pathobionts, produce antimicrobial molecules, and modulate the host's immune response (Gheisary et al., 2022; Hernández et al., 2022; Kamble et al., 2022). Additionally, they decrease the virulence factors of common pathobionts, namely *S. mutans* (Archambault et al., 2021; Hernández et al., 2022; Jung et al., 2022).

Despite lowering the levels of potentially pathogenic microorganisms through competition for binding sites and nutrients, probiotics also increase oral health due to their ability to control inflammation in the oral cavity (Wang et al., 2021; Gheisary et al., 2022; Inchingolo et al., 2022; Seidel et al., 2022; Wang et al., 2022). This anti-inflammatory capacity is reached through the activation of toll-like receptors or blockage of interleukin production (Giannini et al., 2022; Hernández et al., 2022).

Probiotics may also increase the salivary flow, which is directly related to maintaining homeostasis and preventing caries (Babina et al., 2022).

In addition, certain species of probiotics, such as *Lactobacillus acidophilus* LA5, can also

downregulate certain virulence factors of important periodontopathogens, such as the adhesion *P. gingivalis* and *Fusobacterium nucleatum* (Zhang et al., 2022).

Moreover, probiotics can produce peptides with important functions, such as bacteriocins, that increase the environmental pH, counteracting the acid effect of *S. mutans* and activating the immune system (Gheisary et al., 2022; Hernández et al., 2022).

Another important characteristic of some probiotic species is the production of antimicrobial agents, which decrease the levels of pathobionts in the oral cavity. For instance, *Limosilactobacillus reuteri* produces reuterin, a natural antibiotic effective against both Gram-negative and Gram-positive bacteria (Gheisary et al., 2022; Hardan et al., 2022; Salinas-Azuceno et al., 2022; Zhang et al., 2022). Other probiotics, particularly *Lactococcus lactis*, are major producers of nisin that help maintain oral health. This nisin production is essential not only in the formation of caries but also in the reduction of tumors (Hernández et al., 2022; Radaic et al., 2022). Other LAB bacteria, frequently administered as probiotics, produce ammonia, which prevents the growth of *S. mutans* in the oral cavity, and others, usually belonging to *Streptococcus* spp. produce H_2O_2, which is effective in not only decreasing the levels of pathobionts but also in controlling their virulence (Hernández et al., 2022).

Babina and Kijima also demonstrated that probiotics increase the level of specific immunoglobulins (Ig), namely IgA, in the oral cavity. This property reduced the ability of pathogens to adhere to oral mucosa and dental surfaces while controlling the inflammatory process (Babina et al., 2022; Kijima et al., 2022).

Essentially, the activity of probiotics in the oral cavity demonstrated a reduction of dental plaque and a controlled inflammatory response (Alhallak et al., 2022; Hardan et al., 2022; Salinas-Azuceno et al., 2022; Zhang et al., 2022).

Regarding periodontitis, probiotics demonstrated an improvement in clinical parameters, such as bleeding on probing and periodontal pocket depth (Gao et al., 2022; Gheisary et al., 2022; Zhang et al., 2022).

Although some studies have shown that probiotics alone as therapy are not able to reverse the disease state, however, Jansen and collaborators (2021) concluded that if the probiotics reduce only minimally the levels of dental pathogens, it is enough to give the immunological system of the patient the ability to act on the pathogens, thus reducing the infection.

Probiotics from the *Lactobacillus* genus can be applied to treat inflammation in the oral cavity (Hardan et al., 2022) and as an adjuvant in treating some oral diseases, namely periodontitis. This can be correlated with the fact that the application of probiotics improves bone regeneration and repair (Garcia et al., 2022).

Bizzini et al. (2012) demonstrated that *Limosilactobacillus fermentum* inhibited the production of glucans by *S. mutans*, thus reducing the pathogenicity of the biofilm. In addition, in 2022, Zhang et al. demonstrated that the administration of *Lactiplantibacillus plantarum* CCFM8724 was more effective than the treatment with chlorhexidine. In this line, several studies have demonstrated that *L. reuteri* produces antimicrobial molecules (reuterin) against *S. mutans* (Daliri et al., 2021; Garcia et al., 2022). It can also reduce the levels of several pathobionts in the oral cavity (Hasslöf et al., 2022) and the release of inflammatory cytokines by the host (Garcia et al., 2022).

Lin et al. (2018) managed to reduce drastically the levels of *S. mutans* in patients with moderate and severe gingivitis. Bustamante et al. (2020) showed that the ingestion of *L. reuteri* daily reduced the levels of caries and gingivitis in the first 9 years of age. In another study, the same authors demonstrated that after 10 months of ingesting *L Lacticaseibacillus rhamnosus* SP1, the indices of caries lowered. For these reasons, some studies have emphasized that probiotic supplementation is a good strategy to prevent ECC (Staszczyk et al., 2022).

Additionally, a recent study showed that probiotics, specifically *Ligilactobacillus salivarius*, can also favor the salivary buffer ability (Abikshyeet et al., 2022). This probiotic can be attractive due to its resistance to acidic environments (Kijima et al., 2022). *L. salivarius* is also important for maintaining periodontal health (Hardan et al., 2022).

Another study demonstrated that *Lactobacillus gasseri* has probiotic activity *in vitro* towards caries and periodontitis (Kaan et al., 2021). Besides that, the probiotic *Lactobacillus acidophilus* is also able to decrease the number of *S. mutans* (Hernández et al., 2022).

Several studies have demonstrated that *L. rhamnosus* could reduce oral cavity inflammatory molecules and periodontopathogen growth, such as *Fusobacterium nucleatum* (Chen et al., 2022; Giannini et al., 2022; Zhang et al., 2022). Interestingly, this probiotic also showed the capacity to reduce biofilm formation by *S. mutans* (Jung et al., 2022). One study demonstrated that the use of the probiotic *Lacticaseibacillus paracasei* showed a better capacity to recover from gingivitis. In this case, this probiotic inhibited the secretion of IL-1β, a pro-inflammatory cytokine, and modulated the plaque ecosystem (Volgenant et al., 2022). Additionally, *L. paracasei* has antimicrobial activity via the secretion of molecules that damage the *S. mutans* cell wall (Archambault et al., 2021).

Another important probiotic is *L. lactis*, which is usually administered to improve the homeostasis of the biofilm (Radaic et al., 2022). However, according to the study by Wu et al. (2022), one of the most effective probiotics regarding caries control was *Lacticaseibacillus casei*, which was the most effective in lowering the levels of *S. mutans*.

Recently, *Streptococcus salivarius* and *Weisella cibaria* have been gaining attention due to a high number of probiotic properties displayed in the oral cavity (Abikshyeet et al., 2022; Chen et al., 2022; Ebrahim et al., 2022; Inchingolo et al., 2022; Zhang et al., 2022). These species are usually chosen based on their enhanced adherence ability, representing an advantage compared to traditional probiotics, such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Babina et al., 2022). In fact, probiotics such as *Streptococcus salivarius* and *W. cibaria*, present in caries-free individuals, are thought to improve oral health and modulate the immune system (Kang & Park, 2021; Babina et al., 2022; Giannini et al., 2022; Inchingolo et al., 2022; Poorni et al., 2022; Zhang et al., 2022). *S. salivarius* shares multiple characteristics with *S. mutans* and *S. sobrinus*, two important pathobionts in the oral cavity. Consequently, it competes for nutrients and bonding sites, ultimately decreasing the number of pathobionts in the microbiome (Babina et al., 2022). Besides, probiotics belonging to the *Streptococcus* genus may negatively influence the release of pro-inflammatory cytokines, thus controlling the inflammatory response (Hernández et al., 2022). Furthermore, *S. salivarius* shows antibiotic action by releasing bacteriocins into the environment, particularly active against *S. mutans* (Inchingolo et al., 2022; Wang et al., 2022) or used as a strategy to decrease halitosis (López-Valverde et al., 2022).

It is important to state that several *Lactobacillus* species have shown interesting properties against fungal infections, namely those caused by *Candida* species. Interestingly, when probiotics are applied, the symptoms of the infection appear alleviated (Contaldo et al., 2023). Curiously, when probiotic therapy is used concomitantly with fluconazole, the anti-biofilm activity and restoration of the oral equilibrium are reached more quickly (Fusco et al., 2023).

On the other hand, *W. cibaria*, a Gram-positive bacterium (Kang & Park, 2021), has demonstrated antibacterial and antifungal activity towards oral pathobionts, and its presence is associated with healthy individuals (Fanelli et al., 2022). Moreover, it actively inhibits the growth of periodontopathogens, such as *F. nucleatum* and *P. gingivalis*, presents an anti-inflammatory capacity, and reduces dental plaque formation (Kang & Park, 2021; Zhang et al., 2022). Of note, some studies suggest that *W. cibaria* can effectively reduce halitosis (Kang & Park, 2021; López-Valverde et al., 2022). This species also produces bacteriocins and is resistant to a wide range of pH and temperatures, making it an exciting approach for preventive strategies (Fanelli et al., 2022).

A key advantage of probiotic therapy is the fact that it can be used for long periods without the risk of side effects (Gheisary et al., 2022; Staszczyk et al., 2022). However, it is necessary to keep in mind that to be effective, probiotics must be taken continuously (Hernández et al.,

2022). In order to enhance probiotic activity, the use of prebiotics, ingredients that enhance probiotic activity, is highly recommended by several authors (Nguyen et al., 2021; Abikshyeet et al., 2022; Gao et al., 2022; Hernández et al., 2022; Inchingolo et al., 2022; Jung et al., 2022; Konde et al., 2022).

Despite some promising results, some studies failed to identify probiotics as a tool for dysbiosis control effectively. Actually, some studies demonstrate that probiotics belonging to the genus *Lactobacillus* do not present a proper ability to adhere to dental surfaces, and for that reason, they cannot appropriately prevent *S. mutans* colonization (Jung et al., 2022). This elucidates the need for an adjuvant therapy used concomitant with probiotics or the need to find a better strategy.

Chuang et al. (2011) did not find differences in the levels of *S. mutans* in their work on the effect of *L. paracasei* tablets to control cariogenic bacteria. Since there are few long-term studies, there is still no data about how long it takes for *S. mutans* to repopulate and colonize the oral cavity after the end of preventive procedures like probiotic intake (Colombo et al., 2015). A different study showed no increase in immunoglobulin production after a four-week intake (Babina et al., 2022), while a meta-analysis showed that the use of probiotics did not improve the plaque index (Hardan et al., 2022).

An important detail to keep in mind is that probiotic functions are specific to each strain, and they cannot be inferred from one study to another. Notably, Gao et al. (2022) could not find a reduction in periodontopathogens such as *P. gingivalis* and *F. nucleatum* with the application of *L. rhamnosus* GG, one of the best-studied probiotics.

Despite all the efforts to understand the mechanism of action behind probiotic efficacy, a considerable amount of research still needs to be done to fill in the gaps in the current knowledge (Zhang et al., 2022).

Moreover, a reasonable number of studies showed a slight decrease in the number of pathogens but with no statistical significance. This could be due to a wrong delivery mechanism, dose, or even probiotic strain. Also, because probiotics are live bacteria, they could be weakened or damaged during several steps of their preparation and storage (Baral et al., 2021). Probiotic bacteria are rapidly removed from the oral cavity through swallowing and mastication since they lack the ability to adhere to the tooth surface (Ferrer et al., 2020; Jiang et al., 2020; Amargianitakis et al., 2021; García et al., 2021; Nguyen et al., 2021; Hernández et al., 2022; Volgenant et al., 2022). Additionally, several species belonging to the *Lactobacillus* genera have been linked to an increased risk of caries development and even EPS production that aggravates the biofilm (Ferrer et al., 2020; Ishikawa et al., 2020; Jansen et al., 2021; Karaca

et al., 2022; Wu et al., 2022). Moreover, live bacteria cannot be used in immunocompromised patients due to their susceptibility to opportunistic infections (Kwon et al., 2020; Jastrząb et al., 2021; Wang et al., 2021; Basir et al., 2022; Ma et al., 2023). Adding to that, live bacteria demonstrate a very limited shelf life and extreme environmental sensitivity (Basir et al., 2022; Ma et al., 2023). To overcome these limitations, recent studies have now focused on the use of postbiotics.

3.2 The use of postbiotics

Postbiotics are described as inactivated microorganisms, or their cell components and metabolites, that promote health when administered (Jastrząb et al., 2021; Salminen et al., 2021; Liang & Xing, 2023). They can be grouped into two different categories: cell-free probiotics and cell-derived. These postbiotics are usually obtained from fermentation processes (Jastrząb et al., 2021; Scott et al., 2022) being composed of metabolites, either secreted by live bacteria or acquired after the lysis of bacterial membrane or fragments of bacterial cells. These components may positively affect commensal microorganisms and the host's immune system (Salminen et al., 2021).

The most common metabolites found in postbiotics are short-chain fatty acids and organic acids, namely acetic acid, lactic acid, butanoic acid, and propionic acid, antimicrobial molecules, such as bacteriocins (Sornsenee et al., 2021; González-Lozano et al., 2022; Scott et al., 2022; Liang & Xing, 2023), amino acids, such as alanine and leucine (Shin et al., 2022), EPS, cell wall peptides and lysates, varied enzymes (Chang et al., 2021; Scott et al., 2022), flavonoids, and phenolic compounds (Diez-Gutiérrez et al., 2022; Karaca et al., 2022). However, the presence of these components is related to the cultivated strain, the medium culture, and even the selected method to obtain the postbiotics (Liang & Xing, 2023), as well as other factors, such as temperature and pH (Chang et al., 2021).

Recent studies conducted with postbiotics demonstrated their ability to affect commensal microorganisms in the oral cavity, leading them out of a dysbiotic environment (Jastrząb et al., 2021; Ma et al., 2023). Besides that, postbiotics also show the ability to promote the epithelial barrier function, improving the host's protection, as well as the same immunomodulation capacity as probiotics (Liang & Xing, 2023; Ma et al., 2023); this ability is usually attributed to membrane vesicles (González-Lozano et al., 2022). They also demonstrate anti-inflammatory capacity, which is related to the type of postbiotic (Jastrząb et al., 2021; Basir et al., 2022; Liang & Xing, 2023; Ma et al., 2023) (Fig. 2).

Furthermore, postbiotics present an interesting ability to reduce the levels of oral pathobionts in the oral cavity. In this case, short-chain fatty acids, such as acetate, propionate, and butyrate (González-Lozano et al., 2022), are indicated as a possible mechanism of growth inhibition since they disrupt the membrane of bacteria (Yang et al., 2021). Postbiotics obtained from LAB usually contain bacteriocins produced by these bacteria, demonstrating an inhibition activity towards pathogens (Liang & Xing, 2023).

Another characteristic of postbiotics is the anti-inflammatory and antioxidant effects of the EPS of certain bacteria, such as *L. plantarum* and *L. paracasei,* that can reduce reactive oxygen species and limit QS between pathogens (Karaca et al., 2022; Scott et al., 2022; Liang & Xing, 2023). In addition, EPS has been a notable antibiofilm factor (Barzegari et al., 2020; Bengoa et al., 2021; Karaca et al., 2022).

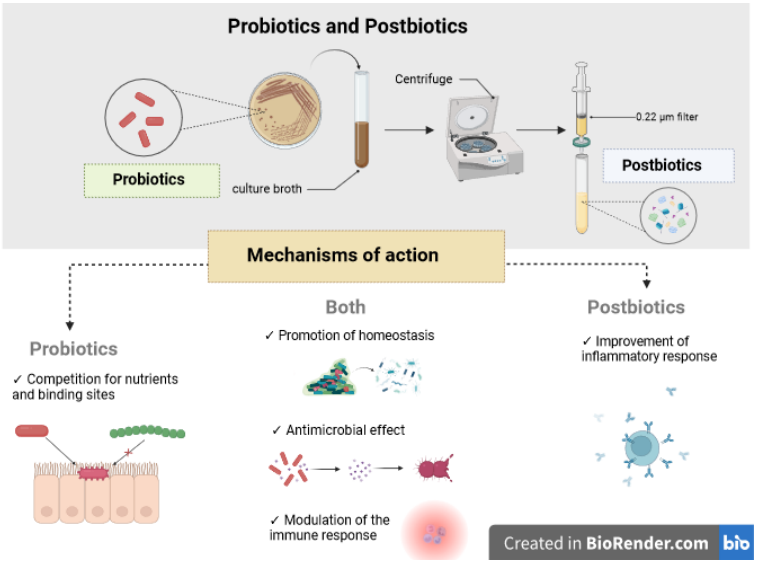

Fig. 2. Mechanism of action of probiotics and postbiotics. As described in the main text, postbiotics are produced metabolites of probiotics, obtained after a cycle of centrifugation and filtration step with a membrane of 0.22 µm. They exert their functions as adjuvants of human health by different mechanisms, such as promoting homeostasis of oral microbiota, disrupting biofilm, and modulating the immune and inflammatory response. Probiotics also compete for nutrients and binding sites, which helps decrease pathobiont levels and produce molecules with antimicrobial effects. Notably, postbiotics also show antimicrobial effects since they disrupt bacterial cell walls. Created with BioRender.com

In fact, several studies have shown promising results against pathogenic biofilms in the oral cavity (Rossoni et al., 2018; Kim et al., 2019; Carvalho et al., 2021). However, the efficacy of the postbiotics against pathobionts is always related to their concentration (Yang et al., 2021). To obtain the postbiotics, the cell wall must be disrupted, releasing the components responsible

for these properties into the culture medium (Ba et al., 2022).

Furthermore, postbiotics have recently also been related to improving oral health in periodontal issues due to their ability to reduce periodontal pathogens, such as *P. gingivalis* (Yang et al., 2021). Moreover, another study demonstrated that postbiotic extracts from *Lactobacillus* spp. have antimicrobial activity against Gram-negative pathogens (Ma et al., 2023). Further, the administration of postbiotics also presented anti-biofilm capacity against *S. mutans*, a desirable characteristic for controlling dysbiosis. This effect can be correlated with the presence of teichoic acids produced by *Lactobacillus* spp. Lipoteichoic acid is also related to the activation of the innate immune system (Bae et al., 2022). The most common metabolite of *Lactobacillus* spp. is lactic acid, which is also considered a postbiotic due to its antibacterial effects (Bengoa et al., 2021). Lactic acid leads to a drop in the environmental pH and promotes the lysis of the pathogen's cell walls (Chang et al., 2021).

All things considered, the consumption of postbiotics should decrease *S. mutans* numbers in the oral cavity, improving oral health and lowering the risk of caries, periodontitis, and even cardiac complications (Lin et al., 2022).

Besides common pathogens related to caries and periodontitis, there are already studies relating the effectiveness of postbiotics to candidiasis, demonstrating a reduction in yeast numbers in the planktonic state and in biofilm (Barzegari et al., 2020; Rossoni et al., 2020).

The most crucial advantage of postbiotic administration is based on the fact that there are no living microorganisms present, this means there is no risk of transmission of resistance genes, and there is no risk of causing infection in immunosuppressed individuals or risk groups (elderly, children and pregnant women) (Malagón-Rojas et al., 2020; Jastrząb et al., 2021; González-Lozano et al., 2022; Liang & Xing, 2023; Ma et al., 2023). Moreover, the postbiotic application circumvents the need for bacteria cells to adhere to tooth enamel (Liang & Xing, 2023). Besides, postbiotics demonstrate a longer shelf life than probiotics and do not require extra care in storage or transportation (Nataraj et al., 2020; Jastrząb et al., 2021; Liang & Xing, 2023; Ma et al., 2023). From a financial perspective, postbiotics demonstrate several advantages, either in the obtaining process or in transportation (Scarpellini et al., 2021). Additionally, another interesting study demonstrated that the application of postbiotics did not result in cytotoxic activity for the cells (Sornsenee et al., 2021).

Regarding the limitations of postbiotics, there have been a few studies that did not show an intense immunomodulation activity (Vinderola et al., 2022; Ma et al., 2023). Besides, there is still a lack of information about identifying the present compounds, which hinders the scale-up process (Jastrząb et al., 2021). In addition, specific guidelines must be provided for the

attainment of postbiotics since it can be noted that their efficacy is highly related to the medium, strain, and growth conditions (Nataraj et al., 2020).

Moreover, the efficacy on the oral health of both postbiotics and probiotics is intrinsically correlated to the form of administration because they determine the time of contact with the oral cavity and how well they are released in the mouth to provide the benefits of their biological properties. As such, the form of administration significantly impacts the effectiveness of the postbiotics. One form of administration might be to vehicle the postbiotics through orodispersible films.

Therefore, understanding oral films' main advantages and disadvantages is paramount, a promising delivery mechanism for both probiotics and postbiotics.

3.2.1 Orodispersible films: Easy vehicles to target oral dysbiosis

There are several different vehicles to administer probiotics and postbiotics in the oral cavity. The most common are tablets and lozenges, but studies report milk, powders, and even ice creams as administration vehicles in the oral cavity (Seminario-Amez et al., 2017).

However, the efficacy of the treatment is intimately related to the chosen vehicle (Gheisary et al., 2022; Wiegers et al., 2022). For that reason, it should be improved regarding pro and postbiotic efficiency (Ribeiro et al., 2020). These standard administration vehicles are effective when the goal is to improve intestinal health. However, dairy options should not be considered when it comes to improving oral health (Staszczyk et al., 2022). Furthermore, to allow the probiotics and postbiotics to act on the colonization sites, the administration vehicles should not be intended for swallowing but instead focused on in-mouth disintegration (Ribeiro et al., 2020; Kaźmierczyk-Winciorek et al., 2021; Lordello et al., 2021; Staszczyk et al., 2022; Hasslöf et al., 2022).

Orodispersible films (ODF) are not a new strategy for the delivery of drugs in the oral cavity since they have been explored for more than 40 years (Irfan et al., 2016). They are described as solid, thin, polymeric preparations with the ability to deliver the active principle quickly and easily. ODF must be non-toxic and biocompatible, which is why there should be no adverse effects expected after its use (Seminario-Amez et al., 2017; El-Bagoory et al., 2021; Lordello et al., 2021; Olechno et al., 2021; Ebrahim et al., 2022; Cornilă et al., 2022; Salawi, 2022). Another attractive property of ODF is its solubility and quick disintegration, usually under five minutes (Desai et al., 2020), without the need for water (Salawi, 2022; Shah et al., 2022). Therefore, there is no choking risk associated with its intake (Irfan et al., 2016; Baral et

al., 2021). This translates to an advantage of application since it broadens its use to paediatrics, geriatrics, and individuals with dysphasia or bedridden patients (Salawi, 2022).

An important characteristic that should be reached is mucoadherence because it allows the active principle to be present in the oral cavity for enough time to be released and to colonize different areas of the mouth (Giannini et al., 2022; Mura et al., 2022). One of the most important advantages of ODF is the elevated tolerability by several different groups of individuals (Ebrahim et al., 2022). This is particularly important since several studies have demonstrated the lack of compliance with traditional oral hygiene methods from both children and adults (Hasslöf et al., 2022). Moreover, the manufacturing of ODF is simple and brief, and the process is not expensive (Salawi, 2022).

On the other hand, it is important to understand that, due to its characteristics, ODFs show effectiveness loss throughout time. Because of that, particular attention should be given to packaging (Salawi, 2022). In addition, there must be extra care in achieving high concentrations of probiotics and postbiotics since several studies demonstrate the need for at least 10^9 CFU/mL to restore homeostasis (Wang et al., 2022).

Regarding its composition, it is observed that ODFs are mainly composed of film-forming polymers, plasticizers, sweetening agents, and saliva stimulators (Alves et al., 2020; Lordello et al., 2021; Olechno et al., 2021; Salawi, 2022). However, the properties of each ingredient and its quantity are crucial to establishing the most favorable preparation. It should be noted that oral dispersible films should be applied at least twice a day in order to obtain maximum results. More than this can be considered inconvenient for daily use (Volgenant et al., 2022).

Polymers, such as hydroxypropyl methylcellulose, maltodextrin, or chitosan, compose nearly 50% of the ODF (Lordello et al., 2021; Olechno et al., 2021; Shah et al., 2022). Interestingly, it has been shown that polymers positively affect oral health even without administering pro or postbiotics (Giannini et al., 2022).

Plasticizers, the second most important excipient of ODFs, optimize the plasticity and handling of the films (Irfan et al., 2016; Alves et al., 2020; Olechno et al., 2021; Chen et al., 2022; Salawi, 2022; Shah et al., 2022). The most used plasticizers are glycerol, sorbitol, or mannitol, among others (Olechno et al., 2021).

It is relevant to add a saliva stimulant, namely organic acids, to improve the characteristics of the ODF and the convenience of its use (Irfan et al., 2016; Olechno et al., 2021). The importance of using citric acid as a stimulator of saliva production is based on the buffering capacity of saliva and its potential to eliminate microorganisms with the salivary flow. Also, it has been demonstrated that saliva stimulators aided with the increase of oral pH (Cugini et al.,

2021; Zhang et al., 2022).

Other ingredients can be used for specific purposes. For example, *Mentha* sp. is used to reduce *S. mutans* biofilm, and it can be applied to postbiotic ODF, and sugars have been used due to their antimicrobial properties (Hernández et al., 2022; Kamble et al., 2022).

Finally, including excipients relevant to caries prevention in ODF formulations, such as fluoride and arginine, is valuable. When arginine is present, some bacteria can produce ammonia and, consequently, raise the oral pH, preventing tooth demineralization (Ferrer et al., 2020; Inchingolo et al., 2022). Another common prebiotic added to ODF preparation is nitrate due to its ability to shift oral communities towards a healthier environment (Rosier, Buetas et al., 2020).

Packaging is another vital aspect to pay attention to since it is crucial to maintain the mechanical and physical characteristics of the ODFs (Salawi, 2022). The most common and effective method of packaging described in the literature is aluminum or foil pouches (Bala et al., 2013; Irfan et al., 2016; Ribeiro et al., 2020; Salawi, 2022). This solution is cost-effective, simple, and reliable (Salawi, 2022).

Although ODFs are a promising strategy for administering pro and postbiotics, presenting key attractive advantages and satisfying the conditions needed to release these molecules in the oral cavity adequately, their actual impact on oral dysbiosis must be addressed and clarified.

In this sense, this work aimed at producing an orodispersible film with an optimized formulation with incorporated postbiotics. For this, several tasks were undertaken, namely:

1-Formulation optimization and production of the orodispersible films

2- Physico-chemical characteristics of the films

3-Evaluation of the antimicrobial and antibiofilm capacity of certain postbiotics: CFS postbiotics obtained from *L. plantarum*, CFS postbiotics obtained from *L. paracasei,* and a mixture of both CFS postbiotics.

4- Biological properties of the postbiotics

Given what was mentioned, it provided a deep understanding of the use of novel strategies for oral health prevention, to avoid the use of antibiotics and to control oral dysbiosis while in a reversible state, showing that these novel approaches exhibit beneficial properties with an interesting outcome in terms of health care.

Materials and Methods

1. Formulation of the orodispersible film

An orodispersible film made from Xantham gum (Sigma-Aldrich, Darmstadt, Germany), Maltodextrin (Sigma-Aldrich, Darmstadt, Germany), Citric acid (Merck, Darmstadt, Germany), and Glycerol (VWR chemicals, USA) was developed. Different polymer concentrations were tested to obtain the optimized formulation, namely, 30%, 25%, and 20% of film-forming polymers and 20%, 15%, and 10% of plasticizers.

It was essential to consider the different excipients needed to achieve the final formulation: Film-forming polymers, plasticizers, sweetening agents/taste masking agents, and saliva stimulants. For that, a list of the most commonly used polymers was made, and a final formulation was selected based on incompatibilities between polymers and safety for human use, as described in the table below.

Table 1 – List of all the components used to develop the orodispersible films with respective amounts. The quantity described below refers to 200 mL of deionized water.

Material	Function	Quantity
Xantham gum	Film-forming polymer	2.0 g
Maltodextrin	Film-forming polymer/ Plasticizer	3.0 g
Citric acid	Saliva stimulant	0.2 g
Glycerol	Plasticizer/Sweetening agent	2.0 mL

According to Shah et al., 2022, the solving-casting method was used with some modifications. In a beaker, 2.0 g of Xantham gum was added to 140 mL of deionized water and left in a magnetic stirrer for at least 4 hours (solution 1). In another beaker with 58 mL of deionized water, 2.0 mL of glycerol was added and stirred for 2 hours. After that, the rest of the excipients were added, Citric acid followed by Maltodextrin, and left to stir for two more hours (solution 2). Finally, solution 2 was poured slowly over solution 1 with constant stirring until complete homogenization. The final solution was then left to rest to allow the disappearance of all the bubbles formed during magnetic agitation.

The final solution was then spread over a plastic container and left to dry for 48 hours. After that period, the samples were cut using a box cutter in a dumbbell shape or 1x1 cm^2 squares for further analysis.

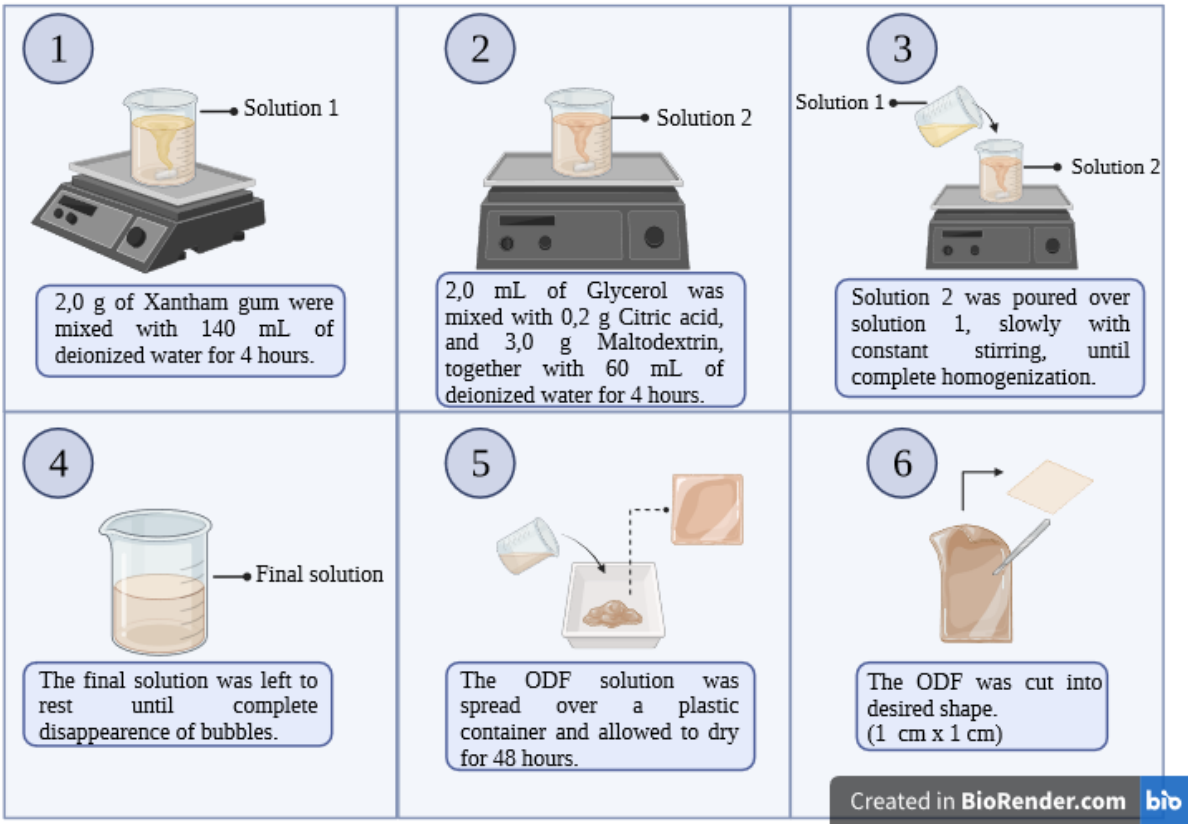

Figure 3 – Methodology for the preparation of orodispersible films, according to Shah et al., 2022, with modifications. The excipients were mixed in a magnetic stirrer for 4 hours in 2 separate solutions and further mixed until complete homogenization. The final solution was then left to rest until all the formed bubbles disappeared. The film solution was spread over a plastic container and dried at room temperature for at least 48 hours. Lastly, the orodispersible film was cut into the desired shapes. Created with BioRender.com

2. *Postbiotic preparation*

To obtain the postbiotic solutions, two probiotics, *Lactiplantibacillus plantarum* 226V and *Lacticaseibacillus paracasei* L.26, were grown in De Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) and isolated in MRS agar (Biokar Diagnostics, Beauvais, France) as previously described by Sornsenee et al. (2021) with modifications. Briefly, after obtaining isolated colonies of both species, each was inoculated in a 15 mL falcon in MRS broth (Biokar Diagnostics, Beauvais, France) and incubated at 37 ºC for 48 hours. To certify that the probiotics had enough growth for the exploitation of the postbiotic solutions, a

sample was taken, diluted, plated by the drop method, and counted after incubation at 37 ºC for 24 hours to confirm that the growth was higher than 10^9 CFU/mL.

After that period, the falcon tubes were centrifuged at 8,000 g for 15 min at 4 ºC using a Hettich centrifuge (Hettich, Tuttlingen, Germany), and the supernatant was then filtered with a membrane of 0.22 µm to obtain the cell-free supernatant (CFS) of the probiotics, which is the postbiotic solution.

To ensure that the supernatant did not contain any cells, the solution was plated in MRS agar (Biokar Diagnostics, Beauvais, France) using the drop method, and the growth was observed after an incubation of 24 hours at 37 ºC.

After confirmation, the CFSs obtained were used to incorporate the orodispersible films and for antimicrobial and antibiofilm analysis. Three conditions were tested: CFS of *L. plantarum*, CFS of *L. paracasei*, and a combination of both CFSs.

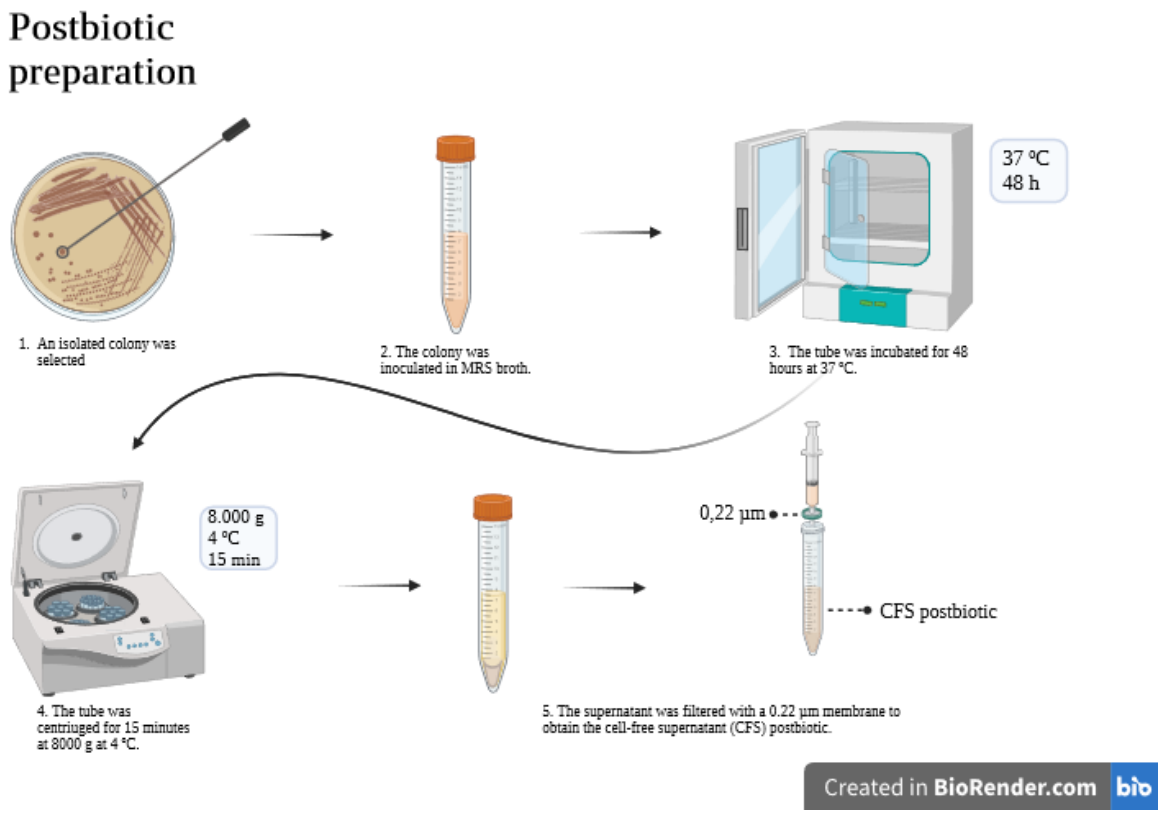

Figure 4 – Process for obtaining the CFS postbiotics as described above. The selected isolated colony was inoculated in MRS broth, followed by incubation for 48 hours at 37 ºC. The tubes were then centrifuged at 8,000 g at 4ºC for 15 minutes and filtered with a membrane of 0.22 µm to obtain the cell-free supernatant (CFS) postbiotic solution. Created with BioRender.com

a. *Incorporation of the postbiotic solution in the orodispersible film*

To incorporate the CFSs postbiotics in the orodispersible films, the postbiotic solutions were added to the polymeric solution 2, adjusting the amount of water to maintain the final volume constant.

b. *Analysis of the physical characteristics of the orodispersible film*

i. Surface morphology and appearance

The surface appearance was observed after the ODFs were dried, before and after cutting, in order to understand if the surface was homogenous, without bubbles, and transparent. The observations were performed for all the samples, with and without the postbiotic solution, and the results were compared.

ii. Disintegration time and pH

The disintegration time was performed according to Shah et al., 2022, with a few modifications. Briefly, the orodispersible films were cut into squares measuring 1x1 cm^2 to measure the disintegration time. In a beaker, 100 mL of deionized water was added. Then, the previously cut ODFs were added, and the stopwatch was started. After the complete dissolution of the film, the stopwatch was stopped.

Each measurement was performed in triplicate, and the mean value was calculated. The results were compared to the orodispersible films without the impregnation of postbiotics, which served as a control.

The pH was then measured, according to Salawi (2022). After the complete disintegration/dissolution of the ODFs in water, the pH was measured using a Crison basic 20 pH probe (Crison, Barcelona, Spain). Each measurement was performed in triplicate, and the average value was calculated. The results obtained with the ODFs impregnated with CFS postbiotics were compared to those obtained with the ODFs without the impregnation of postbiotics to understand the pH variation.

iii. Thickness

The thickness of the ODFs was measured using a My20 micrometer (Adamel lhomargy, Saint-Baldoph, France), according to Batista et al. (2019). For that, using the previously cut orodispersible films in 1x1 cm^2 squares, three distinct points, randomly selected, were measured in the same sample in order to understand whether the orodispersible films were homogenous throughout the entire surface. The mean value was calculated, and the result was compared to the orodispersible films without the addition of postbiotics, which were used as a control.

iv. Film weight

The mass was measured using an analytical scale (Sartorius, Göttingen, Germany) to understand the variation of the ODF's weight with the addition of the three conditions of the postbiotic solution, according to Al-Naamani et al. (2016). Each measurement was performed in triplicate, and the values were averaged to obtain the mean value. The ODFs without the addition of the postbiotics were used as a control.

v. Hydration, moisture loss, and solubility

The percentages of hydration, moisture loss, and solubility were measured according to Al-Naamani et al. (2016) with modifications. The orodispersible films, previously cut into 1x1 cm^2 squares, with and without postbiotics, were weighed under the same conditions (W_1). The ODFs were then submerged in 10 mL of deionized water for 1 hour and weighed again after removing the excess water with a paper towel (W_2). To obtain the solubility and moisture loss percentage values, the ODFs were stored for 24 hours in an incubator at 37 ºC, and one last measure was conducted after that period (W_3).

All the measurements were performed on an analytical scale (Sartorius, Göttingen, Germany) in triplicate, and the values were averaged to obtain the mean values.

The percentages of hydration, moisture loss, and solubility were calculated using the following equations:

$$(1) \qquad \%\, hydration = \left(\frac{W_2 - W_1}{W_1}\right) \times 100$$

$$(2) \qquad \%\, moisture\ loss = \left(\frac{W_2 - W_3}{W_2}\right) \times 100$$

$$(3) \qquad \%\, solubility = \left(\frac{W_1 - W_3}{W_1}\right) \times 100$$

vi. Contact angle

The contact angle of the ODFs was determined through the sessile drop technique using a tensiometer (Attension Theta, Biolin Scientific, Sweden). For that, 5 µL of deionized water was dispensed on the film samples, and the angle formed between the baseline and the lines tangent to the water droplet. The values were recorded for 1 minute, and the average value was

calculated to perform the analysis. The ODFs without the addition of the CFS postbiotic solution were used as a control.

3. *Antimicrobial capacity of the postbiotics against Streptococcus mutans*

    a. *Growth rate measurement of Streptococcus mutans*

To understand the postbiotic solution's antimicrobial activity, the growth rate of *Streptococcus mutans* 45091 was evaluated under distinct postbiotic conditions, according to Jung et al. (2022): those obtained from *L. plantarum,* those from *L. paracasei*, and a mixture of both, in the concentrations of 10% (v/v), 20% (v/v), 40% (v/v), 60% (v/v) and 100% (v/v).

Initially, *S. mutans* was grown in BHI broth (Biokar Diagnostics, Beauvais, France) and isolated in BHI agar (Biokar Diagnostics, Beauvais, France) plates. After obtaining isolated colonies, *S. mutans* was regrown in BHI broth (Biokar Diagnostics, Beauvais, France) until a concentration of 10^9 CFU/mL, confirmed with plating following the drop technique and counting.

Firstly, a co-culture of the *S. mutans* in BHI broth (Biokar Diagnostics, Beauvais, France) and the CFS postbiotic solution were mixed in a 15 mL falcon tube in a 1:1 ratio and incubated for 24 hours at 37 ºC. Following that time, a 2 µL sample was taken from each condition and plated in BHI agar (Biokar Diagnostics, Beauvais, France) in triplicate. The results were expressed as positive or negative growth.

After this first assessment, new inocula of *S. mutans* and CFS postbiotics were prepared. With an inoculum of 10^9 CFU/mL, a 96-well plate with all the different conditions was inoculated in triplicate. The plate was then incubated at 37 ºC while measuring the OD for 24 h in a Multiskan GO plate reader (Thermo Scientific, Massachusetts, USA).

A positive control of *S. mutans* was used to understand the standard growth rate of the microorganism, and negative controls of each medium were also used.

The values were then averaged to obtain the mean results to analyze the effects of the different postbiotics on the *S. mutans* growth rate.

4. *Monitoring flora reduction of Streptococcus mutans in co-culture with postbiotics*

    a. *Minimal inhibitory volume*

The methodology from Drumond et al. (2023) was followed to determine the minimal inhibitory volume. For that, samples of *S. mutans* were grown in MRS broth at 37 ºC until reaching an $OD_{\lambda=600nm}$ of 1.0 which corresponds to a concentration of 10^9 CFU/mL, and further decimal dilutions were performed, to obtain the concentration of 10^6 CFU/mL.

After that, a 96-well microplate was inoculated with 100 µL of *S. mutans* together with 100 µL of the three postbiotic solutions in five different concentrations: 10% (v/v), 20% (v/v), 40% (v/v), 60% (v/v) and 100% (v/v), and incubated at 37 ºC for 24 hours. A negative control of MRS broth and BHI broth was used, as well as a positive control of *S. mutans* and MRS broth. After this period, the OD was measured at λ=600 nm in a Multiskan GO plate reader (Thermo Scientific, Massachusetts, USA). The minimal inhibitory volume was defined as the lowest volume of postbiotic that inhibited the growth of *S. mutans*.

b. *Time-kill assay*

Another vital aspect to be determined was the time-kill assay of *S. mutans* in a co-culture with the CFS postbiotic solutions, conducted according to the methodology described by Jansen et al. (2021), with modifications.

The postbiotics were obtained as described before, and *S. mutans* was grown until a concentration of 10^9 CFU/mL was reached. Afterwards, the bacteria were incubated at 37 ºC with the postbiotic solutions in their respective minimal inhibitory volumes. At different time points (0, 1, 2, and 4 hours), a sample of 1 mL was taken from the mixture, and decimal dilutions were made.

Finally, 2 µL from the samples was plated, in duplicate, in BHI agar and incubated at 37 ºC for 24 hours. Following that time, the plates were counted to determine the values of CFU/mL. Negative (MRS broth and BHI broth) and positive controls (only *S. mutans*) were also added to plates, incubated, and counted.

5. Antibiofilm capacity of the postbiotics against Streptococcus mutans

The antibiofilm capacity of the CFS postbiotic solutions against *S. mutans* was tested according to J. Wu et al. (2022), with slight modifications. Two potential capabilities were tested: the ability to prevent biofilm formation and the ability to destroy mature biofilms, as described below.

a. *Antibiofilm formation inhibition*

Streptococcus mutans was grown in BHI broth until a 10^9 CFU/mL concentration was reached. The CFS postbiotic solutions were obtained in 2 – postbiotic preparation. In a 96-well microplate, 100 µL of *S. mutans* was inoculated, together with the different postbiotics in five different concentrations (10% (v/v), 20% (v/v), 40% (v/v), 60% (v/v) and 100% (v/v). The

plates were inoculated for 72 hours at 37 ºC to allow the biofilm formation of *S. mutans* to occur.

After that time, the content of each well was removed, washed with Ringer solution to ensure only the adhered biofilm would be measured, and treated with 0.1% crystal violet. The plates were left to dry at room temperature for 24 hours. Then, the wells were resuspended in glacial acetic acid (30%), and the OD was measured at λ=630nm.

b. *Mature biofilm inhibition*

Streptococcus mutans was grown in BHI broth until a concentration of 10^9 CFU/mL was reached, and 100 µL of that concentration was inoculated at a 96-well microplate and incubated for 5 days at 37 ºC to allow for biofilm to reach its mature state.

The CFS postbiotic solutions were obtained as described in 2 – postbiotic preparation, inoculated in different concentrations on the formed biofilm, and left to react for 72 hours at 37 ºC. The remaining steps were performed as described above in 5.a – Antibiofilm formation inhibition.

6. Cytotoxicity of the postbiotic-based orodispersible film on human mouth cells

The TR146 cell line was used as an *in vitro* model of the human epithelial mucosa. The cell line was purchased from Sigma-Aldrich (# 10032305), defrosted, and maintained in HAMS F12 medium (BioWest), with 10% of Fetal Bovine Serum (FBS, Biowest), and 1% of Penicillin-Streptomycin-Fungizone solution (Pen-strep, Lonza). TR146 cell line was then maintained in T75 flasks at 37 °C in a 5% CO_2 humidified atmosphere during the experimental time.

TR146 cells were plated onto a 24-well microtiter plate at 10^5 cells/mL of density to perform the cytotoxicity assay and allowed to attach overnight. Afterwards, the medium was replaced, and two pieces of each film with 0,5 cm of diameter were added to the wells in triplicate per condition. The conditions tested were: (1) control of cells (cells were maintained only with medium, without any film); (2) control of the film (sample of the film without postbiotics) (3) film with CFS postbiotics obtained from *L. plantarum*; (4) film with CFS postbiotics obtained from *L. paracasei*, and (5) film with a mix of CFS postbiotics obtained from both probiotics.

After 24 hours of incubation, the metabolic activity of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Sigma-Aldrich). For this purpose, the culture media was removed and replaced with 450 µL of HAMS F12 and 50 µL of MTT solution per well. These were incubated for 3 to 4 hours at 37 °C in a CO_2 incubator.

The culture media was discarded, and 500 μL of DMSO per well was added to dissolve the formazan crystals. Subsequently, the plates were kept under agitation for 10 minutes at room temperature (RT). The absorbance was measured at λ = 570 nm using a microplate reader (Synergy 4, Biotek). Three independent assays were performed, and the cell viability was calculated in percentage.

7. Statistical analysis

All analyses were done with GraphPad Prism version 10.1.1 software (GraphPad Software, Inc., San Diego, CA, USA).

For the analysis of the physical properties of the ODF, the one-way analysis of variance (ANOVA) method was used for multiple comparisons, followed by Tukey's post hoc test after testing for the normal distribution of all data. The antimicrobial and antibiofilm activity was analyzed using a two-way ANOVA followed by Dunnett's multiple comparison test to assess the significant differences.

All experiments were performed in triplicate. The results are expressed as means, and the corresponding standard deviations were calculated. Values were considered statistically different at a p-value of ≤ 0.05.

Results and Discussion

1. *Physical characteristics of the orodispersible film*

    a. *Surface morphology and appearance*

The orodispersible film formulation was optimized for elasticity, appearance, and maneuverability. According to Muta et al. (2022), an ODF that presents elasticity and flexibility ensures a pleasurable sensation in the oral cavity. The optimized formulation was based on Cugini et al. (2021), who stated that film-forming polymers should constitute up to 50% of the total concentration, followed by up to 20% of plasticizers, 10% of sweetening agents and 10% of saliva stimulants. In this sense, the final optimized concentration of the film-forming solution was 25% (m/v) film-forming polymers (Xantham gum and Maltodextrin), 15% (m/v) plasticizer agent (Maltodextrin and Glycerol), 1% (m/v) saliva stimulant (Citric acid), and 1% (v/v) sweetening agent (Glycerol). After drying, the postbiotic-free ODFs presented adequate handling; they were thin and easy to cut. Regarding color, the ODFs were transparent. However, the same was not observed regarding the ODFs impregnated with the postbiotic solutions. During the formulation of the ODF, a significant number of bubbles were formed in the solutions; however, when left to rest before pouring, the complete disappearance of the bubbles was noted. However, the bubbles persisted in the solution impregnated with the postbiotic, even after the rest period; they were darker and not wholly homogenous. Nonetheless, the ODFs impregnated with CFS postbiotics were more accessible to handle since they were thicker and less glutinous.

The choice of natural polymers was based on the fact that they are biodegradable and biocompatible, do not present toxicity for the oral cavity, and the FDA recognizes them as GRAS. (Alam et al., 2014) Maltodextrin was chosen based on the appearance it provides the films; however, its mechanical properties are often limited. (Shah et al., 2022) To overcome this problem, xantham gum was used concomitantly as a film-forming polymer.

    b. *Disintegration time and pH*

An important value to determine regarding ODFs is the disintegration time, i.e., the time it takes to completely dissolve after being in contact with the oral cavity. There are still no guidelines regarding the dissolution time. However, the time should be long enough to ensure that the postbiotics can be delivered in the oral cavity and exert their activity. Still, according to Lordello et al. (2021), the disintegration time is directly related to the polymer concentration of the ODF.

The ODF without the impregnation of CFS postbiotics served as a control and presented a high dissolution time of 24 min. It was observed that there was no significant difference between the disintegration times of the samples when compared to the control; the CFS postbiotic obtained from *L. plantarum* and *L. paracasei* both dissolved in 24 min as well, and the CFS postbiotic obtained from both probiotics dissolved in 25 min. According to these results, there were also no statistically significant differences between the different postbiotics tested. The data is presented below in Fig. 5.

However, it is essential to remember that *in vitro* behavior differs drastically from *in vivo* performance. The time it takes to dissolve the ODFs is expected to decrease if applied in the oral cavity since it will be affected by deglutition, speech, and the normal movement of the tongue.

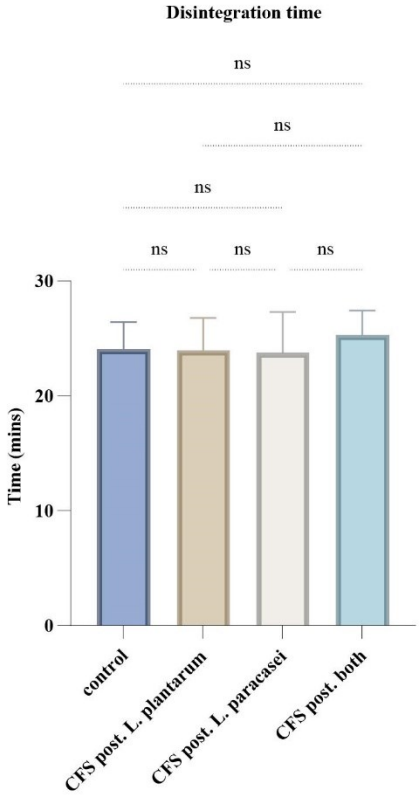

Fig. 5 – Time, in minutes, necessary until complete dissolution of ODFs impregnated with different postbiotic solutions. The data are presented with mean ± SD. ns indicates a lack of statistical differences between the samples.

The pH was measured after the complete dissolution of the ODFs; data is shown below in Fig. 6. The ODF without the impregnation of the postbiotics served as a control and had a pH value of 4.33. The pH value of the ODF impregnated with CFS postbiotics obtained from *L. plantarum* was significantly lower (3.86) than the control ($p<0.01$). The other ODFs were not statistically different from the control. However, an increase in the pH of the ODFs impregnated

with CFS postbiotics from *L. paracasei* was noted (4.58), which was significantly higher than the ODF with postbiotics from *L. plantarum* ($p<0.001$). This demonstrates that, probably during growth, *L. plantarum* produced acidic metabolites, such as lactic acid, which offers more antimicrobial activity, as noted in the antimicrobial assays when compared with *L. paracasei*. The ODF impregnated with CFS postbiotics obtained from both probiotics had a pH value of 4.06, not statistically different from those obtained from *L. plantarum* or the control but significantly lower than those obtained from *L. paracasei* ($p<0.01$).

The pH of the films was supposed to be closer to a neutral value (7 or close) (Bala et al., 2013). However, the low values were expected since the chosen probiotics are LAB, which produce acid during growth. (Lordello et al., 2021) Notably, after assessing the cytotoxicity, it was noted that none of the ODFs tested presented increased cytotoxicity when in contact with cells from the oral cavity.

The low pH of the control was due to the addition of citric acid to the composition to act as a saliva stimulant. These low pH values could be the cause of antimicrobial activity, which may be due to the acidic metabolites excreted to the medium during bacterial growth. However, multiple factors can be at play, and further analysis should be performed to determine if the antimicrobial activity persists after neutralizing the film-forming solutions. Additionally, these pH values can be considered a limitation for the film application since they can aggravate the already acidogenic environment of an unhealthy oral cavity in the presence of *S. mutans*.

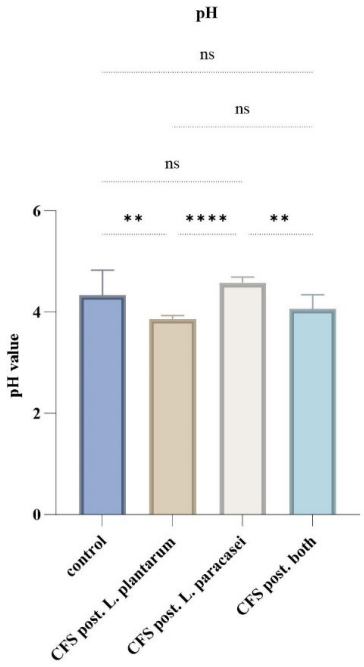

Fig. 6 – pH values of the different ODFs impregnated with postbiotic solutions. The data are presented with mean ± SD. ns indicates a lack of statistical differences between samples. ** indicates significant differences between samples ($p<0.01$) and **** ($p<0.0001$)

c. *Thickness*

Another physical property of the ODFs assessed was the variation of their thickness before and after the addition of the different CFS postbiotic solutions. Data is shown below in Fig. 7. The ODF without impregnating the postbiotic solutions was considered a control, presenting a thickness of 0.175 µm. When compared to the ODFs after impregnation with postbiotics, it was noted a statistically significant increase in the ODFs thickness, namely 0.327 µm for ODF with CFS postbiotic obtained from *L. plantarum* ($p<0.001$) and 0.346 µm for ODF with CFS postbiotic obtained from *L. paracasei* ($p<0.001$). Regarding the ODF impregnated with CFS postbiotics from both probiotics, the ODF was not as thick (0.266 µm) but equally different from the control ($p<0.001$). The thickness increase was directly related to the easiness of maneuvering and cutting the ODFs into 1 x 1 cm^2.

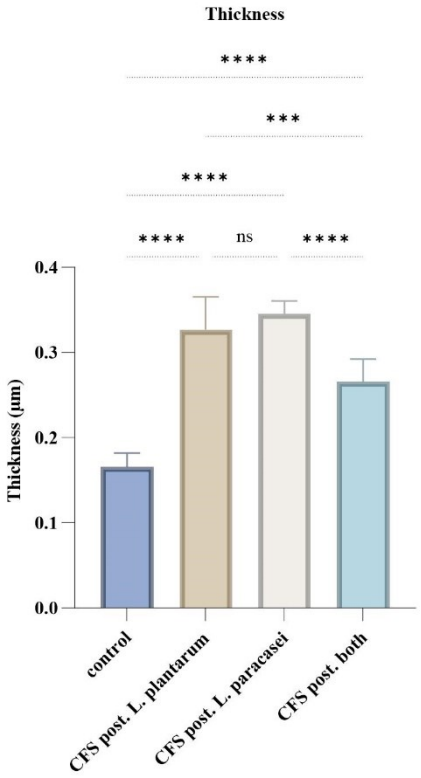

Fig. 7 – Thickness values, in µm, of the different samples of postbiotic-impregnated ODFs. The data are presented with mean ± SD. ns indicates a lack of statistical differences between the samples. *** indicates significant differences between samples ($p<0.001$) and **** ($p<0.0001$).

d. *Film weight*

In order to understand the variations of the film weight before and after the incorporation of the different postbiotic solutions, they were measured on an analytical scale (Sartorius, Göttingen, Germany). For this, the ODF, without the impregnation of any CFS postbiotic

solution, served as a control. The data is shown below in Fig. 8. It was observed that the weight of all the ODFs increased significantly with the incorporation of CFS postbiotics.

The control presented a value of 0.0210 g; the ODF impregnated with CFS postbiotic obtained from *L. plantarum* increased the weight, reaching 0.0427 g ($p<0.001$). The ODF impregnated with CFS postbiotics from *L. paracasei* presented significantly more weight than the control, 0.0492 g ($p<0.001$), but were not statistically different from the previously analyzed sample. Regarding the ODF impregnated with CFS postbiotics obtained from both probiotics, the weight increase was not as high as the other two ODFs, reaching a weight of 0.0346 g ($p<0.05$). However, it did not present statistically significant differences when compared with the ODF impregnated with CFS postbiotics obtained from *L. plantarum*.

It was noted that the weight variations were similar to the thickness changes with the different postbiotic solutions, which was expected. However, according to some authors, the weight obtained in the ODFs after the impregnation of CFS postbiotics and the measured thickness could be higher than the recommended to achieve optimal behavior in the oral cavity. (Batista et al., 2019; Alves et al., 2020)

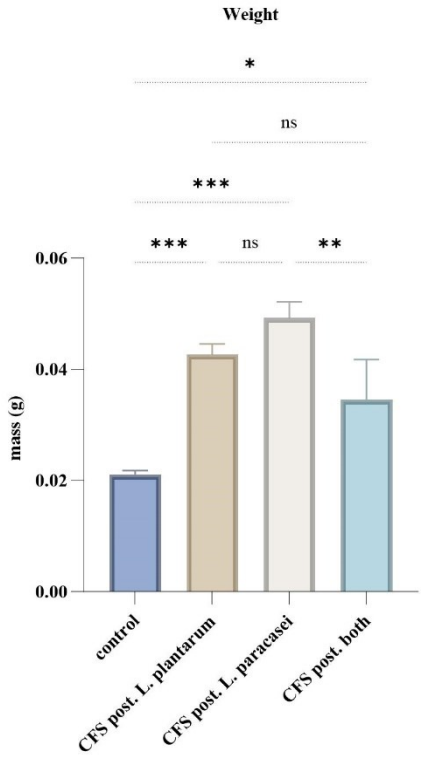

Fig. 8 – Film weight, in grams, of the different samples of postbiotic-impregnated ODFs. The data are presented with mean ± SD. ns indicates a lack of statistical differences between samples. * indicates significant differences between samples ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

e. *Hydration, moisture loss, and solubility*

Another critical factor to consider is the hydration percentage or swelling capacity. This value is directly related to the hygroscopic properties of the ODF. It is affected by the film-forming polymers and can influence the physical characteristics of the final product. (Alves et al., 2020; Shah et al., 2022) It is noted that films with high concentrations of glycerol display lower mechanical stress since it increases their hygroscopy tendency. (Shah et al., 2022)

After observing the results, it was noted that the percentage of hydration significantly dropped with the impregnation of the CFS postbiotics in the film-forming solution ($p<0.001$). In the control ODF, the hydration percentage was 1710%, which clearly indicates the hygroscopic nature of the ODFs; it also explains the long dissolution time since these results show that the ODFs increase in weight before starting to dissolve.

Besides that, it was observed that the ODF impregnated with CFS postbiotics obtained from both probiotics presented the higher hydration percentage of the three samples (800%), compared with 552% and 423% of hydration percentages of ODF impregnated with CFS postbiotics obtained from *L. plantarum* and *L. paracasei* respectively. Data is shown below in Fig. 9.

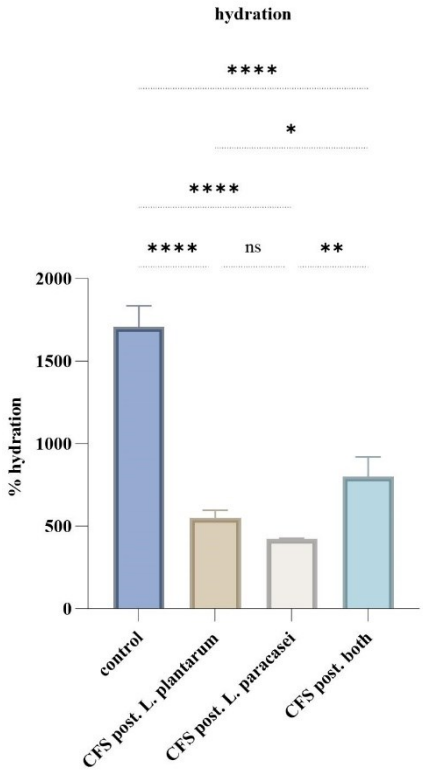

Fig. 9 – Hydration percentage of the different samples of postbiotic-impregnated ODFs. The data are presented with mean ± SD. ns indicates a lack of statistical differences between the control and the samples. * indicates significant differences between samples ($p<0.05$) , ** ($p<0.01$) and **** ($p<0.0001$).

Concomitantly with the hydration, the moisture loss and solubility percentages were also measured. As expected, the solubility was higher in the samples with lower hydration. Increasing solubility is desired since it allows for better dissolution of the postbiotics in the oral cavity. The control ODF presented a solubility percentage of 39%, the ODF impregnated with CFS postbiotic obtained from *L. plantarum* increased to 58% ($p<0.0001$), the ODF impregnated with CFS postbiotic obtained from *L. paracasei* increased to 57% ($p<0.0001$). The ODF impregnated with CFS postbiotics obtained from both probiotics increased to 56% ($p<0.0001$). Additionally, the differences between the three samples were not statistically significant. Data is shown below in Fig. 10. These values of increased solubility are critical for good behavior in the oral cavity since one of the desired characteristics of ODFs is easy dissolution without the need for water.

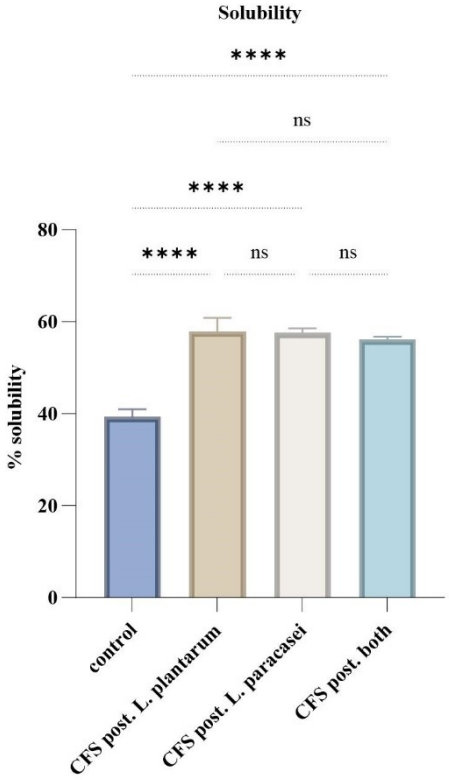

Fig. 10 – Solubility percentages of the different samples of postbiotic-impregnated ODFs. The data are presented with mean ± SD. ns indicates a lack of statistical differences between the samples. **** indicates significant differences between samples ($p<0.0001$).

Regarding the percentage of moisture loss, the variability between samples was not as substantial as hydration and solubility percentages. However, they were statistically significant. The ODF without the impregnation of postbiotics served as a control and presented a moisture

loss percentage of 96%. The ODF impregnated with CFS postbiotics obtained from *L. plantarum* showed a moisture loss percentage of 93% ($p<0.0001$). The ODF impregnated with CFS postbiotics obtained from *L. paracasei* presented a moisture loss of 92% ($p<0.0001$). The ODF impregnated with CFS postbiotics obtained from both probiotics showed a percentage of 95% moisture loss ($p<0.01$). The variation between samples was also statistically significant ($p<0.01$). Data is shown below in Fig. 11.

These values represent the stability of the ODFs over time since they represent the constancy of the weight of the ODF after being placed in the incubator. Despite being incredibly high values, which demonstrates a lack of stability, a desired decrease was noted after adding the CFS postbiotics, regardless of the postbiotic used.

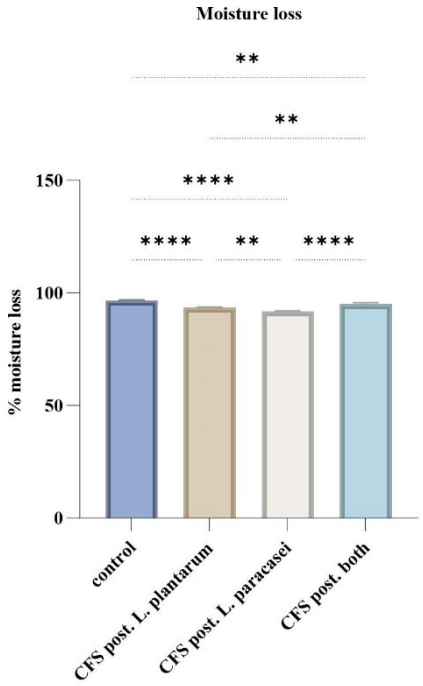

Fig. 11 – Moisture loss percentages of the different samples of postbiotic-impregnated ODFs. The data are presented with mean ± SD. ns indicates a lack of statistical differences between the samples. ** indicates significant differences between samples ($p<0.01$) and **** ($p<0.0001$).

f. *Contact angle*

The last analyzed physical property of the ODFs was the water contact angle, which is the angle formed between the base of the ODF and the tangent to the exterior plane of the water droplet (Fig. 13). This value can influence the dissolution time and hydration percentages of the ODFs since it is directly related to the hydrophilicity of the strips. The lower the contact angle, the higher the hydrophilicity. As expected, the contact angle of the ODFs decreased after

the addition of CFS postbiotics, which was also noted with the increase in solubility. The ODF without the impregnation of CFS postbiotics was used as a control with a contact angle of 54.6º. Data is shown in Fig. 12.

However, the difference was insignificant compared to the addition of CFS postbiotics obtained from *L. paracasei* (47.3º). The lowest contact angle was measured on ODF impregnated with CFS postbiotics obtained from *L. plantarum* (39.7º), followed by the ODF impregnated with CFS postbiotics obtained from both probiotics (44.7º) ($p<0.01$ and $p<0.05$, respectively).

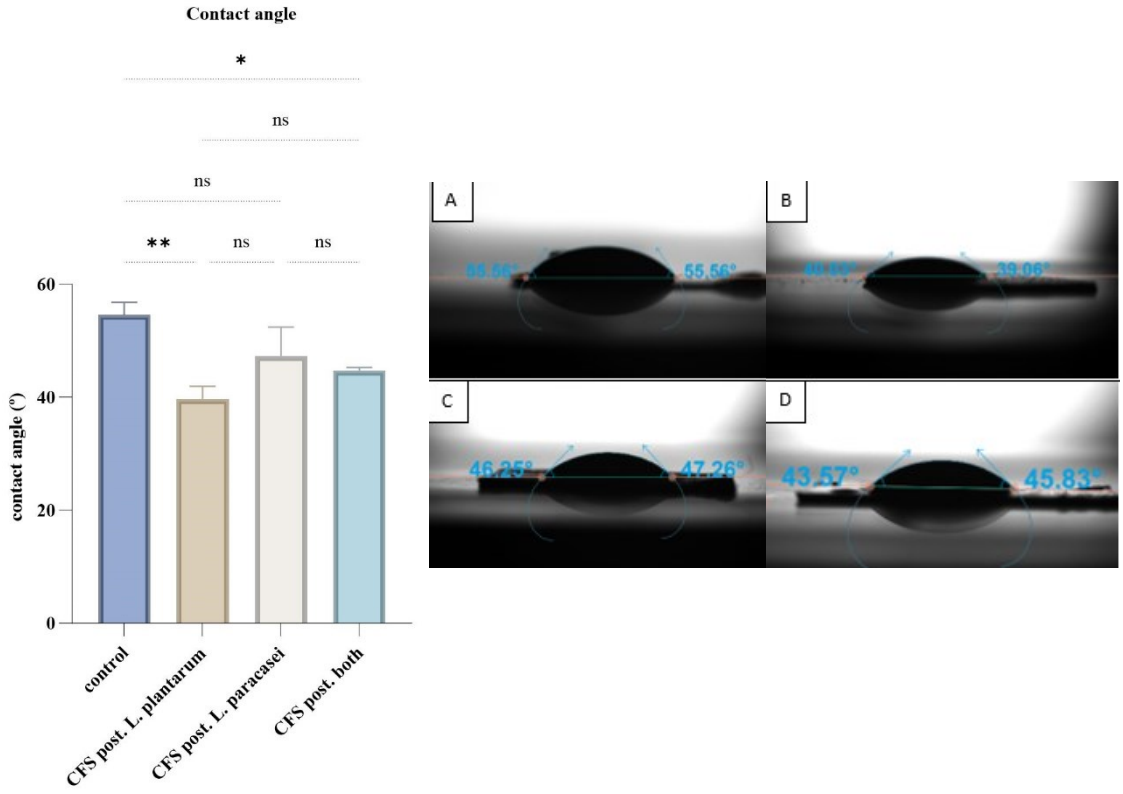

Fig. 12 and 13– Angle of water contact of the different samples of postbiotic-impregnated ODFs. The data are presented with mean ± SD. ns indicates a lack of statistical differences between the samples. * indicates significant differences between samples ($p<0.05$), and ** ($p<001$). Fig 13 shows the photographies recorded while measuring the contact angles of the different ODFs: control without CFS postbiotics (A) impregnated with CFS postbiotics obtained from *L. plantarum* (B) impregnated with CFS postbiotics obtained from *L. paracasei* (C) and impregnated with CFS postbiotics obtained from both probiotics (D).

2. *Monitoring the reduction of viable numbers of Streptococcus mutans in co-culture with postbiotics*

    a. *Minimal inhibitory volume*

As mentioned above, the minimal inhibitory volume assay was performed with all the postbiotic solutions to understand the necessary volume (v/v) at which the postbiotics completely inhibit the growth of *S. mutans*. The minimal inhibitory volume were defined as the lowest postbiotic concentrations that resulted in no visible growth of *S. mutans*.

In this sense, the minimal inhibitory volume defined for CFS postbiotics obtained from *L. plantarum* is 40%. Regarding CFS postbiotics obtained from *L. paracasei,* the minimal inhibitory volume was defined as 60%. The lowest concentration for complete inhibition of the bacteria was observed in CFS postbiotics obtained from both probiotics, with only 20% needed to inhibit *S. mutans'* growth. Data is shown below in Fig. 14.

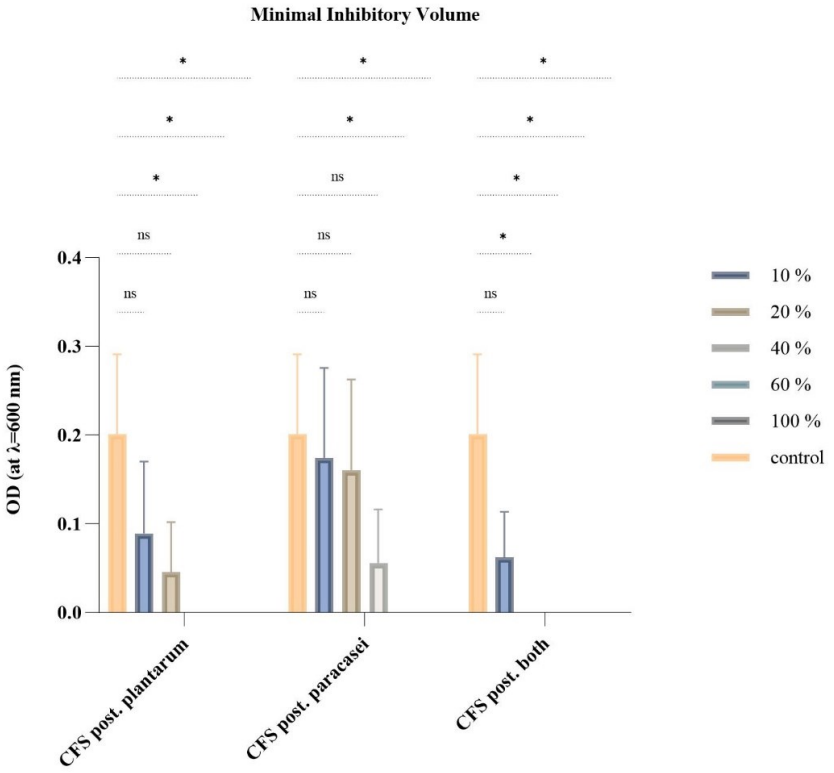

Fig. 14– OD variation of *S. mutans* measured at λ=600nm with different CFS postbiotic concentrations. The data are presented with mean ± SD. ns indicates a lack of statistical differences between the samples. * indicates significant differences between samples ($p<0.05$).

b. *Time-kill assay*

The time-kill assay was also performed to assess the inhibition/death of *S. mutans* in co-culture with the different postbiotics. Notably, the postbiotics that demonstrated a higher antimicrobial activity also showed a lower time to kill *S. mutans*.

In fact, both CFS obtained from *L. plantarum* and those obtained from both probiotics took 2 hours until no growth was observed after plating and counting. Regarding CFS postbiotics obtained from *L. paracasei,* the time until no growth was measured was 4 hours. A control plating without the addition of postbiotics was performed. Data is shown below in Fig. 15.

These are promising results for applying postbiotics in ODFs since they do not require significant time to exert their antimicrobial properties and can be administered in the oral cavity.

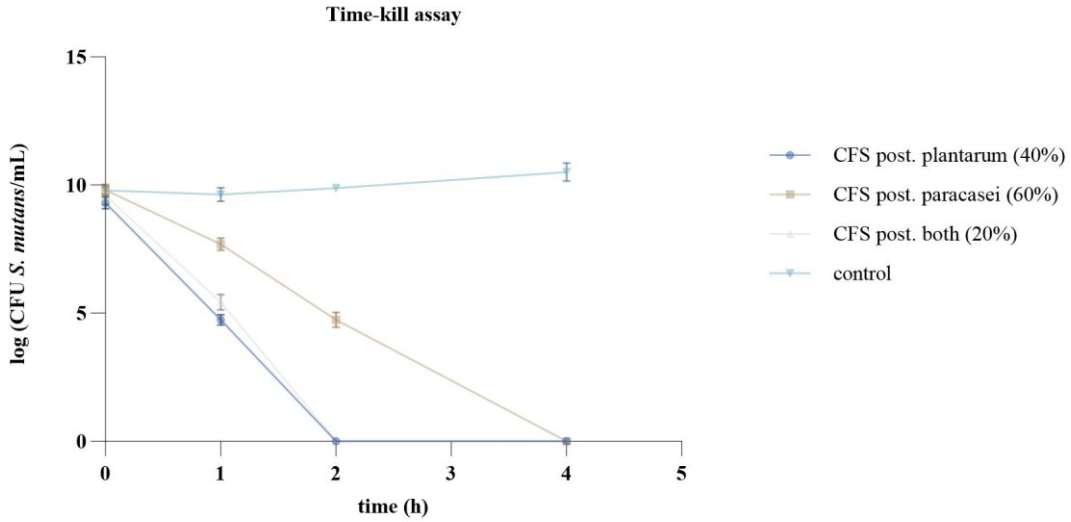

Fig. 15– Values of the logarithm of the CFU of *S. mutans* in coculture with different CFS postbiotics over 4 hours. The data are presented with mean ± SD.

3. *Antimicrobial capacity of the postbiotics against Streptococcus mutans*

Regarding the antimicrobial measurements performed with the postbiotics against *S. mutans*, it was critical to understand how the co-culture of the different postbiotics in distinct concentrations affected the growth rate of the microorganism.

Firstly, a co-culture of different concentrations of *S. mutans* with the three postbiotics (at 50% concentration) was plated, and growth inhibition was observed (data not shown). Based on those results, the growth rate of *S. mutans* at a concentration of 10^9 CFU/mL was evaluated for each postbiotic during 24 hours.

a) *Growth rate measurement of Streptococcus mutans*

In a co-culture with CFS postbiotic obtained from *L. plantarum,* the growth rate of *S. mutans* showed a notable variation in so far as the concentration of postbiotic increased. (Fig. 16) Interestingly, a difference could be observed with only 10% concentration of CFS postbiotic. When a concentration of 40% was reached, the growth was minimal, and no pronounced difference was noted between the higher concentrations (40, 60 and 100%). These results are in accordance with the results obtained in the minimal inhibitory volume (Fig. 14).

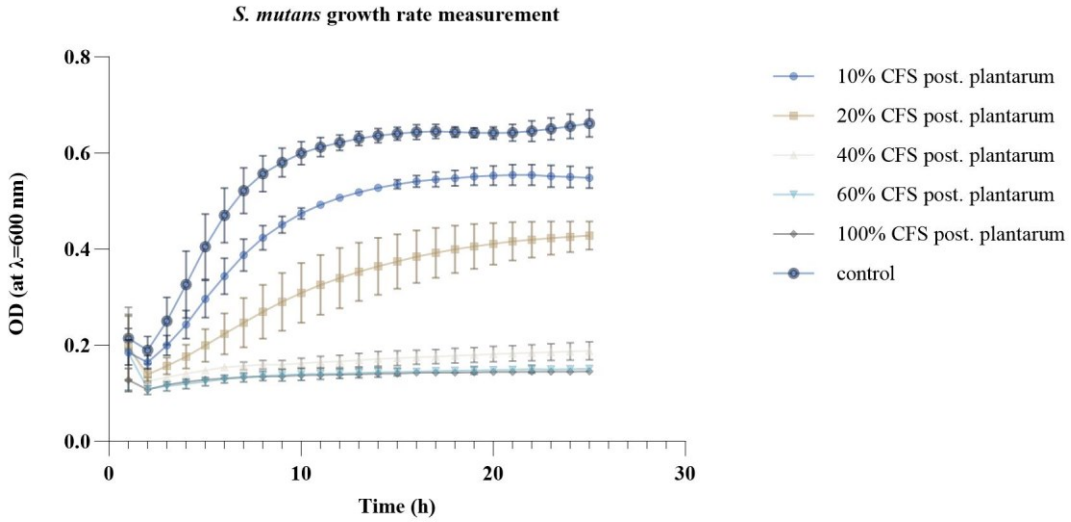

Fig. 16– OD variation of *S. mutans* measured at λ=600nm over 24 hours, representing the growth rate of *S. mutans* in coculture with CFS postbiotic obtained from *L. plantarum*. The data are presented with mean ± SD.

Regarding the results obtained in the co-culture of *S. mutans* with CFS postbiotics obtained from *L. paracasei*, a drop in the growth rate of *S. mutans* was also observed with the increase of postbiotic concentration. However, only a high concentration of postbiotics (100%) could notably reduce the growth of this microorganism (Fig. 17).

These results showed that the antimicrobial activity of the postbiotic from *L. paracasei* was not as effective as the postbiotic obtained from *L. plantarum*. This was also pointed out in the minimal inhibitory volume assay since this postbiotic demonstrated the need for a higher concentration in order to exert its influence on the growth of the bacteria (Fig. 14).

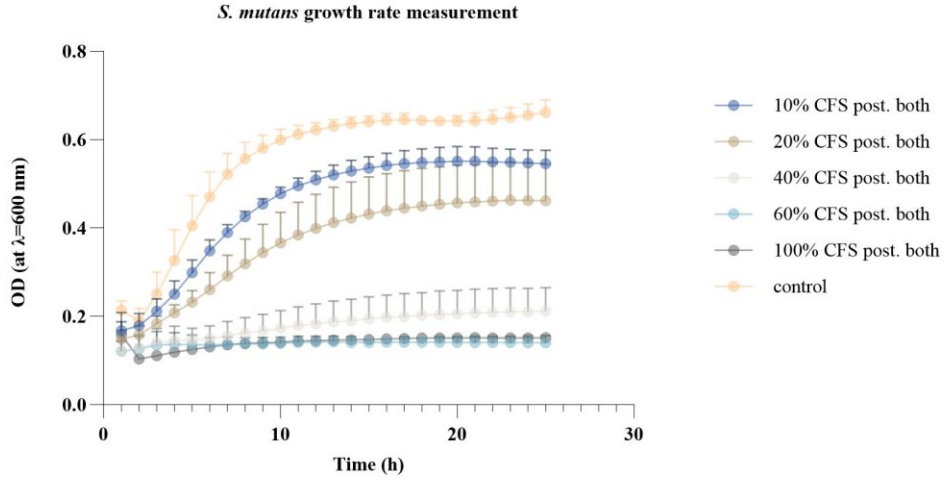

Fig. 17– OD variation of *S. mutans* measured at λ=600nm over 24 hours, representing the growth rate of *S. mutans* in coculture with CFS postbiotic obtained from *L. paracasei*. The data are presented with mean ± SD.

After analyzing the results obtained in the co-culture of *S. mutans* with the postbiotics obtained from both probiotics, it was also noted that the growth rate was highly affected by the addition of the postbiotics in the culture (Fig. 18). In fact, the use of the two probiotics to obtain the postbiotic mixture demonstrated a higher antimicrobial activity than the use of separate postbiotics.

Again, these results are confirmed by the minimal inhibitory volume assay, which demonstrates that a lower concentration of CFS postbiotics obtained from both probiotics was enough to completely inhibit the growth of *S. mutans* (Fig. 14).

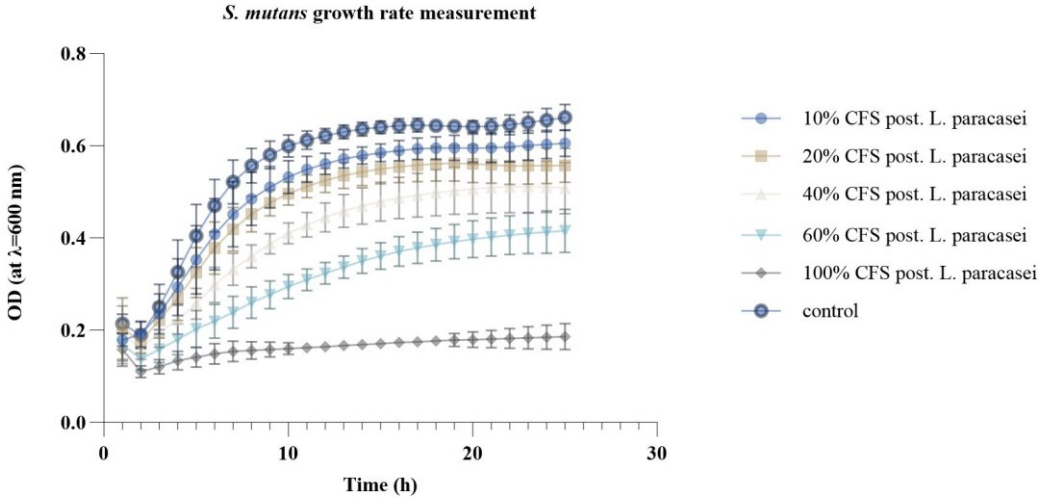

Fig. 18– OD variation of *S. mutans* measured at λ=600nm over 24 hours, representing the growth rate of *S. mutans* in coculture with CFS postbiotic obtained from both probiotics. The data are presented with mean ± SD.

4. Antibiofilm capacity of the postbiotics against Streptococcus mutans

In order to cause disease in the oral cavity, *S. mutans* shows the ability to adhere to the oral mucosa and the dental surfaces, resulting in biofilm formation. With this in mind, it is of the utmost importance to study a potential antibiofilm activity, which can ultimately result in the control of homeostasis in the oral cavity.

Two distinct measures must be considered: the inhibition of the biofilm formation and the disruption of a mature biofilm. The first is critical to allow the postbiotics to be used as a preventive approach, while the second is vital concerning a co-adjuvant therapy. Besides that, the symptoms of the disease take longer to appear than the dysbiosis itself, and the postbiotics could be applied in an oral cavity already presenting a degree of dysbiosis without showing any signs of it. In addition, the microorganisms are more resistant to therapeutic approaches when the biofilm is already formed. For these reasons, it is crucial to understand the mature biofilm disintegration/inhibition capacity of the different postbiotic solutions tested throughout this work.

a) *Biofilm formation inhibition*

Regarding biofilm formation inhibition, it was clear that the antibiofilm activity was directly related to the concentration of the postbiotic used. Data is shown below in Fig. 19. The CFS postbiotic obtained from *L. plantarum* exhibited the highest inhibition percentage when the postbiotic was administered at 100% concentration (79.6%). The second most active postbiotic in inhibiting biofilm formation was the CFS postbiotic obtained from both probiotics, reaching 79.4% inhibition at 100% concentration. The least active postbiotic regarding biofilm formation was the CFS postbiotic obtained from *L. paracasei*. Curiously, it reached the highest inhibition percentage (72.2%) at 60% instead of the maximum concentration. Accordingly, the postbiotic that exerted less antimicrobial activity demonstrated less antibiotic capacity, which is understandable.

Notably, the postbiotic obtained from *L. plantarum* showed a high inhibition percentage (75.5%) at its minimal inhibitory concentration (40%). The same was not observed in the CFS postbiotic obtained from both probiotics since its minimum inhibitory volume (20%) corresponded to 34.3% inhibition of biofilm formation. Curiously, none of the postbiotics tested reached statistical significance regarding biofilm inhibition percentage despite showing considerably high inhibition rates.

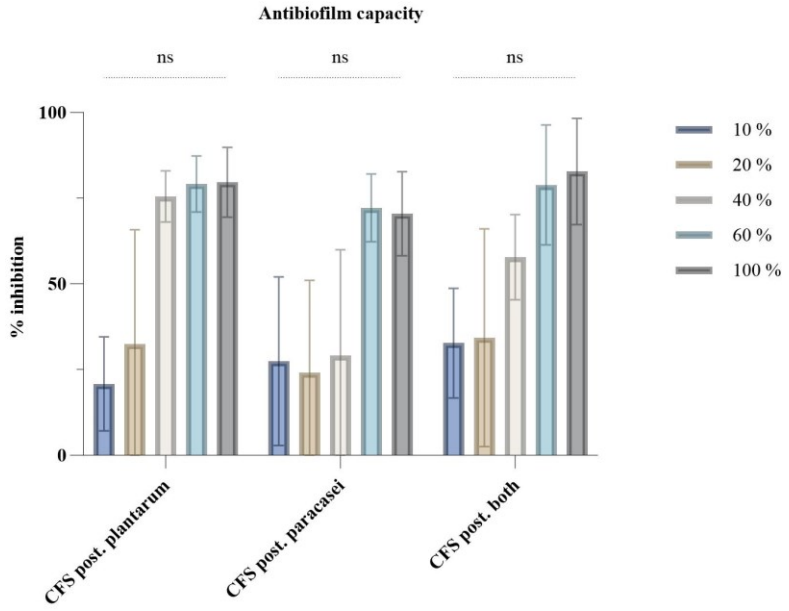

Fig. 19– Inhibition percentage of *S. mutans* biofilm formation with different CFS postbiotics in distinct concentrations. The data are presented with mean ± SD. ns indicates a lack of statistical differences between the samples.

b) *Mature biofilm inhibition*

The mature biofilm inhibitory properties of the postbiotic tested were expected to be lower than the biofilm formation since bacteria in mature biofilm present higher resistance to treatment, and it is considerably challenging to remove biofilm after it reaches its mature state. Accordingly, when analyzing the results shown in Fig. 20 below, it can be noted that no tested postbiotic reached 50% inhibition.

Curiously, the mature biofilm inhibition activity was unrelated to the postbiotic concentration. In fact, the highest inhibition percentage (44.7%) was achieved with CFS postbiotic obtained from *L. plantarum* at only 20% concentration (lower than the minimal inhibitory volume).

Regarding CFS postbiotics obtained from *L. paracasei* and the postbiotic solution obtained from both probiotic bacteria, anti-biofilm activity was only noted against mature biofilm at 100% concentration, with values of 6.1% and 28.8%, respectively. Again, no statistical significance was reached in the mature biofilm inhibition assay. However, CFS postbiotics obtained from *L. plantarum* demonstrated potential activity and should be further analyzed. Anyway, the lack of relation between antibiofilm activity and concentration must be better understood since these results were not expected.

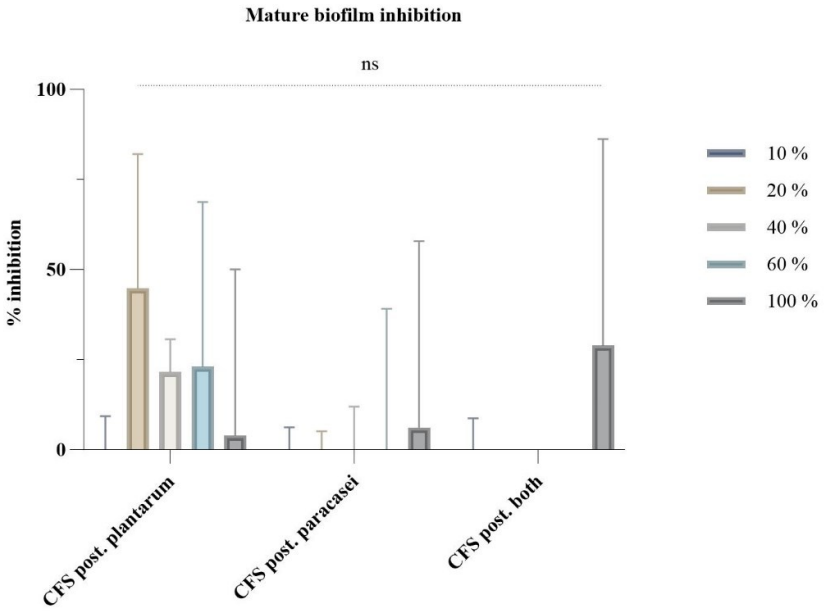

Fig. 20– Inhibition percentage of *S. mutans* mature biofilm, with different CFS postbiotics in distinct concentrations. The data are presented with mean ± SD. ns indicates a lack of statistical differences between the samples.

5. Cytotoxicity of the postbiotic-based orodispersible film on human mouth cells

ODFs are a clear possibility to achieve homeostasis control in the oral cavity, with the aid of postbiotics impregnated in the film-forming solution that exert antimicrobial and potential antibiofilm activity when administered in the mouth. For this reason, a cytotoxicity assay was conducted to ensure cell viability when in contact with the different ODFs tested. Data is shown below in Fig. 21.

Two controls were defined in this assay: a cell control without the addition of ODFs with or without postbiotics and a control of the ODFs without impregnating CFS postbiotics. The loss of cell viability between the controls was not statistically significant (from 100% to 77.9%).

Regarding the three postbiotics tested, there was a decrease in cell viability in the three samples, with the highest loss in cell viability being attributed to the CFS postbiotic obtained from *L. plantarum* (56.2%) significantly lower than the control cells ($p<0.01$). The CFS postbiotic obtained from *L. paracasei* showed a cell viability percentage of 62.2%, again lower than the control cells ($p<0.01$). The CFS postbiotics obtained from both probiotics presented the least cytotoxicity (72.6%), lower than the control cells ($p<0.05$). It is important to note that compared to the control of the ODFs, no statistical significance was reached in the differences in cell viability, demonstrating the lack of cytotoxicity of the ODFs.

Remarkably, no cell viability was lower than 50%, and for that reason, the ODFs can be considered safe for use and nontoxic to mouth cells. With this in mind, the ODFs impregnated with postbiotics should be regarded as an option for a preventive treatment to control the dysbiosis in the oral cavity, especially when the major pathogenic causing the dysbiosis is *S. mutans*.

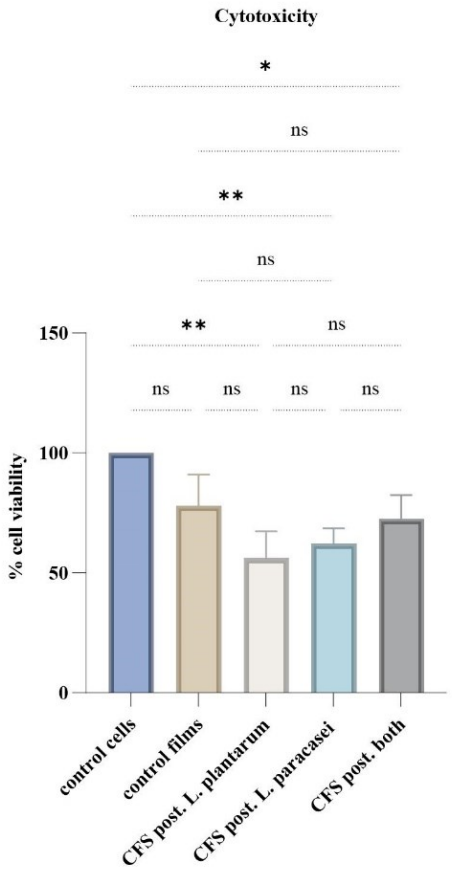

Fig. 21– Percentage of cell viability of TR146 cell line in contact with ODFs impregnated with the different CFS postbiotics, representing the cytotoxicity. The data are presented with mean ± SD. ns indicates a lack of statistical differences between the postbiotic solutions. * indicates significant differences between samples ($p<0.05$), and ** ($p<0.01$).

Conclusions

Oral diseases are deeply related to an unbalanced oral cavity microbiome called oral dysbiosis. Since this lack of homeostasis is the basis for most oral diseases, preventive strategies need to focus on re-establishing the commensal microbiota rather than eliminating it or, in other words, restoring homeostasis. For this, novel strategies, such as probiotics, prebiotics, and postbiotics, have been gaining attention. Probiotics and prebiotics are well-established and accepted in society. While the first is a group of bacteria that provide specific benefits to the human body when taken in considerable amounts, the second is a group of ingredients that enhances the growth and activity of probiotics. On the other hand, postbiotics are considered the metabolites of probiotics, as well as fragments of bacteria itself, that provide benefits to the host.

Both probiotics and postbiotics have been widely studied, and their efficacy has been proven in many studies, either by decreasing the numbers of oral pathobionts, controlling their growth, or simply by modulating the inflammatory and immune response of the host. In such cases, their efficacy has been deeply associated with how they are employed, namely the administration vehicle used.

Orodispersible films are a promising strategy due to their characteristics and lack of toxicity. ODF's composition is simple, mostly consisting of polymers and plasticizers. They dissolve easily without the need for water. Besides, they can adhere to the mucosal membranes, allowing the probiotics or postbiotics to act as desired. Moreover, they can be easily administered to patients without any risk of choking, are inexpensive to manufacture, and have scale-up potential.

In this work, it was possible to produce an ODF with added CFS postbiotics obtained from *L. paracasei* and *L. plantarum*. Regarding the physical appearance of the strips, it was noted that the postbiotics changed the texture and the color perceived. However, it had no impact on the dissolution time.

The pH value was significantly lower ($p<0.05$) when ODFs were impregnated with CFS from *L. plantarum* but not with the other CFS. This is probably due to the production of acidic metabolites during bacterial growth.

The contact angle value did not vary significantly when the ODFs were impregnated with CFS from *L. paracasei*. Still, it reduced with the other postbiotic samples, meaning it becomes a less hydrophobic surface. This can also be confirmed by the significant increase in solubility after the impregnation of ODFs with the postbiotic solutions. It could also be noted that the

impregnation of CFS postbiotics drastically increased the weight of the ODFs. However, despite these differences, the ODFs did not lose the ability to dissolve, which is the key to exerting their functions.

Regarding the antimicrobial ability of the different postbiotics tested, it was noted that all presented anti-microbial activity against *S. mutans*, which could be observed in the growth measurements and was dependent on the postbiotic concentrations. Postbiotics obtained from both probiotics demonstrated the lower minimal inhibitory volume (20%), followed by CFS postbiotics obtained from *L. plantarum* (40%), and the highest minimal inhibitory volume was noted in postbiotics obtained from *L. paracasei* (60%). This can be explained by the differences in the pH values: the lower the pH, the higher the antimicrobial activity. Likewise, the time-kill assay was similar between the postbiotic mixture obtained from both probiotics and those obtained from *L. plantarum* (2 hours) but longer for postbiotics obtained from *L. paracasei* (4 hours). These results may suggest that the CFS from these bacteria act synergistically when applied together.

Notably, the postbiotics presented antibiofilm ability, mainly if the postbiotics were applied during its formation. Again, this activity is highly dependent on its concentration. Regarding mature biofilm disruption, it was noted that the postbiotics obtained from *L. plantarum* demonstrated higher activity. However, it did not reach 50% inhibition in either concentration. These results were also expected since it is well-known that mature biofilms are more resistant than forming biofilms or bacteria in their planktonic state.

Last but not least, it is important to note that ODFs did not present cytotoxicity for human mouth cells (TR146 cell line).

Therefore, the impregnation of ODF with postbiotics should be considered as a potential alternative to target oral dysbiosis, aiming at preventing oral diseases, such as dental caries and tooth decay, through its antibiofilm capacity, reducing not only the virulence of common oral pathogens, like *S. mutans* but also the risk of acquiring antibiotic resistance genes.

However, more detailed studies of ODF physical characteristics with and without postbiotics should be performed to fully understand the mechanisms by which it delivers the active compounds in the oral cavity and the need for special storage conditions and care when handling, as well as understanding the shelf life of postbiotics after impregnation in the ODFs, to accurately determine their viability.

Future Work

Despite the recent advances, some barriers remain that need to be overcome to use ODFs impregnated with either postbiotics or probiotics as preventive approaches for oral dysbiosis. Firstly, intensive investigation is necessary to evaluate and clarify the mechanisms of action of both probiotics and postbiotics. In fact, a more detailed understanding of their mechanisms of action could drive the best option to be used as preventive strategies to avoid oral diseases. Furthermore, regarding the viability of probiotics in oral films, more studies are required to understand the survival rate since some of the probiotics used are not usual commensals of the oral cavity. Besides that, different strains present different characteristics, and it is essential to fully establish their role in other complex oral diseases, such as periodontitis. It is also important to understand the best combination of probiotics to be applied as a preventive strategy, how they interact with each other, and with the oral commensals.

Secondly, regarding postbiotic use, studies to establish and define the accurate composition of postbiotics that are crucial to understanding which components confer antimicrobial activity to postbiotics and to determine the ideal conditions and parameters for large-scale production, as well as the identification of specific molecules with antibiotic properties and other important health-related characteristics, need to be conducted. The ability to produce its effects locally should also be studied since deglutition possibly removes a significant amount from the mouth. Likewise, the impact of postbiotic concentration must be better understood to fully comprehend its relation with the desired obtained effect.

Besides, it is equally important to deepen the knowledge of ODF formulation to ensure the process is optimized. For this, studies must be conducted on the composition and concentration of different components and how to better store and transport ODFs.

In addition, it is important to remember that *in vitro* characteristics significantly differ from *in vivo* behavior. This is because the effect of the environment is not achieved in a Petri dish. The salivary flow and deglutition, mastication and food debris, and the 3D structure of the biofilm are important factors that change the way probiotics/postbiotics perform in the oral cavity. Therefore, it is vital to carry out clinical tests in the future to determine the actual benefits of the use of ODF.

Last but not least, it is essential to define specific postbiotic acquisition and composition guidelines to safely apply this therapeutic strategy for human oral health.

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