Probiotics to counteract biofilm-associated infections: promising and conflicting data

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Altered bowel flora is currently thought to play a role in a variety of disease conditions, and the use of *Bifidobacterium* spp. and *Lactobacillus* spp. as probiotics has been demonstrated to be health-promoting, even if the success of their administration depends on the applied bacterial strain(s) and the targeted disease. In the last few decades, specific probiotics have been shown to be effective in the treatment or the prevention of acute viral gastroenteritis, pediatric post-antibiotic-associated diarrhea, some pediatric allergic disorders, necrotizing enterocolitis in preterm infants, inflammatory bowel diseases and postsurgical pouchitis. The potential application of probiotics is continuously widening, with new evidence accumulating to support their effect on the prevention and treatment of other disease conditions, including several oral diseases, such as dental caries, periodontal diseases and oral malodor, as well as genitourinary and wound infections. Considering the increasingly widespread ability of pathogens to generate persistent biofilm-related infections, an even more attractive proposal is to administer probiotics to prevent or counteract biofilm development. The response of biofilm-based oral, intestinal, vaginal and wound infections to probiotics treatment will be reviewed here in light of the most recent results obtained in this field.

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**INTRODUCTION**

A role of some intestinal bacteria to maintain or restore health conditions was firstly proposed by Elie Metchnikoff more than one century ago when he observed that ‘good’ lactic acid-producing bacteria, particularly those belonging to the genera *Bifidobacterium* and *Lactobacillus*, were beneficial to the host by reducing the growth of toxigenic bacteria within the colon.1,2

The original bowel toxemia theory evolved in the following decades into the intestinal dysbiosis hypothesis, with the term ‘dysbiosis’ being defined as ‘...qualitative and quantitative changes in the intestinal flora, their metabolic activity and their local distribution’.3

Altered bowel flora is currently believed to play a role not only in intestinal disorders but also in a variety of disease conditions.4 The use of *Bifidobacterium* spp. and *Lactobacillus* spp. as probiotics, with this term being defined by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) as ‘live microorganisms which when administered in adequate amounts confer a health benefit on the host’,5 has been demonstrated to be health-promoting, even if the success of specific probiotics depends on the applied bacterial strain(s), the number of species, the microbial concentration and the targeted disease.6–8

In fact, according to the Cochrane Summaries,9 probiotics have been shown to be effective or possibly effective in the treatment or prevention of acute viral gastroenteritis,10 pediatric post-antibiotic-associated diarrhea,11 certain pediatric allergic disorders,12 necrotizing enterocolitis in preterm infants,13 inflammatory bowel diseases14 and post-surgical pouchitis.15

In the post-genomic era, high-throughput methodologies, such as metagenomics, transcriptomics, proteomics and metabolomics, have greatly helped to classify strains as probiotics16 and to understand the mechanisms by which several lactic acid-producing bacteria help to maintain human health and the numerous functions assigned to these species in the gut.17 They provide nutrients, help the host to digest foods, compete for space and nutrients with potential pathogens and induce the secretion of antimicrobial peptides through an interaction with intestinal epithelial cells.18–19

*Bifidobacteria* and *Lactobacilli* are also able to stimulate the development of the immune system, with certain species of gut commensal microbiota being required for immune regulation and tolerance of the large amount of antigens present in the gut.20–21 Perturbations in the microbiota could result in a lack of immune regulation, out-growth of more pathogenic microbes and promotion of tissue inflammation.

The potential application of probiotics is continuously widening, with new evidence supporting their effect on the prevention and treatment of other disease conditions, including urogenital infections,22 cystic fibrosis23 and various cancers.24–25

With regard to oral health, great attention is being given to the use of a probiotic therapy for the treatment of dental caries, periodontal diseases and oral malodor.26–27
With this in mind, recent data provide proof-of-concept that a mixture of streptococcal species applied to canine teeth as an adjunctive therapy along with the probiotic Lactobacillus brevis CD2 (refs. 29–30) can delay the recolonization of periodontal pathogens and reduce inflammation through modulatory effects on the host response and on the periodontal microbiota.

Furthermore, the use of a mouth rinse containing Bacillus subtilis or the oral administration of tablets containing Lactobacillus salivarius reduced the number of periodontal pathogens. While this approach seems promising, it is still a relatively new concept, and more research is needed to determine its clinical efficacy.

Considering the increasingly widespread ability of pathogens to generate persistent biofilm-related infections, an even more attractive proposal is to administer probiotics to prevent or counteract biofilm development. In fact, in vitro investigations on adhesion, bacteriocin production, co-aggregation, growth inhibition and metabolic activity have suggested a potential role of probiotic Lactobacilli and Bifidobacteria in modulating the microbial ecology of biofilms, in particular, those developing at the oral, intestinal, vaginal and wound level.

The ability to adhere to mucus and epithelial cells, as well as to co-aggregate, is proposed as one of the most important selection criteria for potential probiotic strains. Biofilm-growing probiotic strains have the ability to contribute to enhanced thermotolerance and freeze-drying resistance, and to replace resident biofilm-growing pathogens with a non-pathogenic bacteriocin-producing variant. Despite this, the molecular mechanisms controlling biofilm development of probiotic bacteria have so far been poorly investigated when compared with the extensive studies performed on the biofilm formation of several microbial pathogens. In fact, these types of studies could provide important insights into how normal microbiota is maintained, as well as being key starting points for the rational use of probiotics.

The response to probiotics treatment of biofilm-based oral, intestinal, vaginal and wound infections is reviewed here.

COMPETITION AND INTERFERENCE OF PROBIOTICS WITH ORAL BIOFILMS

Dental plaque, as a well-defined multispecies biofilm constituted by a complex microbial community, is known to play a major role in a variety of dental diseases, such as dental caries and periodontal disease. In the healthy oral cavity, beneficial and pathogenic bacteria maintain a delicate balance, while the accumulation of strictly anaerobic Gram-negative bacteria within the biofilm and the resulting microbial imbalance predispose one to the onset of periodontal diseases and transform the dental plaque into a difficult to treat ‘pathogenic’ biofilm.

Thus, oral cavities have been suggested as a relevant target for probiotic applications, through the use of non-pathogenic, bacteriocin-producing Lactobacilli and Bifidobacteria to restore the microbial balance and to counteract pathogenic bacteria. Clinical trials have been carried out to evaluate the ability of different probiotic products to reduce dental caries and caries risk in children, young adults and healthy complete denture wearers. In particular, after recognizing biofilms as the major cause of oral diseases, the extra-intestinal effect of probiotic strains through both specific interactions with dental biofilm and influencing oral health have been recently investigated.

Several studies detailing the role of probiotics in preventing dental caries have been published, with a particular emphasis on their ability to co-aggregate with caries-associated strains and to reduce the number of cariogenic bacteria, especially Streptococcus mutans, within the dental plaque.

In 2002, Cornelli and co-workers encouraged the selection of non-pathogenic dairy bacterial strains that were able to decrease the cariogenic potential of dental plaque. Specifically, they found that Lactococcus lactis NCC2211 was able to successfully incorporate itself into a biofilm, thus mimicking the dental plaque, and was able to modulate the growth of the cariogenic Streptococcus sobrinus OMZ176. Afterwards, the oral bacterium S11, isolated from the saliva of young children without dental caries and with a 99.5% similarity with Lactobacillus fermentum, was demonstrated to inhibit the ability of S. mutans to produce sucrose using glucosyltransferases (GTs) and to grow as biofilm, thus having an anti-biofouling effect.

In recent years, the ability of other commonly used probiotic strains (Lactobacillus acidophilus DSM 20079, Lactobacillus paracasei DSMZ 16671, Lactobacillus plantarum 299v, Lactobacillus rhamnosus GG, Lactobacillus reuteri strains PTA 5289 and L. reuteri SD2112, etc.) to hamper S. mutans growth and biofilm formation in vitro has been evaluated, and these results suggest that the antimicrobial activity of Lactobacilli seems to be strain-specific and pH-dependent. Lactobacilli have also been reported to reduce streptococcal adhesion, not as much on glass surfaces, but in particular on saliva-coated hydroxyapatite.

On the other hand, probiotic species were also shown to possibly cariogenic themselves under specific growth conditions (low pH and concurrent inoculation with the microcosm). For instance, intestinal probiotic L. salivarius W24 was able to affect the compositional stability of the microbial communities derived from individual saliva and appeared to have a cariogenic potential. Furthermore, Schwendicke and co-workers compared to and combined with S. mutans (SM), the probiotic L. rhamnosus GG (LGG), by simulating three biofilm compositions (SM, LGG, SM×LGG) and two dental lesion sites (smooth enamel, dentin cavity). The resulting mineral loss (ΔZ) in dental tissues and bacterial numbers measured after 10 days showed that this probiotic, other than lacking the inhibitory effects on SM, also induced mineral loss especially in dentin cavities and under highly cariogenic conditions, thus contributing to the caries process in a dental biofilm model.

Even if the strong evidence supporting the efficacy of probiotic strains in counteracting gingivitis and periodontitis is still not convincing, data in the literature are also accumulating with regards to this issue. In general, several papers state that probiotics could be useful in the improvement/maintenance of oral health, mostly in subjects at a high risk of periodontal disease. In in vitro studies and clinical trials, a variation in the composition of oral lactoflora in chronic periodontitis or gingivitis and in healthy subjects has been proposed, with both homo- and heterofermentative oral Lactobacilli suppressing the growth of oral pathogens. However, these results are all strain-, species- and origin-specific, and the effectiveness of probiotics on the prevention and treatment of periodontal diseases is therefore questionable. Currently, there is scarce evidence about the possible benefits of a systematic preventive use of probiotics in patients affected by periodontal diseases.

Regarding the ability of probiotic strains to interfere with biofilm-growing periodontal pathogens, the experimental results to date are few and divergent.

In fact, Teanpaisan and co-workers as well as Vuotto and coworkers have recently shown that Lactobacillus SD1–SD6 and L. brevis
and were effective in displacing the enteropathogens Salmonella typhimurium and Aggregatibacter actinomycetemcomitans and in the latter Prevotella melaninogena (Figure 1). Twetman group reported that a daily intake of probiotic lozenges did not seem to significantly affect plaque accumulation, inflammatory reaction or biofilm composition during experimental gingivitis.73

Conflicting results suggesting that probiotic therapy can prevent or combat oral disease is just beginning to evolve, and it is likely that other factors, such as the site of lesion or the availability of fermentable carbohydrates, might also affect caries, in some case more significantly than the bacterial composition of the oral cavity.76

COMPETITION AND INTERFERENCE OF PROBIOTICS WITH INTESTINAL BIOFILM

The most claimed use of probiotics is in contribute to intestinal well-being. In fact, administered probiotics appear to be able, although only transiently for the duration of their administration, to influence the composition and function of the intestinal microbiota,77 therefore providing robust clinical data on the benefits of probiotics in at least 3 main areas: intestinal infection, inflammatory bowel disease and irritable bowel syndrome.78

Probiotic cocktails of different strains have also been demonstrated to be useful in some gastrointestinal diseases. For instance, VSL#3, a cocktail containing 8 different strains, has proven to be effective in the primary prevention79 and maintenance of remission80 among patients with pouchitis.

The anti-biofilm properties of some probiotics against biofilm-growing enteropathogens have also been evaluated, despite the fact that the results obtained so far are few and conflicting. On the one hand, there are studies reporting that probiotics are able to inhibit biofilm formation of intestinal pathogens, but on the other hand different experimental data seem to support the enhancing of enteropathogens biofilm biomass in the presence of probiotics.

In the first case, Collado and colleagues showed that specific probiotic combinations are able to enhance the inhibition percentages of pathogens adhering to intestinal mucus when compared to single probiotic strains.81 Furthermore, single strains (L. acidophilus Bar13, L. plantarum Bar10, Bifidobacterium longum Bar33 and B. lactis Bar30) were effective in displacing the enteropathogens Salmonella typhimurium and Escherichia coli H10407 from a Caco-2 cell layer,82 and the exopolysaccharides released from L. acidophilus A4 were able to drastically decrease enterohemorrhagic E. coli biofilms on 96-well microplates (87%) and on polystyrene and polyvinyl chloride surfaces (94%) by affecting genes related to curli production (crl, csgA and csgB) and chemotaxis (cheY).83 In 2010, Hancock and coworkers investigated the biofilm-forming capacity of Nissle 1917 and found that this strain was a significantly better biofilm former than enteropathogenic, enterotoxigenic and enterohemorrhagic E. coli strains, with the exception of being able to outcompete such strains during biofilm formation.84

On the other hand, exopolysaccharides fractions produced by three probiotic strains (L. rhamnosus GG, B. longum NB667, and Bifidobacterium animalis IPLA-R1) have been demonstrated to increase the adhesion of Enterobacter sakazakii ATCC 29544 and E. coli NCTC 8603, Salmonella enterica serovar Typhimurium ATCC 29631 and Clostridium difficile ATCC 9689,85 while Miyazaki and co-workers86 reported in 2010 on the effects of nine probiotic bacterial strains on the growth, adhesion, and biofilm formation of enteroggregative E. coli (EAggEC), with the supernatants of L. casei ss. casei and L. casei ss. rhamnosus stimulating biofilm formation of EAggEC.

COMPETITION AND INTERFERENCE OF PROBIOTICS WITH BIOFILMS OF GENITOURINARY TRACT

Lactobacilli inhabiting the genitourinary environment seem to play a pivotal role in preventing illnesses, including urinary tract infections87–92 and bacterial vaginosis (BV), that is one the most common vaginal infections in women.93–102

Even at a small level, several clinical studies have demonstrated the potential of probiotics to treat BV, with three of four studies reporting a significant cure rate103–105 and three of five studies describing reduced recurrence rates when probiotics were used following antibiotic treatment.106–108

Most of these clinical trials were performed using high doses of Lactobacilli, thus suggesting that the amount of Lactobacilli could have a role in the effectiveness of the probiotic product.22

In particular, there is considerable evidence supporting the notion that hydrogen peroxide (H2O2) production by Lactobacilli is a key factor in resisting BV, and these particular strains are found in 61% of pregnant women with normal flora, yet in only 5% of women with BV.109 In fact, in vitro studies demonstrated that H2O2 is toxic to
Probiotics and biofilm-associated infections

C Vuotto et al

BV-causative organisms, such as Gardnerella vaginalis, Prevotella bivia and Atopobium vaginae.\textsuperscript{110}

Furthermore, the ability of Lactobacilli to co-aggregate with some urinary pathogens allows them to block the ability of pathogens to adhere and kills the pathogens through the production of antimicrobial substances.\textsuperscript{111–112} As an example, Mastromarino et al.\textsuperscript{113} found that Lactobacillus gasseri 335 and L. salivarius FV2 were able to coaggregate with G. vaginalis, and the combination of these lactobacilli strains with L. brevis CD2 in a vaginal tablet reduced the G. vaginalis adhesion by 57.7%.

It was demonstrated that G. vaginalis forms a biofilm on the vaginal epithelium that is highly tolerant to antibiotics.\textsuperscript{114–115} Biofilm formation, among other factors, allows for G. vaginalis to survive in the presence of lactobacilli-derived H\textsubscript{2}O\textsubscript{2} and lactic acid.\textsuperscript{116}

Despite this, recent experiments suggest that probiotics may still have a place in the treatment of BV alone or in combination with antibiotic therapy.\textsuperscript{117–118}

Additionally, probiotics that produce low amounts of H\textsubscript{2}O\textsubscript{2}, such as L. reuteri RC-14, are able to largely displace G. vaginalis, and deconvolution microscopy shows changes in the structure and viability of the biofilms, with loss of dense G. vaginalis biofilm pods.\textsuperscript{119}

With regard to the ability of specific probiotics to enhance the antibiotic activity against G. vaginalis biofilm, McMillan and colleagues\textsuperscript{120} evaluated changes occurring in a 12-mm-thick confluent A. vaginae and G. vaginalis biofilm after antibiotic and probiotic treatment. Metronidazole produced holes within the biofilm without eradicating bacteria, while L. reuteri RC-14 and L. rhamnosus GR-1 infiltrated BV biofilms leading to a higher bacterial cell death.

Recently, four single probiotics and four probiotic mixtures have been tested for their inhibitory activity against the urinary pathogens E. coli NCTC 9001 and Enterococcus faecalis NCTC 00775, a greater effect of the probiotic mixtures with respect to the single strains being not detected.\textsuperscript{121}

Lately, other probiotic species have been shown to positively impact vaginal infections, with \textit{in vitro} results providing a basis for the use of Pediococcus pentosaceus SB83 as a vaginal probiotic to prevent \textit{Listeria monocytogenes} colonization in pregnant women.\textsuperscript{122}

All of these findings provided evidence of how probiotics could interfere with an aberrant vaginal microbiota and gave strength to the possibility to eradicate pathogenic biofilms by administering probiotics, alone or combination with antimicrobials.

COMPETITION AND INTERFERENCE OF PROBIOTICS WITH WOUNDS BIOFILM

Recent findings indicate that chronic wound pathology may be caused by alterations in skin microbiota. Thus, probiotics could be promising tools to topically prevent and treat non-healing wounds.\textsuperscript{123}

Nevertheless, very little data, although consistent, have been reported to date on the ability of probiotic strains to prevent wound infections by acting against the main biofilm-growing causative agents. In fact, the research in this field is really in its infancy.

First, Valdez and colleagues evaluated the ability of the probiotic organism \textit{L. plantarum} to inhibit the pathogenic activity of \textit{Pseudomonas aeruginosa}, demonstrating that probiotic whole cultures as well as culture filtrates (acid filtrate and neutralized acid filtrate) were able to \textit{in vitro} inhibit \textit{P. aeruginosa} elastase and biofilm by affecting the production of the quorum-sensing signal molecules, acyl-homoserine-lactones. A burned-mouse model was used to test the \textit{in vivo} activity of \textit{L. plantarum} at 3, 4, 5, 7 and 9 days post-infection with \textit{P. aeruginosa}; inhibition of \textit{P. aeruginosa} colonization after 5, 10 and 15 days was proven by analyzing samples from skin, liver and spleen. These results revealed that \textit{L. plantarum} and/or its metabolites could be considered as potential therapeutic agents for the local treatment of \textit{P. aeruginosa} burn infections.\textsuperscript{124}

Following these results, only a few other papers have been published in this area, specifically by \textit{in vitro} studying of the two bacterial species mainly involved in biofilm-based wound infections, i.e., \textit{Staphylococcus aureus} and \textit{P. aeruginosa}. Walencka et al.\textsuperscript{125} evaluated the effects of surfactants obtained from three \textit{L. acidophilus} strains on \textit{S. aureus} and \textit{Staphylococcus epidermidis} adhesion and biofilm formation and demonstrated that both species, even if to a different extent, were inhibited by the tested surfactants. In particular, the probiotic-derived surfactants reduced the bacterial deposition rate and biofilm development without affecting cell growth, most likely by influencing the staphylococcal cell surface hydrophobicity. Furthermore, Sadowska and coworkers observed a limitation of staphylococcal biofilm formation using cell-free supernatants of \textit{L. acidophilus} H-1 and a direct competitive interactions between \textit{S. aureus} strains and the probiotic strain.\textsuperscript{126}

The inhibition of \textit{S. aureus} and \textit{P. aeruginosa} growth and biofilm formation was also evaluated by a co-incubation with \textit{L. fermentum} or culture filtrate of \textit{L. fermentum}. These results showed that \textit{L. fermentum}-secreted compound(s) inhibited both growth and biofilm formation of several \textit{S. aureus} and \textit{P. aeruginosa} strains.\textsuperscript{127} Finally, \textit{L. plantarum} has been demonstrated to be topically effective in preventing skin wound infections in mice,\textsuperscript{128} not only against \textit{P. aeruginosa} but also against \textit{S. aureus}.

CONCLUSIONS

The increasing interest in promoting health in a natural way has intensified the research in the field of probiotic on a global scale over the last two decades, thus leading to the industrial production of an overwhelming number of new products with an estimated 7% annual growth (Global Industry Analysis Report 2012).

To date, clinical confirmations have been obtained on the relevance of the relationship between immune system and probiotic microorganisms in protecting the host from colonization by pathogenic species. In fact, probiotics produce a variety of substances, ranging from relatively nonspecific fatty acids and peroxides to highly specific bacteriocins, which have been widely demonstrated to inhibit or kill other potentially pathogenic bacteria.

\textit{In vitro} studies and clinical trials have accumulated evidence in the recent years on the effect of probiotics, especially \textit{Lactobacilli}, in oral, wound and vaginal infections through a competition and counteraction of pathogens. However, data are still scarce and not always consistent to look at probiotics as tool to avoid biofilm formation and/or to disperse pre-formed pathogenic biofilms.

However, conflicting results also arise from a confounding interpretation of available data, often due not only to the differences in dose, delivery vehicle and evaluation of viability and efficacy, but especially to the variability in strain selection. In fact, no two probiotics are the same and, even within the same species, different strains may have quite different and sometimes contrasting effects.

In other words, an ideal probiotic that is able to compete and interfere with biofilm-growing pathogens needs to be properly identified, especially in the context of specific microbial targets and infection.

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