VNiVERSiDAD
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Facultad de Medicina

Departamento de Cirugía

Oral Microbiota in Caries Prevention, Diagnostics and Management – Challenges and Opportunities

Tesis Doctoral

Pedro André Ferreira Campos Lopes

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Dedicatory

In memory of my beloved brother Décio.

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2. Lopes, P. C., Gomes, A. T. P. C., Mendes, K., Blanco, L., & Correia, M. J. (2024). Unlocking the potential of probiotic administration in caries management: a systematic review. *BMC oral health*, *24*(1), 216. https://doi.org/10.1186/s12903-024-03893-8

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Abbreviations index

A. johnsonii - *Actinomyces johnsonii*

A. naeslundii - *Actinomyces naeslundii*

AAPD CRAs -*American Academy of Pediatric Dentistry Caries Risk Assessment*

ADA CRAs - *American Dental Association Caries Risk Assessment*

B. longum - *Bifidobacterium longum*

C. albicans - *Candida albicans*

C. durum - *Corynebacterium durum*

C. hominis - *Cardiobacterium hominis*

C. matruchotii - *Corynebacterium matruchotii*

CAMBRA - *Caries Management by Risk Assessment*

CIIS - *Centre for Interdisciplinary Research in Health*

CHX - *Chlorhexidine*

CPP-ACP - *Casein phosphopeptide-amorphous calcium phosphate*

DMFT - *Decayed, Missing, and Filled Teeth index*

DDE index - *Enamel Developmental Defects index*

F. nucleatum - *Fusobacterium nucleatum*

FDA - *Food and Drug Administration*

FMD-UCP - *Faculty of Dental Medicine of Universidade Católica Portuguesa*

FV- *Fluoride varnish*

GI- *Gingival index*

HCL - *Hydrochloric acid*

HIV- *Human immunodeficiency virus*

HNP1-3 - *Human neutrophil peptides 1-3*

hβD-1- *Human β-defensin-1*

ICDAS - *International Caries Detection and Assessment System*

IgA - *Immunoglobulin A*

IR -*Infiltrative resin*

JBI - *Joanna Briggs Institute*

L. brevis - *Lacticaseibacillus brevis*

L. paracasei - *Lacticaseibacillus paracasei*

L. rhamnosus - *Lacticaseibacillus rhamnosus*

L. acidophilus - *Lactobacillus acidophilus.*

MI - *Minimally invasive*

MIH - *Molar incisor hypomineralization*

NAD - *Nicotinamide adenine dinucleotide*

N. elongata - *Neisseria elongata*

NaF - *Sodium fluoride*

P. acidifaciens - *Propionibacterium acidifaciens*

P. catoniae - *Porphyromonas catoniae*

P. CW034 - *Porphyromonas CW034*

P. denticola- *Prevotella denticola*

P. FMA5 - *Propionibacterium FMA5*

P. propionicum - *Propionibacterium propionicum*

P. gingivalis - *Porphyromonas gingivalis*

P. histicola- *Prevotella histicola*

P. intermedia - *Prevotella intermedia*

P. maculosa - *Prevotella maculosa*

P. multisaccharivorax - *Prevotella multisaccharivorax*

P. pallens - *Prevotella pallens*

P. sp OT-279- *Porphyromonas sp OT-279*

ppm- *Parts per million*

PICO - *Population, Intervention, Comparison and Outcome strategy*

PRISMA - *Preferred Reporting Items for Systematic reviews and Meta-analysis*

PROSPERO - *International prospective register of systematic reviews*

R. aeria - *Rothia aeria*

R. dentocariosa - *Rothia dentocariosa*

RCT - *Randomized controlled trials*

ROB- *Revised Cochrane risk-of-bias tool for randomized trials*

rRNA- *Ribossomal RNA*

S. cristatus - *Streptococcus cristatus*

S. dentisani - *Streptococcus dentisani*

S. gordonii - *Streptococcus gordonii*

S. infantis - *Streptococcus infantis*

S. parasanguinis - *Streptococcus parasanguinis*

S. mitis - *Streptococcus mitis*

S. mutans- *Streptococcus mutans*

S. oralis- *Streptococcus oralis*

S. salivarius- *Streptococcus salivarius*

S. sanguinis- *Streptococcus sanguinis*

S. sobrinus - *Streptococcus sobrinus*

SAPP11-4 - *Self-assembling peptide P11-4*

T. forsythia - *Tannerella forsythia*

TCLE - *Free and Informed Consent Form*

V. dispar - *Veillonella dispar*

V. parvula - *Veillonella parvula*

WHO - *World Health Organization*

WSL - *White spot lesions*

Abstract

The oral microbiome plays a critical role in oral pathologies, namely in caries and periodontal disease. Although most of these pathologies are currently understood as the result of dysbiosis, knowledge about the specific microbial and ecological events that precede the development of the disease and/or support health is scarce. In this context studying biological mechanisms underlying oral and dental pathologies becomes crucial. In this work a thorough review of the molecular data published regarding the oral microbiome as well as the intricate interactions within polymicrobial communities is done and information is extracted and applied to the study of oral microbiome changes in adults and its relation to caries risk and management.

Three systematic reviews were conducted, to extract knowledge on the microbial communities associated with caries in adults; on the influence of diagnosis tools of white spot lesions in the treatment of such lesions; and on targeted interventions for management caries, such as the use of probiotics to restore the microbial balance in population with caries.

In the first review on the microbiome associated with caries in adults the major conclusion drawn is that although there are several studies published which use the molecular characterization of the oral microbiome, there is a great discrepancy between studies which hampers the comparisons and weakens the conclusions drawn. Data analysis shows that the species in the oral microbiota of individuals with caries are most reported as significantly increased are *Cryptobacterium curtum, Prevotella denticola, Shuttleworthia satelles* and *Streptococcus mutans*. These species have been mentioned in 2/7 papers on oral biofilm analysis. On the other hand, *Actinomyces johnsonii, Cardiobacterium hominis, Corynebacterium durum, Corynebacterium matruchotii, Fusobacterium nucleatum, Gemella sanguinis, Neisseria elongata* and *Rothia dentocariosa* were reported as significantly increased by 1/8 articles which analysed oral biofilm. The analysis of the 13 articles analysing the oral microbiome in caries vs healthy groups shows that considering identification to the genus level might be erroneous (*Prevotella* and *Streptococcus* are good examples). Furthermore, there is evidence that species associated with caries and health may be considered in an index to assess caries risk. This index may assist in clinical decisions thought an assessment of caries risk.

In the second systematic review, a comparison between the treatments applied for white spot lesions (usually caused by bacterial plaque accumulation in cervical region of the tooth) using different diagnosis tools were studied. The main conclusion drawn is that regardless of the use of conventional or more recent methods, the treatment options were the same, and as far as our results showed, the clinical outcome was also similar.

In the last review, evidence for the effect of probiotic administration on caries outcomes directly related to caries risk and development, in individuals with caries was searched. The results showed a beneficial and promising effect on dental caries outcomes using milk supplemented with *Lacticaseibacillus rhamnosus* as an adjuvant approach to clinical intervention and daily oral hygiene routines. However, once more the heterogeneity of study designs and study methodologies hampers comparison of large number of studies.

With the knowledge gathered from the afore mentioned reviews a longitudinal study was done to identify the changes in oral microbiome during an orthodontic treatment with clear aligners. This study aimed at contributing to the scares body of information on the impact of the use of clear aligners in the development of white spot lesions, the first sign of demineralization in orthodontic patients. Biofilm and saliva samples were collected from patients undergoing aligner orthodontic treatment at three distinct time-points and were then sequenced using a metagenomic approach. The results showed that the impact of the use of clear aligners in oral microbiome of these patients is not relevant. Although the number of patients analysed is limited, no significant differences in biodiversity neither on the increase in cariogenic species were observed. On the other hand, the species associated with health are present in several samples, even after the use of the clear aligners.

In sum, this work provides important information on the current knowledge on how the oral microbiome is affected by caries, by using clear aligners, and by using probiotics. Furthermore, an assessment of how caries lesions and caries risk might be managed is also discussed both using probiotics and by the early diagnosis of white spot lesions.

1. Introduction

Human Microbiome studies have attracted much attention since the first publication of the Human Microbiome Project results [1]. This fact stems mainly from the immense possibilities envisioned for microbiome modulation and the impact that this may have on health and wellbeing of the human host. The microorganisms present in oral cavity may indicate the state of health or disease; hence oral microbiota can be used as a biomarker in diagnosis of several pathologies with high prevalence and impact on quality of life such as dental caries, one of the most prevalent chronic diseases in the world [2]. Molecular approaches might reveal genera/species associated with caries and could identify potential microbial "markers" associated with this condition. This could enable the development of a cariogenic/carioprotective index, complementing existing tools, for a more effective assessment and management of caries risk. An improved index of microbial dysbiosis associated with dental caries considering the different niches in the oral community would be a key tool, capable of assisting in clinical decisions, for example, in creation of strategies for prevention and treatment of caries, using, microbiome modulators, such as prebiotics or probiotics, to reverse microbial dysbiosis. This index could be a complement to other caries risk tools currently used which are more focused on clinical and behavioural factors. Therefore, taking the whole microbial community into account has the potential of leading to more effective caries management approaches.

1.1. Oral microbiome

The oral microbiome, as other human microbiomes, contributes to the host's health and prevents infections by providing resistance to the colonization by exogenous opportunistic or pathogenic microorganisms and by eliciting an appropriate immune response [3]. The relationship between the microbiota and the host exists in a dynamic balance called eubiosis [4]. This state results from a dynamic cross talk between the microorganisms and the host and is not represented by a steady community or static factors rather by constant changes in local and systemic factors, which impact and determine the resident microbial communities present at each moment. When the changes in the factors are considerable, microbial interactions may be perturbed and the microbe-host balance lost, leading to dysbiosis. This imbalance frequently heightens the risk of disease [5]. Dysbiosis in the oral cavity is frequently associated with dental caries,

periodontal diseases and fungal infections [5]. These oral diseases and the associated physiopathological changes often lead to an increased risk or severity of several systemic diseases, including diabetes, cardiovascular disease and rheumatoid arthritis [5,6]. Although most oral pathologies are currently understood as the result of dysbiosis, little is known about the specific microbial and ecological events that precede the development of the disease or support health. Studying biological mechanisms that are at the origin of oral and dental pathologies is necessary to reveal yet unidentified oral pathogens and their polymicrobial interactions, as well as taxa that help maintain homeostasis and are associated with health [7].

The oral cavity of an adult human generally hosts more than 200 species of bacteria at a time [8]. Through culture-dependent methods, some specific bacteria have been identified as responsible for dental caries and periodontal disease [9]. With new methodologies and new identification methods, like microbiome analysis of the 16S Ribosomal RNA (rRNA) gene, we can understand that oral microbiome is much more complex than that identified by culture-dependent methods. With these new methods the relation of oral microbiota with oral diseases can be accurately determined [10]. The 16S rRNA sequencing method is particularly valuable as it significantly enhances our understanding of the diversity within the oral microbiome [11,12].

The integrated results from culture dependent and independent studies show that the oral bacterial community is dominated by six main bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes and Fusobacteria, representing approximately 94% of identified taxa. Among them, the first four - Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria - play crucial roles in maintaining oral health. The main bacterial genera in the oral cavity are *Streptococcus*, *Prevotella*, *Haemophilus*, *Rothia*, *Veillonella*, *Neisseria*, *Fusobacterium* and *Porphyromonas* [11–16]. Fungi are represented by approximately 101 species in the mouth of healthy individuals and the most prevalent genus is *Candida*, followed by *Cladosporium*, *Aureobasidium*, *Saccharomyces*, *Aspergillus*, *Fusarium* and *Cryptococcus* [17].

The Archaea domain constitutes only a small part of the oral microbiome and is represented by a limited number of species. The most frequently observed belong to *Thermoplasmatales, Methanobrevibacter, Methanobacterium, Methanosarcina* and *Methanosphaera*, all methanogenic [18]. Recently *Nanopusillus massiliensis* was isolated

from oral samples, by co-culture methods [19]. Microorganisms from this domain can be observed in healthy individuals but are more prevalent in disease states such as periodontitis [11,18]. According to Nguyen-Hieu, *et al* [18], *Methanobrevibacter oralis* is present in 41% of patients with periodontitis and 55% of their periodontal pockets, when compared to healthy individuals. Although some studies report the molecular detection of Archaea in patients with periodontitis, this association requires further studies to be established, as knowledge about the diversity of Archaea is still scarce and preliminary [11,18]. Their small size escapes microscopic observations and their unique molecular signature of the 16 S rRNA gene makes them undetectable using current Archaean universal primer systems. Also, the culturing of these nanoorganisms continues to pose a significant challenge [11,18].

Viruses are important for maintaining oral health and microbiome homeostasis and longitudinal analysis of oral virome demonstrated that, in healthy subjects, it is characterized by high diversity, individual specificity, and temporal stability [20–22]. But viruses are generally associated with oral pathologies [21,23,24]. *Herpes simplex* virus can cause primary herpetic and mucocutaneous gingivostomatitis and recurrent lesions on the face and lips [11]; the human *papillomavirus* is responsible for several lesions in oral cavity, including oral papillomas and condylomas, increasing hyperplasias. Furthermore, infection caused by human immunodeficiency virus (HIV) can also indirectly lead to the appearance of numerous oral manifestations, such as oral candidiasis, hairy leukoplakia, linear gingival erythema, acute necrotizing ulcerative periodontitis and Kaposi's sarcoma [22]. Viruses also could cause local immunosuppression, which can lead to subgingival colonization and the multiplication of periodontal bacteria [25]. Epstein-Barr virus, Cytomegalovirus, and Herpes simplex virus have been clearly identified as more prevalent in patients with severe periodontitis than in healthy individuals. However, the association between periodontitis and human oral virome, specifically those in the *Herpesviridae* family [22], requires further studies to be established.

The microorganisms described above exist in various habitats of the mouth, forming part of a complex microbial community that grows as biofilms. Biofilm formation is a gradual process involving the sequential addition of distinct bacterial groups to the glycoprotein complex on tooth surfaces. Initially, Gram-positive facultative anaerobic bacteria like *Actinomyces* spp and oral streptococci, constitute over 80% of the

initial biofilm [26]. Streptococci, as primary colonizers, play a crucial role in interacting with other early colonizers and adhering to the tooth surface, thereby influencing the composition of late colonizers in oral biofilm. The attachment of these initial colonizers to the pellicle is facilitated by adhesins on the bacterial surface, which interact with receptors on the dental pellicle [27]. This process is dynamic, leading to a transition from an early aerobic environment, characterized by Gram-positive facultative anaerobic species, to a later stage dominated by highly oxygen-deprived conditions where Gram-negative anaerobic microorganisms prevail. This transition not only shapes the microbial composition of the oral biofilm but also has implications for the health or disease status of the host [28–30].

In the process of oral biofilm formation, secondary colonizers such as *Prevotella intermedia* (*P. intermedia*), *Prevotella loescheii*, *Capnocytophaga* spp, and *Fusobacterium nucleatum* (*F. nucleatum*) adhere to early colonizers, attracting late colonizers like *Porphyromonas gingivalis* (*P. gingivalis*) [31]. This adherence is facilitated by coaggregation, a phenomenon documented in laboratory studies, involving specific interactions on bacterial cell surfaces and less specific forces like hydrophobic, electrostatic, and *van der Waals* forces [32,33].

The oral ecosystem is quite complex and consists of several habitats with unique conditions provided by each environment [11,34]. The best-known differences exist between the supra and sub gingival communities. The supragingival environment is more aerobic, and pH and temperature vary between all the environments. For example, the occlusal surface of a molar has oxygen levels that differ significantly from those in crypts on the dorsum of the tongue. The diversity in these habitats is determinant for the microbial colonization which depends on the abiotic and biotic conditions of each habitat [28].

The supragingival microbial community is characterized by a stratified organization with Gram-positive cocci and short rods dominating the tooth surface, while Gram-negative rods, filaments, and spirochetes prevail in the outer surface of the mature biofilm mass. Highly specific cell-to-cell interactions form "corncob" structures, observed between rod-shaped bacterial cells as *Bacterionema matruchotii* or *F. nucleatum* and coccal cells like streptococci or *P. gingivalis* [28]. Species of the *mitis* group streptococci, including *Streptococcus oralis* (*S. oralis*), *Streptococcus mitis* (*S. mitis*), and *Streptococcus sanguinis* (*S. sanguinis*), are early colonizers associated with

oral health. During initial oral biofilm formation, *Veillonella* species like *shutonella atypica* (*V. atypica*), *Veillonella denticariosa*, *Veillonella dispar* (*V. dispar*), *Veillonella parvula* (*V. parvula*), *Veillonella rogosae* and *Veillonella tobetsuensis* are major early colonizers. The interaction of *Veillonella* with other bacteria, such as *Streptococcus gordonii* (*S. gordonii*), *Streptococcus mutans* (*S. mutans*) or *Streptococcus salivarius* (*S. salivarius*), enhances biofilm development [35].

When the mucogingival line is passed towards the root, conditions change. The subgingival environment is more anaerobic, and pH and temperature may change. Concerning temperatures, recent studies do not find statistically significant differences between the supra and subgingival regions. However, when pathologies such as periodontal disease and inflamed subgingival tissues are present, there is a significant increase in temperature in this region and the pH is substantially lower [36]. Mature biofilms in this region consist of anaerobic, assaccharolytic bacteria. Crevicular fluid, rich in nutrients, bathes this area, and host inflammatory cells play a crucial role in influencing bacterial establishment and growth. In subgingival regions nine different bacteria have been described, including Saccharibacteria (formerly known as TM7), *Deferribacteres*, Spirochaetota, Fusobacteria, Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes [37]. Recent studies highlight *Fusobacterium* and *Treponema* as abundant subgingival genera, with *Spirochetes* increased in periodontitis. *Streptococcus* species exhibit heterogeneity, with *S. sanguinis* increased in health and *Streptococcus constellatus* associated with disease [28,38].

Colonization also happens in soft tissues of the mouth. The buccal mucosa and the palate have monolayers of bacteria, which regularly peel off. In contrast, the surface of the tongue has multiple layers of bacteria, very similar to biofilm. Therefore, it is thought that the tongue, compared to the remaining mucous membranes of the oral cavity, has a greater quantity and diversity of microorganisms. The predominant bacteria in healthy individuals in these structures are *Aggregatibacter*, *Haemophilus, Prevotella, Moryella, Oribacterium, Eubacterium, Rothia dentocariosa* (*R. dentocariosa*), *Rothia mucilaginosa* and *S. salivarius* [39,40]. Oral microorganisms can also be retrieved from saliva as a result, mainly from the removal of biofilm from the surface of hard dental tissues. The predominant organisms are *Streptococcus*, *Veillonella* and *Prevotella* [41].

1.2. Caries

The capacity of some microorganisms in oral biofilm to produce (acidogenicity) and tolerate acid (acid tolerance) may, under the right conditions, lead to tooth decay. This common chronic infectious disease results from a synergistic and complex interaction between bacteria, diet, and susceptible host factors (such as teeth anatomy and saliva composition) [2]. The proximal cause is a pH imbalance, but the niches of species involved in this imbalance are not fully understood [2,42].

The caries lesions development on tooth tissues involves dynamic biological processes, in which acids produced by bacterial fermentation of dietary carbohydrates affect the demineralization of tooth tissues. Repeated acidification can lead to the selection of acid-producing and highly acid-resistant organisms, which in turn destroys the pH homeostasis and causes the demineralization-remineralization balance to proceed in the direction of tooth mineral loss [2,42]. More recently, processes such as arginine catabolic pathways have been recognized as important in counteracting the lowering of pH in the mouth, even though the magnitude of such impact is not clear and the implications for clinical purposes not fully ascertained [43]. Therefore, changes in the composition and biochemical activity of oral biofilms are important determinants of the etiology of dental caries [44].

Excessive oral environment acidification by aciduric species such as *S. mutans* is directly associated with dental caries development. However, species with low acid tolerance, such as *S. salivarius* and *S. gordonii*, produce a large amount of alkali, which plays an important role in the acid-base physiology of the oral cavity. Another important feature of certain oral streptococci is their ability to produce hydrogen peroxide, which can inhibit the growth of *S. mutans* [43]. Therefore, and contrary to what is often considered in the design of management strategies for caries, pH homeostasis depends on a complex set of microbial interactions in the oral biofilm and not solely on acid producing bacteria [43].

Studies have been published reporting species associated with samples collected in several types of caries. However, it remains unclear whether these species or microbial groups can serve as reliable indicators of susceptibility, prognosis, or caries activity. Results are usually shown in diversity indices and long lists of genera and species, difficult to interpret and impossible to use in a clinical setting. Having a more accurate knowledge of the communities that are associated with health or tooth decay presents a

significant challenge, primarily due to the complexity of the oral microbiota, one of the more diverse in the human body [45]. Microbiome analysis of the 16S rRNA gene from biofilm, saliva or carious dentin samples characterizes a unique microbial signature associated with dental caries much more complex than that identified by other bacterial identification methods, such as culture-based studies and DNA probe. Much information has been published regarding the molecular knowledge of microbial species present in the oral cavity and it is important to verify if there is potential for their use as prognostic indicators [10].

1.2.1. Caries risk index

Caries risk assessment is essential to find individuals who are more likely to develop these lesions and thus individualize the treatment plan, providing preventive measures to avoid the onset or progression of disease. In addition, it helps to recommend the frequency of medical appointments and the need for complementary diagnostic means [46,47]. Nowadays, several caries risk assessment indexes are available, including Cariogram, Caries Management by Risk Assessment (CAMBRA), American Dental Association Caries Risk Assessment (ADA CRAs) and American Academy of Pediatric Dentistry Caries Risk Assessment (AAPD CRAs). For example, CAMBRA® protocol was developed in 2002 in California, being oriented towards prevention, reversal and intervention when necessary [48]. CAMBRA is a visual representation of the relationship between risk factors, protective factors and disease indicators. The balance or imbalance between these factors will determine whether the disease stabilizes, progresses, or is reversed. When pathological risk factors prevail, the probability of the disease progress is high, when protective factors prevail, caries lesions stabilize or reverse, depending on the stage lesion. CAMBRA does not indicate the number of caries that may occur in the future, but the potential risk scenario. It serves as a tool to educate patients through the discussion of risk factors, but also to plan, to promote protective habits and serves as a motivation to modify behaviours [48].

Caries diagnosis, which is usually made through clinical visual detection and carried out on clean, dry and illuminated surfaces, makes it possible to detect the carious process at an early stage. To make the classification of carious lesions less subjective, some classification systems for these lesions have emerged, such as the International

Caries Detection and Assessment System (ICDAS) [49]. These are based on the visual inspection of dry and clean plaque surfaces, in which the activity (active or inactive) and the clinical features of the lesion are evaluated. The ICDAS is an evaluation method that stands out for including the identification of caries lesions in their most initial stage, the white spot, and simultaneously determine the existence of appropriate treatment and the stage of caries development, using seven scores:

0: No change in enamel translucency after drying for 5 seconds

1: Visible opacity after drying for 5 seconds and pigmentation restricted to the bottom of the fissure

2: Visible opacity even in the presence of moisture and diffuse pigmentation

3: Cavity located in opaque or pigmented enamel

4: Shading of the underlying dentin

5: Cavity in opaque or pigmented enamel with exposure of underlying dentin

6: Cavitation in opaque or pigmented enamel with exposure of underlying dentin, involving more than half of the surface.

Considering dental caries and their impact on quality of life, it is essential to identify patients with these lesions and to detect those potentially at high risk. In addition to pain and discomfort caused by these injuries, dental caries may also be associated with potential systemic effects [5]. Thus, it is important to have methods for early assessment of the risk of caries and objective ways to evaluate these lesions and minimize their appearance.

1.2.2. White spots lesions

White spot lesions (WSL) represent the first stage of dental caries, and their prevalence has been increasing in recent years, corresponding to a value between 10% and 49% [50], especially in patients undergoing orthodontic treatment. They are usually caused by bacterial plaque accumulation in the cervical region of the tooth [51]. Epidemiological indices are used to aid diagnosis, such as the Enamel Developmental Defects index (DDE index) and this index allows the classification of lesions as demarcated, diffuse and hypoplastic and histopathological changes [51].

WSL are signs of demineralization under a layer of intact, highly mineralized enamel, which may or may not lead to the development of caries. The clinical aspects of discoloration are related to the optical properties described by the tooth itself: value, hue and chroma are responsible for tooth color, however, its appearance is minutely affected by its translucency, opacity and fluorescence [51]. Clinically, the appearance of the lesion is opaque white, due to the optical phenomenon caused by mineral loss and the difference in the refractive index of water and air that fill the spaces formed in the enamel [50,52,53]. In this way, the lesion will be *whitish* and with little translucency since there is an increase in enamel porosity. This surface irregularity leads to a loss of brightness, resulting in a diffuse reflection of light [54,55]. Regarding formation time, lesions develop relatively quickly. However, they are only visible after the tooth surface has dried [54,55]. Thus, the white spot is related to the loss of minerals by the enamel which, when diagnosed in its initial phase, is still partially demineralized and is subject to remineralization [56].

Several factors can determine white spots appearance, such as traumatic hypomineralization, fluorosis and molar incisor hypomineralization (MIH). MIH are considered pre-eruptive lesions, due to defects in enamel development, differing from dental caries or post-orthodontics lesions considered post-eruptive lesions, that are caused by a disturbance of the mineral balance. Either type of white spot is characterized by a local decrease or loss of enamel translucency or by hypomineralization [57]. Although all risk of enamel demineralization lesions has enamel hypomineralization as a common feature, they can be distinguished by the shape and depth that the lesion assumes. There is a significant correlation between the intensity of the color of WSL, their volume, and the depth of enamel demineralization [57,58].

Traditionally, the diagnostic methods for WSL involve visual and photographic examination, to detect the depth and extent of the lesions. However, in recent years, new techniques have emerged such as fluorescence, the DIAGNOdent mechanism, microradiography and microcomputed tomography. These last two techniques can be used *in vivo*, but they are predominantly employed as research tools for *in vitro* studies which contribute to the acquisition of knowledge, which holds the potential for translation into clinical applications. The aim is to make a more accurate diagnosis of the lesion, understanding it in terms of depth and extent. Therefore, the treatment will be more directed towards each injury to improve the results and prognosis and, when possible, this treatment should be as conservative as possible.

With the evolution of Dentistry, several treatments for white spot lesions are now available, ranging from more invasive methods to more conservative solutions aimed to preserve the dental structures. Within this conservative concept various strategies can be used, including oral hygiene instructions, prescription of topical fluorides, including fluoride varnish (FV) and sodium fluoride mouthwash (NaF mouthwash), use of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) and infiltrating resin [59].

There are several forms of diagnosis and suitable treatments for WSL, due to different clinical situations. Knowing how to identify a white spot lesion and ensure correct and effective treatment is crucial to promote good care and achieve clinical success.

1.2.3. Orthodontic correction and caries

Orthodontic treatment is a common method of correcting dental malocclusion, which has evident aesthetic and functional benefits [60]. During orthodontic treatment, microbial biofilms can form on braces, similarly to natural teeth [61]. The constituents of fixed appliances promote a greater accumulation of food waste and, in turn, greater difficulty in cleaning, leading to an increased risk of developing caries and periodontal diseases, compared to patients without fixed orthodontic appliances [62]. Indeed, is a challenge to properly clean teeth, with bacterial plaque accumulation being common, leading to an increased risk of oral pathologies [63], because bacteria have affinity for this surface due to electrostatic forces [64,65]. Furthermore, the increase in periodontal pathogens in dental plaque biofilm causes inflammation, with increased gingival bleeding and gingival hyperplasia, which is a common complication of orthodontic treatment [66]. The clinical changes mentioned above are often associated with shifts in the communities that colonize tooth surface, with previous studies of changes in the microbiome showing that there is generally an increase in the abundance of key species and a decrease in the diversity of the oral microbiome after appliance placement [60,67]. When using an orthodontic appliance, there is an increase in colonization by *S. mutans* and *Lactobacillus*, both in the orthodontic appliance itself and in the oral cavity in general, since the insertion of this appliance creates new retentive areas that favor the growth of these microorganisms [68]. *S. mutans* plays an important role in the beginning of caries lesions,

although it does not necessarily mean that its presence carries out a cariogenic process. Lactobacilli are more prominent in the progression of caries and not in its appearance [69]. Therefore, it can be inferred that the colonization and increase in prevalence of these two organisms, together with all the shifts in biofilm activity, can lead to an increase in the enamel demineralization, with an incidence rate of between 30 to 70% for the development of WSL [6,70]. In addition to these shifts in the biofilm communities, *Streptococcus sobrinus* (*S. sobrinus*) has been identified as a major contributor in the pathogenesis of dental caries, and its presence also contributes to the risk of enamel demineralization [71].

As previously mentioned, the insertion of the device is followed by a qualitative change in the composition of the subgingival bacterial population in orthodontically treated patients [72] and several articles [72–81] discuss the increase of several pathogenic microorganisms, such as *Campylobacter retus, F. nucleatum, P. Tannerella forsythia* (*T. forsythia*), *P. intermedia, Prevotella pallens, Prevotella nigrescens, Treponema denticola, TM7, Rothia* and *Mycoplasma* in patients during the orthodontic treatment .

Regarding the pathological changes, it is now assumed that the replacement of conventional fixed appliances with clear aligners, increase patient compliance allowing adequate oral hygiene and, thus, reducing the risk of dental and periodontal complications [7,82]. Scientific evidence on the use of clear aligners as an alternative to fixed appliances is still scarce, but several studies show that, in comparison with traditional orthodontic treatment, these orthodontic systems showed a significantly lower value in the bleeding index on probing and in the bacterial plaque index during treatment [63]. This suggests that this method is more conducive to the maintenance of periodontal health [63]. Nonetheless, knowing that the incidence of periodontitis increases with age and that today more and more adult patients are looking for orthodontic solutions, one of the great advantages of this type of appliance is the ease of access to all dental surfaces, as they can perform oral hygiene without any limitations or restrictions. So, clear aligners may offer value for patients with periodontal problems or high caries risk [82].

Clear aligners arose from the development of new dental materials and 3D technology applied in dental medicine, when several manufacturers wanted to meet the patients' comfort and aesthetic demands [61,83,84]. One of these brands stood out: Invisalign®, created in 1997 by Zia Chishti and Kelsey Wirth, but only in 1998, was

approved by the Food and Drug Administration (FDA) [85]. Currently, there are several brands of aligners that differ mainly in the material´s composition, the time of use and the design of the gingival margin. With the introduction of new thermoplastic materials, clear aligners have become more flexible, adaptable to the dental arch, ensure constant orthodontic strength allowing the use of aligners also in complex orthodontic cases [86]. Produced in polyurethane that fits the buccal, lingual/palatal, and occlusal surfaces of dental pieces, clear aligners are usually used for a period of 20 hours a day, removed during meals and for cleaning, and are replaced every one or two weeks [87–89]. Note that certain studies show that plaque can colonize the surface of the aligners after 48 hours of use and argue that the use of the aligner for more than 22 hours is related with an increased risk of caries and periodontal disease due to reduced cleaning by the action of saliva [83,87].

Scientific evidence on the use of clear aligners as an alternative to fixed appliances is still scarce. Regarding dental caries, the first sign of demineralization in orthodontic patients is the white spot lesion [81,162], but little has been described regarding changes related to clear aligners. So, the lack of outcomes raised the question of whether the use of orthodontic devices such as clear aligners, will also cause changes in oral microbiome, leading to the appearance of caries. This information is crucial for the definition of oral hygiene instructions to the patient and for the design of preventive intervention strategies for dental caries, which are often a side effect from the orthodontic treatment, for example modulating the microbiome with prebiotics and probiotics to promote the maintenance of a healthy microbiome.

1.3. Oral Microbiome Modulation for Caries Prevention and Management – Probiotics

An alternative approach to control or reduce the risk of caries is by using prebiotics and/or probiotic bacteria [90–92]. They have the potential to modulate the oral ecosystem and may play an important role in the prevention and management of dental caries. This topic has attracted the interest of several research groups in the last decades. The World Health Organization defines probiotics as “live microorganisms which, when

administered in adequate amounts, confer a health benefit to the host" [93]. Traditionally, probiotic bacteria mainly *Lactobacillus* spp. and *Bifidobacterium* spp, have been used in prevention and treatment of gastrointestinal infections (caused by *Salmonella typhimurium*, *Clostridium difficile* and *Mycobacterium tuberculosis*) or other diseases (such as acute colitis) including caries [94–96]. The mechanism of action underlying probiotic therapy in the oral cavity is based on the hypothesis that harmless bacteria could occupy the niche of pathogenic or opportunistic microorganism in the biofilm [90]. However, this effect cannot be generalized, as everyone's microbiome is unique, and probiotic effects are difficult to predict. It is thought that probiotic bacteria interact not only with the commensal microbiota (excluding or inhibiting pathogens) but also with the host by modulating the immune responses with local and systemic effects [90]. Pathogen exclusion or inhibition occurs both by the production of antimicrobial substances which affect specific community members and through the competition for nutrients or attachment receptors [92]. Nevertheless, the specific mechanisms of action are not clearly identified and understood. In the case of caries, the effect of the probiotic in the oral cavity should result from the interaction of probiotic bacteria with the biofilm, inhibiting and hindering the growth of pathogens through the production of hydrogen peroxide and bacteriocins, and by stimulating the immune response that locally results in increased production of immunoglobulin A (IgA) and stimulation of phagocytosis [91]. Another mechanism suggested is that probiotics may prevent cariogenic dental plaque formation directly by adhering to the tooth surface [97] or indirectly by neutralizing free electrons [98].

Probiotics are often associated with prebiotics. The definition of prebiotic has evolved and is currently described as "substrate that is used selectively by host microorganisms conferring a health benefit" [99]. In terms of caries prevention and management, this would include nutrients for microorganisms that inhibit acidogenic and aciduric microbes and/or enhance pH recovery by generating alkali from these nutrients [100]. The two main sources of alkali in the oral cavity are urea and arginine, which, when metabolized by some oral bacteria, result in the production of ammonia and lead to an increase in pH [101]. Thus, prebiotics and probiotics demonstrate the potential to have preventive and therapeutic effects and can be used to prevent or even to treat the disease after it is installed. The potential of probiotics' use in caries prevention has been addressed by some researchers in systematic reviews. Recent reviews considering the impact of

probiotics in children have shown that the use of probiotics presented a positive effect in decreasing *S. mutans* counts in saliva [102,103]. However, Meng and coworkers [103] found that the effect existed when counts of *S. mutans* were done in saliva but not in plaque samples. Meta-analysis has reported that while *S. mutans* counts decreased with the use of probiotics, the same was not observed for *Lactobacillus* counts, neither in saliva nor in plaque [102,103]. There are also some reviews focusing on a specific probiotic microorganism such as Hao *et al* [104], which indicates that use of *Bifidobacterium* based probiotics has shown to be ineffective in reducing *S. mutans* or *Lactobacillus* counts in saliva or dental plaque, nor in reducing the occurrence of caries in deciduous teeth. Poorni *et al* [105] analysed *Streptococcus* strains as probiotics and showed that *in vitro* promising results do not translate into *in vivo* clinical benefits. Regardless of the population studied, the strains included in the analysis, or the main conclusions drawn from the reviews, authors unanimously emphasize the need for more data to provide stronger support the use of probiotics in enhancing oral health.

2. Material and Methods

2.1 Systematic reviews

Systematic reviews were conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [106] and were recorded in an international database of prospectively registered systematic reviews in health and social care, the International prospective register of systematic reviews OSF Registries.

The focused question was determined for each review according to the Population, Intervention, Comparison and Outcome (PICO) strategy and an extensive systematic literature search was conducted in the electronic databases PubMed, Web of Science, Scopus and Cochrane to identify articles with relevant data to answer the PICO question. Search results were imported into Rayyan [107] to help visualize and operationalize the article's selection and to evaluate methodological quality of the studies, the critical assessment tools Joanna Briggs Institute (JBI) [108] or the RoB tool [109] were used, since they are both well accepted in systematic reviews.

The query's, PICO questions, inclusion and exclusion criteria, as well as the bias assessment tools used are summarized in Table 1

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Table 1- Summary of systematic review search details. All reviews followed the PRISMA guidelines [106].

Registration	Search	PICO Question	Filters	Inclusion criteria	Exclusion criteria	Bias
1- Caries microbiome: What Do Molecular Microbial Results on Oral Biofilm Reveal Regarding Caries? **OSF Registries:** osf.io/eqc8a	Search performed in **Pubmed** and **Cochrane** databases with the following query: **"microbiome[Title/abstract] * AND Caries, dental [Mesh Terms]".**	"Which are the most prevalent microbial genera/ species in oral microbiota of individuals with caries scored by DMFT index or ICDAS when compared to individuals without caries experience?	Articles published until March 2024 were included.	- cross-sectional, case control studies; - studies performed in humans; - published in English; - with a valid caries diagnostic with an identified scoring method; - microbial identification method based on metagenomic analysis.	- systematic reviews of clinical trials; - *in vitro* studies; -population with systemic pathologies that could influence results: diabetics, immunosuppressed or polymedicated patients; - studies in individuals under 13 years old; - studies that did not meet all the inclusion criteria.	JBI
2- WSL: Diagnosis and Treatment. **OSF Registries:** osf.io/9k8fw/	Search conducted in **Pubmed** and **Scopus** databases with the following query: **("WSL" OR "White spots") AND (dental caries OR caries) AND (diagnose AND treatment).**	"In patients with WSL, the new diagnostic tools in comparison with conventional ones, there is a different treatment performed?"	Articles published between 2012 and 2023.	- RCT, cross- sectional and longitudinal studies; - studies performed in humans; - published in English; - being about WSL; - discuss both diagnosis and treatment.	- systematic reviews of clinical trials; - *in vitro* studies; - studies that did not meet all the inclusion criteria.	RoB tool
3- Unlocking the Potential Of Probiotic Administration In Caries Management **OSF Registries:** osf.io/d2wa4	Search performed in **Pubmed, Scopus, Web of Science** and **Cochrane** database with the following query: **(caries OR dental caries OR tooth decay) AND (probiotics OR prebiotics).**	"In individuals with caries, after probiotic administration, is there an improvement in outcomes directly related to caries risk and development?"	Articles published between November 2012 and November 2023.	- RCT and clinical trials; - studies performed in humans; - published in English; - studies on patients with caries; - clear indication of probiotic used; - measurement of outcomes directly involved with cariogenic process and quantification of bacteria with cariogenic potential: *Streptococcus, Lactobacillus, Actinomyces, Prevotella* and *Scardovia*.	- systematic reviews of clinical trials; - *in vitro* studies; - population with systemic pathologies that could influence results: diabetics, immunosuppressed or polymedicated patients; - Studies that did not meet all the inclusion criteria.	JBI

RCT - *randomized controlled trial;* RoB- *Revised Cochrane risk of bias tool*; JBI- *Joanna Briggs Institute*

In all the systematic reviews at least 2 reviewers independently performed the electronic and manual search. Search results were imported into Rayyan [107] where duplicates were identified and deleted. Studies that did not meet all the inclusion criteria were excluded. Articles were screened by tittle and abstract for eligibility and when necessary further exclusions were done. Finally, extraction tables were built with the relevant information to answer each PICO question.

2.2 Oral microbiome profile of patients using clear aligners: the cariogenic and carioprotective species

2.2.1 Study design

This study was carried out in three private clinics, in Viseu, Leiria and São João da Madeira, with the support of SalivaTec, from the Centre for Interdisciplinary Research in Health (CIIS) and the Faculty of Dental Medicine of the Universidade Católica Portuguesa (FMD-UCP).

This longitudinal study was approved by the Ethics Committee for Health of the Universidade Católica Portuguesa in the scope of the project "Evolução da Microbiota Oral durante o tratamento ortodôntico" (Project number 62). All patients eligible for this investigation completed and signed the Free and Informed Consent Form (TCLE), which is attached in the supplementary materials. The biological sample collection protocols do not cause any discomfort to the patient and are described in this study. Subjects were screened and recruited by the investigators at their practice.

Twenty-four participants (11 men and 13 women with an average age of 27.6 years) starting orthodontic treatment with clear aligners were recruited and informed consent was obtained from each subject. All patients used the same brand of clear aligner (Invisalign®).

The inclusion criteria required participants in good general health. Participants with active dental caries, periodontal disease, chronic systemic diseases and other medical conditions or who used antibiotics within 30 days of their treatment were excluded from the study, as well as pregnant women and smokers.

The study visits were planned at the start of orthodontic treatment (T0), with follow-up appointments scheduled at 1 (T1), 3 (T2) and 6 months (T3), as seen in Figure

1. Saliva samples and supragingival plaque samples were collected from the anterior and posterior teeth, following the protocols established in SalivaTec [110]. Patients who did not complete at least 2 visits, who received antibiotic therapy or who started smoking during the treatment were excluded from the study. The final sample consisted of 12 patients, with a drop-off of 50%. Patients drop out resulted from antibiotic therapy (6), patient started smoking (3), patient didn't continue treatment (2) and patient became pregnant (1). In all patients and sampling times, the risk of caries was assessed using the CAMBRA protocol [48], with no record of any caries.

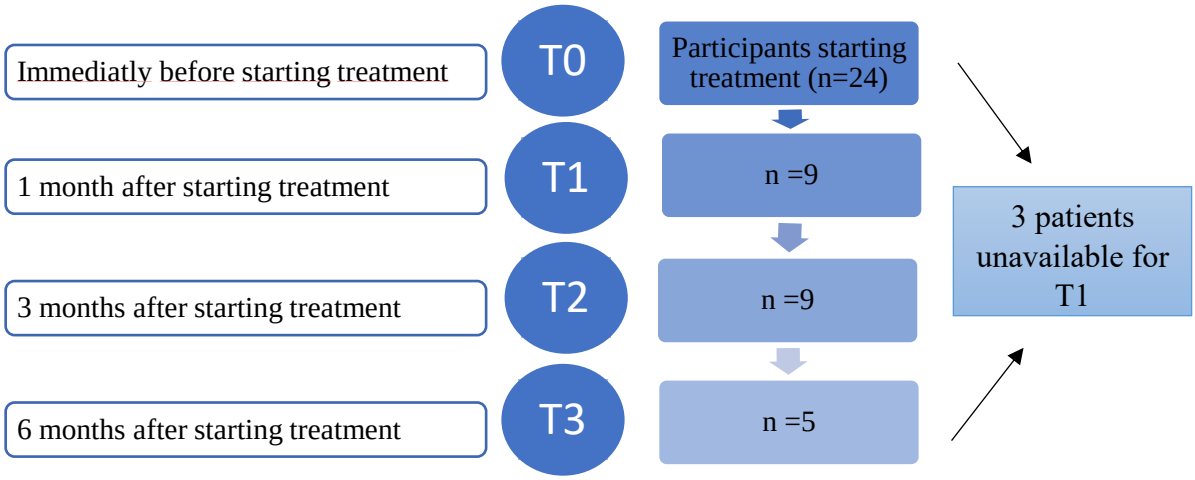

Figure 1- Flow diagram showing the progression of patients through the longitudinal study.

2.2.2 Sample's characterization

Demographic and clinical data were collected (Table 2), as well as biological samples of saliva and oral biofilm that comply with current standards. Saliva and biofilm samples were analysed exclusively in the trusted laboratories of the UCP Faculty of Dental Medicine "SalivaTec" and guaranteed the maintenance of the confidentiality of data collected.

Table 2- Observational study sample characterization.

Characteristics	Initial sample	Final sample
Entire sample	24 (100%)	12 (100%)
Age (Mean, Range)	27.6 (11-47)	27.1 (12-40)
Gender		
- Male	11 (46%)	6 (50%)
- Female	13 (54%)	6 (50%)

2.2.3 Saliva and biofilm collection and processing

All biological samples collected were handled in accordance with the guidelines approved by the National Health Authorities and whose procedure was approved by the UCP Health Ethics Committee. Saliva samples were collected by the "drooling" method, the user expelled (one or more times) saliva into a 50 ml Falcon-type tube until it reached approximately 3 ml. Tubes were well sealed and disinfected externally with sodium hypochlorite solution, in the original concentration of free chlorine at 5 %, at a dilution of 1/50 (1 part of bleach in 49 equal parts of water) and then with alcohol at 70 %. Finally, the lid of the tubes was sealed with Parafilm® Sealing Film, and the tube duly identified and placed in its own container.

Biofilm collection was carried out using a sterile toothpick, which was used to scrape the dental plaque (oral biofilm) in predefined areas of the patient's oral cavity.

Samples were collected from the following locations/points: gingival margin on lower incisors, by lingual; gingival margin on upper incisors, buccally; gingival margin on upper first molar buccally; gingival margin on lower first molar by lingual.

In case of absence of permanent molars, collection was carried out on the most posterior tooth. In no instance was there a total absence of incisor teeth.

The toothpicks with the collected biofilm were subsequently inserted into 1.5 mL microtubes containing phosphate-buffered saline solution. All samples were immediately placed on ice to minimize the degradation of genetic material. In SalivaTec laboratory, biological samples were aliquoted into microtubes, with each tube containing a maximum of 500 µL and a minimum of 200 µL. All this process was carried out in a flow chamber to avoid any contamination. After this procedure, all the samples were stored at -80 °C.

2.2.4 Sequencing and analysis

Bacterial DNA was extracted from saliva and biofilm samples using the KingFisher magnetic particle processor (Thermo Electron, Vantaa, Finland) with MagMAX Microbiome Ultra Nucleic Acid Isolation Kit accordingly with manufacturer instructions. A volume of 400 µL or 500 µL of saliva and biofilm, respectively, were added to the bead tubes containing 800 µL of lysis buffer. To allow a complete sample lysis, two successive rounds of 30 seconds bead beating were performed using a 4-Place Mini Bead Mill Homogenizer (VWR®). After centrifugation at 14 000 x*g* for 2 min, bacterial DNA was purified following the kit manufacturer's instructions. In the end, DNA was quantified using the µDrop Plate (Thermo Fisher Scientific) and sent to sequencing of the 16S rDNA.

Before sequencing, hypervariable regions of the 16S rDNA gene were amplified by PCR using 2 sets of primers (primer set V2-4-8; primer set V3-6,7-9) and the kit Ion 16S Metagenomics Thermo Fisher (Catalog number: A26216) according to the manufacturer instructions. Then, PCR fragments were sequenced using the Ion PGM™ Sequencing 400 Kit (~400 000 reads/sample) on the Ion PGM™ platform. Sequencing data analysis was performed using the Ion 16S™ metagenomics analyses module within the Ion Reporter™ software (Thermo Fisher, https://ionreporter.thermofisher.com/ir/).

3. Results

3.1 Is oral biofilm different in caries?

One of the main goals of this thesis was to assess how the growing knowledge on Oral Microbiology and specifically Oral Microbial Ecology can be used to manage caries. Hence, the undertaken work focused on a literary search on current knowledge on oral biofilm composition and dynamics. The objective was to know whether molecular knowledge of oral microbiome reveals differences between the microbiome of individuals with and without caries. A systematic review on the microbial biofilm associated with caries was performed to answer the PICO question formulated as "Which are the most prevalent microbial genera/species in oral microbiota of individuals with caries scored by DMFT index or ICDAS when compared to individuals without caries?"

The search performed in Pubmed and Cochrane databases returned 289 and 27 unique entries respectively. To carry out a comparative analysis of the results, only "case-control" studies were considered. Therefore, 49 articles were excluded to wrong study design, since they are not case control studies and 90 were excluded because they were reviews. Sixteen articles were excluded for being *in vitro* and 15 studies were excluded because they did not use culture independent microbial identification methods. Thirty-six studies on populations with diseases or conditions identified such as diabetes, HIV or any other systemic or oral disease were not included. One article was excluded because it was not in English, 6 were excluded because they were not conducted in humans and 19 because they were not related to caries research. In total, after 2 reviewers examined the title and abstract, 77 articles were selected for full text analysis. From these, 64 were further excluded and 13 remained (Figure 2). Articles were excluded based on various criteria: 18 were disregarded due to utilizing study designs other than case-control, 13 were not considered because they did not assess caries or identify microbial species, 11 were rejected for using culture-based microbial identification methods, and 10 were excluded for involving populations with systemic diseases. Finally, a total of 13 studies (Table 3) met the inclusion criteria and were considered in the review.

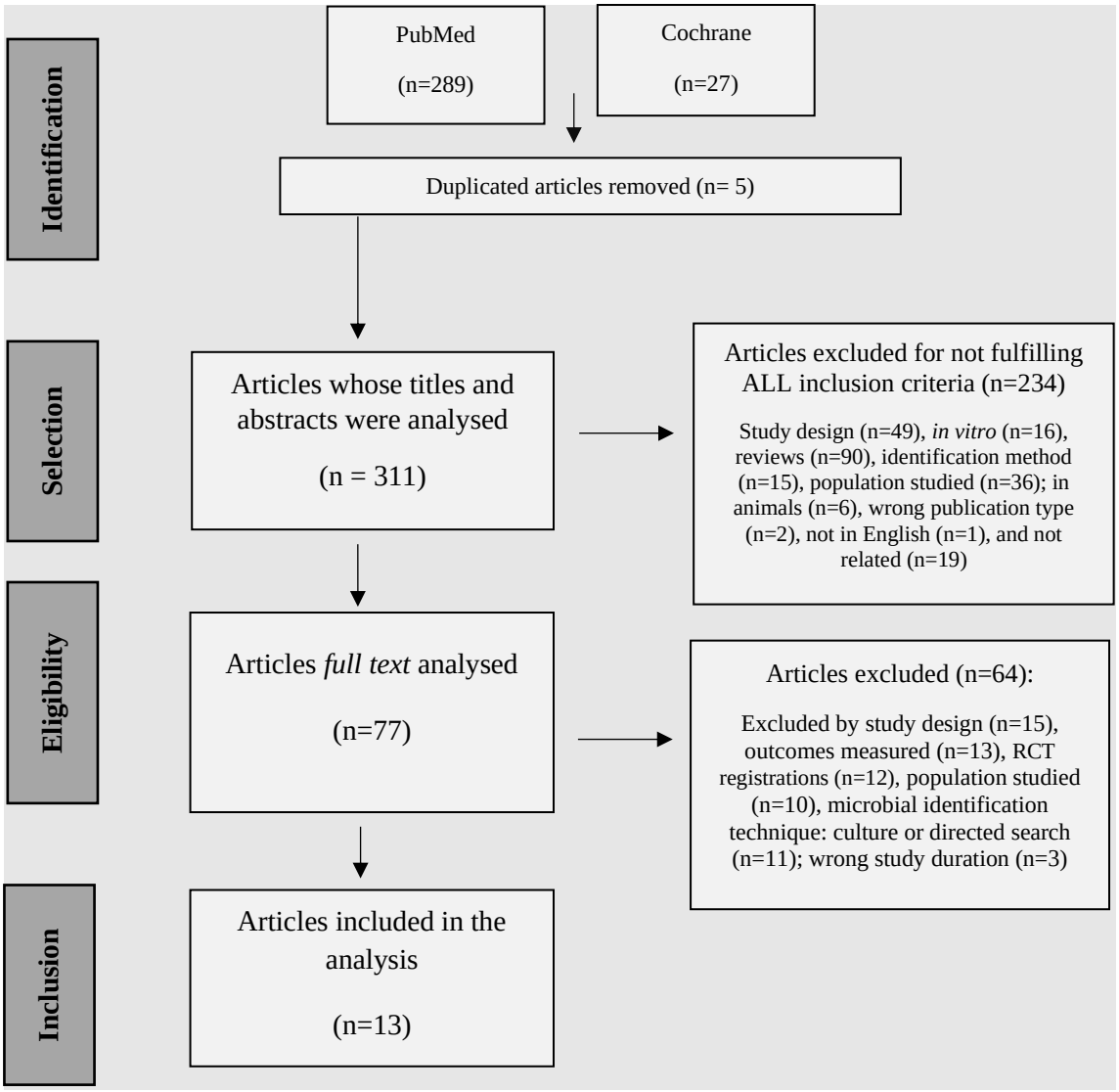

Figure 2- Overview of article selection procedure according to PRISMA guidelines [106], answering the PICO question: "Which are the most prevalent microbial genera/ species in oral microbiota of individuals with caries scored by DMFT index or ICDAS when compared to individuals without caries?".

Table 3- Summary of the studies included in the analysis.

Author, Year	Age	Sample	Caries scoring criteria	Sequencing approach	Sequencing platform
Shao *et al*, 2023	18-29	Biofilm	ICDAS II	16s rRNA (V3-V4 region)	Illumina
Corralo *et al*, 2021	13-76	Biofilm	WHO	Whole genome metagenomic library	Illumina HiSeq 3,000
Havsed *et al*, 2021	14-18	Biofilm	WHO	16s rRNA (V3-V4 region)	Illumina® MiSeq
Johansson *et al*, 2016	17	Biofilm	WHO	16s rRNA (V1-V4 region)	FLX+ pyrosequencing
Celik *et al*, 2021	18-50	Biofilm	ICDAS II	16s rRNA (V3-V4 region)	Illumina® MiSeq V3
He *et al*, 2018	22-55	Biofilm	WHO	16s rRNA	Illumina MiSeq
Wolf *et al*, 2019	20-68	Biofilm	WHO	16s rRNA (V4 region)	Illumina MiSeq
Pang *et al*, 2021	12-13	Biofilm	WHO	No info	Illumina Nova Seq
Foxman *et al*, 2016	1-53	Saliva	WHO	16s rRNA (V6 region)	Illumina® MiSeq
Yasunaga *et al*, 2017	20-28	Saliva	WHO	16s rRNA (V1-V2 region)	Barcoded pyrosequencing
Yama *et al*, 2023	43.7 (mean healthy) and 42.4 (mean caries)	Saliva	WHO	16s rRNA (V1-V2 region)	Illumina® MiSeq V3
Erikson *et al*, 2017	17-19	Saliva + Biofilm	Color and X ray	16s rRNA (V3-V4 region)	Illumina MiSeq
Frese *et al*, 2022	>18	Saliva	ICDAS II	16s rRNA (V4 region)	Illumina MiSeq

In the articles considered in the review only one analyzed simultaneously saliva and biofilm samples [111] although identification was done only to the genus level. The article from Foxman and co-workers 2016 [112] compared the caries free and caries group but only achieved identification to the phylum level and therefore is not represented in the data extraction tables.

The quality of 13 studies included was analyzed using the JBI critical appraisal checklist for analytical cross-sectional studies [108]. All aspects of the analysis were fulfilled by all articles except for the consideration of the confounding factors in the analysis, in which 1 of the 13 articles Johansson and coworkers (2016) [113], does not include the confounding factors as stratification variables in the analysis, as reported in Table 4.

Table 4 - Quality analysis of the studies, using the critical assessment tool - Joanna Briggs Institute.

Author	1. Were the criteria for inclusion in the sample clearly defined?	2. Were the study subjects and the setting described in detail?	3. Was the exposure measured in a valid and reliable way?	4. Were objective, standard criteria used for measurement of the condition?	5. Were confounding factors identified?	6. Were strategies to deal with confounding factors stated?	7. Were the outcomes measured in a valid and reliable way?	8. Was appropriate statistical analysis used?
Celik *et al*, 2021	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Corralo *et al*, 2021	**Yes**	**In part**	**In part**	**Yes**	**In part**	**In part**	**Yes**	**Yes**
Eriksson *et al*, 2017	**In part**	**In part**	**In part**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Foxman *et al*, 2016	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Frese *et al*, 2022	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Havsed *et al*, 2021	**Yes**	**Yes**	**In part**	**Yes**	**Yes**	**In part**	**Yes**	**Yes**
He *et al*, 2018	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Johansson *et al*, 2016	**Yes**	**In part**	**In part**	**Yes**	**No**	**No**	**Yes**	**Yes**
Pang *et al*, 2021	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**In part**	**Yes**	**Yes**
Shao *et al*, 2023	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Wolff *et al*, 2019	**Yes**	**In part**	**Yes**	**Yes**	**Yes**	**In part**	**Yes**	**Yes**
Yama *et al*, 2023	**Yes**	**Yes**	**In part**	**In part**	**Yes**	**Yes**	**Yes**	**Yes**
Yasunaga *et al*, 2017	**Yes**	**In part**	**In part**	**Yes**	**Yes**	**In part**	**Yes**	**Yes**

Upon the analysis of the genus lists provided in the articles it was possible to determine that microorganisms most often mentioned in biofilm as increased in caries status are *Actinomyces*, *Lactobacillus*, *Olsenella*, *Prevotella*, *Propionibacterium*, *Streptococcus*, *Treponema*, and *Veillonell*a as seen in Table 5. These genera are referred by more than one article. However, as shown in Table 5 there are genera found as significantly increased by only one study. On the other hand, the majority microorganism genera cited present in biofilm of caries free individuals are *Actinomyces*, *Cardiobacterium*, *Fusobacterium*, *Selenomonas, Haemophilus*, *Leptotrichia*, *Prevotella* and *Streptococcus*[114–116]. It is interesting to note that 3 genera are common for the two groups (*Actinomyces, Prevotella,* and *Streptococcus*) although in the case *Streptococcus* and *Prevotella* are most often cited as present in individuals with caries. Regarding saliva samples of individuals with and without caries (Table 6), only two articles referred the genus increased in the study population. Thus, *Alloprevotella*, *Fusobacterium*, *Gemella*, *Haemophilus*, *Neisseria*, *Prevotella* and *Veillonella* were increased in healthy individuals and *Fretibacterium*, *Lactobacillus*, *Leptotrichia*, *Rothia*, *Spirochaetes*, *Streptococcus* and *Veillonella* were increased in caries status. In this case, only *Veillonella* appears as increased in the two groups analyzed.

Table 5- Genera increased in biofilm samples of caries *vs* healthy group. Caries group includes individuals with active caries and healthy group individuals with no active caries regardless of caries experience.

Genus	Healthy	Caries
Actinomyces	Celik *et al*, 2021, Johansson *et al*, 2016, Corralo *et al*, 2021	Shao *et al*, 2023, Johansson *et al*, 2016
Atopobium	He *et al*, 2018	
Bergeriella		He *et al*, 2018
Bifidobacterium		Celik *et al*, 2021
Campylobacter	Johansson *et al*, 2016	
Capnocytophaga		Johansson *et al*, 2016
Cardiobacterium	Pang *et al*, 2021, He *et al*, 2018, Celik *et al*, 2021	
Corynebacterium	Corralo *et al*, 2021	
Fusobacterium	Pang *et al*, 2021, Shao *et al*, 2023, Johansson *et al*, 2016, Corralo *et al*, 2021	Johansson *et al*, 2016
Granulicatella	Celik *et al*, 2021	
Haemophilus	Celik *et al*, 2021, Shao *et al*, 2023	
Lactobacillus		Pang *et al*, 2021, He *et al*, 2018, Celik *et al*, 2021
Leptotrichia	Shao *et al*, 2023, Johansson *et al*, 2016	
Microcell	Pang *et al*, 2021	
Neisseria		Corralo *et al*, 2021
Olsenella		Pang *et al*, 2021, Celik *et al*, 2021
Parascardovia		Pang *et al*, 2021
Porphyromonas		Johansson *et al*, 2016
Prevotella	Johansson *et al*, 2016, Corralo *et al*, 2021	Pang *et al*, 2021, Celik *et al*, 2021, Johansson *et al*, 2016
Propionibacterium	Corralo *et al*, 2021	Pang *et al*, 2021, Celik *et al*, 2021
Rhodocyclaceae		Shao *et al*, 2023
Rothia	Celik *et al*, 2021	
Ruthenibacterium	Pang *et al*, 2021	
Scardovia		Pang *et al*, 2021
Schwartzia		Celik *et al*, 2021
Selenomonas	Shao *et al*, 2023, Corralo *et al*, 2021, Johansson *et al*, 2016	He *et al*, 2018
Shuttleworthia	Celik *et al*, 2021	
Streptococcus	Celik *et al*, 2021, Johansson *et al*, 2016	Pang *et al*, 2021, Johansson *et al*, 2016, Corralo *et al*, 2021
Succiniclasticum	Pang *et al*, 2021	
Tannerella	Corralo *et al*, 2021	
TM7		Shao *et al*, 2023
Treponema		He *et al*, 2018, Celik *et al*, 2021, Johansson *et al*, 2016
Veillonella		Johansson *et al*, 2016, Corralo *et al*, 2021

Table 6- Genera increased in saliva samples of caries *vs* healthy group. Caries group includes individuals with active caries and healthy group individuals with no active caries regardless of caries experience.

Genus	Healthy	Caries
Alloprevotella	Frese *et al*, 2022	
Fretibacterium		Frese *et al*, 2022
Fusobacterium	Yasunaga *et al*, 2017	
Gemella	Yasunaga *et al*, 2017	
Haemophilus	Yasunaga *et al*, 2017	
Lactobacillus		Frese *et al*, 2022
Leptotrichia		Frese *et al*, 2022
Neisseria	Yasunaga *et al*, 2017	
Prevotella	Frese *et al*, 2022	Yasunaga *et al*, 2017
Rothia		Yasunaga *et al*, 2017
Spirochaetes		Frese *et al*, 2022
Streptococcus		Yasunaga *et al*, 2017

Regarding microbial species quantified in biofilm samples (Table 7) of caries situations *vs* healthy (caries free) group, it was possible to find different species significantly increased in the caries group such as *Cryptobacterium curtum*, *P. denticola*, *Shuttleworthia satelles* and *S. mutans*. In caries free group, the most prevalent species found in biofilm samples were *Actinomyces johnsonii (A. johnsonii)*, *Cardiobacterium hominis* (*C. hominis)*, *Corynebacterium matruchotii (C. matruchotii)*, *F. nucleatum*, *Gemella sanguinis*, *Neisseria elongata* (*N. elongata*) and *R. dentocariosa*.

In saliva samples, data on the species found increased in individuals with and without caries are scarce with only one article presenting this information [117](Table 1 in Supplemental materials). Thus, *Atopobium parvulum*, *Atopobium rimae*, *Bifidobacterium dentium*, *Dialister invisus*, *Filifactor alocis*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Megasphaera micronuciformis*, *Parascardovia denticolens*, *Parvinomonas micra*, *Porphyromonas endodontalis*, *Prevotella multiformis*, *Prevotella multisaccharivorax (P. multisaccharivorax*), *Prevotella ceroralis*, *Prevotella histicola* (*P. histicola*), *Prevotella oris*, *Prevotella oulorum*, *Prevotella salivae*, *Prevotella veroalis*, *Selenomonas dianae*, *Shaalia odontolytica*, *Streptococcus cristatus* (*S.cristatus*), *Streptococcus lactarius*, *Streptococcus parasanguinis* (*S. parasanguinis*), *S. salivarius*, *T. forsythia*, *V. parvula* and *V. dispar* are the species referred as increased in saliva samples in the caries group [117].

The analysis of the results shows that *P. denticola* [117–119] is the only specie found in both biofilm samples and saliva from individuals with caries.

Table 7- Species increased in biofilm samples of caries *vs* healthy group. Caries group includes individuals with active caries and healthy group individuals with no active caries regardless of caries experience.

Species	Health	Caries
Abiotrophia defectiva		(Havsed et al, 2021)
Actinomyces johnsonii	(Pang et al, 2021)	
Anaeroglobus geminatum		(Wolff et al, 2019)
Atopobium parvulum		(Wolff et al, 2019)
Atopobium rimae		(Wolff et al, 2019)
Bifidobacterium dentium		(Wolff et al, 2019)
Cardiobacterium hominis	(Pang et al, 2021)	
Comamonas testosteroni		(Wolff et al, 2019)
Corynebacterium durum	(Havsed et al, 2021)	
Corynebacterium matruchotii	(Havsed et al, 2021)	
Cryptobacterium curtum		(Wolff et al, 2019), (Havsed et al, 2021)
Dialister invisius		(Wolff et al, 2019)
Dialester pneumosintes		(Havsed *et al*, 2021)
Eubacterium infirmum		(Wolff et al, 2019)
Fusobacterium nucleatum	(Pang et al, 2021)	
Gemella sanguinis	(Havsed et al, 2021)	
Lactobacillus casei		(Wolff et al, 2019)
Lactobacillus parafarraginis		(Wolff et al, 2019)
Lactobacillus salivarius		(Wolff et al, 2019)
Lactobacillus vaginalis		(Wolff et al, 2019)
Lactobacillus gasseri		(Wolff et al, 2019)
Leptotrichia bucallis		(Havsed et al, 2021)
Leptotrichia shahiii		(Havsed et al, 2021)
Mogibacterium muscum		(Wolff et al, 2019)
Neisseria elongata	(Pang et al, 2021)	
Olsenella profusa		(Wolff et al, 2019)
Parascardovia denticolens		(Wolff et al, 2019)
Peptostreptococcus stomatitis		(Havsed et al, 2021)
Porphyromonas endodontalis		(Havsed et al, 2021)
Porphyromonas pogonae		(Shao et al, 2023)
Prevotella multisaccharivorax		(Wolff et al, 2019)
Prevotella aurantiaca		(Shao et al, 2023)
Prevotella denticola		(Wolff et al, 2019) , (Havsed et al, 2021)
Prevotella histicola		(Wolff et al, 2019)
Prevotella maculosa		(Havsed et al, 2021)
Prevotella oralis		(Wolff et al, 2019)
Prevotella salivae		(Wolff et al, 2019)

Species	Health	Caries
Propionibacterium acidifaciens		(Wolff et al, 2019)
Pseudomonas pseudoalcalinogenes		(Wolff et al, 2019)
Pseudopropionibacterium rubrum		(Shao et al, 2023)
Pseudoroamibacter alactolyticus		(Wolff et al, 2019)
Rothia dentocariosa	(Havsed *et al*, 2021)	
Selenomonas putigena		(Shao et al, 2023)
Shuttleworthia satelles		(Wolff et al, 2019), (Havsed et al, 2021)
Staphylococcus warnerii		(Wolff et al, 2019)
Streptococcus mutans		(Wolff et al, 2019), (Shao et al, 2023)
Treponema vincentii		(Havsed et al, 2021)
Veillonella dispar		(Wolff et al, 2019)

3.2 White spot lesions: Diagnosis and Treatment – a Systematic Review

Detecting white spot lesions, which are the initial lesions of caries, is crucial for minimizing the impact of the carious process and may influence treatment decisions. To comprehensively assess current methods and strategies for identifying these initial caries lesions and to evaluate the effectiveness of various treatment options available, a systematic review on the diagnosis and treatment of WSL was performed. The objective of this study is to offer insights into optimal approaches for early detection and intervention, with the ultimate goal of enhancing patient outcomes and reducing the advancement of caries. The search results are presented in

Figure 3. One hundred and forty-three potentially relevant references were identified, with 99 publications from the PubMed database, 33 from Scopus and 11 from manual search to consider grey literature. Two duplicates were found and excluded. Based on the information provided in the title and abstract, 90 articles were considered ineligible. The main reasons for non-inclusion were: (1) being a systematic review, (2)

being *in vitro* and (3) not being about WSL. Fifty-one articles were analysed in full to collect more detailed information and further 29 studies were excluded for the following reasons: (1) not having full text available and (2) not having diagnosis and treatment. Finally, after applying quality assessment tools (k = 0.91), 20 studies were included in the following review, with publication dates between 2012 and 2021 were included, 3 of which were published in 2012, 4 in 2013, 2 in 2014, 2 in 2016, 1 in 2017, 3 in 2018, 4 in 2020 and 1 in 2021. Table 8 shows the characteristics of the 20 studies selected. The different studies were carried out in different and varied countries such as the United States, Switzerland, Turkey, Norway, Egypt, and India. The number of individuals in each study varied from just 5 to 115 people. The participants age varied, from children aged 8 to adults aged 27. Intervention times were also not uniform and varied between a week and 18 months.

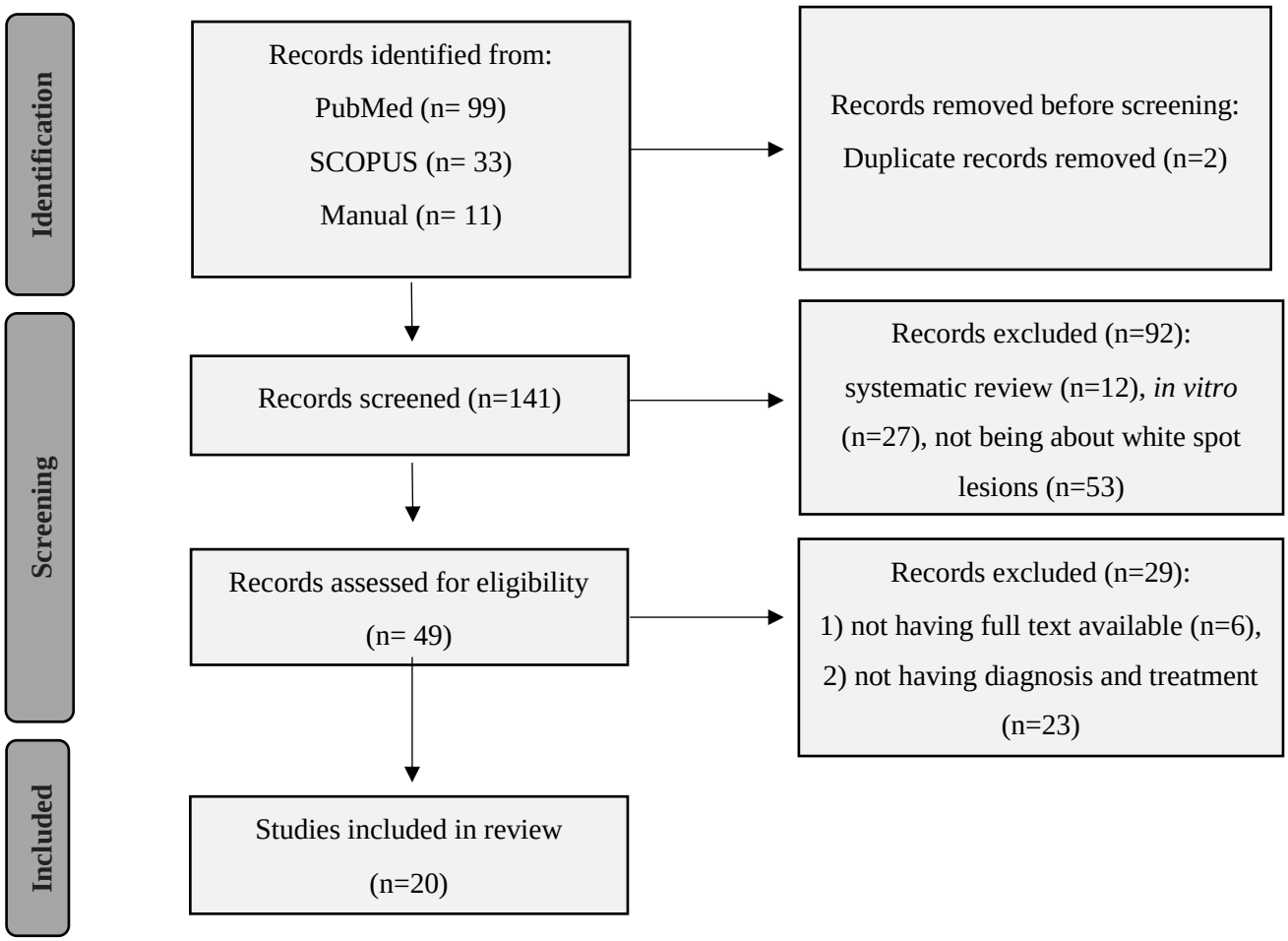

Figure 3- Overview of article selection procedure according to PRISMA guidelines [106], to address the PICO question: “Will new diagnosis tools have the potential to change white spot’s conventional treatment?”.

For the risk of bias analysis, RoB tool was used [109], evaluating articles in five different domains. As seen in Table 8, most of the studies selected are RCT [122–133]. There are also cross-sectional studies [134–138], longitudinal studies [139,140] and only one case report [141]. Visual and fluorescence are the most frequent diagnostic techniques, but other techniques such as clinical photographs [122,123] and DIAGNOdent [131–133] are also used. Interestingly, one study reported the use of two techniques: visual and fluorescence and fluorescence and transverse microradiography [130].

Most of the studies reported the use of infiltrating resin and FV as treatment techniques [123,124,126,130–132,136–141]. However, the treatment with self-assembling peptide P11-4 (SAPP11-4) [122], home care [134], Elmex® fluid [125], CPP-ACP [127,133], fluoride gel [128], minimally invasive (MI) paste plus and MI varnish [129] and hydrochloric acid (HCL) [135] were taken in to account.

Regarding the intervention time, the discrepancy between the studies is obvious. The study with lower intervention time reported the use of treatment techniques for 1 week [141]. However, more robust studies within 3, 5, 6, 9, 12 or 18 months of using the treatment techniques [122,124,125,128,129,132–135,140] are the majority of cases.

The impact of treatment considering diagnostic techniques was evaluated in a range of different cohorts, as children, adolescents, and adults all in good state of general systemic health. The studied population has several characteristics related to orthodontic treatment [125,127,128,131,133,134,136,138,141], MIH [138,141] and caries [122,137,140]. The caries identification and evaluation method were mostly accessed by the ICDAS system, however other clinical evaluation criteria were also considered.

In the various studies included, different ways of carrying out the diagnosis and treatment were used. Thus, most studies showed positive results, in which the treatment methods used improved or even reversed the white spot lesion on tooth surfaces. Only one study did not show significant statistical differences in treatment with the use of fluoride gel [128].

The use of SAPP11-4 was found to be successful compared to the use of FV alone in regressing dental caries [122]. In all studies carried out with infiltrating resin, it presented good clinical results in the aesthetic improvement of the white spot lesion and tooth remineralization [123,136–138,141,142]. Compared to the use of FV, it may even provide better evidence than FV [124], although a study mentioned the opposite [130]. Some studies report that lesion improvement with infiltrating resin is related to its depth [139].

Regarding the use of FV, the study that resorted only to this form of treatment showed good results, stating that there is a positive change at the mineral level and that it is a good way to prevent the existence of dental caries [131]. Although the difference is not very significant when applied to patients with good oral hygiene [132]. This is also reported in another study which refers that the use of FV compared to home care does not present major differences in the treatment of WSL [126]. Likewise, it is reported that an application of FV before orthodontic treatment as prevention is not relevant [125]. Fluoride-based products and home care have proven to be helpful in treating injuries, but compliance by the patients themselves also must be good [129,134].

The use of CPP-ACP had good remineralization effect in WSL [127,133]. The use of HCL also showed good results for the treatment of WSL, despite the percentage of HCL being related to the amount of enamel removal [130].

Table 8- Characteristic of the 20 studies selected to answer this PICO question.

Author	Study design	Type of participants	Intervention time	Diagnostic	Treatment	Outcomes	Conclusions
Sedlakova Kondelova *et al*, 2020	RCT	Patients presenting 2 teeth with WSL	3 months	Clinical photographs	SAP P11-4	SAP P11-4 lesions showed significant WSL size reduction compared to FV alone	Treatment of early buccal carious lesions with SAPP11-4 led to superior regression of caries decay compared to either placebo or FV
Senestraro *et al*, 2013	RCT	Orthodontic patients	8 weeks	Clinical photographs	IR	The results showed a mean reduction in WSL area of 61.8 percent immediately after treatment and 60.9 percent eight weeks later	Resin infiltration significantly improved the clinical appearance of WSL.
Ciftci *et al*, 2018	RCT	No info	3 months	Visual	IR, FV	A significant decrease in DIAGNOdent Pen scores was observed in all the groups	The IR application was more successful than FV on WSL
Hadler-Olsen *et al*, 2012	Cross-sectional	Orthodontic patients	18 months	Visual	Home care	23% of treated patients showed good compliance, 68% moderate compliance, and 9% poor compliance.	Individuals with good adherence developed fewer new WSL than individuals with poor adherence
Kirschneck *et al*, 2016	RCT	Adolescent orthodontic patients	20 weeks	Visual	Elmex® fluid and Fluor Protector S	Each treatment group showed a significant increase of the ICDAS index	A one-time application of FV at the start of orthodontic treatment did not provide any additional preventive advantage
Huang, *et al* 2013	RCT	Orthodontic patients	8 weeks	Visual	MI paste plus, FV	improvements in the affected surface were 16%, 25% and 17% in the MI Paste Plus, FV and control groups.	MI Paste Plus and FV do not appear to be more effective than normal home care for improving the appearance of WSL over an 8-week period.
Hammad *et al*, 2012	Longitudinal	Orthodontic patients	No info	Visual	IR	Results after Icon application showed that around 65-76% of the surface area of the WSL was masked	The masking effect depends on lesions depths
Krithikadatta *et al*, 2013	RCT	Patients w/occlusal WSL	30 days	Visual	CPP-ACP, CCP-ACP with fluoride, NaF mouthwash	All three remineralizing agents heal WSL	All three remineralizing agents heal WSL.

RCT - randomized controlled trials; WSL – White spot lesions; SAPP11-4 - self-assembling peptide P11-4; FV – fluor varnish; IR – infiltrative resin; ICDAS - International Caries Detection and Assessment System; MI – Minimally invasive ; CPP-ACP – Casein phosphopeptide-amorphous calcium phosphate; NaF- sodium fluoride.

Table 8- Characteristic of the 20 studies selected to answer this PICO question. (cont.).

Author	Study design	Type of participants	Intervention time	Diagnostic	Treatment	Outcomes	Conclusions
Bock *et al*, 2017	RCT	Orthodontic patients	24 weeks	Visual	Fluoride gel	No statistically significant group difference existed	No significant positive effect of high-dose fluoride on post-orthodontic WSL development could be detected
Rechmann *et al*, 2018	RCT	Orthodontic patients	12 months	Visual	MI paste plus, MI varnish	Salivary fluoride levels were significantly higher at 12 months for the experimental than for the control group	Applying daily MIPP resulted in no statistically significant differences in ICDAS
Roig-Vanaclocha *et al*, 2020	Cross-sectional	No info	No info	Visual	HCL	When each application was evaluated with the initial situation of the untreated tooth, we observed that 6.6% HCl removes more enamel than 15% HCL	Both HCl-based products are adequate options for treating WSL
Giray *et al*, 2018	RCT	Permanent teeth in children	6 months	Visual and fluorescence	IR, FV	The values of the RV group were statistically lower than those of the FV group	IR and FV are clinically feasible and efficacious methods for the treatment of anterior WSL
Marouane *et al*, 2021	Cross-sectional	Patients with MIH on anterior teeth	No info	Fluorescence	IR	A non-linear correlation was observed between resin application, and MIH decreasing over time	MIH-lesion type and the 'ethanol test' were reliable predictive factors for the application time needed for infiltrating MIH lesions on permanent anterior teeth
Marouane *et al*, 2020	Case report	Patients with enamel opacities	1 week	Fluorescence	IR	The lesions showed a clearly improved aesthetic integration and had almost disappeared	Transillumination was also reliable in monitoring the progression of the infiltration until complete saturation of the porous enamel

RCT - randomized controlled trials; MIH - Molar incisor hypomineralization; MI – Minimally invasive; HCL - Hydrochloric acid; FV – fluor varnish ;IR – infiltrative resin; WSL – WSL ; ICDAS - International Caries Detection and Assessment System.

Table 8- Characteristic of the 20 studies selected to answer this PICO question. (cont.).

Author	Study design	Type of participants	Intervention time	Diagnostic	Treatment	Outcomes	Conclusions
Bock *et al*, 2017	RCT	Orthodontic patients	24 weeks	Visual	Fluoride gel	No statistically significant group difference existed	No significant positive effect of high-dose fluoride on post-orthodontic WSL development could be detected
Rechmann *et al*, 2018	RCT	Orthodontic patients	12 months	Visual	MI paste plus, MI varnish	Salivary fluoride levels were significantly higher at 12 months for the experimental than for the control group	No statistically significant differences in ICDAS between groups
Roig-Vanaclocha *et al*, 2020	Cross-sectional	No info	No info	Visual	HCL	When each application was evaluated with the initial situation of the untreated tooth, we observed that 6.6% HCl removes more enamel than 15% HCl	Both HCl-based products are adequate options for treating WSL
Giray *et al*, 2018	RCT	Permanent teeth in children	6 months	Visual and fluorescence	IR, FV	The values of the experimental group were statistically lower than those of the FV group	IR and FV are clinically feasible and efficacious methods for the treatment of anterior WSL
Marouane *et al*, 2021	Cross-sectional	Patients with MIH on anterior teeth	No info	Fluorescence	IR	A non-linear correlation was observed indicating that at the beginning of resin application, it will decrease over time	MIH-lesion type and the 'ethanol test' were reliable predictive factors for the application time needed for infiltrating MIH lesions on permanent anterior teeth
Marouane *et al*, 2020	Case report	Patients with enamel opacities	1 week	Fluorescence	IR	The lesions showed a clearly improved aesthetic integration and had almost disappeared	Transillumination was also reliable in monitoring the progression of the infiltration until complete saturation of the porous enamel

RCT - randomized controlled trial; MI – Minimally invasive; HCL - Hydrochloric acid; FV – fluor varnish; IR – infiltrative resin; ICDAS - International Caries Detection and Assessment System; WSL – WSL; MIH - Molar incisor hypomineralization.

In the following figures, it is possible to observe, the number of studies that used the different diagnostic methods (**Erro! A origem da referência não foi encontrada.**) and treatments (**Erro! A origem da referência não foi encontrada.**). Regarding diagnostic methods, 2 of the 20 used clinical photographs as a diagnostic method, 9 used visual examination, 5 used fluorescence, 1 used microcomputed tomography and 3 used DIAGNOdent. A combination of two methods, visual examination, and fluorescence was also used.

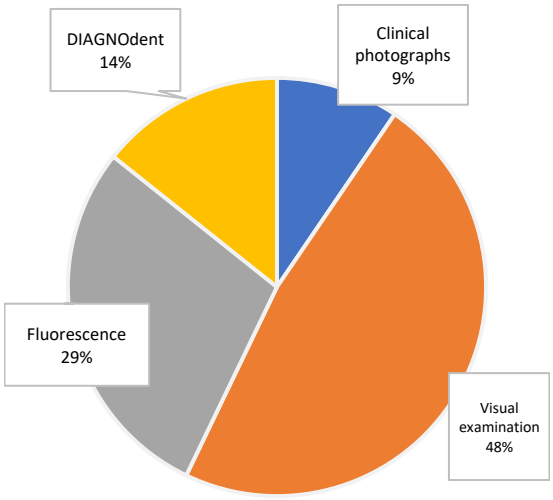

Figure 4- Diagnostic techniques included in this study.

Regarding treatment methods, 7 out of 20 used infiltrating resin, 5 used fluoride-based products, 1 used SAPP11-4, 1 used home care, 2 used CPP-ACP and 1 used HCL. Combination therapies were also considered: 2 used FV and 2 used infiltrating resin and FV.

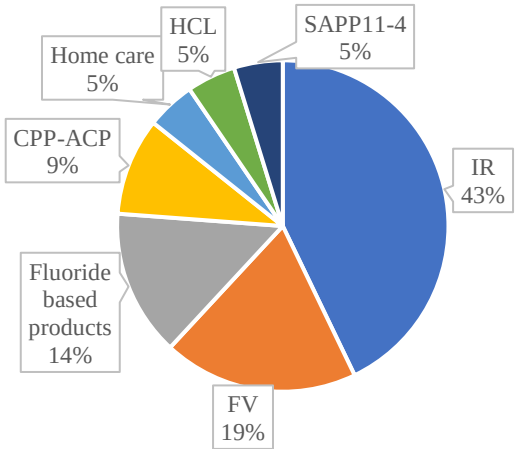

Figure 5- Treatment techniques included in this study. HCL - Hydrochloric acid; FV – fluor varnish ;IR – infiltrative resin; CPP-ACP – Casein phosphopeptide-amorphous calcium phosphate; SAPP11-4 - Self-assembling peptide P11-4.

Table 9**Erro! A origem da referência não foi encontrada.** shows the risk of bias analysis using the RoB tool. This tool consists of evaluating articles in five different domains.

Table 9- Risk of bias analysis according to the RoB tool to RCT studies.

Included articles	D1	D2	D3	D4	D5	Overall
Giray et al, 2018	+	+	+	−	+	+
Ciftci et al, 2018	+	+	−	−	+	+
Kirschneck et al, 2016	+	+	−	−	+	+
Perrini et al, 2016	+	+	+	−	−	+
Huang, et al 2013	+	+	−	−	+	+
Bock et al, 2017	+	+	+	−	×	−
Du et al, 2012	+	+	−	+	+	+
Aykut-Yetkiner et al, 2014	+	+	+	−	−	+
Kondelova et al, 2020	+	+	+	+	−	+
Rechmann et al, 2018	+	+	+	−	−	+
Senestraro et al, 2013	−	+	−	+	−	−
Krithikadatt et al, 2016	+	+	+	−	−	+

The first domain evaluates bias arising from the randomized process. In this domain, 11 articles have a low risk of bias and 1 article has some concerns. Domain two refers to Bias due to deviations from intended intervention. In this domain, all articles have a low risk of bias. Regarding the third, Bias due to missing outcome data, 7 articles have low risk and 5 article has some concerns. Domain four is about Bias in measurement of the outcome. In this domain, 9 articles present some concerns and 3 low risk. In the last domain, which addresses Bias in selection of the reported result, 5 articles present

low risk, 6 present some concerns and only 1 presents high risk. Overall, this analysis resulted in 10 of the 20 articles with low risk of bias and the remaining 2 articles with some problems.

Cross-sectional studies, longitudinal and case reports were analysed regarding the quality of the study according to the JBI criteria and the results of the analysis are presented in Table 10, Table 11**Erro! A origem da referência não foi encontrada.** and Table 12**Erro! A origem da referência não foi encontrada.Erro! A origem da referência não foi encontrada.**. Almost all aspects of the analysis were fulfilled except regarding confounding factors. In most articles this aspect was not identified and strategies to deal with confounding factors were not always stated.

Table 10-Risk of bias according to JBI, the critical assessment tool, to cross sectional studies.

Author	1. Were the criteria for inclusion in the sample clearly defined?	2. Were the study subjects and the setting described in detail?	3. Was the exposure measured in a valid and reliable way?	4. Were objective, standard criteria used for measurement of the condition?	5. Were confounding factors identified?	6. Were strategies to deal with confounding factors stated?	7. Were the outcomes measured in a valid and reliable way?	8. Was appropriate statistical analysis used?
Roig-Vanaclocha *et al*, 2020	Yes	No	Yes	Yes	In part	In part	Yes	Yes
Marouane *et al*, 2021	In part	No	Yes	Yes	In part	In part	Yes	Yes
Sezici *et al*, 2020	Yes	Yes	Yes	Yes	In part	In part	Yes	Yes
Tassery *et al*, 2013	Yes	No	Yes	Yes	In part	No	Yes	Yes

Table 11-Risk of bias according to JBI, the critical assessment tool, to longitudinal studies.

Author	1. Were the criteria for inclusion in the sample clearly defined?	2. Were the study subjects and the setting described in detail?	3.Was the exposure measured in a valid and reliable way?	4.Were objective, standard criteria used for measurement of the condition?	5.Were confounding factors identified?	6.Were strategies to deal with confounding factors stated?	7.Were the outcomes measured in a valid and reliable way?	8.Was appropriate statistical analysis used?
Kabaktchieva *et al*, 2014	Yes	Yes	Yes	Yes	In part	In part	Yes	In part
Hammad *et al*, 2012	Yes	Yes	Yes	Yes	In part	In part	Yes	Yes

Table 12-Risk of bias according to JBI, the critical assessment tool, to case reports.

Author	Were patient's demographic characteristics clearly described?	Was the patient's history clearly described and presented as a timeline?	Was the current clinical condition of the patient on presentation clearly described?	Were diagnostic tests or assessment methods and the results clearly described?	Was the intervention(s) or treatment procedure(s) clearly described?	Was the post-intervention clinical condition clearly described?	Were adverse events (harms) or unanticipated events identified and described?	Does the case report provide takeaway lessons?
Marouane *et al*, 2020	In part	In part	Yes	Yes	Yes	In part	In part	Yes

3.3 Do the biofilm modulation strategies (probiotics) promote a less cariogenic biofilm?

The results in section 3.1 show evidence that there are differences in the microbiome associated with health and caries. Therefore, the question of whether the oral biofilm could be modulated to become less cariogenic was posed. The use of prebiotics and/or probiotic bacteria to modulate the oral ecosystem has been tested by several researchers and to assess the evidence of the potential of pre/probiotics both in the prevention and treatment of dental caries, a systematic literature search was conducted to identify articles with relevant data to answer the PICO question: “In individuals with caries, after probiotic administration, is there an improvement in outcomes directly related to caries risk and development?". This search identified de 850 articles potentially relevant, with 183 publications from PubMed database, 365 from Scopus, 240 from Web of Science and 62 from Cochrane. After removing 166 duplicates, 694 articles were considered and with the information provided in the title and abstract, and after article selection and full text analysis, 14 articles were considered (Figure 6).

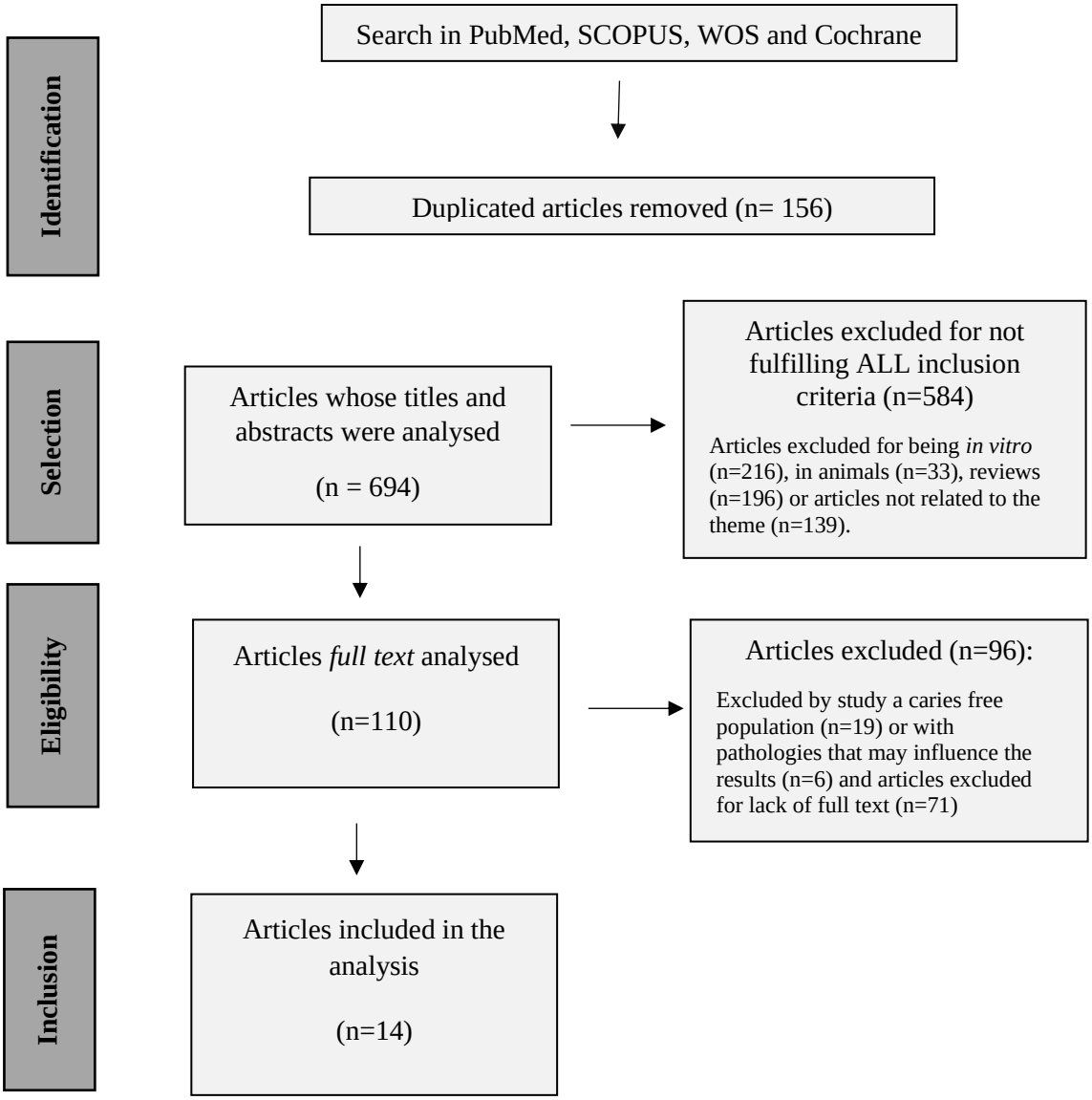

Figure 6- Overview of article selection procedure according to PRISMA guidelines [106], to address the PICO question: In individuals with caries, after probiotic administration, is there an improvement in outcomes directly related to caries risk and development?".

The main reasons for non-inclusion were *in vitro* studies (n=216), articles were not related to the topic of probiotics and caries (n=139), animal studies (n=32) or used bovine enamel on devices used by humans (n=1), reviews (n=196) and articles focused on a caries-free population (n=19). Six studies addressed diabetics or polymedicated geriatric patients and were also excluded. Moreover, 71 articles with no full text available were also excluded. Most of the excluded studies are randomized controlled trial (RCT) protocols registered but without published results.

The studies selected were screened using the JBI criteria for quality assessment (Table 13). The studies selected were generally compliant with the quality criteria. Only 5 articles did not fully comply: 4 for not mentioning the follow up of subjects [143–147], and one article [148] lacking real blinding. In Shaalam *et al*, 2021 [148], to control group is given chewing gum, and the experimental group is given yogurt, therefore both subjects and researchers are aware who was in the control and who is in experimental group. Another article [149] indicates that a double blind, randomized controlled trial was done, but does not describe in detail how selection, randomization and blinding were performed.

Table 13- Results of the analysis of Joanna Briggs Institute appraisal checklist for critical evaluation of RCT studies.

	1. Was true randomization used for assignment of participants to treatment groups?	**2. Was allocation to groups concealed?**	**3. Were treatment groups similar at the baseline?**	**4. Were participants blind to treatment assignment?**	**5. Were those delivering the treatment blind to treatment assignment?**	**6. Were treatment groups treated identically other than the intervention of interest?**	**7. Were outcome assessors blind to treatment assignment?**	**8. Were outcomes measured in the same way for treatment groups?**	**9. Were outcomes measured in a reliable way?**	**10. Was follow-up complete and, if not, were differences between groups in terms of their follow-up adequately described and analysed?**	**11. Were participants analysed in the groups to which they were randomized?**
Campus *et al*, 2014	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	In part	Yes
Rungsri *et al*, 2017	Yes	In part	In part	In part	In part	In part	In part	Yes	Yes	Yes	Yes
Villavicencio *et al*, 2018	Yes	Yes	In part	Yes	In part	Yes	Yes	Yes	Yes	No	Yes
Zare Javid *et al*, 2019	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Manmontri *et al*, 2019	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ferrer *et al*, 2019	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Gedam *et al*, 2019	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Shaalan *et al*, 2021	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	In part	Yes
Wattanarat *et al*, 2021	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Piwat *et al*, 2020	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ratna Sudha *et al*, 2020	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Gandhi *et al*, 2020	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes
Sandoval *et al*, 2021	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

The analysis of the results shows that *Lacticaseibacillus* and *Bifidobacterium* are the probiotic most frequently studied, and the most common species are *Lacticaseibacillus rhamnosus* (*L. rhamnosus*) [143,146,149–151], *Lacticaseibacillus paracasei* (*L. paracasei*) [147,152,153] and *Bifidobacterium longum* (*B. longum*) [143,146]. Interestingly, two studies reported the comparison of probiotics (*Bifidobacterium*, *L. rhamnosus* and *Lacticaseibacillus plantarum*) with the prebiotics Xylitol and Cinnamon Bark Oil [144,148].

Most of the studies, reported the consumption of the probiotic as an ingredient of probiotic milk [143,146,149,151,152,154,155], however, the administration via yogurts [147,148], lozenges and oral tablets [145,156], adhesive gel or patch [92,144] and even mouth rinse [150] were found. The daily intake of such probiotics varies between 10^5 and 10^9 CFU/mL during the different intervention times.

A daily intake of one dose of probiotic was the most frequent application [145,147,150,151] whereas Campus *et al* [156] opted for a frequency of administration of two doses of probiotic daily. Two other studies reported the intake of one dose of probiotic five times a week [143,146]. The probiotic bucco-adhesive gel [92] was administered every 48 hours, while the mucoadhesive patch [144] was administered two times per day. In 4 studies probiotic intake regimens were compared: one group took one dose daily and the other groups three doses per week [148,152–154].

A summary of the analysis of the RCTs and a quasi-experimental pilot study [143] are presented in Table 14;

Table 14-Primary characteristics of the 14 studies included in this study.

Entry	Author	Study Desing	Participants	Participant age (years)	Probiotic/Prebiotic	Administration	Frequency of administration	Intervention time
1	**Campus *et al*, 2014**	RCT	181	6-8	*L. brevis CD2*	lozenges	Two doses daily	6 weeks of use + 2 weeks follow-up
2	**Rungsri *et al*, 2017**	RCT	41	20-25	*L. rhamnosus SD11*	milk	One dose daily	4 weeks of use + 8 weeks follow-up
3	**Villavicencio *et al*, 2018**	RCT	363	3-4	*L. rhamnosus, B. longum*	milk	One dose 5 times per week	9 months
4	**Angarita-Díaz *et al*, 2019**	Quasi-experimental pilot study	63	3-5	*L. rhamnosus, B. longum*	milk	One dose 5 times per week	3 months
5	**Zare Javid *et al*, 2019**	RCT	66	18-30	*Bifidobacterium lactis Bb12*	yogurt	One dose daily	2 weeks
6	**Manmontri *et al*, 2019**	RCT	286	1-5	*Lactobacillus paracasei SD1*	milk	One dose daily or 3 times per week	6 months of use + 6 months follow-up
7	**Ferrer *et al*, 2019**	RCT	59	18-65	*S. dentisani*	Bucco-adhesive gel	Every 48h	4 weeks of used + 2 weeks follow-up
8	**Gedam *et al*, 2019**	RCT	51	8-12	*L. rhamnosus, L. acidophilus, B longum, Saccharomyces boulardii*	mouth rinse	One dose daily	2 weeks of use + 4 weeks follow-up

RCT - randomized controlled trials*; L. brevis - Lacticaseibacillus brevis; L. rhamnosus - Lacticaseibacillus rhamnosus; B. longum - Bifidobacterium longum; S. dentisani - Streptococcus dentisani; L. acidophilus - Lactobacillus acidophilus.*

Table 13- Primary characteristics of the 14 studies included in this study (Cont.).

Entry	Author	Study Desing	Participant #	Participant age (years)	Probiotic/Prebiotic	Administration	Frequency of administration	Intervention time
9	**Shaalan *et al*, 2021**	RCT	96	>65	*Bifidobacterium, Xylitol*	Yogurt/chewing gum	One dose daily or 3 doses daily	3 months
10	**Wattanarat *et al*, 2021**	RCT	286	1-5	*Lactobacillus paracasei SD1*	milk	One dose daily or 3 times per week	6 months of use + 6 months follow-up
11	**Piwat *et al*, 2020**	RCT	469	1-5	*Lactobacillus paracasei SD1*	milk	One dose daily or 3 times per week	6 months of use + 6 months follow-up
12	**Ratna Sudna *et al*, 2020**	RCT	48	5-15	*Bacillus coagulans Unique IS2*	Oral tablets	One dose daily	2 weeks
13	**Gandhi *et al*, 2020**	RCT	60	7-10	*Cinnamon Bark Oil, L. rhamnosus, L. plantarum*	Mucoadhesive patch	Two times per day	2 weeks
14	**Sandovalet *et al*, 2021**	RCT	42	2-3	*L. rhamnosus SP1*	milk	One dose daily	10 months

RCT - randomized controlled trial*; L. rhamnosus - Lacticaseibacillus rhamnosus; ; L. plantarum – Lactobacillus plantarum.*

Regarding intervention time, the discrepancy between studies is notorious. Studies with lower intervention times reported the use of probiotics for 2 weeks [145,147,151] or 2 weeks of probiotic usage, followed by an evaluation of the microbial counts or clinical signs after a period of 4 weeks without probiotic use [150] to assess if the changes introduced by the probiotic are maintained. However, more robust studies with 6 months of probiotic usage and an additional 6 months before follow-up [152,154–156] or even 9 and 10 months of probiotic usage [146,151] are the most common design.

The impact of probiotic administration on dental caries outcomes was evaluated in a range of different cohorts, such as children, adolescents, and adults, all in good general systemic health. The studied population has different previous caries experience: from children with at least 1 decay [143] to individuals with 3-10 carious active lesions, including WSL and non-cavitated lesions on enamel surface [92]. Caries identification and evaluation methods were mainly the International Caries Detection and Assessment System (ICDAS), however other clinical evaluation criteria were also considered, such as plaque and gingival indexes. In Table 15 the parameters evaluated, and the respective outcomes achieved in the studies selected are summarized.

Table 15- Evaluated parameters and the respective outcomes achieved in the 14 studies included in this study.

Entry	Study	Clinical Examination	Microbiological analysis	pH evaluation	Other parameters	Main findings
1	Campus *et al*, 2014	Reduction of bleeding on probing	Reduction on salivary *S. mutans* concentration	Reduction of plaque pH	N/A	*L. brevis* CD2 lozenges were effective in reducing important oral health variables.
2	Rungsri *et al*, 2017	No effects in DMFT, GI	Reduction on total bacteria and *S. mutans* concentration and increase of Lactobacilli. Persistence of *L. rhamnosus* SD11 in test group	No effects on pH	N/A	Daily consumption of fermented milk containing *L. rhamnosus* SD11 for 4 weeks may have beneficial effects on oral health
3	Villavicencio *et al*, 2018	No significant differences were attained	*Lactobacillus* spp. concentration reduction	Increase of saliva buffer capacity	N/A	Daily milk intake supplemented with *L. rhamnosus* and *B. longum* reduces the *Lactobacillus* spp. counts and increases the saliva buffer capacity
4	Angarita-Díaz *et al*, 2019	Positive effect on carious lesions Remineralization	No significant effect on *S. mutans* concentration	No significant effect on pH variation	N/A	Clinical studies should continue to determine the functional foods effect of supplemented with probiotics with low acid production to promote children oral health.
5	Zare Javid *et al*, 2019	N/A	*S. mutans* and *Lactobacillus* spp. concentration reduction	N/A	N/A	Consumption of probiotic yogurt with may modify the oral biofilm
6	Manmontri *et al*, 2019	N/A	Reduction on salivary and plaque *S. mutans* concentrations. Increase of Lactobacilli spp. In saliva and plaque.	N/A	N/A	Daily or triweekly consumption of milk supplemented with *L. paracasei* SD1 may help prevent preschool children dental caries
7	Ferrer *et al*, 2019	Decrease in plate index, gingival index. Increase salivary flow	Efficient colonization of *S. dentisani*	No significant effect on pH variation	Increase of salivary calcium and ammonium	The application of *S. dentisani* 7746 improved several clinical and microbiological parameters associated with oral health, supporting its use as probiotic to prevent caries
8	Gedam *et al*, 2019	N/A	No significant differences in *S. mutans* concentration between groups	N/A	N/A	Probiotic mouth rinse was equally efficacious in mouth rinses against *S. mutans*.
9	Shaalan *et al*, 2021	N/A	Decrease on *S. mutans* concentration between groups	N/A	N/A	Probiotic yogurt can be used as an alternative to xylitol in enhancing the oral condition and prevention from caries of geriatric patients

DMFT - *Decayed, Missing, and Filled Permanent Teeth index;* GI- gingival index; *S.mutans- Streptococcus mutans; L. rhamnosus - Lacticaseibacillus rhamnosus; S. dentisani- Streptococcus dentisani; L. brevis - Lacticaseibacillus brevis; B. longum - Bifidobacterium longum.*

Table 14- Evaluated parameters and the respective outcomes achieved in the 14 studies included in this study (Cont.).

Entry	Study	Clinical Examination	Microbiological analysis	pH evaluation	Other parameters	Main findings
10	Wattanarat *et al*, 2021	No significant differences were attained	Decrease in *S. mutans* concentration between groups. Increase of *Lactobacillus* spp. Concentration.	N/A	Elevated salivary HNP1-3 levels in children with early childhood caries upon probiotic supplementation	In the severe caries status, consumption of *L. paracase*i significantly enhanced salivary HNP1-3 levels and but reduce *S. mutans* levels, resulting in reduction of caries progression
11	Piwat *et al*, 2020	Transitions of active caries to inactive caries and decrease of caries risk in probiotic group	N/A	N/A	N/A	Probiotic milk consumption can modestly prevent new caries, but considerably transform active caries to inactive lesions
12	Ratna Sudna *et al*, 2020	N/A	Reduction on salivary and plaque *S. mutans* and Lactobacilli spp. Concentrations	No significant effect on pH variation	N/A	14-day administration chewable tablets with probiotic *B. coagulans* Unique IS2 can reduce cariogenic bacteria
13	Gandhi *et al*, 2020	N/A	Decrease in *S. mutans* concentration.	N/A	N/A	Cinnamon bark oil incorporated mucoadhesive patch is comparable to the probiotic incorporated patch due to its similarity in the reduction of salivary S. mutans counts.
14	Sandovalet *et al*, 2021	Increase in the number of teeth with carious lesions in the control group	N/A	N/A	Increase of salivary hβD-3 in probiotic group	Regular intake of probiotic-supplemented milk in preschool children with high caries risk decreased the occurrence of caries and the salivary levels of hβD-3

S.mutans- Streptococcus mutans; HNP1-3 - Human neutrophil peptides 1-3; hβD-1- Human β-defensin-1.

Several clinical parameters related to oral health were evaluated and compared between control and probiotic groups in these studies, including plaque and gingival indexes, salivary flow, and bleeding on probing. Among the studies were verified statistical differences between groups in the increase of salivary flow [92] and in plaque and gingival indexes [92] were noted, as well as the reduction of bleeding on probing [92,156]. In some cases, authors went one step further by evaluating clinical parameters strictly related to caries management. These studies showed that the administration of probiotics caused positive effects on carious lesion demineralization or remineralization [143] in the transition of active to inactive caries, and in the decrease of caries risk [152]. Moreover, one study reported the increase of the number of caries lesion in the control group [151].

Several studies analysed the concentration of cariogenic *S. mutans* by culture methods. Most reported the decrease of salivary and/or plaque *S. mutans* counts in the probiotic group after the intervention period [144,145,147–149,154–156]. Statistical differences between these groups were not observed in only two studies [143,150]. Some authors have compared the levels of *Lactobacillus* spp. between groups. The increase of *Lactobacillus* spp. was observed in three studies [149,153,154] however, the same number of studies reported a decrease in these bacterial genera [145–147]. Interestingly, both *L. rhamnosus* [149], *Streptococcus dentisani* (*S. dentisani*) and *Propionibacterium FMA5* (*P. FMA5*) [92], were still detected in samples collected in the follow up period. Moreover, when oral microbiome of individuals of each group was analysed by sequencing strategies, a beneficial shift in bacterial composition was observed in the probiotic group, characterized by a reduction in several cariogenic organisms. [92].

Salivary and plaque pH were also evaluated by some authors. Only one study reported the decrease in the plaque pH in the probiotic group [156], and four studies observed an increase in pH after the probiotic intervention [92,143,145,149]. One study also reported the increase of buffer capacity in the probiotic group [146].

At least, two studies had evaluated the levels of antimicrobial peptides defensins that provide the first line of host defence against a broad spectrum of microorganisms, HNP1-3 (Human neutrophil peptides 1-3) and hβD-1 (Human β-defensin-1) [151,155]. In both cases, an increase of these defensins levels was achieved in the probiotic group.

3.4 Oral Microbiome profile in Clear Aligner Patients During Treatment: the cariogenic and carioprotective species

To investigate changes in oral microbiome during an orthodontic treatment with clear aligners, namely in bacteria with cariogenic and carioprotective potential, biofilm and saliva samples were collected from patients undergoing aligner orthodontic treatment at three distinct time-points. These samples were then sequenced using a metagenomic approach.

The bacterial species diversity within each sample was assessed through different alpha diversity metrics such as Shannon, Simpson and Chao1 combining species richness and evenness (Shannon and Simpson indexes) or only species richness (Chao1 index). The alpha diversity metrics for both sample types (saliva and biofilm) and comparison of the initial (T0) and final (T2 or T3) treatment time points are shown in Figure 7. The data indicate that upon treatment there is a trend for a reduction sample mean alpha diversity in saliva and an increase in biofilm samples, respectively. Although, these changes are more pronounced for the Shannon and Simpson indexes (usually characterized using the total number of species (species richness) and the relative abundances of the species (species evenness), the observed differences are not statistically significant.

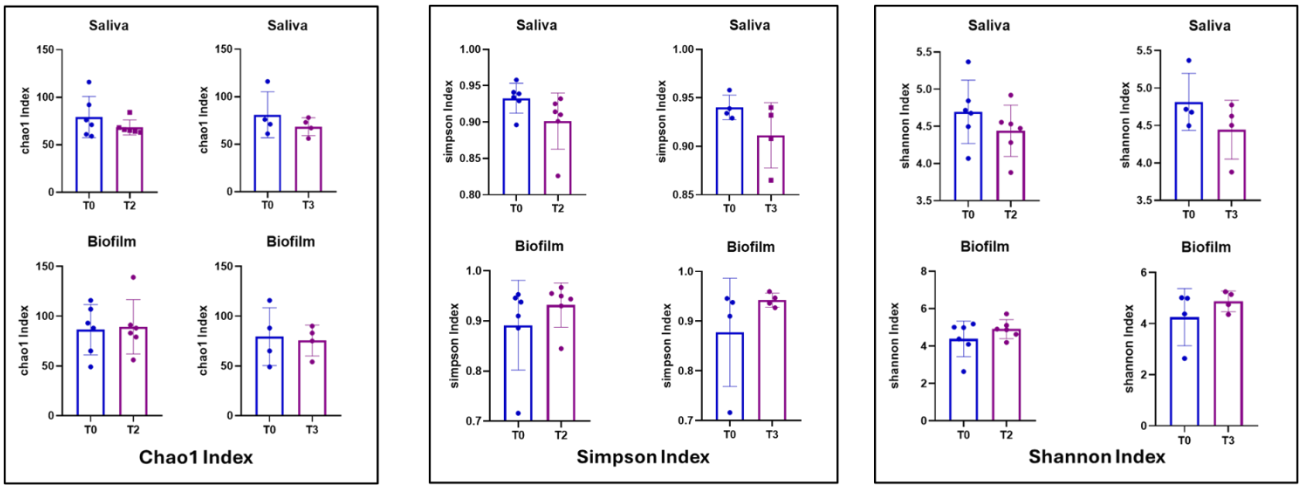

Figure 7- Alfa diversity metrics (Chao1, Simpson and Shannon) for saliva and biofilm samples comparing T0 and T2/T3 (3/6 months) orthodontic treatment time points. Data are shown as mean +- SD (Standard deviation).

To access the microbiome profile of these patients during the orthodontic treatment, only the relative abundance of cariogenic and carioprotective bacteria

previously referred (section 3.1 in Table 5,Table 6, Table 7 and Table 1 in Supplementary Materials) were considered. Figure 8 shows the relative abundances variations of cariogenic and carioprotective bacteria present in saliva (A) and biofilm (B) samples of such patients between T0 and T2 timepoints. In the case of saliva samples Figure 8A, the microbiome profile is distinct in the two timepoints, being T0 (the beginning of the treatment) more diverse than the T2, regarding these particular species. At this timepoint the predominant species are *Rothia aeria* (*R. aeria*), *S. mitis*, *S. sanguinis* and *Actinomyces naeslundii* (*A. naeslundii*). From the analysis of Table 5,Table 6, Table 7 and Table 1 in the supplemental material, these species do not have a significant cariogenic role. It is important to note that the cariogenic bacteria content is low in the two sampling moments, but patients 1, 9 and 17 presented 1-2% of relative abundance of *V. dispar* at T0 and patient 25 with an increase of 2% of the relative abundance of this bacterium at T2. In the case of patients 1, 9 and 17, this cariogenic bacterium was not detected after 3 months of treatment. Regarding biofilm samples (Figure 8B), a higher diversity of bacteria is observed, both in T0 and T3 when compared to saliva samples of the same timepoints. However, even in this case, T0 presented more bacteria diversity and higher abundances than T2, being the most predominant bacteria *C. matruchotii*, which is associated with health (as described in table 6). After 3 months of orthodontic treatment, it is possible to observe that both bacteria diversity and relative abundances diminished with no detection of cariogenic bacteria.

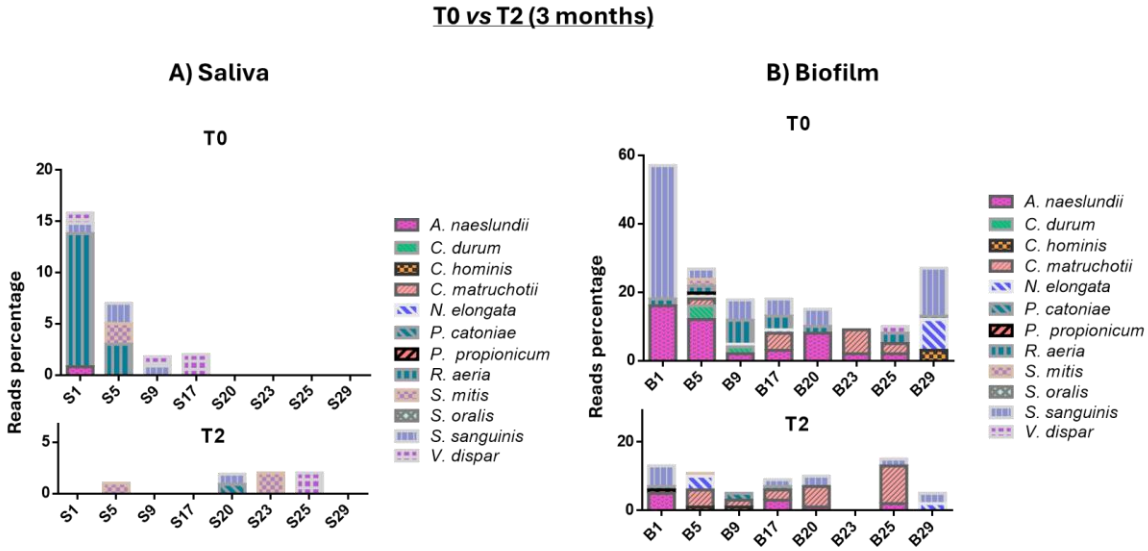

Figure 8- Relative abundances of cariogenic and carioprotective bacteria present in saliva and biofilm samples of patients undergoing orthodontic treatment between T0 and T2 timepoints.

When saliva sampling was extended to 6 months of orthodontic treatment (A) the diversity of the cariogenic species also decreased over the course of treatment. In this case, T0 exhibits greater diversity compared to T2, the cariogenic *V. dispar* was detected in 3 patients, however this bacterium was not detected after 6 months of treatment. In biofilm (Figure 9B), a different profile was achieved. Patients not only presented a more diverse profile but also increased relative abundances. After 6 months of orthodontic treatment the relative abundances *C. matruchotii* (patient 27) increased.

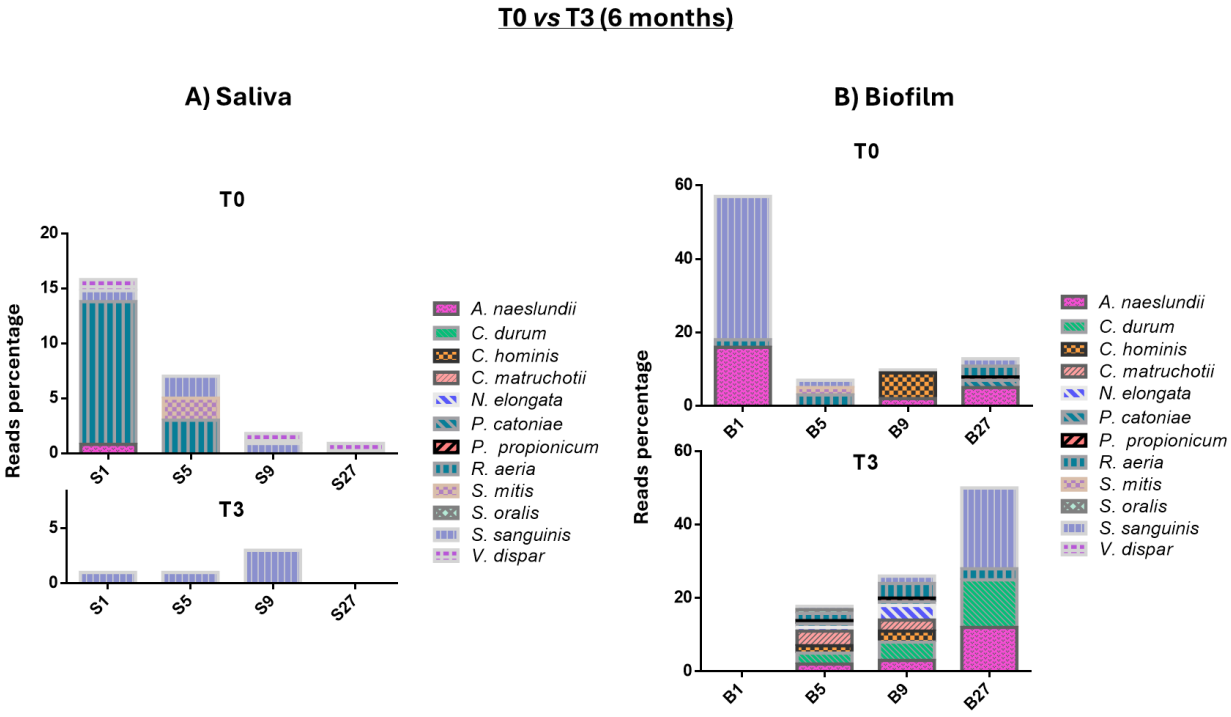

Figure 9- Relative abundances of cariogenic and carioprotective bacteria present in saliva and biofilm samples of patients undergoing orthodontic treatment between T0 and T3 timepoints.

It is important to note, that some of these species were detected in saliva and biofilm samples in lower relative abundances (lower than 0.5 %) such as *N. elongata* (S17, S20, S25 and S29), and the cariogenic *V. dispar* (S1, S9, S23 and S25).

4. Discussion

It seems obvious that analysing the oral microbiome is essential to unravel the mechanisms behind caries. Delving into the intricate ecosystem of bacteria in the mouth allows the identification of the specific players responsible for the dynamic pH balance and to design targeted preventive and management strategies. The development of molecular techniques including sequencing has enabled researchers to probe microbial communities more intensively and extensively.

The microbiology of caries has been studied for a long time before molecular techniques were available for microbial identification. It is widely accepted that streptococci and lactobacilli are the culprits lowering the oral pH and lead to caries. However, molecular microbial identification studies have shown that the complexity of the oral microbiome is enormous and finer analysis reveals that other species are involved. It is thus important to use molecular approaches to assess which species participate in the critical pH balance in the mouth, and to consider all aspects of the equilibrium, rather than only the pH lowering processes and the presence of specific species. Although several studies have been conducted and published since the beginning of the century with molecular analysis of the oral microbiome, there is a huge heterogeneity among the different studies regarding design and the groups compared, types of samples collected, genes analysed and even when the 16sRNA is chosen which region is used for identification, DNA sequencing platform, and databases used for amplicon annotation. These facts make comparison between the results of the different studies difficult because the heterogeneity in the dimensions mentioned above are confounding factors for the outcome of the identification. In other words, it is unadvisable to compare groups including children with groups of adults, or to aggregate results from a metagenomic approach with studies in which certain genera or species were the target of the research, or even oral microbiome characterization using saliva with characterization using biofilm. As was seen in the systematic reviews included in this thesis, in spite of the large number of articles found with the queries, once stratification of the studies is done, a few are really comparable. Therefore, one of the main conclusions of this work is that there is the need of some standardization of the studies analysing oral microbiome to drive a better and evidence supported understanding of the oral microbiome composition and changes in oral diseases such as caries.

In the studies identified in the reviews conducted often the oral microbiome is identified solely at the genus level. There are 2 main reasons for this: the depth of

sequencing necessary to distinguish genera is lower and it is easier to distinguish taxonomic units between genera than within the genus. However, when the identification is done to the species level, it becomes obvious that certain species within genera such as *Streptococcus* have different abundances in health and disease and therefore our results show that solely relying on *Streptococcus* as the single microbial biomarker for assessing caries risk, does not offer a comprehensive insight and might even be erroneous. It is known that not all streptococci are cariogenic, and the species most frequently associated with caries are *S. mutans, S. parasanguinis, S. salivarius* [117,118,121].

The results from the systematic review on the microbiology of caries also indicate that besides *Streptococcus,* other genera, such as *Actinomyces, Fusobacterium, Prevotella,* and *Propionibacterium,* are also reported as increases both in health and in caries situations, which means that at the genus level they cannot be used as caries risk biomarkers Table 4. At least for the *Prevotella*, the previous statement is true regardless of the sample type, saliva or biofilm.

Besides *Streptococcus*, *Prevotella* showed to be one of the more diversified genera, where *P. multisaccharivorax* and *P. denticola* were found to be abundant in caries samples (in the case of P. *denticola* found both in saliva and biofilm samples) Table 6. In fact, the presence of a high content of *Prevotella* species such as *P. denticola*, *Prevotella pallens* (*P. pallens*), *Prevotella verovalis*, *Prevotella salivae*, *P. histicola*, *Prevotella DO039, Prevotella maculosa* (*P. maculosa*), *Prevotella loescheii* have been associated with higher caries risk [157]. Also, in the case of *Prevotella*, some minority species were found in the samples of participants. These species include *P. denticola* (S20, B17, B20 and B23), *P. salivae* (S1, S5, S9, B5, B17, B22, B23 and B25), *P. pallens* (S1, S5, S9, S17, S20, S23, S25, B1, B9, B17, B20 and B25), *P. histicola* (S1, S5, S9, S17, S20, S23, S25, S27, B1, B5, B17, B20 and B25) and *P. maculosa* (S1, S23, S25, B1, B5, B9, B20, B23 and B27). A strong symbiotic relationship between *P. denticola* and *S. mutans* enhances caries-associated virulence of plaque biofilms [157].

Another *genus* referred as increased in caries is *Propionibacterium* where *P. acidifaciens* is the most abundant specie. The high content of *Propionibacterium acidifaciens* (*P. acidifaciens*) is assigned to its capacity to bind and to survive inside dentinal tissue, and its acid production at low pH condition is involved in the development of dentinal caries [158]. However, the study by Corralo and others (2021) also finds this genus increased in health [159]. Although more often associated to caries free samples,

the genus *Actinomyces* also appears increased in some caries groups [160]. The role of this genus in root caries development is complex and might be attributed to the existence of certain species capable of surviving and adapting their metabolism in order to use the available substrates. This suggests that these species have developed mechanisms to survive in that inhospitable environment [161]. *Lactobacillus* has long been considered a cariogenic bacterium, due to its acidogenic and acid tolerant profile. It is particularly abundant in deep dentin and root caries [162].

In the caries free individuals, *Cardiobacterium, Corynebacterium, Actinomyces, Fusobacterium, Haemophilus, Leptotrichia, Prevotella, Selenomonas* and *Streptococcus* are the genera most mentioned as increased. The *Porphyromonas* increase referred by some authors in caries free individuals is expectable since this genus includes alkalinogenic species with optimal growth at pH around 7.4 [163]. The fact that most species are assaccharolytic and proteolytic results in an increase in pH which impacts the overall pH equilibrium in the mouth. In fact, some studies reported *P. catoniae*, *Porphyromonas CW034* (*P. CW034*) and *Porphyromonas sp OT-279* as the more abundant species in caries free individuals, being this genus one of the most diversified in the non-caries group [164–166]. Besides, *Porphyromonas, Corynebacterium* genus is increased in caries-free, where the species *C. durum* and *C. matruchotii* were found to be the most abundant in biofilm samples. In fact, *C. matruchotii* has been referred as an indicator of "caries-free" and seems to play an important role in the formation and stability of a healthy dental biofilm [167]. This protection role is due to its capacity to support a highly organized 3D structure where "symbiotic" streptococci such as *S. gordonii* and *S. sanguinis* are favoured and aid in the control of the cariogenic activity of *S. mutans* [167].

As previously referred, besides being associated to caries, *Streptococcus* genus also appears associated to caries-free situation. Although the results from tables 4-6 and table 1 in the supplemental material do not report and increased presence of any *Streptococcus* in health, some streptococci such as *S. sanguinis* are abundant in individuals free of caries and this bacterium is commonly found in the oral cavity as part of the normal oral microbiota. *S. sanguinis* is associated with oral health as it is an early colonizer of dental plaque, facilitating the development of a stable oral biofilm and the attachment of other beneficial oral bacteria to the tooth, contributing to the general stability of biofilm structure [168]. It produces several antimicrobial substances that

inhibit oral pathogens, which may explain that higher levels of *S. sanguinis* are related to a lower incidence of dental caries and periodontal disease. The antimicrobial properties and biofilm-forming abilities of *S. sanguinis* are thought to contribute to maintaining a healthy balance within the oral microbial community, conferring it a carioprotective effect [169].

Like *S. salivarius*, *A. johnsonii* is also involved in the initial formation of dental plaque and appears to have synergistic relationships with *S. sanguinis* and *S. gordonii*, contributing to the stability and functionality of the oral biofilm. *A. johnsonii* may also be involved in inhibiting the colonization of pathogenic bacteria by occupying and using available resources, limiting their growth. This bacterium is also capable of increasing local pH by producing ammonia and alkali what can lead to a decrease in lactic acid production, when interacting with *S. m*utans [170]. It was demonstrated that *A. naeslundii* strains degrade lactate into pyruvate by a NAD-independent lactate dehydrogenase which can contribute to preserve pH homeostasis in root surfaces biofilm. This mechanism of lactate degradation was shown to also be upregulated in *A. johnsonii* [161].

Current caries risk indices use *Streptococcus* as the genus or *S. mutans* as the main cariogenic agents, leaving out other genera and species that are consistently increased in caries. This systematic review on oral microbiome of caries provides valuable insights on the diversity genera and species that can be found in biofilm and saliva samples of individuals with and without caries. It became clear that basing the risk of caries on the targeted search for just one cariogenic genus and/or specie may not be coherent neither accurate. As seen in this work, *Streptococcus* is found increased in the caries and healthy groups and *S. mutans* is not the only species increased in individuals with caries.

In the future, it would be interesting to complement a generic caries risk index such as CAMBRA [48], with the direct search (by qPCR, for example) for some of the microorganism refereed as increased in caries situation, other than *S. mutans* such as *P. denticola*, that for the reasons mentioned above, could be an useful biomarker of caries risk. Furthermore, considering both acidogenic and alkalinogenic species when designing this kind of management strategies is also important. The presence of carioprotective genera and species, found increased in caries-free group, should also be considered since they also contribute to the pH balance of the biofilm. The proposal an index of microbial dysbiosis in caries based in this assumption that could be capable of assisting in clinical decisions, for example, in the creation of strategies for the prevention and treatment of

caries, using, microbiome modulators to reverse microbial dysbiosis, seems very promising. However, as seen in this work, few studies report the increased genera in caries *vs* healthy populations and even fewer report the species presented in these groups.

Regarding the choice of saliva or dental plaque from teeth (biofilm), as the source of information on the oral microbiome, the goal of the analysis is important. On the one hand saliva is an advantage since it is easy to collect, is non-invasive and allow the collection of sufficient sample volume for direct analysis. These properties make saliva an optimal sample for chairside analysis and clinical decision support. However, saliva and biofilm do not match in terms of the species found as would be expected. The distinct microenvironments of the oral cavity have distinct microbial residents and depending on where the sample is collected a different community is represented. For biofilm an ideal sampling scenario would examine several multiple sites [171] which makes clinical chairside applications time consuming and not feasible. Therefore, it seems essential to understand what saliva reflects of the oral microbiome in different situations. In this work it was possible to find that *P. denticola* was found in saliva and biofilm samples of individuals with caries, but it is only one species. This fact does not mean that it is indeed the only species since the number of studies using both sample types was very reduced. More studies which in the same situation use both sample types are essential to provide more evidence on what species are present in saliva samples and could be used as biomarkers.

White spot lesions are an initial and often reversible stage of caries. Therefore, timely identification is essential to apply suitable treatment at an early stage. The choice of diagnostic technique to be used for detecting caries and white spot lesions is important and depends on the clinical case. It is crucial to understand whether these tools have enough scientific evidence to justify their preference over conventional diagnostic techniques, Additionally, it is important to consider whether they enable differentiated and less invasive treatment methods compared to current approaches. New forms of diagnosis have emerged, such as fluorescence and DIAGNOdent, as well as microradiography and microcomputed tomography that facilitate and accelerate the diagnostic process. Fluorescence was useful in detecting demineralization and remineralization of lesions. This method has been shown, in several studies, to be favourable in assessing the size of the lesion and in evaluating its progression throughout the treatment. The use of fluorescence is quite accurate and ends up surpassing the visual

examination. Nonetheless, it remains important to preserve visual inspection skills to ensure that clinicians maintain their diagnostic sensitivity when inspecting and detecting a lesion [137,140,141].

Articles selected for this study reported different types of treatments depending on the diagnosis methods used. For conventional diagnoses the treatments proposed were: SAPP11-4, infiltrating resin, FV, hygiene care at home, CPP-ACP, HCL and even chlorhexidine (CHX) varnish. Therefore, there was no consensus in all articles concerning the treatment associated with conventional diagnostic techniques, although the results were satisfactory in all studies. On the other hand, the treatments reported in the studies in which the diagnoses were differentiated consisted of infiltrative resin, FV, hygiene care at home with fluoride products and CPP-ACP. The treatment's choices applied after using different diagnostic techniques was also not consensual, as there was variation in the choice of treatment and the results were also all positive. Thus, all means of treatment promote good evidence in the teeth of patients. Therefore, both through a conventional diagnosis and through a differentiated diagnosis, the form of treatment does not present great differences and may be the same. This means that there is no scientific evidence of less invasive treatment techniques than the current ones in the face of a differentiated diagnosis. There are several options for the diagnosis, as well as for the appropriate treatment of WSL, with a positive prognosis and good treatment longevity and it is possible to conclude that the diagnostic tool chosen does not have the potential to change the treatment option, whether it is a conventional tool or a different one. Therefore, there are no differences in the therapeutic approach for the treatment of WSL, regardless of the type of diagnosis used.

It's important to remind that, as the main motivation of patients with WSL is fundamentally aesthetic, follow-up is very important. In terms of treatment, the patient values the result and especially the stability of the treatment carried out, and the professional must be able to guarantee this to patients.

Advancements in technology have given rise to new, minimally invasive, and more comfortable methods for orthodontic correction. In this context, clear aligners have emerged as an alternative to traditional fixed braces, addressing patients' aesthetic preferences. From an oral hygiene perspective, clear aligners offer advantages due to their removability. However, it is essential to consider that prolonged wear of aligners on tooth surfaces may impact the tooth's microenvironment and the biofilm present [172].

The observational study conducted allowed us to identify slight variations in the oral microbiome during orthodontic treatment with aligners but did not find significant differences in the biodiversity between T0 and T2/T3, neither in saliva nor in biofilm. This result is in accordance with that described in literature, suggesting that alpha diversity indices were relatively stable at the first 3 months of orthodontic treatment with clear aligners [173]. Although a change in diversity is not observed it should be considered that the time considered in this study is limited and that ideally the study should follow the patient even after treatment is complete. Results suggest that the use of clear aligners does not have a negative impact on patients' oral microbiome, which is in line with what is described in literature [174–177], with better periodontal indicators, less risk of white spot development than fixed appliances.

In this observational study it was also possible to verify that the species profiles of saliva samples are very different from those present in the biofilm samples. This observation is supported by other studies [178,179] and is expected due to the nature of the samples.

The use of prebiotics and/or probiotic bacteria, which have the potential to modulate the oral ecosystem and may play an important role in the prevention and management of dental caries. Prebiotics and probiotics are well-known in health promotion and have been extensively studied [180] and demonstrate the potential to have preventive and therapeutic effects. Probiotics can prevent the oral biofilm from being environmentally “stressed,” enhancing the symbiosis associated with health, as well as “repairing” a dysbiotic biofilm associated with disease [181]. In oral health promotion, oral probiotics should be able to adhere and colonize oral tissue including hard non-shedding surfaces and become a part of the biofilm. Moreover, oral probiotics should not be able to perform sugar fermentation, avoiding pH decrease and therefore caries development [182]. Several studies have reported the potential of probiotic use in caries management and development; however, it is not easy to find comparable data to support the generalized use of probiotics as adjuvants for treatment and/or prevention of caries. As stated before, there is no lack of studies in the literature, and according to PubMed, in the last 5 years, 8 systematic reviews were published focusing on the study of probiotics’ use in oral health management, namely in caries prevention and/or treatment of preschool children with or without caries [102–105,183–186]. However, reviews with results for

other age groups (not exclusively children), and which focus on individuals with a previous caries experience are not available, so we had this goal.

The results of the review on the potential of probiotics to prevent caries show that the probiotic most often referred as having beneficial results in dental caries outcomes is *L. rhamnosus* [146,149,151], being the most recommended to be included in clinical studies related to oral health. *L. rhamnosus* has been the object of several studies for its application as a powerful probiotic for human health [187,188]. This fact is due to its capacity to endure to stressful environments, such low pH, to adhere or compete for colonization in the oral cavity, modulating the innate and adaptive immune responses [149]. Moreover, *L. rhamnosus* strains are capable of secreting antimicrobial substances that can inhibit other bacteria strains and can be incorporated into varied delivery food vehicles.

A species which has only recently been considered as a potential oral probiotic is *S. dentisani* [92]. This species is significantly more abundant in caries free individuals [189] and its impact on the oral microbiome has been tested "*in vitro*"[92]. This bacterium seems to modulate the oral microbiome, promoting a beneficial shift in bacterial composition and leading to a reduction of cariogenic organisms, probably by the production of bacteriocins and increasing the pH buffering capacity of saliva through ammonia production[92]. Despite the promising results obtained *in vitro* and in pilot studies, further randomized clinical trials assessing administration regimens and vehicles are needed to support the use of *S. dentisani* to prevent tooth decay [190]. Although most studies refer a significant positive effect of probiotics administration in caries prevention, more scientific evidence is needed to support these findings.

5. Conclusion

A better understanding of the oral microbiome is essential to comprehend the onset and progression of caries and to decide on what biomarkers to use for targeted preventive and management strategies. Caries depends on a dynamic pH balance in the mouth and microorganisms are fundamental players responsible for this balance. This work provides evidence to understand the reasons why *Streptococcus* is not useful as the only microbial biomarker to be analysed when accessing the caries risk. It is also essential to consider both acidogenic and alkalinogenic species when designing management strategies.

Caries management depends on early diagnosis and the identification of WLS seems crucial for good prognosis. Analysis of the published evidence on WSL diagnosis and treatment revealed that the treatment options for these lesions did not depend on the type of diagnosis method used. Regardless of the use of conventional or more recent methods the treatment options were the same, and as far as our results showed the clinical outcome was also similar.

Analysing the oral microbiome of patients undergoing clear alignment orthodontic treatment showed that the impact of this treatment on the microbiome is not relevant. Although the number of patients analysed is limited there is no evidence on the differences in biodiversity (measured by diversity indexes) neither on the evidence of increase in cariogenic species. The species associated with health, on the contrary are present in several samples, even after the use of the clear aligners.

There is evidence of a beneficial and promising effect on dental caries outcomes by the usage of milk supplemented with *Lacticaseibacillus rhamnosus* as an adjuvant approach to clinical intervention and daily oral hygiene routines. Knowledge in this field would also benefit from well-designed studies with a systematic assessment of caries, caries risk and microbial quantification and identification, to elicit a systematic comparison between probiotic composition, vehicles, and administration strategy.

In sum, knowledge on the oral microbiome has great potential for applications in caries management. However, strong evidence on what are the best samples, methods, and indicator species or genera to use in the analysis of the oral microbiome in the clinical setting is still lacking. More studies with better designs

and greater number of individuals are needed for the potential of the microbiome to be fully realized.

6. References

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7. Supplementary materials

Supplementary material 1: Consent form

DECLARAÇÃO DE CONSENTIMENTO INFORMADO, LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO EM INVESTIGAÇÃO

De acordo com a Declaração de Helsínquia e a Convenção de Oviedo.

Por favor, leia com atenção a seguinte informação. Se achar que algo está incorreto ou que não está claro, não hesite em solicitar mais informações. Se concorda com a proposta que lhe é feita, queira assinar este documento.

Título do estudo: *"Evolução da Microbiota Oral durante o tratamento Ortodôntico"*

Enquadramento: A estética do sorriso é cada vez mais valorizada pelos indivíduos e a procura por alternativas estéticas, mais eficientes e confortáveis ao tratamento ortodôntico convencional, principalmente por adultos, fez disparar a popularidade dos alinhadores transparentes, pela conveniência de serem removíveis para atividades como comer, beber e realizar a higiene oral. Apesar dos efeitos dos aparelhos ortodônticos fixos na saúde oral estarem descritos, os efeitos dos alinhadores são pouco estudados. A oclusão provocada pelos alinhadores tem o potencial de modificar as condições à superfície do dente e, portanto, o equilíbrio no biofilme oral. Será relevante proceder a um estudo prospetivo para conhecer a evolução da microbiota oral durante o tratamento ortodôntico com alinhadores, verificando se ocorre disbiose durante o tratamento, com potencial para surgirem sinais e sintomas de doença.

Condições: O estudo será composto por três momentos distintos. Numa primeira fase será realizado um questionário para recolha de dados demográficos e clínicos. Após o preenchimento destes questionários e numa segunda fase, será realizado a todos os inquiridos, um exame clínico para avaliação da condição oral, com recurso ao índice de CPOD que terá a duração aproximadamente de 15 minutos. Seguidamente proceder-se-à recolha e processamento das amostras de saliva e biofilme oral de pacientes com alinhadores, que serão analisadas por metagenómica.

Este estudo não envolve procedimentos que não se enquadrem na prática clínica normal nem pretende testar novos produtos ou medicamentos. A participação neste estudo é totalmente voluntária, não acarretando quaisquer custos, podendo retirar o seu consentimento em qualquer etapa do estudo, sem necessidade de facultar explicações aos seus responsáveis, e com a total ausência de prejuízos, assistenciais ou outros, caso não queira participar. Ao decidir participar pode colocar todas as questões que considerar necessárias para o seu esclarecimento.

Confidencialidade e anonimato:Os dados recolhidos são de uso exclusivo dos responsáveis envolvidos no estudo e serão tratados de modo a garantir a sua confidencialidade. A análise dos dados será efetuada em ambiente que garanta a privacidade dos mesmos.

ESTE DOCUMENTO É COMPOSTO POR DUAS PÁGINAS E FEITO EM DUPLICADO:

UMA VIA PARA O INVESTIGADOR, OUTRA PARA A PESSOA QUE CONSENTE

Para qualquer esclarecimento adicional deve contactar:

Investigador principal: Pedro Campos Lopes Telemóvel:939281284

Email: pedrocampus@gmail.com

Data Protection Officer – UCP :

Dra. Frederica Campos de Carvalho Contacto telefónico: +351 217214179

E-mail: compliance.rgpd@ucp.pt

Consentimento informado

Declaro ter lido e compreendido este documento, bem como as informações verbais que me foram fornecidas pela pessoa que acima assina. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Desta forma, aceito participar neste estudo e permito que os dados recolhidos sejam divulgados sob a forma de publicação científica, desde que a minha identidade seja mantida confidencial.

SE NÃO FOR O PRÓPRIO A ASSINAR POR INCAPACIDADE

NOME:

_____

BI/CC No: _____ DATA OU VALIDADE: ___ /___ /_____

GRAU DE PARENTESCO OU TIPO DE REPRESENTAÇÃO: _____

ASSINATURA _____

Nome do participante no estudo.

Assinatura: _____. Data: _____/_____/_____

Nome do investigador responsável.

Assinatura: _____. Data: _____/_____/_____

Nome do orientador responsável.

Assinatura: _____. Data: _____/_____/_____

Supplementary material 2: Table 1

Table 1- Species increased in saliva samples of caries vs healthy group. Caries group includes individuals with active caries and healthy group individuals with no active caries regardless of caries experience. Only species significantly increased in caries were found and all by Yama and coworkers, 2023 [117].

Species
Atopobium parvulum
Atopobium rimae
Bifidobacterium dentium
Dialister invisus
Filifactor alocis
Lactobacillus fermentum
Lactobacillus gasseri
Megasphaera micronuciformis
Parascardovia denticolens
Parvinomonas micra
Porphyromonas endodontalis
Prevotella multiformis
*Prevotell**a** multisaccharivorax*
Prevotella ceroralis
Prevotella denticola
Prevotella histicola
Prevotella oris
Prevotella oulorum
Prevotella salivae
Prevotella veroalis
Selenomonas dianae
Shaalia odontolytica
Streptococcus cristatus
Streptococcus lactarius
Streptococcus parasanguinis
Streptococcus salivarius
*Tannerell**a** forsythia*
Veillonella parvula
Veillonella dispar