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What do we know about the mechanisms of action of probiotics on factors involved in the pathogenesis of periodontitis? A scoping review of *in vitro* studies

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ABSTRACT

Objective: Probiotics are increasingly used in oral prevention and treatment conditions, but little is known about their abilities. The aim of this review is to clarify, summarize and disseminate current knowledge about the mode of action of *in vitro* probiotics on factors involved in the pathogenesis of periodontitis.

Method: 2495 articles were identified in three databases (Medline, Web of Science, SpringerLink) and 36 studies included in this scoping review.

Results: Twenty-four probiotic species were identified, the majority of which were Lactobacilli or Bifidobacteria. *Lactobacillus rhamnosus* (38.8%) and *Lactobacillus reuteri* (38.8%) were found to be the two predominantly studied probiotic species and three main mechanisms of action of probiotics could be classified as: (i) modulation of the immuno-inflammatory response, (ii) direct actions of probiotics on periodontopathogens by adhesion or nutritive competitions and/or the secretion of antimicrobial molecules and (iii) indirect actions through environmental modifications. A combination of several probiotic strains seems to be beneficial via synergistic action amplifying the functions of each strain used. However, heterogeneity of the methodologies and probiotic species included in studies leads us to consider the following avenues for future research: (i) implementation of standardized periodontal models as close as possible to *in vivo* periodontal conditions to identify the functions of each strain for appropriate medication, (ii) updating data about interactions within oral biofilms to identify new candidates and to predict then analyze their behavior within these biofilms.

Conclusion: Probiotics may have their place in the response to inter-individual variability in periodontitis, provided that the choice of the probiotic strain or combination of them will be personalized and optimal for each patient.

Keywords: Probiotics, Periodontal disease, Laboratory research, Scoping review

Abbreviations: *A.naeslundii, Actinomyces naeslundii; B. animalis)/ bifidum/ breve/ dentium/ longum/ pseudolongum,, Bifidobacterium animalis/ bifidum/ breve/ dentium/ longum/ pseudolongum; C. albicans*, *Candida albicans*; CD, cluster of differenciation; DCs, dendritic cells; EPS, exopolysaccharides; *F. nucleatum, Fusobacterium nucleatum;* G-MSSCs, gingival mesenchymal stromal stem cells; GM-CSF, granulocyte macrophage colony stimulating factor; H_20_2, hydrogen peroxyde; hBD-2, human beta-defensin 2; HLA-DR, human leukocyte antigen – DR isotype; IL, interleukine; LPS, lipopolysaccharide; *L. acidophilus/ casei/ delbrueckii (bulgaricus)/ fermentum/ gasseri/ mucosae/ oris/ paracasei/ plantarum/ reuteri/ rhamnosus/ salivarius/ vaginalis; Lactobacillus acidophilus/ bulgaricus/ casei/ delbrueckii (bulgaricus)/ fermentum/ gasseri/ mucosae/ oris/ paracasei/ plantarum/ reuteri/ rhamnosus/ salivarius/ vaginalis*; *L. lactis, Lactococcus lactis*;

MDMs, monocyte derived macrophages; MIP, macrophage inflammatory protein; MOI, multiplicity of infection; Nf-kB, nuclear factor- kappa B; NK cells, natural killer cells; NO, nitric oxide; *P. gingivalis, Porphyromonas gingivalis; P. intermedia / nigrescens, Prevotella intermedia / nigrescens;* PBMCs, peripheral blood mononuclear cells; PSD, polymicrobial synergy and dysbiosis; PDL, periodontal ligament; ROS, reactive oxygen species; *S. dentisani/ mutans / sanguinis / thermophilus/ salivarius, Streptococcus dentisani/ mutans / sanguinis / thermophilus/ salivarius; T. forsythia, Tannerella forsythia;* TLR, toll-like receptors; TNF, tumor necrosis factor.

1. Introduction

Periodontitis is a polymicrobial infection of surrounding and supporting tissues of the teeth. This disease consists of inflammatory lesions modulated by general and environmental factors (Slots, 2017). Indeed, periodontitis results from the formation of a dysbiotic and synergistic polymicrobial community which uses immune-system-induced inflammation to persist by recovering nutrients from the breakdown of periodontal tissues and to invade them deeply, thus perpetuating the periodontal pathology (Hajishengallis & Lamont, 2012; White *et al*., 2016; Van Dyke *et al*., 2020).

Periodontal therapies are based on the implementation of complementary medical, behavioral and mechanistic strategies. Their success involves controlling the periodontal infection by reducing the total bacterial load, in order to restore a compatible microbial flora with the host's periodontal health (Graziani *et al*., 2017). Evidence-based studies suggest that periodontal treatments significantly improve clinical patient outcomes, such as periodontal pocket depth, clinical attachment level or bleeding on probing, but this effectiveness is, nevertheless, limited and temporary (Mombelli, 2018; Suvan *et al*., 2020). These limitations include (i) restricted access to deep and/or complex lesions (Heitz-Mayfield & Lang, 2013), (ii) the presence of specific periodontopathogens which can invade cells and escape mechanical or immune system action (Ji *et al*., 2015), (iii) the persistence of extra-periodontal bacterial niches serving as bacterial reservoirs (Zhang *et al*., 2018) and (iv) patient compliance with personal plaque control and supportive periodontal therapy (Mombelli, 2019).

In an attempt to redress some of these limits and to improve infection control, local or systemic antimicrobial adjuvants are widely used in mechanistic periodontal strategies. The two main ones used are antibiotics and antiseptics. In addition to mechanical professional debridement, systemic antibiotics have shown (i) medium-term clinical and microbiological efficacy, (ii) eradication or decrease of periodontopathogens levels considered to be the most virulent types (*Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*) to undetectable levels and (iii) control of extra-periodontal microbial niches (Chambrone *et al*., 2016). However, antibiotics are non-specific, have some side effects (gastrointestinal, drug interactions, hepatic intolerance), demonstrate low biodisponibility in periodontal tissues and bacterial resistances increase steadily (Martinez & Baquero, 2014; Mahuli *et al*., 2020). The same applies to topical antiseptics as chlorhexidine, the gold standard, which has also side effects such as dental staining, dysgeusia, burning and mucous membrane lesions and its efficacy is limited in the subgingival area (Slot *et al*., 2014; James *et al*., 2017).

Probiotics, defined as living microorganisms that have beneficial effects on the health of the host when a sufficient amount is administered (World Gastroenterology Organization [WGO], 2017), have been proposed for several years as a possible alternative to standard antimicrobial adjuvants and have opened up a promising horizon in the fight against dysbiotic biofilm. Historically, probiotics were first used to prevent or treat diseases of the gastrointestinal tract. Over the past 20 years,

researchers have been interested in their usefulness in the prevention and treatment of periodontal disease. The rationale for using probiotics as an adjunct to conventional periodontal therapy is based on their potential ability to compete with pathogens and promote the recolonization of "good bacteria" and thus constitute an alternative "ecological" approach to broad-spectrum antimicrobials (Butera *et al*., 2020; Li *et al*., 2017; He *et al*., 2009). Indeed, it is suggested that they are able to induce changes in the structure of the bacterial community, which could lead to changes in interbacterial interactions (cooperation, competition), decrease the most virulent periodontopathogens and restore the balance of the oral ecosystem (Rosier et al., 2018). In a recent randomized controlled clinical trial, the use of a probiotic-based toothpaste, Lactobacilli and Bifidobacteria, as well as its combination with a chewing gum also based on probiotics reduced the number of copies per microliter of pathogens belonging to the orange complex such as *Prevotella intermedia* and *Fusobacterium nucleatum* (Butera *et al*., 2020). In the case of periodontitis, several clinical studies have observed an improvement in clinical periodontal parameters, after different probiotic strain administrations at varied concentrations, concomitantly with non-surgical periodontal treatment, and may have reduced the need for surgical treatment compared to scaling and root planing alone. However, although these results seem to be valid 3 months later, they are less homogenous at 6, 9 or 12 months (Matsubara *et al*., 2016; Ho *et al*., 2020). These discrepancies may be related to the diversity of the administration route, the dose, the assessment of efficacy and viability, and notably to the variability in strain selection. It is indeed known that the impact of probiotics is strain-specific as described under simulated intestinal environmental conditions. (Chamignon *et al*., 2020; Barzegari *et al*., 2020). Finally, recent systematic reviews of the literature and meta-analyses have concluded that the current evidence is favorable towards the use of probiotics as adjuvants in the management of periodontitis, but nevertheless consider the evidence as yet insufficient to formulate clinical recommendations and argue that further fundamental and clinical studies are required (Gruner *et al.,* 2016; Martin-Cabezas *et al.*, 2016; Ikram *et al*., 2018; Ho *et al*., 2020).

Among other things, it has been shown that the host-microorganism interface, widely recognized as an individualized community, presents varying degrees of resistance to colonization in different individuals. In-keeping with this idea, the action of probiotics is not universal and specific oral diseases would require specific probiotic interventions/combinations to produce desired effects (Chugh et al., 2020). A better understanding of the mechanisms of action of each strain of probiotics is therefore necessary to better target uses.

The aim of this scoping review was to summarize current knowledge and provide for the first time a comprehensive understanding about the mode of action of *in vitro* probiotics on factors involved in the pathogenesis of periodontitis in order to inform future researchers in this area.

2. Methods

2.1. Question of the review

The research question of the current scoping review was presented as follow:

Based on the analysis of outcomes from *in vitro* periodontal models in the field of probiotics, what are the abilities and mechanisms of action of these probiotics and future recommendations for *in vitro* research?

2.2. Search strategy

Medline (Pubmed), SpringerLink and Web of Science databases were screened for entries between January 1 2009 and November 30 2020. Several search term combinations were used in the electronic databases: "probiotics + oral biofilms"; "probiotics + periodontal diseases"; “probiotics + periodontitis”; "probiotics + inflammatory response" and "probiotics + *in vitro* + cytokines". A complementary search in the grey literature and in the bibliography of the articles selected by the search strategy was also carried out.

2.3. Criteria for study selection and inclusion

Articles were considered if they met the following inclusion criteria: (i) *in vitro* studies, (ii) written in English or French language, (iii) about periodontal bacteria or pro-inflammatory cytokines as elements associated with probiotics, (iv) used in *in vitro* periodontal models as described as any model using primary periodontal cells and/or mono or pluri-species biofilms with periodontopathogens (Koch *et al*., 2020). Clinical studies, animal models, literature reviews and meta-analyses were excluded. Two independent reviewers first reviewed the articles selected. Full reports of potentially eligible articles were carefully screened.

2.4. Screening methods and data extraction

Articles were analyzed in this review according to the PRISMA-ScR guidelines for writing and reading scoping reviews (Peters *et al*., 2015; Moher *et al*., 2009). A total of 2495 articles were identified using the search strategy. After article titles and summaries were screened by two independent reviewers, 1732 duplicates and 592 off-topic papers were removed. A total of 171 full-text articles were further analyzed according to the inclusion/exclusion criteria, assessed by two independent reviewers. 26 of these were retained and included in this scoping review. 133 articles were excluded for irrelevant probiotics’ species, clinical or animal studies. Two studies were not available in English language and 10 studies did not use an *in vitro* periodontal model. The flow chart of the study is described in **Fig. 1.** For each article, the following data were extracted: general

characteristics (title, author and publication date), study objective(s), material (probiotic(s) and markers of periodontal disease), cells and culture medium support, microbiological analysis technique, judgment criteria and results (**Appendix Table 1**). No additional information was added by searching in the grey literature or by searching the bibliographic references of the selected articles. Indeed, the elements only concerned gastroenterological models or the selection processes of probiotics and not the study of their *in vitro* capacities.

3. Results

3.1 Selected studies

A total of 26 articles, published between January 1 2009 and November 30 2020, were included in this study. Twenty-three species of probiotics were evaluated during this period, all lactic acid bacteria, mostly Lactobacilli (24 studies – 92.3%) (*Lactobacillus acidophilus (*15.4% of the studies*), Lactobacillus casei (*11.5%*)*, *Lactobacillus delbrueckii (*7.7%*)*, *Lactobacillus fermentum (*15.4%*), Lactobacillus gasseri (*3.8%*), Lactobacillus mucosae (*3.8%*), Lactobacillus oris (*3.8%*), lactobacillus paracasei (*7.7%*), Lactobacillus plantarum (*11.5%*), Lactobacillus reuteri (*42.3%*), Lactobacillus rhamnosus (*30.8%*)*, *Lactobacillus salivarius (*11.5%*), Lactobacillus vaginalis (*3.8%*)*), Bifidobacteria (4 studies – 15.4%) (*Bifidobacterium animalis (*11.5%*)*, *Bifidobacterium bifidum (*3.8%*)*, *Bifidobacterium breve (*7.7%*), Bifidobacterium dentium (*3.8%*), Bifidobacterium longum (*7.7%*), Bifidobacterium pseudolongum (*3.8%*))* and streptococci (3 studies – 11.5%) (*Streptococcus dentisani (3.8%), Streptococcus salivarius (*3.8%*), Streptococcus thermophilus (*3.8%*)*) with the only exception being *Lactococcus lactis* (1 study – 3.8%). Santos *et al.* (2020), Castiblanco *et al*. (2016) and Caglar *et al.* (2010) are the only studies to have worked with the same two strains of *L. reuteri* (*DSM17938* and *ATCC PTA 5289*). Concentrations of probiotic strains varied between studies ranging from 10^1 to 10^9 CFU/ml.

Different probiotic functions were identified by the authors, including modulation of the immuno-inflammatory response, production of antimicrobial substances, bond to dental surface models or competitive adhesion with other bacteria, inhibition of the growth of periodontal pathogens, changes in environmental conditions and cytotoxicity towards periodontal cells.

3.2 Modulation of the immuno-inflammatory response / genetic expression

3.2.1 Aims and in vitro models

The ability to modulate immuno-inflammatory responses was found in 7 studies, which used co-cultures respecting the nutritional needs, physico-chemical and temperature conditions necessary for cell viability.

Five of these studies were aimed to evaluate immuno-inflammatory responses from several cell types such as human gingival fibroblasts or human gingival epithelial cell, found in periodontal tissues and induced by the presence of a probiotic strain (Zhao *et al.*, 2012; Mendi *et al.*, 2016; Castiblanco *et al*., 2017a-b; Albuquerque-Souza *et al*., 2019; Esteban-Fernandez *et al*., 2019). In addition to this, 5 articles studied the immuno-modulatory activities of their probiotic strain in co-culture with periodontal bacteria (Zhao *et al*., 2012; Mendi *et al.,* 2016; Shin *et al*., 2018; Widyarman *et al*., 2018; Albuquerque-Souza *et al*., 2019). Pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, chemokine IL-8 or anti-inflammatory cytokine IL-10 are the most quantified cellular mediators of the immune response by the authors. Co-culture times ranged from 2h to 48h with cells:probiotics ratios of mostly 1:100 (MOI).

3.2.2 Main findings

The findings about modulation of immune-inflammatory response's mediators in mono-infection are summarized in **Appendix Table 2** and tend towards a decrease in the production of IL-1β, TNFα and IL-8 and an increase in the production of IL-10 by *lactobacilli* and *bifidobacteria* except for *L. rhamnosus* (Lr32) for which no effect on IL-1β production was found compared to the control (cells without probiotics) in the study from Albuquerque-Souza *et al* (2019). Furthermore, contradictory effects between this study and that from Mendi *et al.* (2016) are found about the TLR2 and TLR4 genes' expressions with no effect for the first study and an increase for the latter study compared to the control. Moreover, one study investigated the effects of a multi-strain probiotic mixture of *L. reuteri (ATCC PTA 5289* and *DSM 17938)* on the immunoinflammatory response from human gingival fibroblasts and found a dose-dependent stimulation of the production of PGE2, a potent mediator of inflammation (Castiblanco et al., 2017).

Finally, the ability of probiotics to modulate the immune-inflammatory response in co-infection with periodontopathogens is summarized in **Table 1**. *Fusobacterium nucleatum, Treponema denticola* and *Tannerella forsythia* were used in only one study whereas *Porphyromonas gingivalis* was used in the 5 studies. In the presence of *L. rhamnosus* (*Lr32*, $2x10^8$ CFU/ml or *ATCC9595*, 10^8 CFU/ml) and after infection by *P. gingivalis* 33277, a decrease of TNF-α, IL-10 and TLR-4 expression by human gingival epithelial cells (Albuquerque-Souza *et al*., 2019) or gingival mesenchymal stromal stem cells (Mendi *et al*., 2016) was observed when compared with the infection of *P. gingivalis* alone (MOI 1:1000 or MOI 1:100). In contrast, an increase of IL-8 and TLR-2 expression was observed, whereas Widyarman et al. (2018) found a decrease in IL-8 and hBD-2 expression in epithelial cells after co-infection by *L. reuteri* (ATCC55730) and *P. gingivalis* compared to mono-infection by the periodontopathogen (MOI 1:100). Shin *et al*. (2018) found that *L. lactis* decreased TFN-α and IL-6 production by THP-1 monocytic cell line in the presence of *P. gingivalis, F. nucleatum, T. denticola*

and *T. forsythia* and Zhao *et al*. (2012) that *L. acidophilus* decreased IL-6, IL-8 and IL-1β concentrations by gingival epithelial cells in the presence of *P. gingivalis* (MOI 10:1, 1:1 or 1:100). This last result was in accordance with those of Albuquerque-Souza *et al.* (2019), who found that three Bifidobacteria (*B. animalis (BB-12), B. pseudolongum (1191A)* and *B. bifidum (1622A)*) and *L. acidophilus (LA-5)* decreased the IL-1β and TNF-α concentrations induced by *P. gingivalis*.

3.3 Production of antimicrobial substances / their effects

3.3.1 Aims and in vitro models

Four studies investigated the direct production of antimicrobial substances by probiotics (Kang *et al*., 2011; Mendi & Aslım., 2014; Saha *et al.*, 2014; Cornacchione *et al*., 2019). Probiotic bacteria were grown for 24-72 h before the antimicrobial substances were identified and quantified through, for example, tetramethylbenzidine (TMB) oxidation, optical density or pH measurements.

3.3.2 Main findings

The produced antimicrobial substances were: (i) exopolysaccharides by *L. rhamnosus GD11*, *L. plantarum LA3* and *B. breve A 28* and *A10* (Mendi & Aslım, 2014), (ii) reuterin and (iii) organic acid by *L. reuteri KCTC3594* (Kang *et al.,* 2011) and (iv) nitric oxide (NO) through nitric oxide synthase activity in particular by *L. reuteri NCIMB701089* (Saha et al., 2014). Only hydrogen peroxide production was assessed in 2 studies, but with different strains of *Lactobacilli*: *L. reuteri KTCT 3594*, *3678, 3679* (Kang *et al.,* 2011) and *L. debrueckii STYM1* (Cornacchione *et al*., 2019). All these productions were dependent on strains.

3.4 Binding to dental surfaces / adhesion competition with periodontopathogens

3.4.1. Aims and in vitro models

The adhesion of probiotics is also one of the observed abilities, in particular their integration and colonization into a biofilm. Among the six concerned studies, the authors investigated the adhesion of probiotics to some dental surface models, as well as the effects of probiotics on the adhesion of periodontopathogens to dental surfaces or host cells. Several types of dental surfaces and/or cells were used: (i) saliva-coated hydroxyapatite discs (Stamatova *et al*., 2009; Jiang *et al*., 2016), (ii) human gingival epithelial cells from the Tujia line (Saha *et al*., 2014) or OBA-9 line (Albuquerque-Souza *et al*., 2019), (iii) human gingival fibroblasts (Esteban-Frenandez *et al*., 2019;) and (iv) gingival mesenchymal stromal stem cells (Mendi *et al*., 2016).

3.4.2. Main findings

In contact with dental surfaces or host cells, probiotic strains presented an adhesion function. This was reported for *L. rhamnosus GG* to saliva-coated hydroxyapatite discs (Stamatova *et al*., 2009; Jiang *et al.,* 2016), *L. reuteri NCIMB11951* at a MOI of 1:100 (Saha *et al*., 2014) or *B. longum subsp infantis ATCC15697* at a MOI of 1:1000 to human gingival epithelial cells (Albuquerque-Souza *et al*., 2019). Some authors sought to identify changes in the adhesion of periodontopathogens to host cells in co-infection with a probiotic. The outcomes were a decrease, even an absence of pathogen adhesion to host cells compared to the periodontopathogen alone. Thus, Esteban-Fernandez *et al*. (2019) observed an adherence to human gingival fibroblasts close to 0% for *P. gingivalis* and *F. nucleatum* in the presence of *S. dentisanii* at a MOI of 1:1. In addition to the decrease of *P. gingivalis W83* adhesion to OBA-9 cells, that of *B. animalis BB-12* is increased compared to mono-infection (Albuqueurque-Souza *et al*., 2019). These results support the adhesion competition between probiotics and periodontopathogens.

3.5 Growth inhibition of periodontal pathogens

3.5.1. Aims and in vitro models

The growth's inhibition of periodontal pathogens by probiotics is one of the functions most often assessed by authors (Zhu *et al*., 2010; Kang *et al*., 2011; Teanpaisan *et al.,* 2011; Chen *et al.,* 2012; van Essche *et al.,* 2013; Saha *et al*., 2014; Baca-Castanon *et al.,* 2015; Jäsberg *et al*., 2016; Jiang *et al*., 2016; Shin *et al*., 2018; Cornacchione *et al.,* 2019; Esteban-Fernandez *et al*., 2019; Higuchi *et al*., 2019; Moman *et al*., 2020). Growth inhibition was assayed in a co-culture model composed of a probiotic mixture and one periodontopathogen for 72h (Zhu *et al*., 2010; Geraldo *et al*., 2019; Santos *et al*., 2020) or models containing a probiotic strain and a multi-species biofilm for 16 to 42h (Jäsberg *et al*., 2016; Jiang *et al*., 2016). The 3 multi-species models involved were (i) a 3 species biofilm model with *P. gingivalis*, *F. nucleatum* and *A. naeslundii* (Jäsberg *et al*. 2016), or (ii) a 4 species biofilm model S. sanguinis ATCC10556, *A. actinomycetemcomitans ATCC43718, F. nucleatum ATCC25586* and *C. albicans ATCC10231* or (iii) 5 species biofilm model composed by the 4 species biofilm model with, in addition, *S. mutans* (Jiang *et al*., 2016). The main periodontopathogens assessed were: *P. gingivalis* (68,7%), *F. nucleatum* (50%) and *T. forsythia* (43,7%). Only two studies used the same probiotic and periodontopathogen strains (Moman *et al.,* 2020; van Essche *et al.,* 2013).

3.5.2. Main findings

The reported outcomes showed probiotics' strains, alone or associated as a mixture, were able to slow down the growth of periodontal bacteria. The most important findings are provided in **Table**

2. Probiotics' effects on pathogens seems to be strain-specific. For example, a significant growth's inhibition of *P. gingivalis* by *L. delbrueckii STYM1* and *GVKM1* strains was found whereas the three others one (*SYB7/SYB13/ATCC 11842*) had only a little impact after 48h of incubation (Cornacchione et al., 2019). Three studies (Zhu et al., 2010; Geraldo *et al*., 2019; Santos *et al.,* 2020) investigated growth inhibition of a periodontopathogen by a mixture of probiotics. Zhu *et al*. (2010) found growth inhibition of *P gingivalis, F. nucleatum, P. nigrescens, P. intermedia* and *S. sanguinis* by the multistrain probiotic formulation present in fresh yogurt *(L. bulgaricus + S. thermophilus + L. acidophilus + B. lactis lm26 + B. lactis Lm3r*) while the heat-treated yogurt failed to inhibit *F. nucleatum* and *P. gingivalis*. The association of *L. reuteri PTA5289* and *DSM17938* (*Prodentis*®) seems to result in the inhibition of the growth of *P. gingivalis* and *F. nucleatum* (Geraldo *et al*., 2019; Santos *et al.*, 2020). Finally, growth inhibition of pathogens was also observed in multi-species models of periodontal pathogens (Jäsberg *et al.,* 2016; Jiang *et al.,* 2016). Jäsberg *et al.* (2016) reported strain-specific outcomes in a subgingival biofilm model. After 42h, all Bifidobacteria strains used inhibited the growth of *P. gingivalis*, that of *F. nucleatum* (except *B. animalis BB-12, B. dentium NH4-1* and *B. longum MU-92)* whereas only *B. dentium* strains inhibited *A. naeslundii* growth.

3.6 Modification of environmental conditions

3.6.1 Aims and models

Two studies have investigated the potential actions of probiotics on the environmental conditions when incorporated into biofilms and, in particular, on the persistence of probiotics developing into biofilms and pH changes, which are important for optimal bacterial growth (Madhwani & McBain., 2011; Jiang *et al*., 2016). Biofilms of single-, bi- or 4-5 species of periodontal bacteria including *S. sanguinis* (*ATCC10556*), *A. actinomycetemcomitans* (*ATCC43718*), *F. nucleatum* (*ATCC25586*), *S. mutans* (*ATCC2751*), *C. albicans* (*ATCC10231*) or Gram-negative or facultative anaerobes were induced in models of hydroxyapatite discs impregnated with artificial or unstimulated human saliva from healthy donors for 16.5h to 30 days.

3.6.2. Main findings

Madhwani & McBain (2011) observed that the introduction of two strains of *L. reuteri* (*ATCC 55730* and *ATCC PTA 5289*) resulted in alterations of nascent and mature biofilms with an increase in exogenous *Lactobacilli* and with a persistence of at least 20 days. This *Lactobacilli* persistence is accompanied by a change in the pH value. Concerning *L. rhamnosus GG*, a resistance and proliferation into 4 (*A. actinomycetemcomitans + F. nucleatum + S. sanguinis + C. albicans*) or 5

multi-species biofilms (*A. actinomycetemcomitans* + *F. nucleatum* + *S. sanguinis* + *C. albicans* + *S. mutans*) after 16.5h of culture was also observed (Jiang *et al*., 2016). Moreover, Jäsberg *et al.* (2016) reported in the subgingival model an increase in the number of *B. longum* strains accompanied by a decrease in the number of periodontal pathogens and pH value after 42h of incubation.

3.7 Cytotoxicity

3.7.1 Aims and models

The potential cytotoxic activity of probiotics was investigated in 5 studies (Caglar *et al*., 2010; Moman *et al.,* 2010; Castiblanco *et al*., 2017; Albuquerque-Souza *et al*, 2019; Widyarman *et al.,* 2018) as well as their protective effects against the toxicity of periodontopathogens on host cells in 3 studies (Mendi *et al.,* 2014; Albuquerque-Souza *et al*., 2019; Zhao *et al*., 2019). All the *in vitro* models were co-cultures for a few minutes to 24 hours (probiotics and periodontal cells or periodontopathogens, probiotics and periodontal cells). Several cell types were used: (i) periodontal ligament cells from avulsed teeth (Caglar *et al*., 2010), (ii) human gingival epithelial cells (Albuquerque-Souza *et al*., 2019; Zhao *et al*, 2019), (iii) human epithelial cells of the HaCat keratinocyte lineage (Widyarman *et al.,* 2018), (iv) human oral keratinocytes (Moman *et al.,* 2010) and (vi) human gingival fibroblasts from one or more donors (Mendi & Ashm*,* 2014; Castiblanco *et al.,* 2017).

3.7.2 Main findings

The results tend to show that the probiotics used in the studies do not significantly affect host cell viability (Caglar *et al*., 2010; Castiblanco *et al.,* 2017; Widyarman *et al*., 2018), even at doses up to 10^7 or at an MOI of 1:1000 (Moman, *et al.,* 2010). Albuquerque-Souza *et al*. (2019) even observed an increase in gingival epithelial cell viability with *L. rhamnosus Lr-32*, *L. acidophilus LA-5* and *B. bifidum 1622A* at an MOI of 1:1000 (Albuquerque-Souza *et al.,* 2019). In addition to the absence of cytotoxicity, certain probiotics seem to preserve the viability of host cells against toxic agents such as hydrogen peroxide (Mendi & Ashm*.,* 2014) or periodontal pathogens (Albuquerque *et al*., 2019; Zhao *et al.,* 2019). Thus, the decrease in gingival epithelial cell viability caused by *P. gingivalis* (*33277* or *W83*) was neutralized by probiotics and even increased with *L. rhamnosus Lr-32*, *L. acidophilus LA-5, B. bifidum 1622A* (Albuquerque-Souza *et al*., 2019) and *L. acidophilus ATCC4356* (Zhao *et al*., 2019).

4. Discussion

Bacteria, used as probiotics, arguably present some abilities for periodontal health purposes. This scoping review proposed to explore these functions through *in vitro* studies using probiotics.

Among the wide range in reported outcomes, probiotics *in vitro* have been shown to (i) modulate the immuno-inflammatory response through modulation of the production of cellular mediators in the presence of periodontal pathogens, (ii) inhibit the proliferation and adhesion of these pathogens to dental surfaces and (iii) modulate environmental conditions by secreting various anti-microbial molecules. Previously, several mechanisms of action of probiotics have been proposed and classified into three groups (Laleman & Teughels, 2015; Teughels *et al.,* 2011): (i) modulation of the immuno-inflammatory response, (ii) direct effects and (iii) indirect effects on periodontal pathogens. Based on this statement and the present results, several mechanisms have been highlighted and summarized in **Fig. 2**.

The introduction of probiotics into an *in vitro* periodontal model induces in mono-infection, as in any micro-organism, a modulation of the immuno-inflammatory response. This modulation is mostly represented by a decreased in the production of pro-inflammatory cellular mediators (cytokines, chemokines) secreted by the periodontal cells. Probiotics can modulate this production through action on different levels of the inflammatory activation cascade, such as on TLR-2 expression (Albuquerque-Souza *et al*., 2019). TLR-2 is a receptor mainly implicated in the recognition of microbial components, such as peptidoglycans, and which initiates signaling transduction pathways which induce the genetic expression of these cytokines (Arancibia *et al*., 2007). Thus, Albuquerque-Souza *et al*. (2019) observed a decrease in the TLR-2 expression pathway with *L. rhamnosus (Lr32)* and leads to descrease in the production of pro-inflammatory cytokines and chemokines. However, in contrast, Mendi *et al.* (2016) observed an increase in the activation of the TLR-2 pathway with another strain *L. rhamnosus GD11*. These outcomes confirm the strain-specific functions of probiotics and, thus, the importance of knowing these functions before setting up *in vivo* preclinical studies.

In periodontitis, a disturbance of the immuno-inflammatory response consecutive to the presence of a community of dysbiotic periodontopathogens, and in favor of a pro-inflammatory response, leads to the destruction of periodontal tissues. In this scoping review, the modulation of immune-inflammatory response by probiotics was also observed in co-infection *in vitro* models with a probiotic or a probiotic mixture and one or more periodontopathogens. Whether pathogens (live bacteria or LPS) were inoculated prior to the introduction of probiotics, concomitantly or after, the pro-inflammatory response seems to be attenuated with such introduction. However, some results were ambivalent. For example, Mendi *et al.* (2016) observed an increase in IL-8 production by gingival mesenchymal stromal stem cells during co-infection of *P. gingivalis (ATCC33277)* and *L. rhamnosus (ATCC9595)* while Zhao *et al.* (2012) and Widyarman *et al.* (2018) found a decrease in the production of this cytokine by human gingival epithelial cells in the presence of the same periodontopathogen but with different probiotics (*L. acidophilus (ATCC4356)* and *L. reuteri*

(ATCC55730), respectively*)*. The increase in IL-8 production in the study of Mendi *et al*. was explained by the authors as being due either to (i) direct degradation of the enzymes, the *P. gingivalis'* gingipains by the probiotic, which would degrade chemokines (Uehara *et al*., 2008), or (ii) an indirect inhibition of the action of gingipains by co-aggregation between *P. gingivalis* and *L. rhamnosus.* The absence of Il-8, which is involved in the recruitment of immune cells could be beneficial for the expression of virulence factors of *P. gingivalis*. Differences in the production of IL-8 concentrations in the two other studies (Zhao *et al.*, 2012 and Widyarman *et al*., 2018) could be related to differences in the probiotic strains, the concentration of probiotics (lower for *L. reuteri*), time of incubation or the type of cells used.

Among the main results of the review, direct effects of probiotics on other microorganisms were identified. In addition to the bacterial co-aggregation mentioned above, the production of antimicrobial substances by probiotics is another of these direct mechanisms. Probiotics have, in fact, been able to secrete certain antimicrobial substances such as NO, reuterin or lactic acid (Saha *et al*., 2014; van Essche *et al*., 2013). The production of NO, for example, has been found, in previous studies, to correlate directly with the host's ability to suppress microbial growth and contain infection (MacMicking *et al*., 1997). This substance is known for its bactericidal activity against a wide range of bacteria, including anaerobic bacteria such as *P. gingivalis* and *F. nucleatum (*Allaker *et al.,* 2001; Ghaffari *et al*., 2006). Reuterin is a mixture of monomeric and dimeric forms of β-hydroxypropionaldehyde. This antimicrobial substance has a broader spectrum of inhibitory activity, including fungi, protozoa, Gram-positive and Gram-negative bacteria (Suskovic *et al.*, 2010). It can be noted that even if these molecules or bacteriocins produced by the lactic acid bacteria have an inhibitory action against certain periodontopathogens, this action is not specific and an elimination of protective bacteria of the oral flora cannot be excluded *in vivo*. This non-specific action is already used in the mechanical treatment of periodontitis associated or not with the use of broad-spectrum antimicrobials such as chlorhexidine (Mombelli, 2018; Chambrone *et al.,* 2016). Some authors have also reported modification in immune cell behavior caused by probiotics, contributing indirectly to their action on periodontopathogens such as the production of reactive oxygen species by macrophages (Rocha-ramirez *et al*., 2017), which have deleterious effects on anaerobic bacteria requiring very low oxygen environments such as *P. gingivalis* (Fang, 2011).

Another reported indirect action of probiotics is based on the principle of competitive exclusion. This principle is characterized by competition between two micro-organisms to use habitat resources: the most competitive micro-organism eventually dominates until the other completely disappears. Periodontal pathogens and probiotics could have affinities for the same cellular receptors or source of nutrients. For example, both *P. gingivalis* strains (*ATCC33277* and *W83*) and *B. animalis BB-12* were able to adhere to gingival epithelial cells OBA-9 in mono-infection. In co-infection, a

reduction of the adhesion of both *P. gingivalis* strains to these cells suggests a competition for the same receptors (Albuquerque-Souza *et al*., 2019).

Finally, the introduction of probiotics into an *in vitro* biofilm leads to modifications in the environmental conditions. Probiotics have the ability to integrate and proliferate in *in vitro* biofilm models by preventing other bacteria from doing the same (Madhwani & McBain., 2011). Probiotics are mostly lactic acid bacteria. These bacteria are known to produce antimicrobial substances, such as organic acids (lactic acid, acetic acid), which can lead to a decrease in the pH value. Therefore, the behavior of certain periodontopathogens could also be altered for *P. gingivalis W83*, the growth of which is considerably slowed down at pH=5 compared to a neutral or alkaline pH value (Xu *et al*., 2017; Van Essche *et al.,* 2013). Recently, Schultze *et al*. (2021) concluded that the initial pH value influences the formation of supra and subgingival biofilms. The modification of the pH level in the subgingival biofilms could be an alternative concept in the prevention of periodontitis and thus potentially in the recurrence of periodontitis. Indeed, the authors recall that after an initial periodontal treatment, periodontopathogens decrease while *Streptococcus mutans* increases, a bacterium with an acid pH (≈5.0), which suggests a dependence of the oral biofilm composition on the surrounding micro-environment with an important potential factor: the pH value of the oral biofilm (Schultze *et al*., 2021). In addition, the stability and organization of bacterial communities within biofilms is orchestrated by the interactions and communication between bacteria, also called *quorum sensing*. *Quorum sensing* is increasingly recognized as an important factor in the development of pathogenic oral biofilms, with genes related to this cell-to-cell communication having been identified as regulating the development of biofilms in many oral pathogens *in vitro* (Guo *et al*., 2014; Muras *et al*., 2020). The adhesion and co-aggregation functions of probiotics could have a role to play on these, leading to modifications in environmental conditions. Probiotics could potentially disrupt this communication through their ability to adhere and/or co-aggregate and associated with changes in environmental conditions.

In order to express their beneficial effects and all the mechanisms identified *in vitro*, probiotics could be delivered in appropriate quantities directly into the periodontal lesions in contact with the dysbiotic bacterial communities and therefore into the periodontal pockets. Advances in drug delivery systems have considerably improved the delivery of active pharmaceutical ingredients in the treatment of human diseases. Indeed, the development of innovative vectors can help to overcome the low bioavailability of an active ingredient at the desired site of action to ensure a safe and controlled administration at the delivery site (Chitkara *et al*., 2006; Hatefi and Amsden, 2002). Several authors have researched local delivery systems for a subgingival administration of probiotics (Mirtic *et al*., 2018; Solanki *et al*., 2013; Sohail *et al*., 2011 Muthukumarasamy *et al*., 2006). Mirtic *et al*. (2018) have notably tested the properties of a delivery system of probiotic bacteria in the form

of microcapsules, vegetative cells or spores, promoting their prolonged survival and their effective reactivation, as well as the successful colonization of the target surface for local administration in periodontal pockets (Mirtic *et al*., 2018). However, to date no preclinical studies on periodontitis have been found using this system.

It is also possible that the action of probiotics on the periodontium is more general and complex than the simple local effect and that the mechanistic study which starts from the assumption that probiotics act when they are in contact with the local target in the oral cavity does not consider this dimension. A recent study of the interconnections between the periodontium and the intestine concluded that oral inflammation exacerbates intestinal inflammation by providing the intestine with both pathobionts and pathogenic T cells (Kitamoto *et al*., 2020). Based on a potential oral-gut route linking periodontal and systemic diseases, Kobayashi *et al.* showed that oral inoculation of probiotic reduced periodontal tissue destruction and modulated the immune response through the gut in a periodontitis murine model (Kobayashi *et al*., 2017). A concern could then be adverse effects in healthy patients. In a recent randomized controlled trial, consumption of high doses of multi-strain of probiotics (*lactobacilli* and *bifidobacteria* at 5.10^9 and 25.10^9 CFU per day for 28 days) by patients with digestive health and general wellness appeared to minimally influence microbiota composition with no change in microbiota diversity, as expected in the absence of dysbiosis, and did not adversely affect gastrointestinal function (Tremblay *et al*., 2021).

5. Limitations and implications for research

5.1. Limitations of the scoping review results

In our scoping review, all the experiments were carried out under *in vitro* conditions. However, due to significant methodological disparities, difficulties were encountered in comparing and interpreting the main findings. The review concerned both the conditions of growth and the expression of these results. Indeed, differences were observed in the amount of periodontal bacteria, probiotics and host cells used; periodontal pathogens, probiotic strains and host cellular types; incubation time; composition of the culture medium; form of cellular contamination and study design. Concerning the expression of the results, disparities were noted in periodontal determinants, the unit of measurement for the same periodontal determinant and microbiological analysis techniques. Furthermore, a lack of accuracies was found in the identification of strains, probiotic concentrations and study replications in certain studies such as those of Santos *et al*., 2020 and Cornacchione *et al*., 2019. The present conclusions must therefore be nuanced because of these important disparities. Moreover, the advantages, disadvantages and limitations of *in vitro* studies should be kept in mind. Even if *in vitro* studies, a fundamental part of preclinical research, can (i) be performed at a lower

cost, (ii) with less ethical concerns than *in vivo* studies, (iii) with results obtained more quickly because of the availability of materials and (iv) are reproducible, these studies have little to do with clinical reality. Most of the in *vitro* models are static, include a limited number of simulated parameters, and are dedicated to a particular application. Even the most sophisticated models do not completely reflect the *in vivo* conditions of the disease because they cannot faithfully reproduce the complexity of the periodontal disease which is multifactorial. The human body is a dynamic environment where the many pathways and cells are in continuous transmission. *In vitro* studies are beyond the scope to predict the complexities of potential interactions (Weinreb and Nemcovsky, 2015). These limitations make it difficult to compare and interpret studies' outcomes, but highlight elements for future research.

5.2. Implications for future research

In order to facilitate the exploitation of findings and confirm the mechanisms of action highlighted in this scoping review, the use of a standard periodontal model seems necessary. *In vitro* monolayer cell culture models do not consider cell-cell interactions, because cells are grown on synthetic surfaces and may form unnatural cell attachments (Kim, 2005). However, 3D *in vitro* cell models have been developed with the aim of considering these complex cellular interactions (Artegani and Clevers, 2018; Amelian *et al*., 2017). They have been described as more closely mimicking the physiology and phenotypes of natural tissues and organs than 2D cultured cells and enabling communication and cell signaling, which are essential for cell function (Antoni *et al*., 2015; Kuchler-Bopp *et al.,* 2016; Bugueno *et al*., 2018). For example, an organotypic mucosal model with a well-organized multilayered epithelium and underlying connective tissue characterized by collagen-embedded fibroblasts has been developed and has been identified as suitable for the analyses of pathophysiological processes involved in periodontitis especially molecular mechanisms related to either innate immune response, role of bacterial virulence factors occurring at the epithelium-connective tissue interface and therapeutic properties of drugs (Dabija-Wolter *et al*., 2013; Pinnock *et al*., 2014; Bugueno *et al.,* 2018; Aveic *et al.,* 2021). These models could therefore be considered for further *in vitro* studies of the periodontal mechanisms of action of probiotics. Several studies suggest also that cell stimulation in *in vitro* models should be performed with human primary cells rather than with cell lines. In these 3D periodontal models, primary periodontal cells, i.e. gingival epithelial cells, gingival connective cells and periodontal alveolar bone cells can be reliably differentiated into major cell types, more closely mimicking tissue development and have a response to bacterial virulence factors such as LPS closer to *in vivo* conditions (Schweinlin *et al*., 2016; Pan *et al*., 2009). Moreover, the use of a multi-species oral biofilm model seems to be required (Sham *et al.,* 2019) in order to simulate the complex interaction between oral bacteria. Different *in vitro* tests may

then be considered depending on the objectives of the study. It makes more sense that if the objective is to determine the effects of probiotic strains in prevention of periodontal disease, bacterial pathogenic mature biofilms should be preferentially introduced in a second stage, after probiotics, in a model simulating the healthy periodontium. Notably, a study investigating the ability of probiotics to prevent cell damage induced by anti-cancer treatments introduced probiotics up to 3 days before the drug (Prisciandaro *et al*., 2012). Conversely, if the objective is based on the curative in periodontitis, then the introduction of the probiotic strains should be done in a second time, in a model simulating the physicochemical conditions of the periodontal pocket.

One of the questions faced researchers in the field of probiotics is the exact dose needed to initiate a dose-response reaction. The concentrations necessary to achieve the desired results for curative or preventive use in the field of the periodontal disease have not been widely studied. Clearly, much stronger evidence on the dose-response must be provided in rigorously controlled studies, which must also aim to establish possible risky levels (Guarino *et al.,* 2013). The vast majority of probiotic studies evaluating various oral health parameters have used concentrations in the 10^6-10^9 CFU range, similarly to studies in the field of gastrointestinal diseases (Ho *et al.,* 2020). Even if high doses seem to be well tolerated clinically, the high doses used *in vitro*, allowing the implementation of the mechanisms of action identified in this scoping review, might not be transferable clinically. Furthermore, the expression of the main outcomes with the same units of measurement, such as CFU, would facilitate their exploitation.

Another question is the duration of action of probiotics. *In vitro*, the effects of probiotics are analyzed in the very short term (24-48h) whereas clinically a long-term effect would be expected. However, most probiotics seem do not clinically permanently adhere in the oral cavity, but as observed for intestine, could exert their effects as they metabolize and grow during their passage through the oral cavity (Kopp-Hoolihan, 2001). Yli-Knuuttila *et al*. investigated whether *L. rhamnosus GG* could only temporally be detected, but did not colonize the oral cavity after discontinuation of administration of the probiotic (Yli-Knuuttila *et al*., 2006). In a clinical study the colonization of *L. reuteri* was identified as persisting temporarily after oral ingestion but gradually decreasing every week in a 5-week post-treatment period (Alforaidi *et al*., 2020). Thus, daily consumption of probiotics is probably the best way to maintain their effectiveness in case of oral consumption such as with chewing-gum or tablets.

5.3. Checklist for reporting in vitro studies on probiotics

Based on the present analysis in this scoping review of *in vitro* studies about probiotics in the field of periodontal diseases, a Checklist for Reporting *In vitro* Studies on Probiotics (CRISP) is proposed in **Table 3** in order to promote transparency and quality in these studies. The proposed

Checklist was based on the CONSORT 2010 checklist of information to include when reporting a randomized trial (Schulz *et al*., 2010) adapted with the Minimum Information and Quality Standards for Conducting, Reporting, and Organizing *In Vitro* Research (Emmerich and Harris, 2019) and on the concept note for standardized guidelines for improving quality and transparency in reporting in-vitro studies in experimental dental research provided by Krithikadatta *et al*. in 2014. Thirty-six items, divided into 10 groups, have been identified and will require further validation.

6. Conclusions

This scoping review reveals that probiotics have interesting abilities for the promotion of periodontal health in *in vitro* models. Several mechanisms of action have been suggested, involving the modulation of the immune-inflammatory response by immune or resident periodontal cells and direct or indirect action on the dysbiotic microbiota. It also revealed that a single probiotic does not systematically present these three mechanisms of action. Each strain has its own characteristics. For this reason, a precise identification of the strains is necessary for their appropriate use. Future studies should therefore focus on tests under similar *in vitro* and *in vivo* conditions in order to confirm strain-specific mechanisms of action.

Competing interests

The authors declare that they have no competing interests.

Author contributions

M.D., and K.A. designed the search protocol and the study. A.R. and M.D. contributed to the literature research. The manuscript was written by A.R., and M.D. All authors (A.R., A.B., K.A., M.D.) reviewed, edited and approved the final manuscript.

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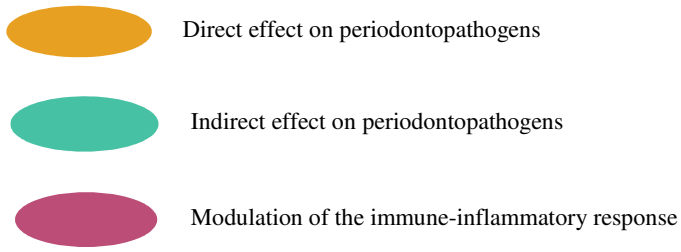
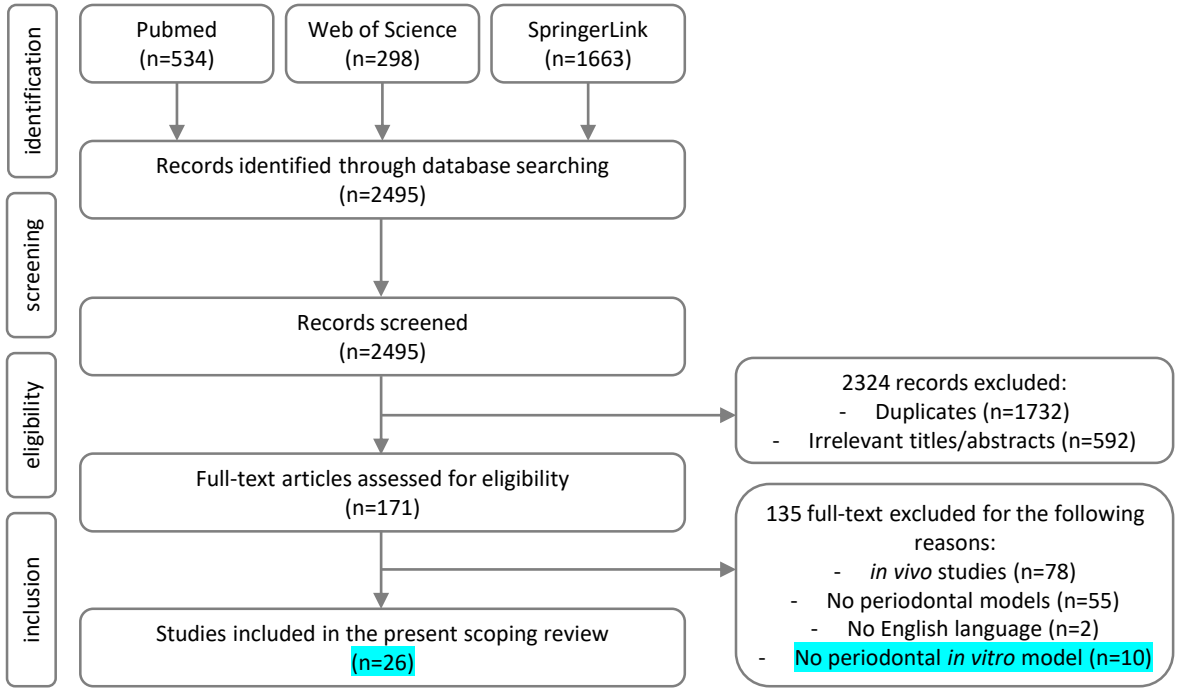
CAPTIONS OF THE FIGURES

Figure 1. Flow chart

Figure 2. Mechanisms of action of probiotics in the rehabilitation of periodontal homeostasis




identification
screening
eligibility
inclusion
Pubmed
(n=534)
Web of Science
(n=298)
SpringerLink
(n=1663)
Records identified through database searching
(n=2495)
Records screened
(n=2495)
2324 records excluded:
- Duplicates (n=1732)
- Irrelevant titles/abstracts (n=592)
Full-text articles assessed for eligibility
(n=171)
135 full-text excluded for the following reasons:
- in vivo studies (n=78)
- No periodontal models (n=55)
- No English language (n=2)
- No periodontal in vitro model (n=10)
Studies included in the present scoping review
(n=26)

Dysbiotic microbiota

Symbiotic microbiota

Dysbiotic microbiota

Cell response

Production of antimicrobial substances

Modification of immune cells behaviour

Competition for adhesion receptors / nutrients / chemical products

Aggregation probiotic-bacteria

Probiotics

Environmental modification

Action on immune cells

Modulation of the induction of immune mediators by bacteria

neutrophil

macrophage

osteoclast

natural killer cell

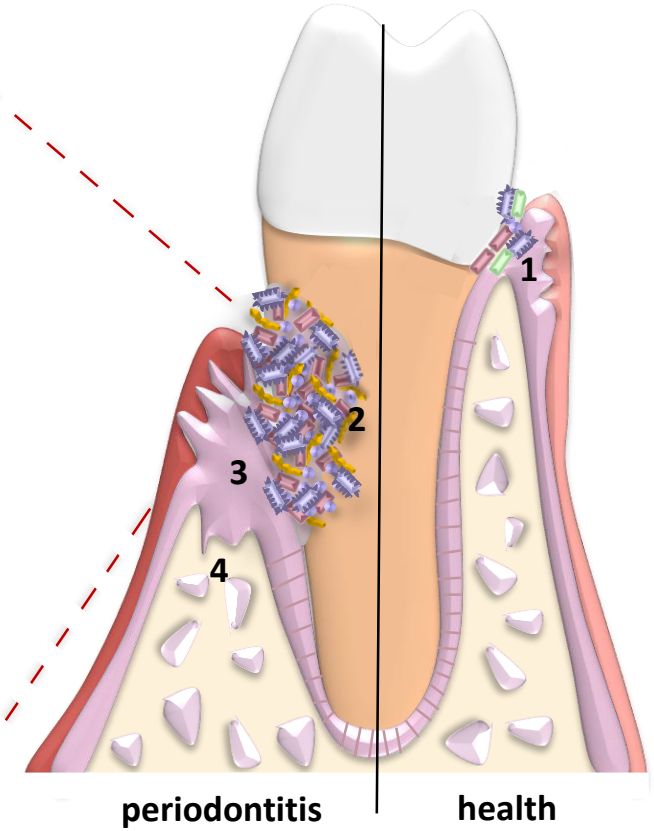
cytokines

fibroblast

dendritic cell

lymphocyte

Disturbed immuno-inflammatory response



1 – Symbiotic microbiota
2 – Dysbiotic microbiota
3 – Immuno-inflammatory response
4 – Bone resorption

Table 1 – Modulation of immuno-inflammatory response in co-infection by probiotics and periodontopathogens

Studies	Cells	Periodontopathogens	Probiotics	Main outcomes compared to periodontopathogens alone
Zhao *et al.,* 2012	Human gingival epithelial cells	*P. gingivalis (ATCC33277)*	*L. acidophilus (ATCC4356)*	IL-1β, IL-6 and IL-8 concentrations' decrease
Mendi *et al.,* 2016	Gingival mesenchymal stromal stem cells	*P. gingivalis (ATCC33277)*	*L. rhamnosus (ATCC9595)*	IL-8 and TLR2 expressions' increase IL-10 and TLR4 expression's decrease
Shin *et al*., 2018	Monocytic cell line (THP-1)	*P. gingivalis (ATCC 33277)* *F. nucleatum (ATCC25586)* *T. forsythia (ATCC43037)* *T. denticola (ATCC35405)*	*L. lactis (HY449)*	TNF-α and IL-6 concentrations' decrease
Widyarman *et al.,* 2018	Human keratinocytes cells line (HaCat)	*P. gingivalis (ATCC33277)*	*L. reuteri (ATCC55730)*	IL-8 and hBD-2 expressions' decrease
Albuquerque-Souza *et al*., 2019	Human gingival epithelial cells (OBA-9)	*P. gingivalis (W83 and ATCC33277)*	*L. rhamnosus (Lr32)*	TNF-α concentrations' decrease TLR4 expression's decrease
	Human gingival epithelial cells (OBA-9)	*P. gingivalis (W83 and ATCC33277)*	*B. animalis (BB12)* *B.pseudoplongum (1191A)* *B. bifidum (1622A)* *L. acidophilus (LA-5)*	IL-1β and TNF-α concentrations' decrease

Table 2. Growth inhibition of periodontal pathogens by probiotics

Studies	Periodontopathogens	Probiotics	Main outcomes (inhibition zone or growth reduction)
Van Essche *et al*., 2013	*P. gingivalis ATCC 33277*	7 lactobacilli strains evaluated separatly[1]	4.07 +/- 0.84 mm
Zhu *et al*., 2010	*P. gingivalis ATCC 33277*	Probiotic mixture [2]	10,6 +/- 1,2mm (MOI 1:1)
Chen *et al.,* 2012	*P. gingivalis ATCC 33277*	*L. fermentum SG-A95 / L. salivarius SG-M6*	9.7±0.6mm / 14±1.0mm (MOI 1:100)
Teanpaisan *et al*., 2011	*P. gingivalis ATCC 33277*	10 lactobacilli strains evaluated separatly[3]	0,5±0.6mm (*SD7*) to 30±2,8mm (*SD5*) (MOI 1:1)
Kang *et al*., 2011	*P. gingivalis ATCC 33277*	*L. reuteri KCTC 3594 / KCTC 3678 / KCTC 3679*	Growth reduction > 90% (MOI 1:1)
Moman *et al.,* 2020	*P. gingivalis ATCC 33277*	*L. rhamnosus GG / L. reuteri ATCC55730 / S. salivarius K-12*	19±1 mm / 15±2 mm / 20±2 mm (MOI 1:1)
Geraldo *et al.,* 2019	*P. gingivalis ATCC 33277*	*L. reuteri Prodentis® (PTA5289 + DSM17938)*	Growth reduction = 86,6%
Shin *et al.,* 2019	*P. gingivalis ATCC 33277*	*Lc. Lactis HY449*	Growth reduction = 50% (MOI 1:10)
Esteban-Fernandez *et al*., 2019	*P. gingivalis ATCC 33277*	*S. dentisani 7746 (CECT8313)*	Growth reduction = 35% (MOI 1:1)
Jäsberg *et al*., 2016	*P. gingivalis ATCC 33277*	10 bifidobacteria strains evaluated separatly[4] (MOI 1 :1)	Growth reduction = 100% (all strains)
Cornacchione *et al*., 2019	*P. gingivalis W83*	*L. delbrueckii STYM1/ GVKM1/ SYB7/SYB13/ATCC 11842*	Growth reduction by *STYM1* and *GVKM1*
Higuchi *et al*., 2019	*P. gingivalis JCM8525*	*L. salivarius WB21*	Growth reduction = 100% at 6h
Van Essche *et al*., 2013	*F. nucleatum ATCC 49256*	7 lactobacilli strains evaluated separatly[1]	0.14± 0.15 mm
Zhu *et al*., 2010	*F. nucleatum ATCC 25586*	Probiotic mixture [2]	11,4 +/- 0,9mm (MOI 1:1)
Santos *et al*., 2020	*F. nucleatum ATCC 25586*	*L. reuteri Prodentis® (PTA5289 + DSM17938)*	Growth reduction = 0.4999 log 10 CFU/mL
Shin *et al.,* 2019	*F. nucleatum ATCC 25586*	*Lc. Lactis HY449*	Growth reduction = 50% (MOI 1:10)
Moman *et al*., 2020	*F. nucleatum ATCC 10953*	*L. rhamnosus GG / L. reuteri ATCC55730 / S. salivarius K-12*	11±2mm / 15±1mm/ 20±0mm
Kang *et al*., 2011	*F. nucleatum ATCC 10953*	*L. reuteri KCTC 3594 / KCTC 3678 / KCTC 3679*	Growth reduction > 90% (MOI 1:1)
Esteban-Fernandez *et al*., 2019	*F. nucleatum DSMZ15643*	*S. dentisani 7746 (CECT8313)*	Growth reduction = 38% (MOI 1:1)
Kang *et al*., 2011	*T. forsythia ATCC 43037*	*L. reuteri KCTC 3594 / KCTC 3678 / KCTC 3679*	Growth reduction > 90% (MOI 1:1)
Baca-Castanon *et al*., 2015	*T. forsythia ATCC 43037*	*L. reuteri ATCC55730*	10±1, 8.5 ± 0.54 mm
Shin *et al*., 2019	*T. forsythia ATCC 43037*	*Lc. Lactis HY449*	Growth reduction = 50% (MOI 1:10)
Van Essche *et al.,* 2013	*P. intermedia ATCC 25611*	7 lactobacilli strains evaluated separatly[1]	1.71± 0.39 mm
Zhu *et al*., 2010	*P. intermedia ATCC 25611*	Probiotic mixture [2]	11,5± 1,4mm (MOI 1:1)
Zhu *et al*., 2010	*P. nigrescens ATCC 33563*	Probiotic mixture [2]	13.7 ±2.6mm (MOI 1:1)
Zhu *et al*., 2010	*S. sanguinis ATCC 10556*	Probiotic mixture [2]	7,9 ± 1,1mm (MOI 1:1)
Teanpaisan *et al*., 2011	*S. sanguinis ATCC 10556*	10 lactobacilli strains evaluated separatly[3]	0mm (*SD7/SD8/SD10*) to 19± 4,2mm (*SD5*)
Baca-Castanon *et al.,* 2015	*A. naeslundii ATCC 51655*	*L. reuteri ATCC55730*	5.8 ± 4.53 mm
Jäsberg *et al*., 2016	Subgingival biofilm: *P. gingivalis ATCC33277* + *F. nucleatum ATCC10953* + *A. naeslundii ATCC12104*	10 bifidobacteria strains evaluated separatly[4]	Growth inhibition of *P. gingivalis* = 100% (MOI 1:1) Groxth reduction of *F.nucleatum* except for *BB-12 / NH 4-1* and MU*92-2* Growth reduction of *A. naeslundii* by *B. dentium* strains
Jiang *et al.* 2016	4 species or 5 species model [5]	*L. rhamnosus GG*	Growth reduction of *S. sanguinis* = 30 to 70%, (MOI 1:1) Growth reduction of *F. nucleatum* and *C. albicans*

Notes:

[1] Lactobacilli strains: *L. rhamnosus* /*L. rhamnosus* GG/ *L. casei* (yogurt)/*L. casei shirota* (milk drink)/ *L. casei* (*ATCC393*)/*L. fermentum* (*LMG8900*)/ *L. paracasei* (*L07-21*)

[2] Multistrain probiotic formulation of *L. bulgaricus* + *S. thermophilus* + *L. acidophilus* + *B. animalis subsp lactis lm26* and *B. animalis subsp lactis Lm3r*

[3] Lactobacilli strains: *L. paracasei SD1*/*L. casei SD2*/*L. salivarius SD3*/*L. plantarum SD4*/*L. rhamnosus SD5*/*L. fermentum SD6*/*L. gasseri SD7*/*L. mucosae SD8*/*L. oris SD9*/*L. vaginalis SD10*

[4] Bifidobacteria strains: *B. animalis* subsp *lactis BB12/ B.* dentium (*AJ 32-1 / AJ 47-1 / NH 4-1 / NH 6-1 / RC-12*) / *B. longum* (*MU 57-1 / MU 86-7 / MU 92-2 / MU 93-4*)

[5] 5 species model = *S.mutans ATCC 2751* + 4 species model : *S. sanguinis* ATCC10556, *A. actinomycetemcomitans ATCC43718*, *F. nucleatum ATCC25586* and *C. albicans ATCC10231*

Table 3. Checklist for Reporting *In vitro* Studies on Probiotics (CRISP)

Section / Topic		Item N°	Checklist Items	Explanation	Reported on Page N°
Title		1a	- Identification as an *in vitro* study	To appropriately allow the indexation and identification of an *in vitro* study in an electronic database and enable the reader to quickly identify the tested probiotic(s).	----------
		1b	- Inclusion of the probiotic(s) used		----------
Abstract	Aim	2a	Clear formulation of the main objective of the *in vitro* study	To assess the coherence between the objective, the methodology used and the results obtained.	----------
	Methods	2b	Identification of the type of *in vitro* model with probiotics strains used and cell lines and/or periodontopathogens		----------
	Results	2c	- Presentation of the main outcomes with precise values and significance		----------
		2d	- Identification of the limitations of the study		----------
	Conclusion	2e	General interpretation of the results of the *in vitro* study summarized in one sentence with perspectives		----------
Funding		3	Open declaration of any funding received, role of funders and of any other sources of support for the *in vitro* study	To identify potential conflicts of interest that may have influenced the results of the *in vitro* study	----------
Introduction	State of the art	4a	Presentation of the scientific background on probiotics and periodontal disease and of the rationale of the study in this context	To identify previous knowledge in the field of study of probiotics in periodontal disease, persistent gaps that require further research and therefore how the proposed *in vitro* study will at least partially address these gaps.	----------
	Aim	4b	Clear formulation of the objectives of the *in vitro* study		----------
	Hypothesis	4c	Clear formulation of the tested hypothesis in the *in vitro* study		----------
Materials and methods	Ethical statement	5a	Indication of permissions to use any materials derived from human volunteers	To be able to easily reproduce the work with a precise understanding of the order of the	----------
	Materials	5b	Precision, for each commercial material (probiotics, cell lines, bacterial		----------

			strains, bacterial virulence factors such as lipopolysaccharide), of the precise denomination / strains, provenance, lot number	experiments, their protocols and the materials used	
	Study design	5c	- Precision of the type of *in vitro* model and/or culture used		----------
		5d	- Indication of experimental and control groups and their size		----------
		5e	- Accuracy of randomization steps such as spatial control of samples		----------
		5f	- Illustration using diagrams especially if the design of the *in vitro* study is complex		----------
	Experimental procedures	5g	- Presentation in the order of sample collection and processing		----------
		5h	- Indication of all parameters about buffer (e.g., cell culture medium) and lysis conditions (e.g., for cell studies), sample preparation, handling and blinding, volumes, concentrations and multiplicity of infection (MOI), temperatures, and incubation times		----------
		5i	- If necessary, illustration using a flow chart or flow diagram, especially for complex or new procedures.		----------
		5j	- Indication of the objective of the experimentation		----------
	Statistical analysis	5k	- Indication of all statistical analyses used and justification (verification of the test conditions of application)		----------
		5l	- Precisions of the parameters analyzed		----------
		5m	- Clear indication of the number of excluded experiments and/or data points		----------
		5n	- Indication of the number of analyses performed and the number of repetitions of each experimental procedure		----------
Results	Organization	6a	Subdivision of the outcomes according to the same objective (e.g. the same mechanism of probiotic analyzed)	To present all the results obtained in a simple,	----------

	Transparency of the outcomes	6b	Impartial analysis of each outcome obtained even non-significant results or those from unsuccessful experiments	comprehensible and transparent manner	----------
	Figures and Tables	6c	Presentation of the main outcomes through figures or tables with the precise values and significances specified in the text of the manuscript.		----------
Discussion	Summary of the main results	7a	Summary of the outcomes by highlighting the main outcome of the study meeting the objective and hypothesis of the *in vitro* study	To objectively present the contributions of the study and its limitations with the purpose of helping future research in the field of probiotics	----------
	Interpretation	7b	Interpretation consistent with the results, based on the most recent scientific literature, balancing the advantages and disadvantages and taking into account other relevant elements		----------
	Generalisability	7c	Presentation of the extent to which the results obtained can be applied to other circumstances.		----------
	Limitations	7d	Exposure of the limitations of the study, potential sources of bias, inaccuracies, and difficulties encountered.		----------
Conclusion		8	Conclusion on the contributions of the study	To provide the key points to be retained from the study	----------
References		9	Inclusion of all studies and/or documents used to support the rationale for the study, the methods used, and interpretations of the results	To declare all sources used in a transparent way	----------
Other information	Supplementary materials	10a	If the instructions to the authors of the journal do not permit some of the items of this checklist to be detailed, inclusion of the description not appearing in the main text should feature in in the supplementary materials/appendix.	To provide all the information about the study that cannot appear in the main text	----------
	Acknowledgments	10b	Acknowledgement of study contributors who do not meet the criteria of authorship of the study.		----------
TOTAL NUMBER OF ITEMS COMPLIED WITH				To determine the quality of the report of the *in vitro* study on probiotics	----- / 36