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Master's Thesis

Three *Lactobacillus* probiotic strains contain the oral pathobionts *Aggregatibacter actinomycetemcomitans* and *Streptococcus mitis*: an *in vitro* analysis.

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1. General Information

The following experimental thesis has been written by Alessia Grossetti, student of the Master's Degree Course in Medical Biotechnology. The internship has been carried out at the Center for Research on Autoimmune and Allergic Diseases (CAAD) at Università del Piemonte Orientale (UPO) in Novara. Supervisor of this thesis was Barbara Azzimonti, associate Professor of Medical Microbiology, assistant Professor in Microbiology and Clinical Microbiology (MED/07), and Head of the Applied Microbiology Laboratory, Department of Health Sciences (DISS), UPO in Novara. Co-Supervisor was Dr. Paola Zanetta, PhD in "Food, Health, and Longevity Studies" in the same Laboratory.

2. Summary

Aggregatibacter actinomycetemcomitans and *Streptococcus mitis* are human oral microbiota commensals that normally contribute to the host's health state maintenance. In dysbiotic promoting conditions they can overgrowth, express their virulence factors, induce tissue damage, and enter the bloodstream. These events can cause not only local diseases like periodontitis and oral lichen planus, but also systemic ones like endocarditis, as well as some cancer types. The antibiotics commonly used to counteract these infection-mediated conditions are leading to a global emergence of multidrug-resistant strains, thus justifying the urgent request of new alternative prevention and therapeutic approaches.

On this premise, the aim of this research thesis was to evaluate the potential of the probiotic *Levilactobacillus brevis* LBR01, *Lacticaseibacillus rhamnosus* LR04, and *Limosilactobacillus fermentum* LF26 strains (Probiotical Research S.p.A., Novara, Italy), and of their cell-free supernatants (CFSs), in the containment of the pathobionts *A. actinomycetemcomitans* and *S. mitis*. The Applied Microbiology Laboratory already demonstrated that these probiotics and their cell-free supernatants (CFSs) show different degree of efficacy in containing the above pathogens in the standard animal-derivative based De Man, Rogosa, and Sharpe (MRS) medium. On this basis, we explored if it changed in the novel animal-derivative free medium Terreno Industriale Lattobacilli (TIL). Probiotic CFSs were produced and their lactic acid, short-chain fatty acids (SCFAs), and protein content was characterized via mass spectrometry. To evaluate the ability of alive *Lactobacillus* strains, grown in TIL, and of their CFSs in the oral pathobionts containment, agar spot, viability, biofilm formation, and co-aggregation assays were performed. While the probiotic CFSs were analyzed with all the experimental approaches except for the agar spot assay, the viable strains were tested only in the agar spot and co-aggregation assay.

Results showed that TIL medium influenced the protein types and SCFAs amount in the CFSs, as well as the lactic acid production. Live probiotics significantly reduced the pathogens growth, both alone and in co-culture, and co-aggregated with the two oral pathobionts indicating their ability to interact with them. All the CFSs tested reduced pathobionts growth, metabolism, and biofilm formation, but did not significantly changed their co-aggregation. Similar results were obtained also in the more complex pathogens co-culture.

In conclusion, postbiotics promise to be an alternative strategy to antibiotics. Moreover, probiotics showed a protective activity even when cultured in the novel TIL medium. However, further studies, both *in vitro* and *in vivo*, are needed to deeply investigate their beneficial properties.

3. Abbreviation List

APCs = antigen presenting cells; *14*

BCA = bicinchoninic acid; *23*; *34*

BSA = bovine serum albumine; *23*

C- = negative control; *34*

CAB = co-aggregation buffer; *22*; *40*

CDT = cyto-lethal distending toxin; *14*; *17*

CFSs = cell-free supernatants; *5*; *17*; *18*; *20*; *21*; *22*; *23*; *25*; *29*; *30*; *32*; *33*; *34*; *35*; *39*; *40*; *41*

CV = crystal violet; *22*; *30*; *32*; *33*

CV_{570} = Crystal violet absorbance at 570 nm; *30*

DCs = dendritic cells; *15*

D-fru = D-fructose; *22*

D-gal = D-galactose; *22*

D-glu = D-glucose; *22*

EPM = extracellular polymeric matrix; *9*

EPSs = extracellular polymeric substances; *9*; *11*

HACEK = *Haemophilus* spp., *Aggregatibacter actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, *Kigella kingae*; *14*

HMP = Human Microbiome Project; *9*

HOMD = Human Oral Microbiome Database; *9*

Ig = Immunoglobulin; *15*

IgA = Immunoglobulin A; *8*; *15*

IL-1 = interleukin 1; *15*

IL-6 = interleukin 6; *15*

iTILF = incubated TIL with fructose; *20*; *21*; *22*; *29*

iTILG = incubated TIL with glucose; *20*; *21*; *22*; *29*

LAB = Lactic acid bacteria; *16*; *17*; *24*

LDH = Lactate dehydrogenase; *24*

LPS = lipopolysaccharide; *14*

LtxA = Leukotoxin A; *14*; *39*

MRS = De Man, Rogosa, and Sharpe; *5*; *18*; *26*; *35*; *36*; *37*; *38*; *39*; *40*; *41*

NAD = Nicotinamide; *24*

NIH = Nation Institute of Health; *9*

nw_{halo} = normalized width halo; *20*; *27*

OD_{600} = optical density at 600 nm; *19*; *20*; *21*; *22*; *30*

OLK = Oral leukoplakia; *8*

OLP = Oral lichen planus; *8*; *13*; *16*

ON = overnight; *19*; *20*; *22*; *23*

OSCC = Oral squamous cell carcinoma; *8*; *13*

PBP1A = Penicillin binding protein 1A; *36*; *41*

PES = polyethersulfone; *20*

QS = quorum sensing; *10*

rpm = revolutions per minute; *19*; *20*; *22*

RT = room temperature; *20*; *22*

SCFAs = Short chain fatty acids; *3*; *5*; *18*; *25*; *36*; *37*; *41*

TIL = Terreno Industriale Lattobacilli; *5*; *19*; *20*; *26*; *29*; *30*; *32*; *34*; *35*; *36*; *37*; *38*; *40*; *41*

TNF-alpha = tumor necrosis factor alpha; *15*

TSB = tryptic soy broth; *19*; *20*; *21*; *22*; *29*; *30*

UHPLC = Ultra high-performance liquid chromatography; *23*

UTIs = Urinary tract infections; *16*

VGS = Viridans Group Streptococci; *15*

4. Introduction

4.1. Oral Cavity

The oral cavity, commonly called mouth or buccal cavity, serves as the initial portion of the digestive system, contributing to the first part of the metabolic pathway. In addition to its mechanic and enzymatic role, it is also essential for the phonation, and normal respiration[1]. All these functions are allowed by the several different anatomical sites that work together. As defined by the US National Cancer Institute, its main components are the lips and the cheeks, the palate, that compose the roof of the mouth, the tongue, its surface papillae, the teeth and gingiva, as well as the oral mucosa[2]. Between the teeth (hard calcified structures in close contact with the oral mucosa) and the gingiva there is the gingival sulcus, where bacterial biofilm is often retrieved (**Figure 1**)[3].

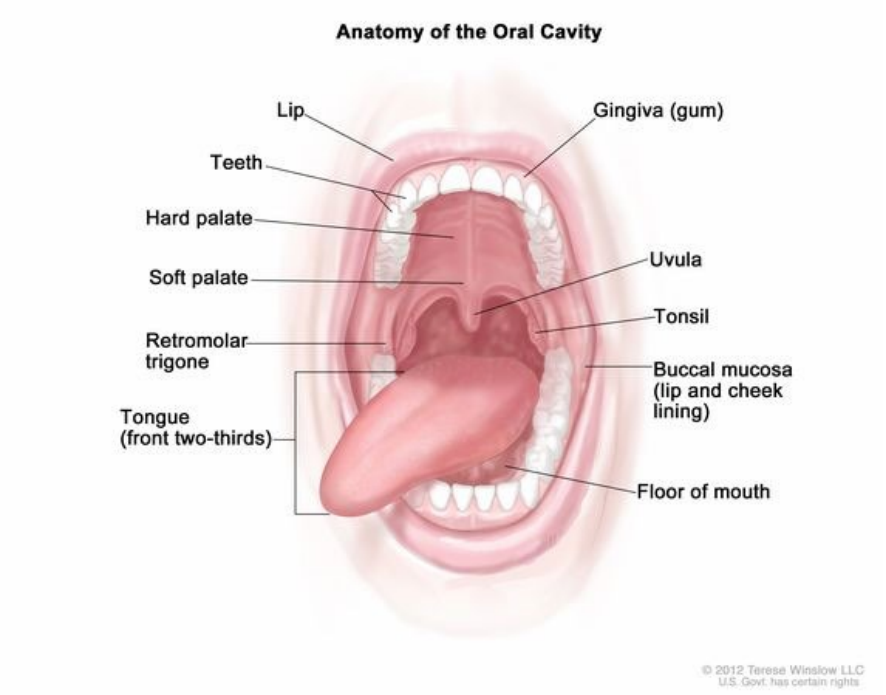

Figure 1 *Representative image of Oral Cavity anatomy*[2].

The oral epithelium, like the skin one, is the most protective and resistant and consists of a physical barrier with integrated immunological elements that prevent the invasion of primary and opportunistic pathogenic organisms. In contrast to the skin epithelium, it is highly vascularized and permeable[4,5]. It defends the host from external stimuli due to its organization in two main components: a multilayered epithelium, that composes the physical barrier, and an immune barrier that takes part in immune responses or tolerance against microbial antigens[6].

The physical barrier is divided in two layers, the surface stratified squamous epithelium and the deeper lamina propria. Depending on its localization, the degree of keratinization can vary. Four are the layers of the keratinized oral mucosa: basale, spinosum, granulosum, and corneum, while in the non-keratinized epithelium the stratum basale is followed by the filamentosum and the distendum ones. Moreover, three different phenotypes can be distinguished: the lining mucosa, the masticatory mucosa, and the specialized mucosa. The lining mucosa is localized over mobile structures such as soft palate, cheeks and lips, while the rigid masticatory mucosa protects the gingiva and the hard palate. Regarding the specialized mucosa, it is located on the dorsum of the tongue, showing a keratinized epithelium which includes lingual papillae and taste buds[5].

Because of its high permeability, there is the frequent contact of both potentially harmful and harmless antigens with local immune cells[4]. These cells distinguish harmful microbes and toxic macromolecules and prevent epithelial damage. In case of infections and chronic inflammation, the epithelial barrier can be disrupted, leading to oral mucosa diseases, such as oral lichen planus (OLP) and oral leukoplakia (OLK). One of the causes of the gingival barrier disruption is the oral microbiota dysbiosis that, together with the inflammatory response, can lead to bone loss in periodontitis[6].

Another important aspect of the oral cavity is represented by the saliva, which is produced by major and minor salivary glands. The main components of saliva are water, electrolytes, mucus, antibacterial compounds (i.e., IgA), and enzymes, that help to digest food and reduce pathogen loads. Saliva contributes to the ecosystem stability, while its absence can induce a significant increase of bacterium-related diseases, such as dental caries, gingivitis, and periodontitis[3,7]. Recent studies suggest that destructed oral epithelial barrier and defects in keratinocytes differentiation may contribute to the development of oral squamous cell carcinoma (OSCC)[6].

4.2. Oral Microbiota

Oral microbiota is defined as the ensemble of microorganisms (bacteria, viruses, fungi, and archaea) that resides in the oral cavity, while the oral microbiome is represented by the sum of genetic information deriving from these microorganisms, influenced by the environment to which they are exposed[8,9]. The microbial communities that colonize the hard surfaces and the soft tissues of the oral mucosa are one of the most heterogeneous, second only to the gastrointestinal microbiota. They are characterized by a high complexity and biodiversity, due to the distinct environmental conditions that differ depending on the oral cavity sites, whose diverse characteristics provide different habitats for microbial colonization and growth, leading to heterogeneity between communities[10].

In 2007, the National Institute of Health (NIH) launched the Human Microbiome Project (HMP), a collection of multiple projects carried out in multiple parts of the world, including USA, European Union (EU), and Asia[8,11]. Through 16S rRNA sequencing it was possible to identify many different microorganisms present in the oral cavity. Among them, the oral bacterial taxa found were categorized in the Human Oral Microbiome Database (HOMD) a descriptive repository of oral bacterial genome sequences and taxa[3,8,11]. The main genera found in the oral microbiota were *Streptococcus*, *Haemophilus*, *Leptotrichia*, *Porphyromonas*, *Prevotella*, *Propionibacterium*, *Staphylococcus*, *Veillonella*, and *Treponema*[3,10]. Primary colonizers of oral surfaces are predominantly facultative anaerobes, such as *Streptococcus* spp. and *Actinomyces* spp., while in the subgingival area, where the oxygen tension is lower, there are strict anaerobes such as *Bacteroidaceae* spp. and Spirochaetes[7]. the oral microbiota composition changes with the different life phases, for example, in children the main phyla are Bacillota[12] (previously known as Firmicutes), Proteobacteria, Acinetobacter, Bacteroidetes, Fusobacteria, and Spirochaetes. Thereafter, with the replacement of primary teeth, a significant modification of the oral microbiota habitat occurs. This leads to an increased proportion of the Prevotellaceae and Veillonellaceae families, and Spirochaetes[13]. In healthy adults the composition is similar, with a relative abundance of different genera included in the phyla Acinetobacter, Fusobacteria, Proteobacteria, Bacillota, and Bacteroidetes. Conversely, the main variations observed among adults are due to demographic, anthropometric and environmental factors[10].

The microorganisms within a community interact in a synergic and dynamic way to enhance colonization, persistence, or pathogenicity, as well as play an important role in modulating health and disease conditions[7,14,15]. To persist in the oral cavity, many oral bacteria produce polysaccharides called extracellular polymeric substances (EPSs), specific for a particular oral niche. These products promote bacterial aggregation and surface attachment, and protect bacteria from desiccation, predation, antimicrobial agents, antibodies, and bacteriophages[16]. Microbes in planktonic state can also associate together to form a complex matrix-like structure known as biofilm, in which EPSs hold microorganisms together. In mature biofilm the extracellular polymeric matrix (EPM) is composed of water, polysaccharides, proteins, lipids, and DNA, that altogether promote the microorganism colonization and stabilization[10,17]. EPM is also able to maintain biofilm tightly bound with the host tissue and facilitates the interactions among microorganisms, providing also protection against host defense and drugs[18]. In biofilms, Gram-positive and Gram-negative bacteria are able to modify their phenotype via the production of different small diffusible signal molecules, that allow communication among each other, in a mechanism known as

quorum sensing (QS). In this way, they regulate different activities, like biofilm formation and growth, adaptation to changes, acquisition of a competitive advantage against other microorganisms, and expression of virulence factors[10,19].

In normal conditions, the resident microbiota contributes to normal tissue and immune system development, and competes with exogenous primary and opportunistic pathogens, helping the maintenance of balance among microorganisms with different degree of pathogenicity, in a state called eubiosis[7]. The alteration in the composition and function of the resident oral microbiota can result in a reduction of the symbiotic interaction with the host, leading to different consequences for the oral and general health[10]. The modification of this highly regulated equilibrium is called dysbiosis, and results in the growth advantage of pathogens which show their disease-promoting potential and determine pathological conditions[20]. With the alteration of the microbial environment, some indigenous species expand and provide ideal conditions for the growth of opportunistic microbes, leading to an altered microbial diversity[21]. However, changes in microbial composition of the oral microbiota between eubiosis and dysbiosis are still controversial[10]. In **Figure 2** some of the bacteria that normally compose the oral microbiota. When their balance is altered (dysbiosis), local and systemic pathological conditions can occur.

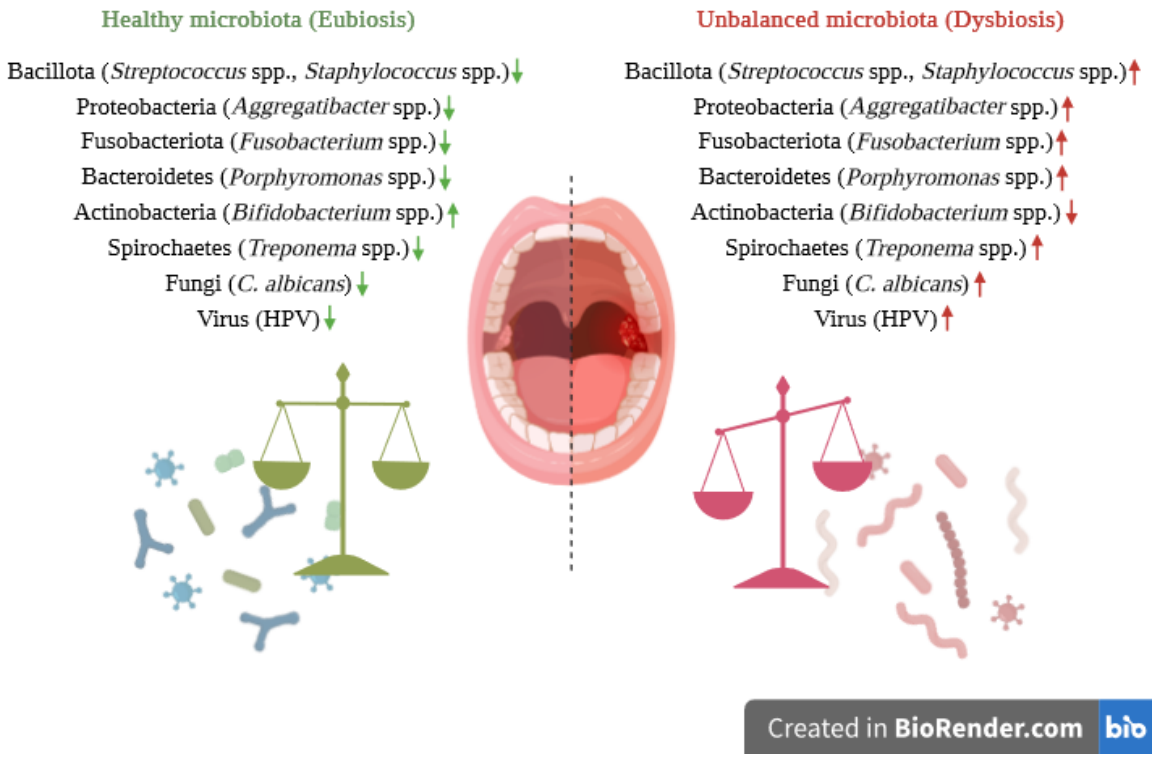

Figure 2 *Schematic representation of the oral microbiota in eubiotic and dysbiotic conditions*. Image reports some of the main bacteria, fungi, and viruses that respectively compose the oral eubiotic and dysbiotic microbiota. The two conditions depend on the microbial load balance among species (created with BioRender.com).

There are many factors, both external and internal (the so called exposome), that can alter the microbiota community balance (**Figure 3**)[7,22]. Among the external ones, worth of mention are drug use, excessive alcohol consumption, cigarette smoke, unbalanced diet, and antibiotic misuse. For the internal factors, the main ones are hormonal changes (e.g. puberty, pregnancy, and menopause), and the host's disease status[7,9,23]. As in every other pathology, a single factor is not sufficient for its development, but a combination of them is required. For example, the overexposure to dietary carbohydrates, together with host's factors, promotes the production of EPSs and acidic metabolites, causing the accumulation of acidogenic and aciduric microorganisms which drive the transition to a pathogenic biofilm community[7,15].

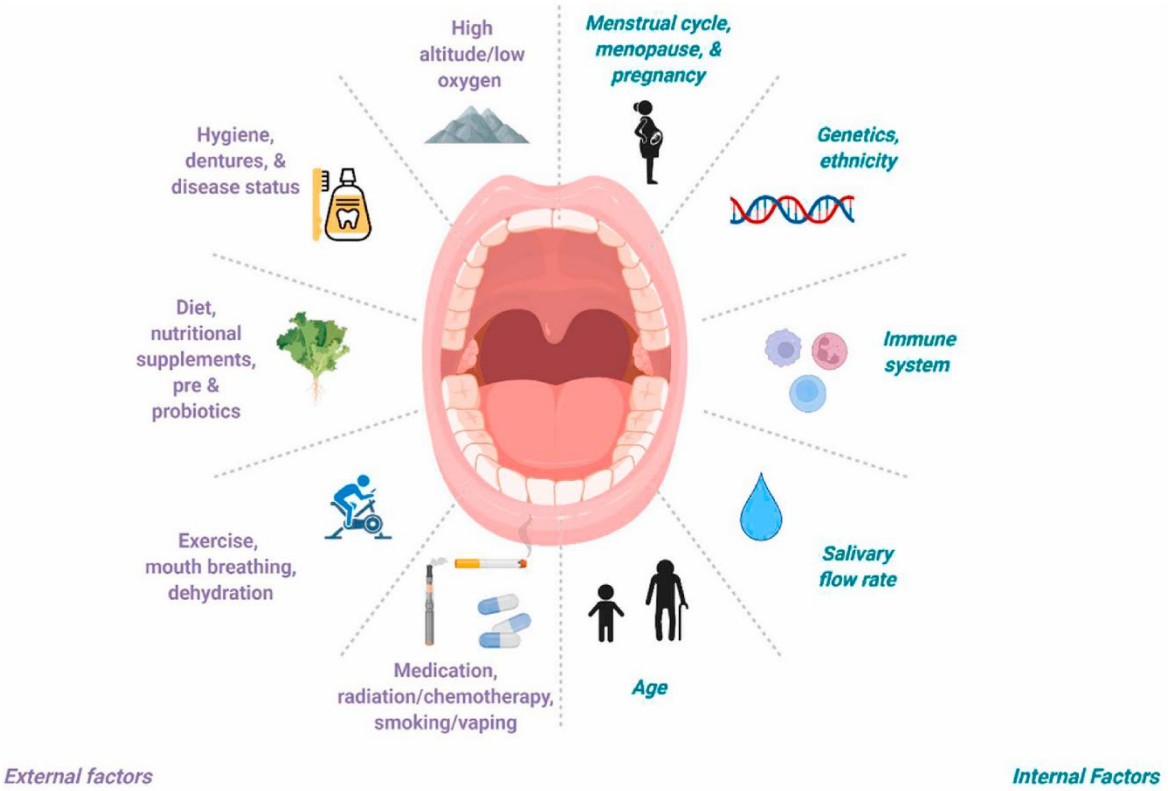

Figure 3 *Schematic representation of oral exposome.* Examples of internal and external factors that contribute to oral microbiota dysbiosis[26].

The microbiota associated with diseases is composed by microorganisms that possess specific specialized metabolic functions and an elevated virulence potential, usually absent in healthy status[7]. The alteration of the dynamic balance between commensals and opportunistic pathogens can, at the end, lead to different oral pathologies, as well as systemic ones, when not properly managed[15].

4.3 Oral Microbiota Dysbiosis-associated Diseases

Human oral microbiota dysbiosis is always associated with several pathological conditions, such as dental caries and periodontitis. Dental caries is a polymicrobial biofilm disease that destroys mineralized tooth tissue through interactions between the microbiota and nutrition. The presence of oral pathogens is required, but not sufficient, for its formation, as pathogenic biofilm is dependent on frequent sugar consumption. There are also other factors that contribute to the formation of dental caries, like poor or wrong oral hygiene, salivary flow and composition, and enamel defects. The main bacteria associated with this condition are mutans streptococci (especially *Streptococcus mutans*) and *Lactobacillus* spp. (*Lactobacillus acidophilus*). However, more recent analyses have revealed the existence of a pathogenic community that includes non-streptococcal bacteria (like *Bifidobacterium* spp.,

Scardovia spp., *Actinomyces* spp., *Porphyromonas gingivalis*, and *Filifactor alocis*) and fungi (*Candida albicans*)[7,23].

Periodontitis is a biofilm-associated inflammatory disease caused by alterations in the local ecology[3,24]. It usually starts with gingival bleeding in response to inflammation due to plaque accumulation around the tooth marginal gingival surfaces, and develops over years, leading to the loss of connective tissue and bone around the teeth[3,25]. The presence of dental plaque is a key factor in the initiation of this common chronic inflammatory disease, with lifestyle and hygiene as promoting agents[23,25]. The main microbial biomarkers of periodontitis are *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Aggregatibacter actinomycetemcomitans*, *Filifactor alocis*, and *Fusobacterium nucleatum*, together with several Bacteroides, *Campylobacter* and *Prevotella* spp.[3,25,27]. Other researchers also confirmed the important role played by Herpes Simplex, Human Papilloma and Epstein-Barr viruses not only in the development of periodontitis, but also in oral cancer (OSCC)[28–30].

Apart from dental caries and periodontitis, oral lichen planus (OLP), leukoplakia, and erythroplakia are the most frequent manifestations associated with oral dysbiosis[3]. OLP is a T-cell mediated chronic inflammatory mucosal disease of multifactorial origin. It is potentially malignant, and up to 12% of the cases progress to OSCC. High levels of *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Solobacterium*, and *Prevotella melaninogenica* have been observed in OLP patients, with a significantly lower abundance of *Haemophilus, Corynebacterium, Streptococcus*, and *Campylobacter* spp. when compared to healthy controls[31,32]. Leukoplakia is a whitish lesion on the oral mucosa not related to any other specific disease and mainly asymptomatic. Around 15-40% of the cases progresses to OSCC. On the other hand, erythroplakia lesions are similar, even if red, with a higher OSCC development rate[3].

Lots of research have demonstrated that oral microbes also play an important role in tumor cell proliferation, invasion, and metastasis, as they are able to induce DNA damage and mutations, together with epigenetic modifications of genes involved in phagocytosis and cellular proliferation[7,32–34]. Oral dysbiosis also relates to the development of different cancer types throughout the human body, like those esophageal, pancreatic and colorectal[3,33]. In all these tumor types, periodontitis is a common risk factor. In particular, it has been found a correlation between high detection rates of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* and pancreatic cancer development, while colorectal cancer is mainly related to an abnormal presence of *Fusobacterium nucleatum*[33,34].

Oral dysbiosis is also connected to non-cancer associated systemic pathologies, such as mainly cardiovascular diseases. In fact, the gingival epithelium of periodontitis affected patients' periodontal pocket is more prone to disruption, therefore leading to bacteriemia[33]. Other pathologies to which oral dysbiosis is associated are Alzheimer's disease, diabetes, rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease, liver cirrhosis, polycystic ovary syndrome, and preterm birth[22,33,35].

4.4 *Aggregatibacter actinomycetemcomitans* and *Streptococcus mitis*

Aggregatibacter actinomycetemcomitans is a Gram-negative, facultative anaerobic coccobacillus. It is an oral pathobiont commonly associated with chronic local and systemic inflammatory disorders, like periodontitis, tooth loss, and atherosclerosis[25,36–38]. It is part of the HACEK (*Haemophilus* spp.*, Aggregatibacter actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens,* and *Kigella kingae*) group, mostly composed of Gram-negative bacteria strongly associated with infective endocarditis[25,38,39]. It colonizes and persists in the oral cavity thanks to its virulence factors, like the capsule, fimbriae that confer motility, biofilm polysaccharides, and a certain number of adhesins, that varies depending on the bacterial strain. Moreover, it possesses different genes that encode for proteins involved in microbial interplay, persistence, and pathogenicity[25,36,37]. The toxins produced by *A. actinomycetemcomitans* are the cyto-lethal distending toxin (CDT), leukotoxin (LtxA), and lipopolysaccharide (LPS), that can induce the release of pro-inflammatory cytokines also causing DNA damage[3,36,37]. More precisely, CDT induces double strand DNA breaks, causing damage and genomic instability, leading to host immune response imbalance affecting antigen presenting cells (APCs), and inhibits lymphocyte proliferation[34]. LtxA it is responsible for its β-hemolytic activity on Columbia blood agar plates. However, there are some *A. actinomycetemcomitans* strains that neither produce LtxA nor possess hemolytic properties[25,40]. The main effects of *A. actinomycetemcomitans* toxins are the induction of apoptosis in fibroblasts and immunomodulation[39].

Among the others, it also shows auto-aggregation capacity, that refers to the spontaneous and rapid formation of aggregates in static suspensions. The fimbriae play an important role in this phenomenon, and also in the adhesion to a wide range of solid surfaces, leading to biofilm formation[25].

Even though *A. actinomycetemcomitans* is usually found in the oral cavity, it can be isolated from other body sites thanks to its ability to enter the blood and cause bacteriemia when the oral epithelia are damaged. Because of this, it is also related to the onset and progression of non-oral systemic diseases, like arthritis, endocarditis, osteomyelitis, skin infections, urinary tract infections, and various types of abscesses[25,36]. In fact, once the oral

epithelial barrier is disrupted, *A. actinomycetemcomitans* can pass through it and induce an immune response in the neighboring tissues by activating macrophages and dendritic cells (DCs). When these cells are activated, a set of cytokines and chemokines promotes the activation and the recruitment of B- and T-cells. Moreover, *A. actinomycetemcomitans,* through the release of pro-inflammatory cytokines with potent pro-resorptive action, like tumor necrosis factor alpha (TNF-alpha), IL-1, and IL-6, promotes osteoclast formation and bone resorption[36]. Like other bacteria *A. Aggregatibacter* is resistant to several antibiotics like doxycycline, ampicillin, tetracycline, penicillin, and metronidazole, hence contributing to the reduction of available therapy options [41,42].

Another oral microbiota commensal is *Streptococcus mitis*, a Gram-facultative anaerobic bacterium localized in the human oropharynx, belonging to the viridans group of alpha hemolytic streptococci (VGS). It also colonizes the upper respiratory tract, intestine, skin, and female reproductive niches[43–45]. Although *S. mitis* usually presents a non-virulent behavior, it is able to migrate from the oral cavity, cause bacteremia and septicemia, thus, a series of infectious complications, including infective endocarditis and toxic shock syndrome[38,43,46,47]. Like many other bacteria it produces different virulence factors, that contribute to its effective colonization of the human host, as well as to induce pathogenic states. Some of its virulence factors are adhesins, pili, proteases, toxins, and molecules that modulate the immune system response. Among all, the main ones are immunoglobulin (Ig) A1 protease and bacteriophage lysin which, however, have not been shown to play a direct role in the pathogenesis of *S. mitis* infections[43,45,48]. Some other possible candidates are metalloproteases-like proteins that show a similar enzymatic activity to the ones of *S. pneumoniae* and that have been hypothesized to have a role in dental caries pathogenesis[48]. The IgA1 protease allows it to counteract the host immune defense by blocking IgA activity, thus permitting *S. mitis* to effectively colonize the oral cavity. Moreover, when in presence of *Fusobacterium nucleatum* or *A. actinomycetemcomitans*, *S. mitis* reduces the production of IL-8 in response to pathogens[43]. As observed in different countries, the main problem related to *S. mitis* infections is the resistance to most used antibiotics[49,50]mainly represented by penicillin, erythromycin, and tetracycline[49,51,52]. It is a significant pathogen in elderly and immunocompromised patients, and in those ones undergoing cytotoxic chemotherapy for cancer. Although it is an infrequent opportunistic pathogen in normal healthy infants and adults, it is implicated in a wide range of diseases due to bacteriemia, from dental caries to bacterial infective endocarditis, meningitis, eye infections, and pneumonia[43,44,47].

A higher prevalence of the periodontopathogens *P. gingivalis*, *S. mitis*, and *A. actinomycetemcomitans* in the oral cavity has been found to increase the risk of pancreatic

cancer. In addition, levels of *S. mitis* and *Streptococcus anginosus* have also been detected to be increased in esophageal cancer patients[3].

4.5 Probiotics in Human Health

Probiotics are non-pathogenic living microorganisms naturally contained in certain foods, like yogurt or kimchi, and in dietary supplements, that, when taken in controlled doses, can confer several benefits[10,23,53,54]. Lactic acid bacteria (LAB) and Bifidobacteria are the two most frequently orally administered probiotics to prevent or treat different disorders. Among LAB, *Lactobacillus* is the largest genus that is currently studied and used, comprising *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. reuteri*, *L. salivarius*, *L. fermentum*, and *L. brevis*[32,53,55].

They are used as both preventive and therapeutic tools in different diseases, such as oral infectious diseases, Crohn's disease, prevention of acute diarrhea, cardiovascular and urogenital infections, cancer, lactose intolerance, atopic dermatitis, and cystic fibrosis[23,53,55]. The main benefits that can be observed with their administration are microbial balance modulation, enhancement of the immune system, anti-hypercholesterolemic and anti-hypertensive action, reduction of diarrhea associated with irritable bowel syndrome[10]. They exert many benefits in the human body, like in the gastrointestinal tract, but different studies also highlight their potential in the oral cavity, where they modify the sub-gingival microbiota composition, lowering the concentration of periodontal pathogens like *P. gingivalis*, *T. denticola, P. intermedia*, *F. nucleatum*, and *A. actinomycetemcomitans*[10,53].

In the human body, probiotics interact and stimulate the growth of good commensal microbes, compete for adhesive surfaces, inhibit the growth of pathogens, and also modulate the host's antigenic response[53,54,56]. To counteract the proliferation of other bacteria they mainly produce bacteriocins, organic acids, fatty acids, and hydrogen peroxide[23,55,56]. Certainly, all these effects are not implemented only by a single strain, that is why the best approach should be a combinatorial use of different probiotics strains[57].

In some studies Lactobacilli demonstrated to be effective in protecting women against urinary tract infections (UTIs)[58]. The use of probiotics showed good results also in patients affected by cancer or HIV, as well as OLP patients[32,53]. *L. rhamnosus* and *L. casei* exert a strong anti-fungal activity against *C. albicans*[23]. Moreover, bifidobacterium is able to preserve the integrity of the oral mucosa with the production of glutamine, which also improves the mucosal barrier defenses[53]. *Bifidobacterium* spp., *L. rhamnosus, L. reuteri,* and *L. casei* showed the capability to change the colonization of cariogenic bacteria, preventing tooth decay[56]. Several studies investigated the potential of *Lactobacillus* spp.

cell-free supernatants (CFSs), defined as a liquid containing microbial metabolites and residual nutrients of the culture media used. CFSs are usually composed by a mixture of low and high molecular weight metabolites, like organic acids, fatty acids, proteinaceous compounds, hydrogen peroxide, and bacteriocins, without bacterial structural components[55,59]. For example, a study associated *L. rhamnosus* and *L. acidophilus* CFSs with reduced expression of leukotoxin and CDT by *A. actinomycetemcomitans*[60].

However, the effect of probiotics in oral cavity is still not completely understood. Some studies observed a slight reduction in gum disease, showing that probiotics can eliminate infections by outcompeting bacteria for bonding surfaces and nutrients. Although it was found that some substances produced by certain LAB can cause inflammation[53,55], no author actually indicated the negative effects in the use of probiotics formulations[53].

Since the use of living microorganisms in food products come with challenges and limitations, the use of postbiotics can also be a valid alternative. Postbiotics are defined as soluble agents released by live probiotics, or after their cell lysis. They are mainly cell wall fragments, cytoplasmic extracts, or secondary metabolites with broad bioactivities, such as those immunomodulatory, antitumoral, and antimicrobial[55,56].

5. Thesis objectives

Oral microbiota balance is essential for both local and systemic human health. Due to the antibiotic resistance global emergence, new alternative strategies, to help maintaining this fine-tuned equilibrium are needed.

This thesis is part of a larger project involving the selection of single and blended probiotics able to counteract the proliferation and virulence of oral pathogens and thus reducing antibiotic use while restoring the host's eubiosis.

In this experimental thesis project, three probiotics strains named *Levilactobacillus brevis* LBR01 (DSM 23034), *Lacticaseibacillus rhamnosus* LR04 (DSM 16605), and *Limosilactobacillus fermentum* LF26 (DSM 33402), kindly provided by Probiotical Research S.p.A. (Novara, Italy), were analyzed for their containment capability towards the oral pathobionts *Aggregatibacter actinomycetemcomitans* (DSM 11123) and *Streptococcus mitis* (DSM 12643). Since the Applied Microbiology Laboratory previously demonstrated that the same probiotic strains showed different degrees of efficacy in containing *A. actinomycetemcomitans* and *S. mitis* pathogens when cultivated in the standard animal derivative-based MRS medium, they were here cultured in a novel medium devoid of animal derivatives, named TIL, in order to explore if varying growth conditions could change probiotic efficacy.

To determine pathogens containment by the live strains, agar spot assay was performed, while their cell free supernatants (CFSs) were tested in the viability and biofilm formation assays. Both live probiotics and their CFSs were used in the co-aggregation assay. Both the single pathogens and their co-culture were used to mimic, as close as possible, an in vivo environment in which multiple pathogenic species coexist simultaneously.

Finally, CFSs were characterized by comparing their pH values, proteins type, and concentration of SCFAs produced in the two different growth conditions.

6. Materials and methods

6.1 Bacterial Cultures

The two oral pathobionts *Aggregatibacter actinomycetemcomitans* (DSM 11123, Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, Braunschweig, Germany), and *Streptococcus mitis* (DSM 12643) were aerobically cultivated overnight (ON) at 37 °C and 200 revolutions per minute (rpm) in tryptic soy broth (TSB, Sigma-Aldrich, St. Louis, MO, USA, distributed by Merk Life Science S.r.l., Milan, Italy).

The three probiotic strains *Levilactobacillus brevis* LBR01 (DSM 23034), *Lacticaseibacillus rhamnosus* LR04 (DSM 16605), and *Limosilactobacillus fermentum* LF26 (DSM 33402) were aerobically grown in static conditions ON at 37 °C, using both the traditional animal derivative containing De Man Rogosa and Sharp (MRS; Condalab, distributed by Cabru S.A.S., Biassono, Monza-Brianza, Italy) and the animal derivative-free media referred to generically as "Terreno Industriale Lattobacilli" (TIL) broth (Probiotical Research S.p.A., Novara, Italy; formula in g/L: proteose peptone N-3 10, dextrose 20, dipotassium phosphate 2, magnesium sulphate 0.1, manganese sulphate 0.05, vegetal extract – confidential, sodium acetate 5, Tween-80 1, yeast extract 5, ammonium citrate 2), that contains peptones from plant sources, supplemented with fructose for LBR01 and glucose for the other probiotics. All the bacterial cultures were freshly renewed ON before each experiment.

6.2 Probiotics Growth Curves

A growth curve for each *Lactobacillus* strain was used to evaluate the probiotics' ability to proliferate in each medium and their growth timing. The curve is divided in four phases: latency phase (Lag), where bacteria metabolically adapt to the surrounding nutritional environment, but do not divide; an exponential phase (Log), in which bacteria exponentially grow; a stationary phase, in which the number of bacteria replicating is equal to the dying ones due to the decrease of nutritional components and increase of waste products of bacterial metabolism; death phase, in which there are no more bacteria dividing and the number of living cells decrease exponentially.

The three probiotics ON cultures were diluted to an initial optical density at 600 nm (OD_{600}) of 0.05 and incubated at 37 °C as per usual, using both the innovative TIL medium and the standard MRS one, in order to compare their growth in the two different media. The OD_{600} values were measured (NanoPhotometer NP80, Implen, Munich, Germany) every 2 h for 8 h and at 24 h, using 1 mL of the bacterial culture.

6.3 Agar Spot Test

The agar spot test was performed to evaluate the efficacy of viable probiotic strains in reducing the pathogen growth, following the protocol by Tejero-Sariñena *et al.*, in 2012 with few modifications[61]. Three drops of 10 µL of each probiotic strain were spotted on a 1.5% agarized TIL medium plate, supplemented with fructose for LBR01 and glucose for LF26 and LR04, and let dry at room temperature (RT). A semisolid media of 3 with 0.8% agar was prepared, with the addition of a fresh ON pathogen culture diluted 1:1000. This suspension was subsequently poured onto the spots immediately (T_0), or after 24 and 48 h of incubation of the probiotic spot at 37 °C. The plates with the pathogen suspension were let solidify at RT before incubation at 37 °C, and after 48 h the inhibition halos were measured. The same experimental procedure was used to test a more complex condition, in which both pathogens were diluted in the semisolid suspension.

The normalized width halo (nw_{halo}) was calculated following the formula used by Martí *et al.*[62], in which d_{iz} is the diameter of the inhibition zone (in mm) and d is the spot diameter (in mm).

$$nw_{halo} = \frac{d_{iz} - d}{2 \cdot d}$$

6.4 Probiotic Cell-Free Supernatant (CFS) Production

To determine the effects of their postbiotics on pathogen viability and biofilm formation, probiotic CFSs were produced as reported by Squarzanti *et al.* in 2022 with few modifications[63]. Fresh ON cultures were inoculated with an OD_{600} of 0.05 in TIL and MRS medium and grown ON in proper conditions. Bacterial growth was assessed by OD_{600} measurement. Subsequently the bacterial suspension was centrifuged at 4000 rpm for 20 minutes at 4 °C with Heraeus Megafuge 16R (Thermo Fisher Scientific, Rodano, Milan, Italy). The supernatant was then collected and sterilized using 0.22 µm polyether sulfone (PES) filters (Clearline, distributed by Biosigma, Cona, Venice, Italy), aliquoted and stored at -20 °C.

To replicate the experimental conditions, TIL with fructose and TIL with glucose were incubated as described for the probiotic cultures and used as controls in the experiments (iTILF, and iTILG respectively).

6.5 Viability Assay

To assess the number of viable cells, present after the treatment of the pathobionts with probiotic CFSs, the BacTiter-Glo™ Microbial Cell Viability Assay (Promega Italia S.r.l., Milan, Italy) was used. It is a luciferase-based assay, able to detect the amount of ATP produced by vital and metabolically active bacterial cells. The luciferase uses ATP to convert luciferin in oxyluciferin, in the presence of Mg^{2+} and O_2. The amount of luminescence produced is directly proportional to the amount of ATP, as well as to the amount of viable microbial cells in the culture (**Figure 4**).

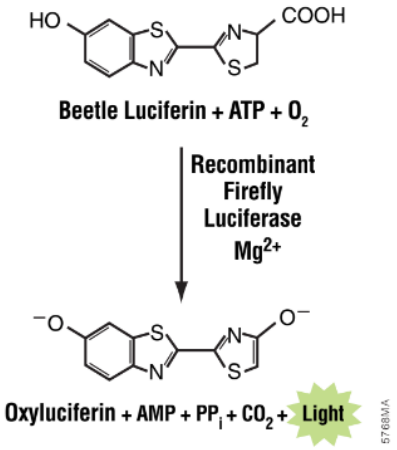

Figure 4 *Luciferase reaction of BacTiter-Glo™ Microbial Cell Viability Assay (Promega Corporation).*

The pathobionts *A. actinomycetemcomitans* and *S. mitis* were plated into a 96-well plate, at an initial OD_{600} of 0.01 (approximatively 5×10^6 CFU/mL) in TSB and immediately treated with probiotic CFSs (50% v/v). The plates were incubated at 37 °C in static conditions and the results were read at 24, 48, and 72 h. A plate for each pathogen and each time point was prepared. The viability assay was performed following the manufacturer's instructions and the luminescence was detected with Spark microplate reader (Tecan Trading AG, Switzerland).

The same experiment was also performed in a more complex environment, in which both pathogens were seeded together, with the same initial OD_{600}, and allowed to adapt for 1 h at 37 °C before the treatment with CFSs. The following passages were the same as described above.

In all experiments TSB, iTILF, and iTILG were used as controls. Each experiment was done with four replicates and repeated three times independently.

6.6 Biofilm Formation Assay

The level of pathogen biofilm formation after CFS treatment was determined following the procedure previously published by Squarzanti *et al.* 2022, with few modifications[63]. Pathogens were plated at an initial OD_{600} of 0.01 into a 48-well plate and immediately treated with probiotic CFSs (50% v/v). A plate for each pathogen and endpoint of 24, 48, and 72 h was prepared. OD_{600} was read before incubation (T_0) and at each endpoint, to evaluate the changes in biofilm formation among probiotic treatment and controls. Per each timepoint, the biofilm was fixed with 4% paraformaldehyde (Bio-Optica S.p.A., Milan, Italy) for 30 min at room temperature (RT). The supernatant was then removed and stained with 1% crystal violet (CV) solution (Sigma-Aldrich) for 15 min at RT. The excess of CV was removed and gently washed with tap water. Images of each well were acquired through EVOS FLoid™ Cell Imaging Station (Thermo Fisher Scientific, Waltham, MA, USA). To quantify the amount of biofilm produced by bacteria, CV was dissolved with 33% acetic acid solution and its absorbance was read at 570 nm using a Spark microplate reader (Tecan Trading AG, Switzerland).

The same assay was performed in a complex environment, by plating the two pathogens together at the same initial OD_{600} 0.01 and allowing them to adapt for 1 h at 37 °C before CFSs treatment. The assay was then performed as described above. In all experiments TSB, iTILF, and iTILG were used as controls and each experiment was performed with four replicates and repeated three times independently.

6.7 Co-aggregation Assay

The interaction between pathogens and probiotic strains was assess through auto- and co-aggregation assay, based on published papers[64–66] with few modifications. ON renewed bacterial and probiotic cultures were centrifuged at 4000 rpm for 15 min at RT (Heraeus Megafuge 16R). The supernatant was removed, and the pellet of each culture was resuspended at a final OD_{600} of 1 in co-aggregation buffer (CAB; 150 mM NaCl, 1 mM Tris, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2 \cdot 2H_2O$). The auto-aggregation of pathogens and probiotics was determined by using 1 mL aliquots of bacterial suspension, while for co-aggregation equal amounts of pathogens, or pathogens and probiotics, were mixed into a tube, vortexed for 30 seconds and then aliquoted into cuvettes for readings. The OD_{600} of each condition was read immediately (T_0) and after 8 h (T_8) of incubation at RT (NanoPhotometer NP80; Implen, Munich, Germany). To assess the potential of CFSs in inhibiting the pathogen co-aggregation, a mixture 50% v/v was used. As positive controls were used solutions of D-glucose (D-glu), D-fructose (D-fru), and D-galactose (D-gal) in CAB at a final concentration of 50 mM. The negative control (C-) was prepared, following Datta *et al.* in 2017

instructions, by using 0.05% Tween-20 in 0.2 M NaCl since it inhibits the aspecific bacterial interactions[66]. To calculate the aggregation percentages the following formula was used:

$$auto\text{- }or\text{ }co\text{-}aggregation\ \% = \frac{OD_{600}T_0 - OD_{600}T_8}{OD_{600}T_0} \cdot 100$$

With $OD_{600}T_0$ the OD at T_0 and $OD_{600}T_8$ the OD at T_8. Each experiment was done in three replicates and independently repeated three times.

6.8 Probiotic CFS Analyses

6.8.1 Probiotic CFS Protein Quantification and pH measurement

The protein concentration of both MRS and TIL produced CFSs was obtained through the bicinchoninic acid (BCA) Protein Assay Kit (Biosciences, St. Louis, USA, distributed by Cabru S.A.S.). This assay involves the reduction of Cu^{2+} to Cu^{1+} by proteins, in an alkaline medium. The combination of two BCA molecules and Cu^{1+} develops a purple-colored product that absorbs at 562 nm, linearly dependent on the quantity of proteins in the sample. The assay was performed following the manufacturer's instructions in a 96-well plate, in which the samples were plated and subsequently the BCA working solution, composed of Cu^{2+} and bicinchoninic acid, was added in each well. The plate was incubated at 37 °C for 30 minutes, and the absorbance was read at 562 nm using the microplate reader Spark (Tecan Trading AG, Switzerland). To obtain the protein concentration, a calibration curve was created with nine serial dilutions of bovine serum albumin (BSA) at a known concentration.

The pH of CFSs was determined with the pHmeter Sension + PH3 (Hach Lange S.r.l., Milan, Italy).

6.8.2 Proteomic Analysis and Data Processing

The analyses have been conducted in collaboration with Professor Marcello Manfredi of the Biological Mass Spectrometry Laboratory (CAAD, Novara, Italy).

The proteins of CFSs produced in both MRS and TIL media were precipitated ON at -20 °C with 4 volumes of ice-cold acetone. The pellets were then collected by centrifugation at 17000 × g for 20 min at 4 °C and then resuspended in 100 mM ammonium bicarbonate (NH_4HCO_3). Proteins were reduced with DTT (dithiothreitol) 200 mM, subjected to alkylation with iodoacetamide (IAM) 200 mM, and then completely digested with 2 µg of trypsin. The peptide digests were desalted on the Discovery® DSC-18- solid-phase extraction (SPE) 96-well plate (25 mg/well; Sigma-Aldrich)[67].

The digested peptides were analyzed with UHPLC Vanquish system (Thermo Scientific, Rodano, Italy) coupled with an Orbitrap Q-Exactive Plus (Thermo Scientific, Rodano, Italy). Peptides were separated by a reverse-phase column (Accucore™ RP-MS 100 × 2.1 mm, particle size 2.6 μm). Mobile A and B phases were water and acetonitrile respectively, both acidified with 0.1% formic acid. The analysis was performed using the following gradient: 0-5 min from 2 to 5% B; 5-55 min from 5 to 30% B; 55-61 from 30 to 90% B and hold for 1 min, at 62.1 min; the percentage of B was set to the initial condition of the run at 2% and hold for about 8 min. The mass spectrometry analysis was performed in positive ion mode with a voltage of 2.8 kV. For the spectra acquisition, a data-dependent (ddMS2) top 10 scan mode was used. Survey full-scan MS spectra (mass range m/z 381 to 1581) were acquired with resolution R=70,000 and AGC target 3×10^6. MS/MS fragmentation was performed using high-energy c-trap dissociation (HCD) with resolution R=35,000 and ACG target 1×10^6. The normalized collision energy (NCE) was set to 30. The mass spectra analysis was carried out using Mascot v. 2.4 (Matrix Science Inc., Boston, USA); the digestion enzyme selected was trypsin, with 2 missed cleavages, a search tolerance of 10 ppm was specified for the peptide mass tolerance, and 0.1 da for the MS/MS tolerance. The following modifications were specified for the analysis: carbamidomethyl cysteine and oxidized methionine as fixed and variable modifications, respectively[68]. Mass spectra were searched against the NCBI sequence databases specific for each probiotic species (2021).

6.8.3 Probiotic CFS Lactic Acid Quantification

Lactic acid is a metabolic product that LAB usually produce; its quantification in CFS samples produced in both media was assessed with the D/L-Lactic Acid Megazyme Assay Kit (NEOGEN Europe Ltd, Ayr, UK), following the manufacturer's instructions. D-lactic acid or L-lactic acid are oxidized to pyruvate by Lactate Dehydrogenase (D-LDH or L-LDH) in the presence of oxidized nicotinamide-adenine dinucleotide (NAD+), with the release of a reduced NAD (NADH). Pyruvate, with the presence of glutamate pyruvate transaminase and D-glutamate, is than converted into D-alanine and 2-oxoglutarate. This reaction allows to avoid the reversibility of the previous one, in this way, the total amount of lactic acid in the sample is proportional to the amount of NADH produced.

Double distilled water (ddH_2O), buffer, NAD+, and GTP were added to cuvettes, and thereafter the sample was added to the cuvette. As a blank the ddH_2O was used. After 3 minutes the absorbance at 340 nm was read, using the NanoPhotometer NP80 (Implen, Munich, Germany). After the first read, 10 μL of D-LDH and L-LDH solutions were added into each cuvette, and after 10 minutes the absorbance was read again. The total concentration of lactic acid in the samples was calculated with the following formula:

$$Lactic\ acid\ concentration\ (mg/mL) = \frac{V \cdot mw}{\varepsilon \cdot d \cdot v} \cdot \Delta A$$

Indicating with V the cuvette volume (1.13 mL), mw the lactic acid molecular weight (90.1 g/mol), ε the molecular extinction coefficient of NADH at 340 nm (6300 L/mol*cm), d the cuvette optical path (1 cm), and v the sample volume (0.05 mL).

6.8.4 Short-chain Fatty Acid Production

The analyses have been conducted in collaboration with Professor Marcello Manfredi of the Biological Mass Spectrometry Laboratory (CAAD, Novara, Italy).

Basal culture media and CFSs produced in MRS and TIL media were assessed for SCFAs content after a liquid–liquid extraction method with methyl tert-butyl ether (MTBE). SCFAs were then analyzed using a gas chromatography-mass spectrometer GC-TOFMS (BT, Leco Corp., St. Josef, MI, USA), as previously described. Briefly, the column adopted was a 30 m DB-FATWAX-UI (Agilent Technologies, Santa Clara, CA), while high-purity helium (99,9999%) was used as the carrier gas. One µL of each sample was injected in splitless mode at 250 °C. The program was as follows: the initial temperature was 40 °C for 2 min, then ramped 7 °C/min up to 165 °C, 25 °C/min up to 240 °C, and maintained for 5 min. The electron impact ionization was applied at 70 eV. The ion source temperature was set at 250 °C, the mass range at 40 to 300 m/z with an extraction frequency of 32 kHz and an acquisition rate of 200 spectra/s.

6.9 Statistical Analysis

One-way and two-way ANOVA tests, with Tukey post-hoc correction, were performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Results were represented as the mean of the replicates ± standard deviation (SD). Significant differences were considered for $p < 0.05$.

7. Results

7.1 Probiotic Growth Curves

The probiotic growth curves were conducted both in MRS and in the novel animal-derivative-free TIL media, to assess if their different nutritional potential could influence their replication. MRS broth is a standard medium for the cultivation and enumeration of *Lactobacillus* spp. usually adopted when working with Lactobacilli, while TIL is an innovative, vegetal based one.

As shown in the graph of **Figure 5**, all the probiotic strains grew better in TIL medium compared to MRS. For all the strains an enhanced growth during the exponential phase, in TIL medium, is highlighted. Particularly, LBR01 (**Figure 5a**) showed a significant growth increase in TIL when compared to MRS, immediately after 4 h of incubation ($p < 0.001$; **Figure 5a**), with a further enhancement over time ($p < 0.0001$ at 6, 8, and 24 h; **Figure 5a**). Similarly, LR04 and LF26 demonstrated a slightly better growth in TIL with respect to MRS after 6 h of incubation, that became significant only after 8 and 24 h, especially for LF26 ($p < 0.0001$; **Figure 5b and Figure 5c**).

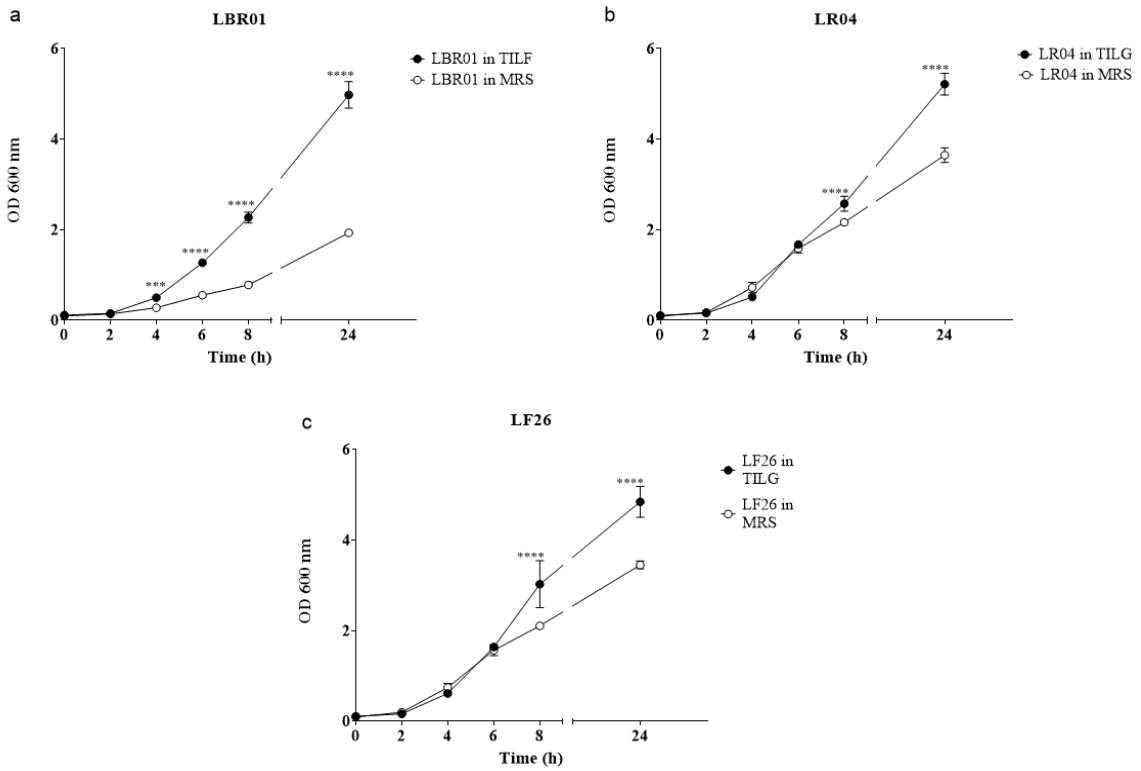

Figure 5 *Probiotic growth curves in MRS and TIL media, of LBR01 (**a**), LR04 (**b**), and LF26 (**c**).* Data are expressed as the mean value of three independent experiments ± SD. OD 600 nm = optical density at 600 nm. ** p<0.01; *** p<0.001; **** p<0.0001.

This allowed to assess the best CFS production and collection time, that is immediately after the end of the exponential phase. Moreover, it was demonstrated that these probiotics strains could adapt and duplicate better in a medium free from ingredients of animal origin. However, this outcome might be only strain specific.

7.2 Agar Spot Test

With the Agar Spot test, it was possible to evaluate if viable probiotic strains were able to contain the pathogen growth. The normalized width halo (nw_{halo}) measurements in mm, together with SD, are reported in **Table *1*** and represented in the graph below (**Figure 6**). Interestingly, LBR01 was ineffective only against *A. actinomycetemcomitans* and the pathogens co-culture at T_0 (**Figure 6a** and **6c**), while against *S. mitis* an inhibition halo was observed (**Figure 6b**). In general, for all the probiotics, the inhibition halo increased over time. Particularly, LBR01 showed a significant increase in growth inhibition from 24 h to 48 h against *A. actinomycetemcomitans* ($p < 0.001$; **Figure 6a**), *S. mitis* ($p < 0.0001$; **Figure 6b**), and the pathogen co-culture ($p < 0.01$; **c**). LR04 showed a significantly increased growth inhibition against *A. actinomycetemcomitans* and the pathogens co-culture only at T_0 versus 24 h ($p < 0.001$, **Figure 6a**; $p < 0.0001$, **Figure 6c**), while against *S. mitis* the inhibition was significant only at 48h ($p < 0.01$; **Figure 6b**). Lastly, LF26 significantly inhibited *A. actinomycetemcomitans* growth at 48 h versus T_0 ($p < 0.0001$; **Figure 6a**). In *S. mitis* the inhibition halo was significant at all time points ($p < 0.01$; **Figure 6b**), while for the pathogens co-culture it was significant only at T_0 and 24 h ($p < 0.01$; **Figure 6c**).

Table 1 *Normalized width measurement in mm of inhibition halo diameters in the Agar Spot test*. Data are reported as the mean values (mm) of three independent measurements at 48 h post incubation ± SD. –: no inhibition observed.

		Probiotic Spot Incubation Time (h)		
Condition	**Probiotic Strain**	**0**	**24**	**48**
A. Actinomycetemcomitans	LBR01	-	0.25 ± 0.06	1.03 ± 0.03
	LR04	0.33 ± 0.04	0.85 ± 0.03	1.27 ± 0.13
	LF26	0.42 ± 0.04	0.8 ± 0.09	1.39 ± 0.06
S. mitis	LBR01	0.21 ± 0.04	0.24 ± 0.03	0.87 ± 0.03
	LR04	0.38 ± 0.06	0.63 ± 0.03	0.93 ± 0.03
	LF26	0.33 ± 0.04	0.65 ± 0.06	1.07 ± 0.03
Pathogen Co-culture	LBR01	-	0.17 ± 0.06	0.92 ± 0.03
	LR04	0.42 ± 0.04	0.82 ± 0.03	0.94 ± 0.00
	LF26	0.46 ± 0.04	0.7 ± 0.06	0.73 ± 0.03

nw_{halo} (mm)

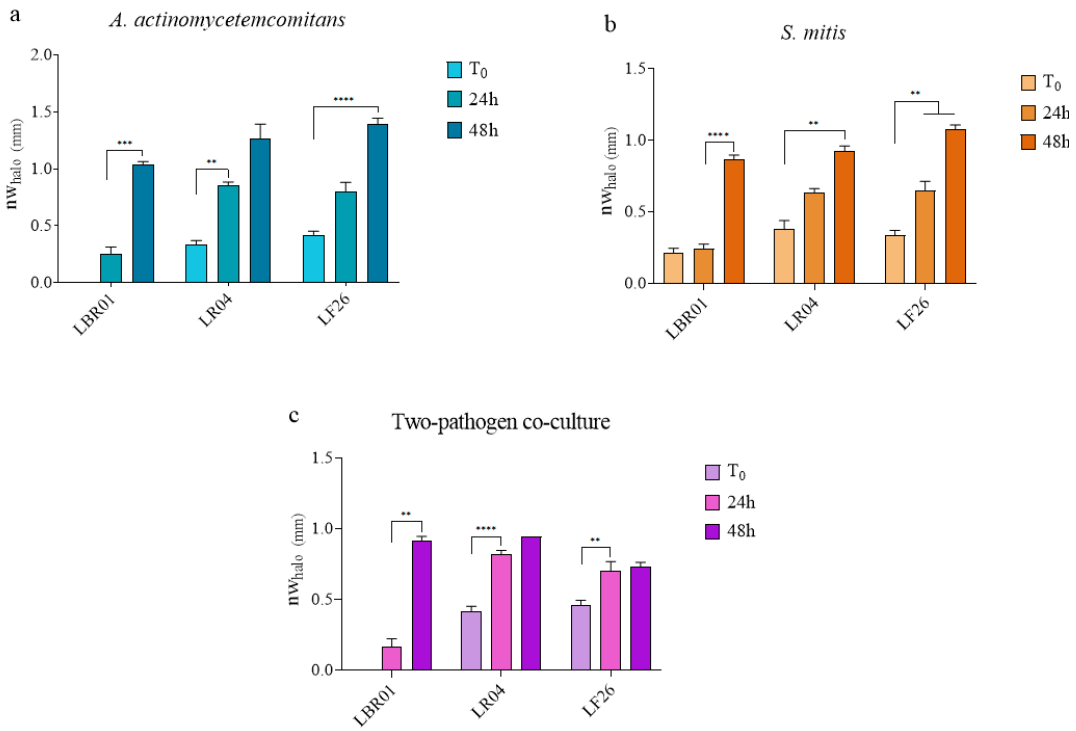

Figure 6 *Agar Spot test.* In the graphs the nw_{halo} is represented for *A. actinomycetemcomitans* (**a**), *S. mitis* (**b**), and the two pathogens co-culture in TIL medium (**c**). Data are expressed as the mean value of three independent experiments ± SD. ** p<0.01; *** p<0.001; **** p<0.0001.

7.3 Viability Assay

To assess the capacity of postbiotics in reducing oral pathogen viability, probiotic CFSs were used in the BacTiter-Glo™ Microbial Cell Viability Assay.

As shown in **Figure 7**, all the probiotic CFSs showed a good capability in reducing bacterial viability during all the time-points evaluated respect to iTILF, iTILG e the TSB media only. Against *A. actinomycetemcomitans* the greatest inhibitory activity was carried out by LBR01, with a slightly decrease in efficacy over time (**Figure 7a**). The same probiotic CFS was also effective against *S. mitis*, with a statistically significant decrease from 24 to 48 h of treatment ($p<0.01$; **Figure 7b**). LR04 and LF26 CFSs demonstrated similar activities, with a slightly better result at 48 h compared to 24 and 72 h of treatment for both pathogens (**Figure 7a** and 7**b**). Among controls, it was possible to observe that TSB, the standard medium for both pathogens, allowed a higher viability despite a small decrease overtime due to the reduction of nutrients ($p<0.001$; **Figure 7a** and 7**b**). The novel TIL medium, both supplemented with glucose or fructose, resulted in a lower viability of both pathogens compared to TSB, with statistically significant increase overtime for *A. actinomycetemcomitans* (**Figure 7a**) and a decrease only at 48 h of treatment for *S. mitis* (**Figure 7b**).

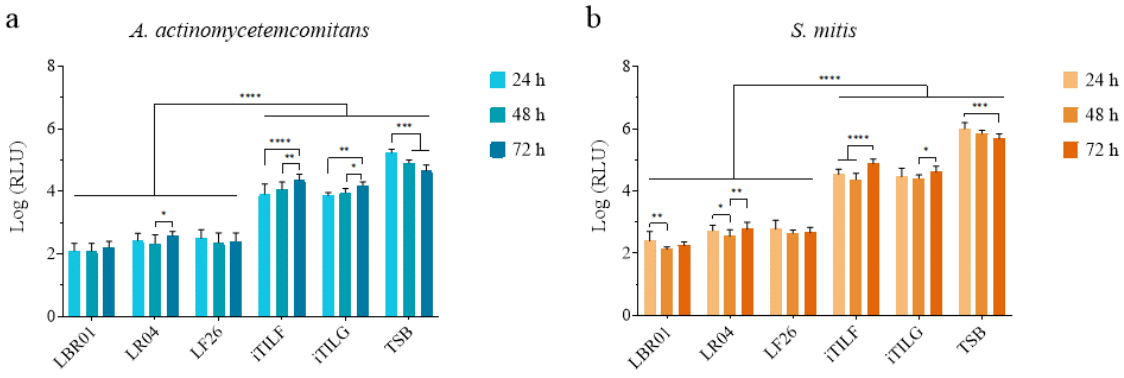

***Figure** 7 Viability Assay.* The viability of *A. actinomycetemcomitans* (**a**) and *S. mitis* (**b**) were determined after 24, 48, and 72 h of probiotic CFS treatment in TIL medium. Data are represented as the Log(mean) of three independent experiments ± SD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Log(RLU) = Logarithm10 (relative luminescence unit); CFS = cell-free supernatant.

The same experiment has been conducted in a more complex environment with both pathogens co-cultured. In this case the probiotic CFSs showed similar results, with a slightly better activity at 24 h for those derived from LF26 and at 48 h of LR04 (**Figure 8**). In contrast to what has been observed in the previous experiments, in this case LBR01 was not the best CFS in reducing pathogens viability, even though only a slightly difference was observed (**Figure 8**). Again, as observable, the elective medium for the growth of the two

pathogens is TSB, that allowed their higher viability when compared to TIL, supplemented with glucose or fructose (**Figure 8**).

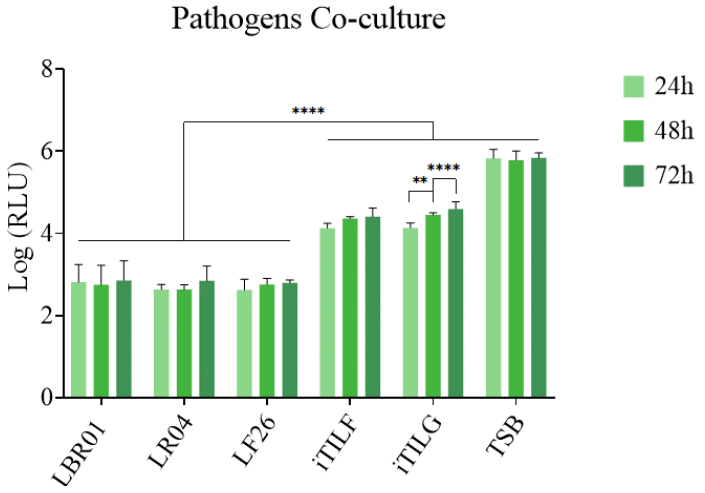

Figure 8 *Two-pathogen co-culture Viability Assay.* The viability of *A. actinomycetemcomitans* and *S. mitis* in co-culture has been determined after 24, 48, and 72 h of probiotic CFS treatment in TIL medium. Data are represented as the Log(mean) of three independent experiments ± SD. ** p<0.01; **** p<0.0001. Log(RLU) = Logarithm10 (relative luminescence unit); CFS = cell-free supernatant.

7.4 Biofilm Formation Assay

This assay was conducted to assess the ability of probiotic CFSs in reducing the formation of pathogen biofilm. In **Figure 9** are represented the mean OD_{600} values and CV_{570} measurement used to assess the quantity of biofilm produced with and without the CFSs treatment. It is possible to observe that all the probiotic CFSs were able to reduce the formation of biofilm at all time-points.

The results of OD_{600} were similar for all the probiotic strains, for both *A. actinomycetemcomitans* and *S. mitis*, with a greater reduction after 72 h of treatment (**Figure 9a** and **9b**). Only for *S. mitis* it was possible to observe an increase in biofilm production at 48 h, despite the lower values at 24 h of incubation (**Figure 9b**). Regarding the controls, it is possible to observe that TSB, in this case, doesn't allow a higher formation of biofilm compared to TIL supplemented with glucose or fructose. This can be mainly observed for *S. mitis* (**Figure 9b**).

The same results were obtained via the CV_{570} quantification, made after the biofilm fixation. All three probiotic strains demonstrated to be effective in preventing biofilm formation in both *A. actinomycetemcomitans* and *S. mitis*, at all time-points (**Figure 9c** and **9d**). Interestingly, while for *A. actinomycetemcomitans* was observed a reduction of biofilm over time (**Figure 9c**), for *S. mitis* an opposite trend was seen, especially for LR04 (**Figure**

9d). Again, the CV staining showed a higher biofilm formation when pathogens were cultured in TILG and TILF. The quantification difference, between TILF and TILG, observable in *S. mitis*, is mainly due to the excessive detachment of biofilm during the

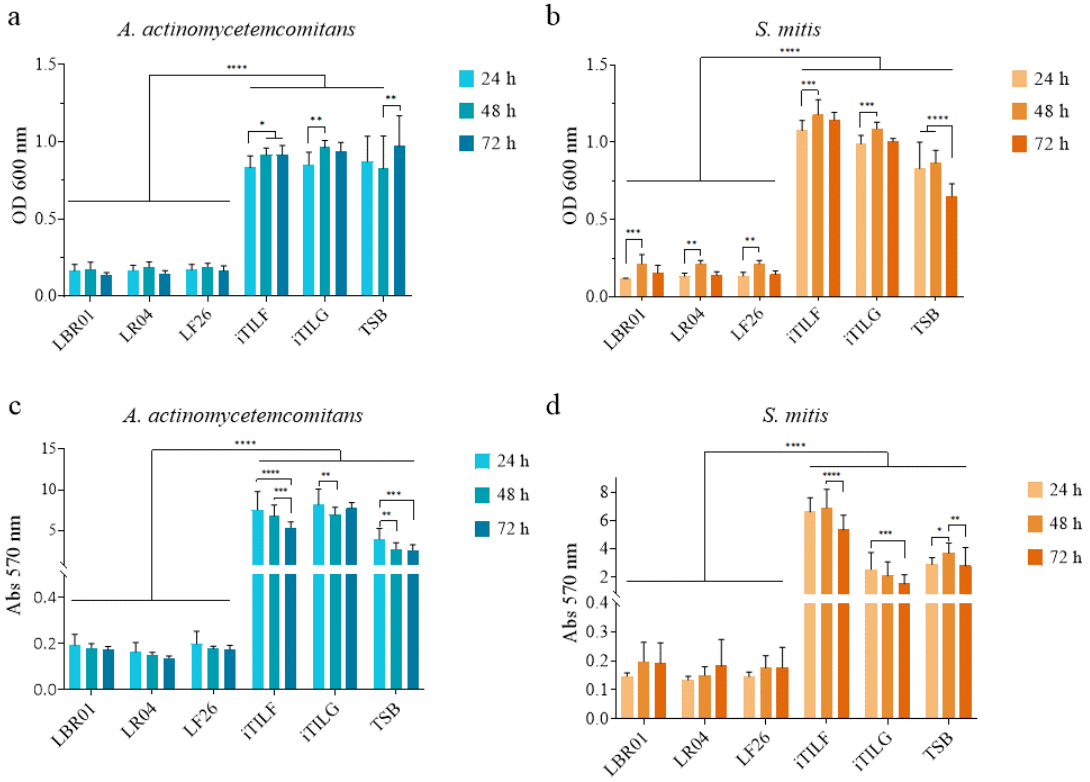

Figure 9 *Biofilm Formation Assay: Optical density (OD) measurement at 600 nm and crystal violet (CV) biofilm quantification.* The OD_{600} measurements were assessed at 24, 48, and 72 h of incubation of the single pathogens with the probiotics CFSs for *A. actinomycetemcomitans* (**a**) and *S. mitis* (**b**); CV quantifications were carried out after 24, 48, and 72 h of incubation for *A. actinomycetemcomitans* (**c**) and *S. mitis* (**d**). Data are represented as the mean value of three independent experiments ± SD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. OD 600 nm = optical density at 600 nm; Abs 570 nm = absorbance at 570 nm; CFSs = cell-free supernatants.

washing step (**Figure 9d**).

Representative images of CV-stained biofilm are shown in **Figure 10**.

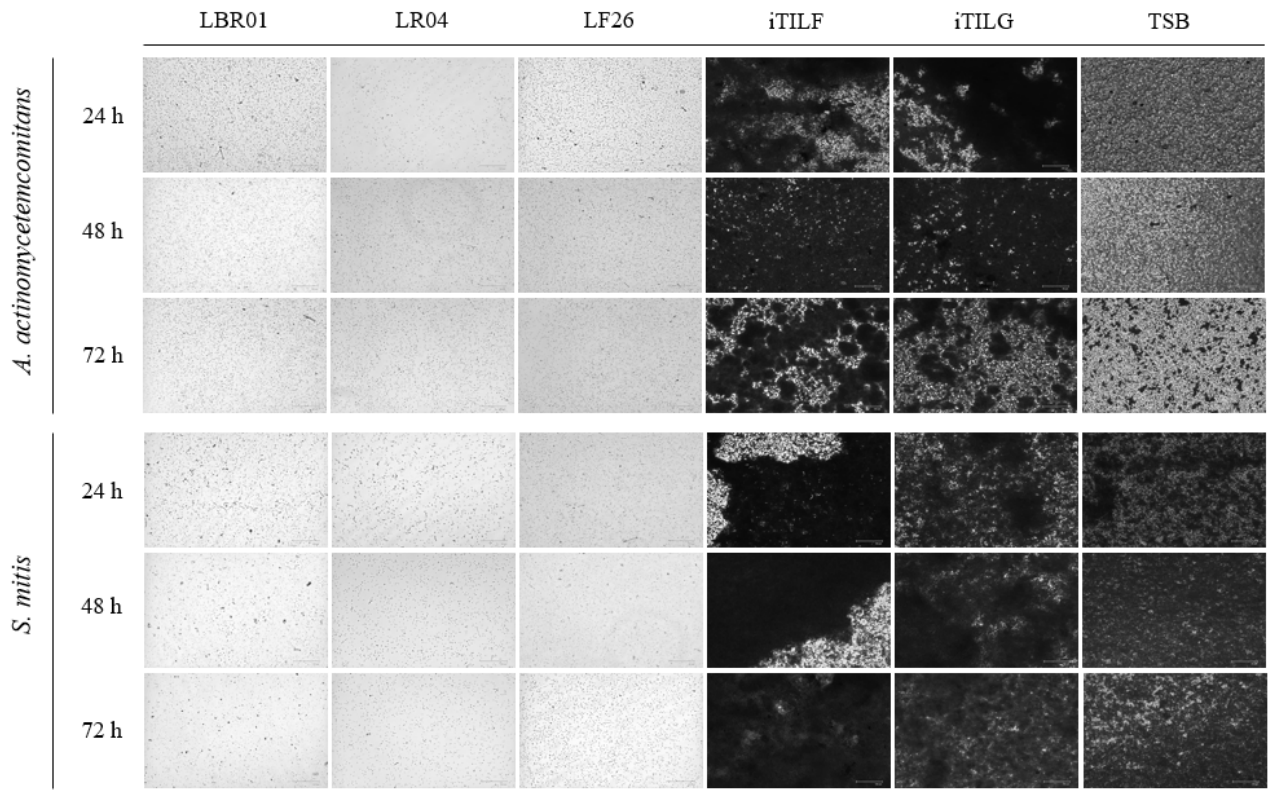

Figure 10 *Crystal violet-stained biofilm images.* Images were obtained at FLoid™ Cell Imaging Station. Magnification 460×.

Also in this case, the biofilm formation assay has been conducted in a more complex environment obtained with the pathogen co-culture. LR04 and LF26 demonstrated to be the most effective in reducing biofilm formation (**Figure 11**). LBR01 was only slightly worse in preventing its formation compared to the other probiotic CFSs. Again, the novel TIL medium proved higher capabilities in favoring biofilm formation, when supplemented with glucose or fructose. As before, the differences in CV staining, between these two conditions, can be related to a greater detachment of biofilm during the washing step (**Figure 11b**).

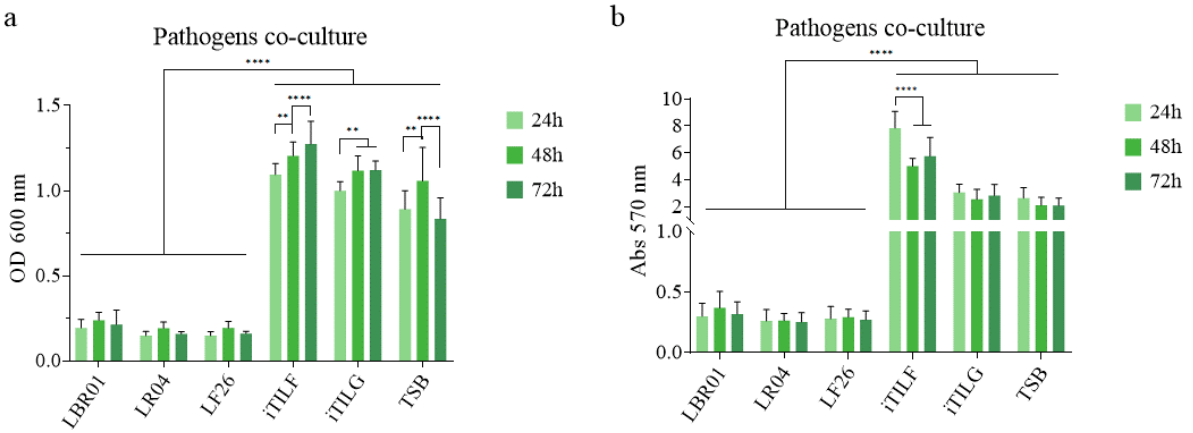

Figure 11 *Biofilm Formation Assay in co-culture: Optical density (OD) measurement at 600 nm and crystal violet (CV) biofilm quantification.* The OD600 (**a**) and CV570 (**b**) measurements were assessed at 24, 48, and 72 h of incubation of co-culture suspension with probiotic CFSs. Data are represented as the mean value of three independent experiments ± SD. ** $p<0.01$; **** $p<0.0001$. OD 600 nm = optical density at 600 nm; Abs 570 nm = absorbance at 570 nm; CFSs = cell-free supernatants.

Representative images of CV-stained biofilm for pathogens co-culture are shown in **Figure 12**.

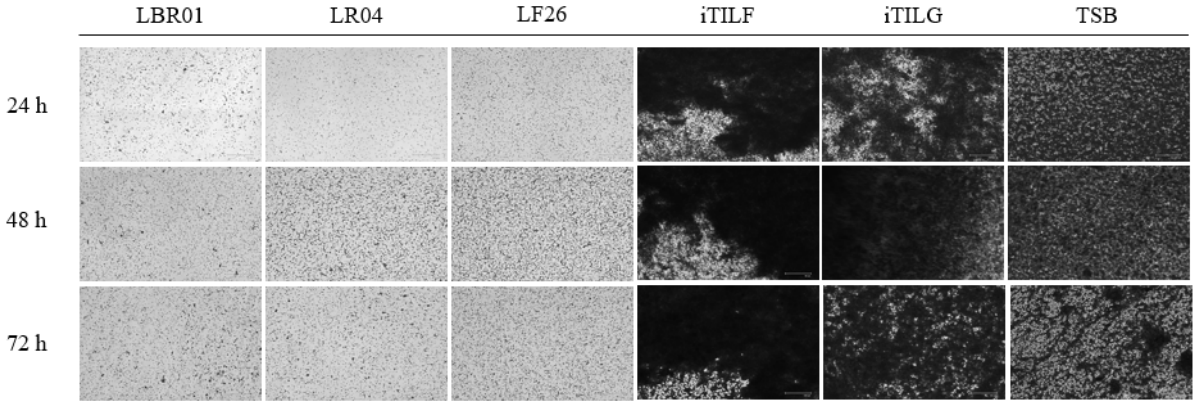

Figure 12 *Crystal violet-stained co-culture biofilm images.* Images were obtained at FLoid™ Cell Imaging Station. Magnification 460×.

7.5 Auto- and Co-aggregation Assays

The auto- and co-aggregation assays were used to investigate the interactions among single pathogens and probiotic strains, with themselves and with each other. It was useful also to investigate whether probiotic CFSs could interfere with the two-pathogens co-aggregation or not. In **Figure 13** are represented the auto-aggregation results for each single bacterial strain, and the co-aggregation obtained after mixing the two pathogens suspension. Their co-aggregation has been tested with both live probiotic strains (**Figure 13a**) and probiotic CFSs (**Figure 13b**).

Among the single pathogen suspensions, *A. actinomycetemcomitans* showed the lowest auto-aggregation capability, with a statistically significant difference only with *S. mitis* of $p < 0.05$ (**Figure 13a**). Probiotic strains auto-aggregation showed the highest rate compared to pathogens auto-aggregation ($p<0.0001$; **Figure 13a**). When probiotics were mixed with pathogens, it was possible to observe that there was only a slight reduction in pathogens co-aggregation, with similar effects among all strains ($p<0.01$; **Figure 13a**).

In **Figure 13b** it is represented the effects of probiotic CFSs in bacterial co-aggregation. None of the CFSs was able to significantly reduced pathogens co-aggregation, on the contrary, it resulted increased. Among the positive controls, it is possible to observe that only D-fructose significantly reduced co-aggregation when compared with the two-pathogens condition ($p<0.001$; **Figure 13b**). D-galactose significantly reduced co-aggregation only when compared to CFSs ($p<0.01$; **Figure 13b**). The negative control C- showed a significant difference only with D-fructose ($p<0.05$; **Figure 13b**).

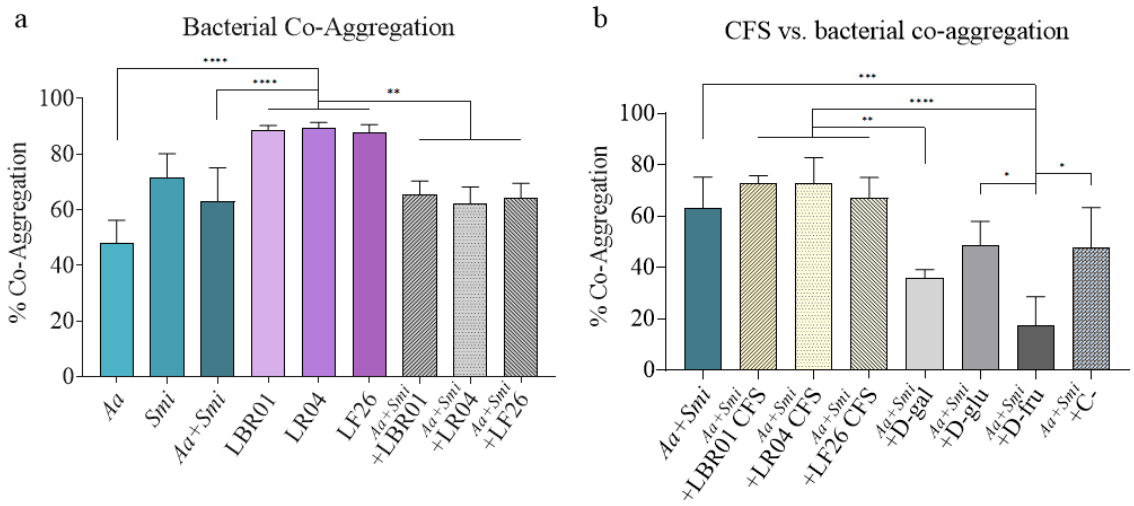

Figure 13 *Co-aggregation Assay.* Auto- and co-aggregation of pathogens and probiotics. Each bacterial strain was first tested in auto-aggregation, and then in co-aggregation together with live probiotics (**a**). CFS effects on two-pathogens co-aggregation (**b**). All data are represented as the mean value of three independent experiments ± SD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Aa = A. actinomycetemcomitans; Smi = S. mitis; D-gal = D-galactose; D-glu = D-glucose; D-fru = D-fructose; C- = negative control; CFS =cell-free supernatant.

7.6 Probiotic CFS Analyses

7.6.1 Probiotic CFS Protein Quantification and pH Measurement

The results of the protein quantification, through BCA Assay, and pH measurement of CFSs are reported in **Table 2**. The results obtained when probiotics were cultured in TIL

were compared to the ones obtained when the same bacterial strains were cultivated in the standard MRS medium.

Table 2 *Protein quantification and pH measurement of MRS- and TIL-produced CFSs.*

	MRS-produced CFSs		TIL-produced CFSs	
Probiotic strain	**pH**	**Protein concentration (mg/mL)**	**pH**	**Protein concentration (mg/mL)**
LBR01	5.22	9.63	4.5	15.41
LR04	4.24	10.79	3.8	13.24
LF26	4.25	8.21	3.9	12.99

7.6.2 Proteomic Analysis

The results of the proteomic analysis are illustrated in **Figure 14** and **Table 3**.

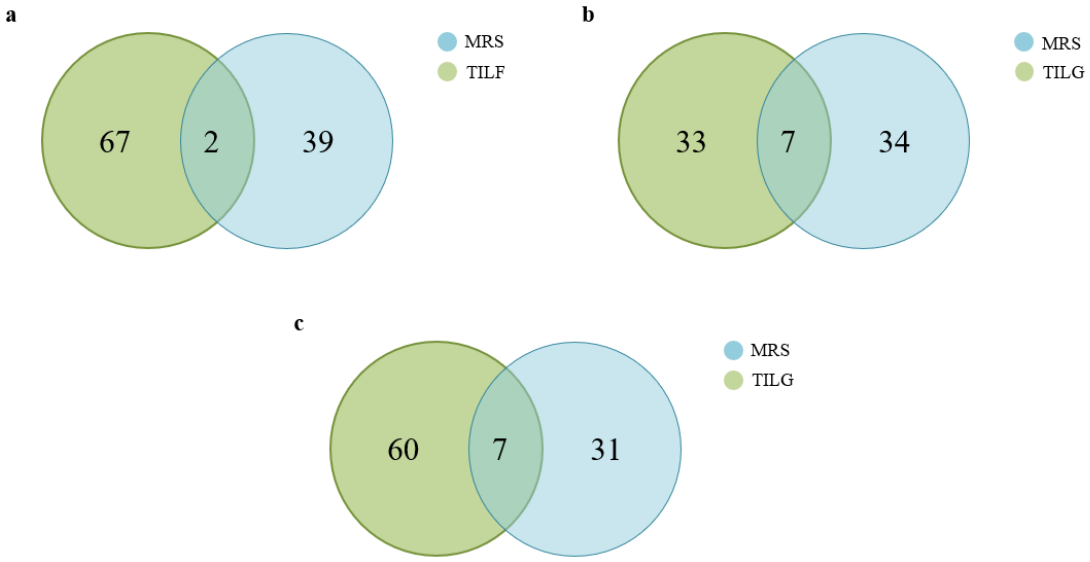

Figure 14 *Protein content of probiotic CFSs cultivated ON in MRS and TIL media.* Protein number identified in MRS and TIL media, respectively in (**a**) LBR01, (**b**) LR04, and (**c**) LF26 CFSs.

The analysis identified 67 proteins produced by LBR01 in MRS medium, and 39 proteins in TILF. Among them, 2 proteins were present in both media (**Figure 14a**). LR04, when cultivated in MRS produced 33 proteins in total, while in TILG 34 proteins were identified, with 7 common proteins produced in both media (**Figure 14b**). LF26 produced 60 different proteins in MRS, and 31 in TILG, while the proteins produced in both media were 7 (**Figure 14c**).

In general, in both media, the proteins characterized were related to ribosomal and transport activities, together with several bacterial enzymes and proteins involved in replication. It was possible to identify also proteins and enzymes for bacterial defense against bacteriophage infections, such as the phage head morphogenesis protein produced by LR04 in MRS and LBR01 in TILF, and phage terminase of LF26 CFS in TILG. Other ones identified were elongation factors and adhesins like elongation factor Ts of LBR01 produced in MRS, and adhesins produced by LF26 in the same medium. LR04, when cultivated in TILG expressed the PBP1A family penicillin-binding protein, beta-lactamase, and antibiotic biosynthesis monooxygenase. All the common proteins identified for each probiotic strain are listed in the **Table 3**. It is possible to observe that the common proteins are mainly ribosomal proteins and enzymes involved in carbon source metabolism, such as 1,5-(carboxyamino) imidazole ribonucleotide synthase, and glyceraldehyde-3-phosphate dehydrogenase.

Table 3 *Identified common proteins produced by the probiotic strains cultured in both MRS and TIL media.*

Probiotic Strains	Identified common proteins	Protein ID
LBR01	2	AYM02917.1 DUF2187 domain-containing protein WAD01306.1 hypothetical protein
LR04	7	EKS50007.1 Glycosyltransferase RDJ92975.1 Hypothetical protein B4Q13_25105 OAU37774.1 Adhesin OAU79553.1 Transposase WP_188434125.1 5-(carboxyamino)imidazole ribonucleotide synthase UTX29435.1 Type I glyceraldehyde-3-phosphate dehydrogenase OAU73993.1 hypothetical protein PY62_14940
LF26	7	QEY00216.1 hypothetical protein F4U91_00840 sp|B2GD71.1|RL27_LIMF3 50S ribosomal protein L27 WP_249665651.1 KxYKxGKxW signal peptide domain-containing protein ARB00227.1 peptidase M23 WCL68818.1 Arm DNA-binding domain-containing protein sp|B2GAC6.1|RL7_LIMF3 50S ribosomal protein L7/L12 ESS02053.1 hypothetical protein NB22_01490

7.6.3 Lactic Acid quantification and SCFAs evaluation

The results of lactic acid quantification are shown in **Table 4**. As shown, when bacteria were cultured in the novel medium, the lactic acid amount was higher.

Table 4 *Lactic Acid quantification (g/L) in both MRS and TIL media.*

	Lactic Acid (g/L)	
Probiotic strain	**MRS-produced CFS**	**TIL-produced CFS**
LBR01	1.68	5.17
LR04	8.86	13.54
LF26	7.98	11.25

The results of the SCFAs analysis are represented in **Figure 15**. Also in this case, generally, TIL medium induced a higher production of these acids. This is notable for all the strains especially for 2-methyl propanoic acid, 3-methyl butanoic acid, and pentanoic acid (**Figure 15c**, **e**, and **15f**). The production of acetic acid by probiotics was higher in TIL only for LBR01, while for the other two it resulted lower (**Figure 15a**). The same can be seen for propanoic acid, for which it resulted higher in TIL medium only for LR04, and for butanoic (also known as butyric) acid production, slightly higher in TIL medium only for LBR01 strain (**Figure 15b**). It is possible to observe that butanoic acid levels were the same in both media for LF26 strain (**Figure 15d**).

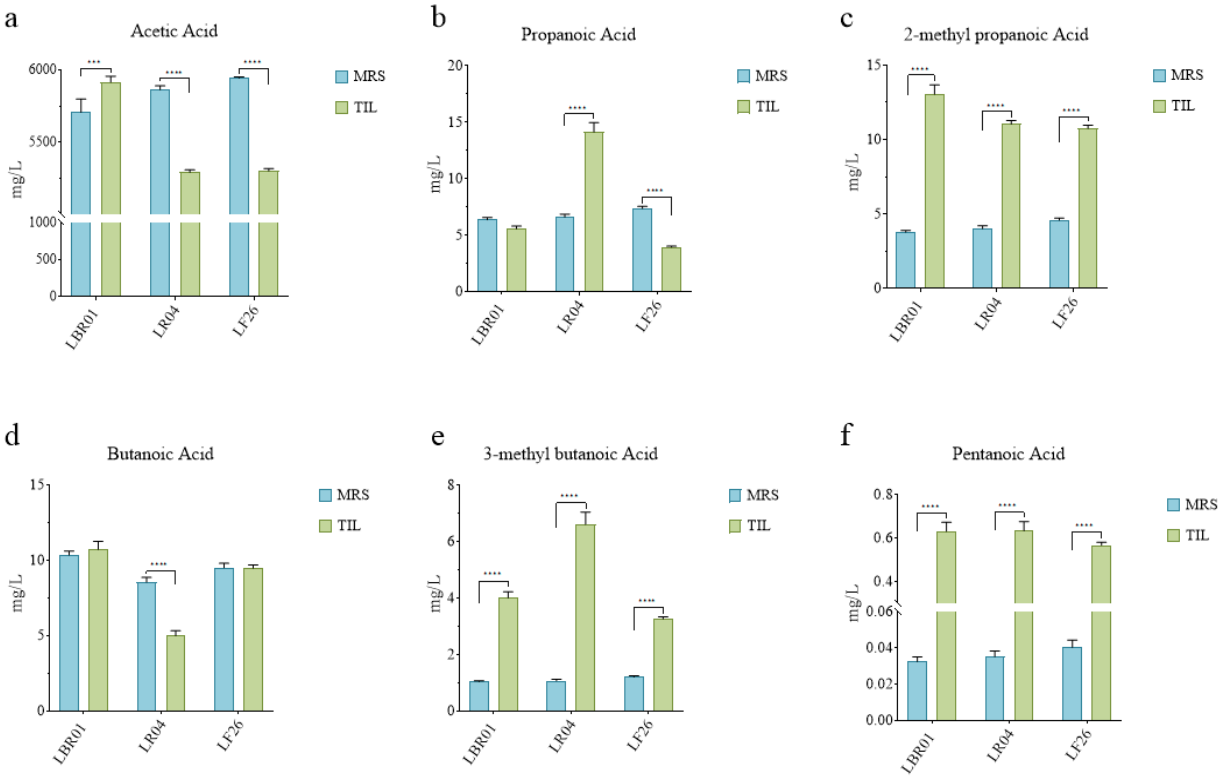

Figure 15 *Short Chain Fatty Acid quantification in MRS and TIL media.* SCFAs identified in MRS and TIL media for all three strains are reported. Acetic Acid (**a**), Propanoic Acid (**b**), 2-methyl propanoic Acid (**c**), Butanoic Acid (**d**), 3-methyl butanoic Acid (**e**), and Pentanoic Acid (**f**). Data are expressed as the mean value of three replicates ± SD. *** $p<0.001$; **** $p<0.0001$

8. Discussion

Aggregatibacter actinomycetemcomitans and *Streptococcus mitis* are two oral pathobionts involved in the pathogenesis of several oral and systemic diseases, [25,43]. In the years, many studies demonstrated the relationship between dysbiosis and diseases development, and this is true also for the oral niche one[69,70].

The inappropriate use of antibiotics to treat a large spectrum of diseases is rapidly leading to ever-increasing number of antibiotic-resistant strains. This aspect represents a global threat which generates an urgent demand for the discovery of new therapeutic approaches to prevent and treat pathologies caused by primary and opportunistic pathogens overgrowth and virulence[71].

Among the possible alternatives to the use of antibiotics, prebiotics, probiotics, and postbiotics were reported for their beneficial effect in pathogens containment. However, the knowledge on their activity toward periodontopathogens has yet to be adequately deepened[56,72].

Of all known probiotics, *Lactobacillus* spp. are the most studied and used in commercially released formulations. For example, some studies demonstrated that *Lactobacillus* spp. can be used for the prevention of eczema in children and young adults, and as food supplements for patients with atopic dermatitis[73,74]. Another example is their use in the treatment of bacterial vaginosis or vulvovaginal candidiasis[75,76]. In oral health probiotics are currently employed for periodontal diseases, and dental caries[56,77].

Based on this premises, the aim of the current experimental thesis was to determine the effects of three *Lactobacillus* strains, *Levilactobacillus brevis* LBR01 (DSM 23034), *Lacticaseibacillus rhamnosus* LR04 (DSM 16605), and *Limosilactibacillus fermentum* LF26 (DSM 33402), against the two oral pathobionts *Aggregatibacter actinomycetemcomitans* (DSM 11123) and *Streptococcus mitis* (DSM 12643).

Firstly, we determined the growth of the probiotic strains in two different media, the animal derivative-based De Man, Rogosa and Sharpe (MRS) and the novel animal-derivative-free TIL (Terreno Industriale Lattobacilli). It was demonstrated that all three probiotics could adapt well and grow better in the medium free from ingredients of animal origin. However, this can be only strain-specific as shown by the results from the research of Squarzanti *et al*., where other *Lactobacilli* strains did not show any significant growth difference between the two media[63].

Subsequently, we demonstrated the effects of the viable probiotics on the growth of *A. actinomycetemcomitans* and *S. mitis*, both alone and in co-culture. Their effects were

evaluated through the agar spot test, that revealed the efficacy of all the probiotic strains in the pathogen growth inhibition. This effect was observed mainly after 48 h of incubation, especially against the single pathogen culture. Between the three probiotic strains, *L. fermentum* LF26 was the most effective against *A. actinomycetemcomitans* and *S. mitis*, while against the pathogens co-culture the best activity was carried out by *L. rhamnosus* LR04. *L. brevis* LBR01 was the least effective in growth inhibition, especially at T_0 for *A. actinomycetemcomitans* and pathogen co-culture. Similar effects for *L. rhamnosus* against *A. actinomycetemcomitans* was observed by Gönczi *et al.* in an agar diffusion assay, and by Squarzanti *et al.* in an agar overlay assay[57,78]. Interestingly, Zanetta *et al.* obtained similar results, but with a higher inhibition halo, when probiotics were cultured in MRS medium. However, in this medium, *L. brevis* LBR01 resulted ineffective at T_0 also against *S. mitis*[38]. In literature it was not possible to find similar data of agar spot against bacterial co-culture.

Probiotics CFSs were used to counteract pathogen viability and biofilm formation. With the viability assay it was possible to assess the efficacy of all probiotic strains in reducing pathogens viability at all the incubation time-points. *L. brevis* LBR01 revealed to be the most effective, especially when the two pathobionts were cultured singularly. Interestingly, Zanetta *et al*. obtained opposite results when the probiotic was grown in MRS medium, while for the other strains results were similar[38]. In another study, Ishikawa *et al.* used two *L. rhamnosus* strains, Lr32 and HN001, against *A. actinomycetemcomitans,* which showed similar effects to what obtained in our experiments. Moreover, they observed the modulation of protein expression in *A. actinomycetemcomitans*. Lr32 was able to downregulate the expression of LtxA while HN001 induced its upregulation. Furthermore, both strains showed the capability to downregulate the production of the cyto-lethal distending toxin B (CdtB)[60].

The biofilm assay results confirmed the inhibitory activity of the *Lactobacillus* strains, since all of them inhibited the biofilm formation, at all time-points, by *A. actinomycetemcomitans* and *S. mitis* both when cultured singularly and in co-culture. Similar results were obtained by Jiang and colleagues, in which *L. rhamnosus* demonstrated to be able to inhibit *A. actinomycetemcomitans* biofilm formation[79]. In a similar study, Jaffar and colleagues demonstrated that both CFSs and live cells of two different *L. fermentum* strains were able to inhibit biofilm formation, and induce its degradation, by three different *A. actinomycetemcomitans* strains[80]. Moreover, Zanetta *et al.* obtained similar results for *L. rhamnosus* LR04 and *L. fermentum* LF26, cultured in MRS medium, that showed good biofilm reduction capability against *A. actinomycetemcomitans* and *S. mitis*, both in single and co-culture. On the other hand, *L. brevis* LBR01, resulted completely ineffective in

preventing biofilm formation when cultured in MRS, both for single pathogens and when co-cultured[38].

The auto- and co-aggregation assay allowed to better understand the interactions between pathogens, and how live probiotics and their CFSs could modulate them. It was possible to observe that probiotic CFSs seemed to slightly increase pathogen's co-aggregation, but none of them in a significant way. The negative control was employed to assess non-specific interactions between bacteria that are unrelated to the specific inhibition being investigated. These interactions may be influenced by factors such as hydrophobicity and ionicity. By comparing the co-aggregation percentage of substances with the negative control, it was possible to determine whether the inhibition observed was specific to the desired effect, as substances that showed a significantly lower co-aggregation percentage would indicate specificity[66]. It is possible to hypothesize that these contrasting results are due to the experimental design and the CFSs metabolites composition. In fact, in viability and biofilm formation assays, the pathogens were plated in their usual growth medium and incubated in optimal growth conditions. In the co-aggregation assay, on the other hand, they were resuspended in CAB, that did not allow bacterial growth and adaption, but only facilitated molecular interactions. Moreover, CFSs are complex substances, composed by metabolites produced by probiotics during their growth and residual components of TIL medium not digested by bacteria, that can interfere with each other. This highlight again the importance of CFS characterization, to identify the molecule responsible for a certain effect. On the other hand, when live probiotic were used, it was possible to observe only a slight reduction in pathobionts co-aggregation. Similar observations were made by Scillato and colleagues, and it is possible to assume that the interaction of probiotics with pathogens can compete for cellular receptor binding, resulting in prevention of pathogen cell adhesion[81]. The same experiment, with the same probiotic strains but cultured in MRS medium, has been performed by Zanetta *et al*., in which they obtained similar results. However, when cultured in MRS, *L. brevis* LBR01 and *L. salivarius* LS03 CFSs were able to significantly reduce pathogen co-aggregation, indicating that the medium affects the metabolites they produce, and thus their activity against pathogens[38]. Similar results were obtained by Mahdizade *et al*., that demonstrated the capability of *L. rhamnosus* 195 and *L. brevis* 205 to co-aggregate with *A. actinomycetemcomitans*, despite with lower percentages[82]. Although the results obtained are conflicting, co-aggregation assay can be useful to understand whether it is worth to study more in deep some probiotic strains, since it is a fast and cheap method.

To better understand the effects of the molecules produced by probiotics, CFSs were analyzed for their proteins and SCFAs composition. It was possible to identify different proteins involved in carbon source metabolism, ribosomal activity, and transporter proteins. Other interesting proteins were the elongation factor T_s produced by *L. brevis* LBR01 in MRS medium, and adhesins by *L. fermentum* LF26 in the same medium. These factors are used by probiotics to bind to human intestinal cells for colonization[83]. *L. rhamnosus* LR04 in the novel TIL medium, was found to produce PBP1A family penicillin-binding protein, beta-lactamase, and antibiotic biosynthesis monooxygenase. These enzymes confer resistance to beta-lactam antibiotics like penicillin and ampicillin. However, Selvin and colleagues found *L. rhamnosus* to be susceptible to these antibiotics, indicating that this characteristic is probably strain specific[84]. The SCFAs analysis revealed that the production of acids in TIL was higher compared to the standard MRS medium, thus leading to broth acidification. The CFSs SCFAs composition can be determinant for the antibacterial effects of the probiotic strains against oral pathobionts. Molecules such as butanoic and propanoic acid are fundamental as energy source for human cells and contribute to pathogen's growth inhibition[85].

In conclusion, *L. brevis* LBR01, *L. rhamnosus* LR04, and *L. fermentum* LF26, both as live bacteria and postbiotics, revealed to be effective in reducing the two oral pathogens viability and biofilm formation, both in single and co-culture. Moreover, they were able to influence pathogens co-aggregation ability by interfering with their surface interaction molecules. Given that multiple studies have demonstrated that the presence of probiotics in the oral cavity can reduce the incidence of caries, tooth decay, and other oral infections, these results imply that probiotics may be useful for the prevention and treatment of several oral pathologies[56]. More recently, postbiotics are becoming more popular also because they are easy to incorporate into a variety of commercial and food formulations and more stable and safer compared to probiotics[53,56,72].

9. Conclusions

From this thesis it is possible to assess those probiotics that counteract pathogens growth, virulence, and metabolism, especially if cultured in the animal-derivative free TIL medium. In fact, by comparing these results with the previous ones obtained in the animal-derivative based MRS medium, it has been demonstrated how different growth conditions can influence what probiotics produce, hence their efficacy. Moreover, it is possible to affirm that they may be a viable alternative to counteract dysbiosis and infections and prevent dysbiosis-associated diseases by reducing antibiotic misuse and the subsequent resistance phenomena. However, despite these findings, further research is required to expand our understanding on probiotics and postbiotics activities and employs due to the strain-specific and strain-combination effects which also depend on the pathogens examined. It is also essential to comprehend which metabolites are engaged in the regulating functions of probiotics and how they act. In conclusion, probiotics and probiotic-derived postbiotics can represent an important resource as potential substitute or support during antibiotic therapies of infection-related diseases.

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