Probiotics for Prevention and Treatment of *Clostridium difficile* Infection

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Abstract

Probiotics have been claimed as a valuable tool to restore the balance in the intestinal microbiota following a dysbiosis caused by, among other factors, antibiotic therapy. This perturbed environment could favor the overgrowth of *Clostridium difficile*, and in fact, the occurrence of *C. difficile*-associated infections (CDI) is increasing in recent years. In spite of the high number of probiotics able to in vitro inhibit the growth and/or toxicity of this pathogen, its application for treatment or prevention of CDI is still scarce since there are not enough well-defined clinical studies supporting efficacy. Only a few strains, such as *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii*, have been studied in more extent. The increasing knowledge about the probiotic mechanisms of action against *C. difficile*, some of them reviewed here, makes promising the application of these live biotherapeutic agents against CDI. Nevertheless, more effort must be paid to standardize the clinical studies conducted to evaluate probiotic products, in combination with antibiotics, in order to select the best candidate for *C. difficile* infections.

1 Introduction

The gut microbiota is a complex and diverse microbial community that has coevolved with humans in a commensal way (Donaldson et al. 2016). In a healthy state, this collection of microorganisms protects the host by inhibiting colonization and growth of pathogens. However, antibiotic exposure strongly perturbs the intestinal microbiota, producing a decrease in microbial abundance and species diversity, as well as a suppression of the innate immune system disrupting the gut barrier and frequently causing antibiotic-associated diarrhea. In some cases, the intestinal dysbiosis followed after antibiotic treatment allows the overgrowth of *Clostridium difficile* given that this perturbed environment has a low abundance of short chain fatty acids, a high abundance of primary bile acids, a high carbohydrate availability, and an immunosuppressed host

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in the absence of microbial competitors in the gut (Lawley and Walker 2013).

C. difficile can be found in the gut microbiota of both healthy infants and adults, the occurrence being higher in infant (70%) than in the adult (17%) population (Ozaki et al. 2004; Jangi and Lamont 2010). In these healthy carriers, the presence of this microorganism does not seem to cause any disease. However, at the same time, *C. difficile* is the main causative agent of antibiotic-associated diarrhea in nosocomial environments (Leffler and Lamont 2015). As previously indicated, the antimicrobial therapy affects the endogenous gut microbiota diminishing colonization resistance, allowing the overgrowth of this pathogen and causing *C. difficile*-associated diarrhea (CDAD). This problem has been traditionally linked to elderly and institutionalized/hospitalized persons under antibiotic therapy (Rupnik et al. 2009); however, the occurrence of *C. difficile*-associated infections (CDI) seems to be increasing also in traditionally considered low-risk populations (Carter et al. 2012). This change in the epidemiology of CDI has been related to the worldwide distribution of hypervirulent strains (Yakob et al. 2015); besides, foods and animals have been found to act as carriers of this pathogen pointing at *C. difficile* as a zoonotic agent and suggesting potential foodborne transmission (Rodriguez et al. 2016). A range of virulent factors are the cause of colitis during CDI course, the main ones being several toxins, encoded in pathogenicity loci, and the flagella, which are factors allowing mobility and adherence of the pathogen (Abt et al. 2016). Pathogenesis was initially attributed to the production of toxins A (TcdA) and B (TcdB), belonging to the large clostridial toxin (LCT) family, which act as intracellular glycosyltransferases that inactivate Rho family GTPases, thus blocking downstream cellular events (Carter et al. 2012). More recently, strains producing a third toxin, the binary toxin (CDT), have been associated with an increase in the CDI severity; this toxin has two components the CDTa, which acts as an ADP-ribosyltransferase targeting actin, and CDTb that is able to bind to the cell and translocate the first component to the cytosol (Gerding et al. 2014). In spite of recent advances in the identification of processes involved on receptor binding and entry into mammalian cells, the mode of action of clostridial toxins remains to be totally elucidated (Orrell et al. 2017).

The standard treatment for *C. difficile* infection is the administration of antibiotics, mainly metronidazole, vancomycin, or fidaxomicin, but unfortunately, the recurrence rate of the disease is very high and this treatment becomes less effective. Indeed, it has been described that some *C. difficile* subpopulations (ribotypes) have a reduced susceptibility to metronidazole (Moura et al. 2013). In case of multiple recurrent CDI, fecal microbiota transplantation (FMT) is being more frequently used as the ultimate therapy, although the selection of the appropriate donor is a critical issue (Woodworth et al. 2017). These facts have prompted researchers to look for alternative therapeutic options (Fig. 1) which have been recently reviewed by different authors (Mathur et al. 2014; Hussack and Tanha 2016; Kachrimanidou et al. 2016; Kociolek and Gerding 2016; Martin and Wilcox 2016; McFarland 2016; Ofosu 2016; Padua and Pothoulakis 2016; Unal and Steinert 2016). Among them, probiotics have been proposed as a potential tool for preventing the dysbiosis of microbiota, caused by the administration of antibiotics, and for assisting the microbiota restoration after antibiotics or infection (Reid et al. 2011); thus, they have also been evaluated for prevention and treatment of CDI (Na and Kelly 2011).

Probiotics were defined in 2001 by a group of experts joined by FAO/WHO as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"; this definition was recently revised, and accepted after minor grammatical modifications, by members of the International Scientific Association for Probiotics and Prebiotics (ISAPP) which also proposes an overall framework for use of this term, encompassing diverse end uses (Hill et al. 2014). In next sections, we will review the current available data about the efficacy of probiotics in prevention and therapy for CDI, as

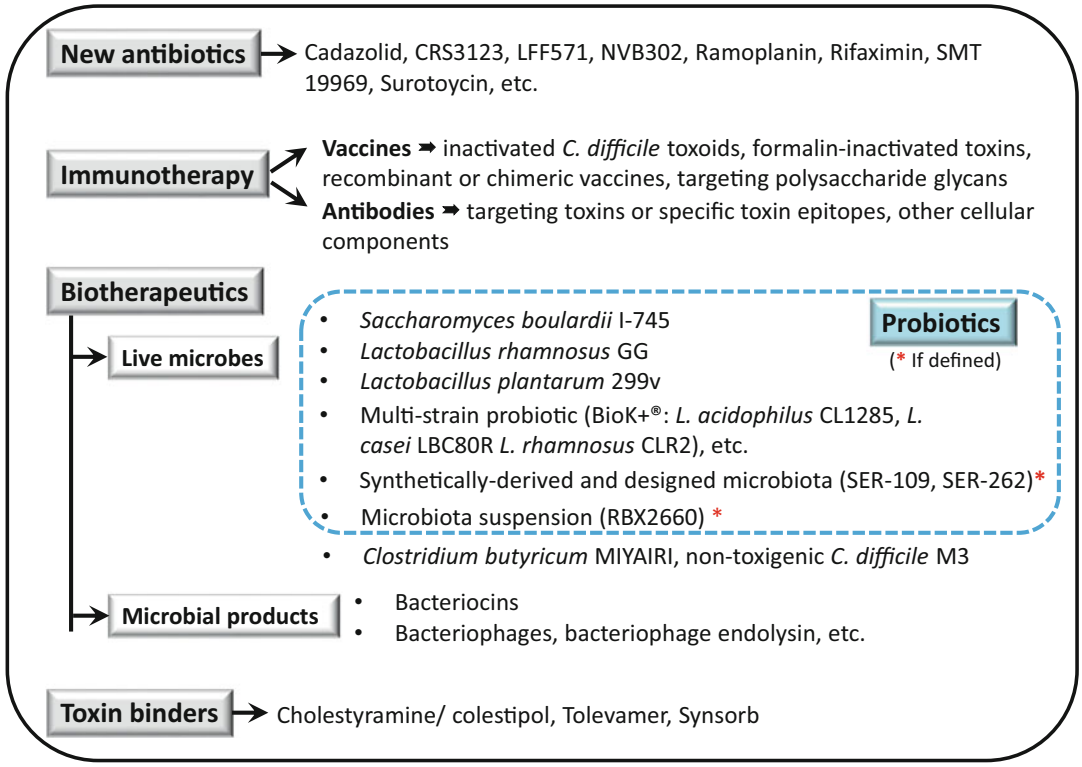

Fig. 1 Some therapeutic options currently under study for the prevention and treatment of *Clostridium difficile* infection

well as some putative mechanisms involved in this anti-*C. difficile* effect.

2 Clinical Studies Evaluating Probiotic Efficacy

The ability of probiotics for inhibiting the growth of *C. difficile* has been characterized by using different experimental approaches (Auclair et al. 2015; Forssten et al. 2015; Valdes-Varela et al. 2016b; Fredua-Agyeman et al. 2017). This use of probiotic microorganisms has long been considered a potential option to combat CDI. However, despise the large number of in vitro studies performed for the selection of probiotic strains with activity against *C. difficile* and for their use for CDI prevention or treatment, the evidence from human clinical trials is still limited. Different probiotic strains have been reported to increase the colonization resistance against *C. difficile* (Hopkins and Macfarlane 2003; Kondepudi et al. 2014; Auclair et al. 2015; Forssten et al. 2015). Certain strains of bifidobacteria and lactobacilli have been found to reduce the adhesion of *C. difficile* to intestinal epithelial cells or intestinal mucus (Collado et al. 2005; Banerjee et al. 2009) or to be able to inhibit its growth (Lee et al. 2013; Schoster et al. 2013; Valdes-Varela et al. 2016b). Moreover, animal studies seem to confirm a potential benefit of probiotics on the inhibition of *C. difficile* colonization (Mansour et al. 2017). Nevertheless, to date most of the clinical studies have focused on prevention, and there is a lack of data on the potential use of probiotics on the treatment of *C. difficile* infection.

During the last couple of decades, several studies have evaluated the usefulness of different probiotic strains in the prevention of CDAD.

However, in spite of the large number of strains screened in vitro, most of the evidence from clinical trials regards only a few bacterial strains, and most often, the studies have focused on the prevention of antibiotic-associated diarrhea, without further confirmation of *C. difficile* etiology. Among the assessed strains, the effect of *Lactobacillus rhamnosus* strain GG (Arvola et al. 1999; Vanderhoof et al. 1999), or the yeast species *Saccharomyces boulardii* (Kotowska et al. 2005; Can et al. 2006), in the prevention of antibiotic associated diarrhea has been widely recognized. Although not so extensively studied, other probiotic strains and probiotic mixes have also been evaluated around the world with positive results (Wullt et al. 2003; Maziade et al. 2015). The availability of a large number of clinical studies focusing on antibiotic-associated diarrhea has provided enough data for carrying out systematic reviews and meta-analysis studies, either considering probiotics as a group, which shows important limitations due to interstrain and/or interproduct variability, or meta-analyses focused on specific strains. The meta-analysis studies on the general use of probiotics for the prevention of antibiotic-associated diarrhea have consistently provided evidence for a beneficial role, especially in children (Cremonini et al. 2002; D'Souza et al. 2002; Sazawal et al. 2006; Johnston et al. 2007; Hempel et al. 2012; Goldenberg et al. 2015). Moreover, meta-analyses conducted for some specific probiotics, such as *S. boulardii* or *L. rhamnosus* GG, have further confirmed the beneficial effect of these strains in the prevention of antibiotic-associated diarrhea (McFarland 2006; Szajewska et al. 2007a, b). This has resulted in recommendations issued by the ESPGHAN (European Society for Paediatric Gastroenterology Hepatology and Nutrition) with regard to the use of probiotics for the prevention of antibiotic-associated diarrhea in children (Szajewska et al. 2016).

Furthermore, some studies have specifically focused on confirmed *C. difficile*-associated diarrhea, and these have also provided positive results for primary prevention (Wullt et al. 2003; Gao et al. 2010; Sampalis et al. 2010; Allen et al. 2013; Dietrich et al. 2014; Maziade et al. 2015). Some practical examples exist as well, such as that of the "Pierre-Le Gardeur" Hospital in Canada, which after a *C. difficile* outbreak began to administer a probiotic mix (BioK+®) together with any antibiotic prescriptions, achieving a significant reduction on the number of *C. difficile* disease cases (Maziade et al. 2015). Recent meta-analyses and systematic reviews have assessed the effects of probiotic administration, most of them administering the strains together with the antibiotic treatment, on the primary prevention of CDAD in different population groups (Table 1). In general the data support a beneficial effect of probiotics on the primary prevention of CDAD. However, the high heterogeneity among the available clinical studies makes difficult defining the best probiotic to be used, its dose, and the administration regime.

Regarding the prevention of the recurrence of the disease, the available data are more limited than in the case of primary prevention. Some clinical intervention studies have been conducted with variable results (McFarland et al. 1994; Surawicz et al. 2000), with reviews and meta-analyses indicating that there is only limited evidence on the benefit of probiotics in secondary prevention of CDI (Allen et al. 2013; O'Horo et al. 2014; McFarland 2015). The limited data available on secondary prevention underlines the need for more clinical intervention trials to be conducted in this topic.

To sum up, the available evidence strongly suggests that probiotics are helpful for primary prevention with only moderate evidence of a role in avoiding disease relapse. However, the potential role of probiotics in the treatment during the active phase of the disease remains largely unknown. Perhaps the major criticism that can be done to the available data is that there has not been a serious standardization effort for the probiotic products, doses, antibiotics, and therapeutic protocols to be used. Moreover, analyses of the cost-effectiveness of probiotic use on the prevention of *C. difficile* disease have not been performed until recently, with variable results, indicating the need for further studies conducted under different healthcare systems (Leal et al. 2016; Starn et al. 2016).

Table 1 Recent meta-analyses and systematic reviews on the use of probiotics in primary prevention of *C. difficile* infection

Target population	Probiotic	N° eligible RCTs	N° volunteers included	Conclusion	References
Elderly	Any	5	>3400	No significant effect	Vernaya et al. (2017)
Adults	Any	19	>6200	Significant reduction	Shen et al. (2017)
Adults	*Lactobacillus* (any)	10	>4800	Inconclusive evidence	Sinclair et al. (2016)
Adults and children	Any	26	>7900	Significant reduction	Lau and Chamberlain (2016)
Adults and children	Any (and by species)	21	>3700	Significant reduction	McFarland (2015)
Adults and children	Any	31	>4200	Significant reduction	Goldenberg et al. (2013)

RCT randomized controlled trial

3 Models to Study Probiotics Against *C. difficile*

Different experimental models have been developed in order to study the interaction of *C. difficile* with the host (recently reviewed by Young (2017)); additionally, these models can be used in the search for new therapeutic alternatives and adjuvant strategies for preventing or treating CDI (Table 2). Investigations using in vitro models of bacterial cultures are valuable systems for the screening of potential probiotics against *C. difficile*, but as disadvantage, they have the lack of feedback mechanisms with host and/or host-microbe interactions (Best et al. 2012). However, these microbial culturing models can be combined with cell culture systems to better mimic the interaction *C. difficile*—probiotic—host (Venema and van den Abbeele 2013). Co-cultures of toxigenic *C. difficile* strains with probiotic candidates have been carried out to determine the potential of the latter for reducing the germination of spores and outgrowth into vegetative toxin-producing cells of the pathogen (Table 2). Models of gut microbiota have been assayed to in vitro evaluate the potential of probiotic candidates for decreasing the growth of *C. difficile* in this complex microbial ecosystem. These models range from simple batch fermentations to complex multi-compartmental continuous systems (Venema and van den Abbeele 2013). Static batch cultures, containing fecal suspensions, have been used to observe the influence of probiotics on the survival of *C. difficile* (Tejero-Sarinena et al. 2013). Continuous culture systems (human "colonic" model) allow the study of the pathogen in an environment closer to the reality, over considerably longer periods than in static batch cultures (Best et al. 2012; Le Lay et al. 2015). Currently, most of the colonic simulators consists of four different units (glass vessels) continuously connected, having different pH and flow rates, thus representing the ascending, transverse, descending, and distal colon (Forssten et al. 2015).

Several in vitro studies investigated the effect of probiotic treatment on the interaction of *C. difficile* with components of the intestinal mucosa, such as mucus or epithelial cells (Table 2). The cytotoxicity of clostridial cell-free supernatants (obtained from co-cultures of probiotic vs. *C. difficile*) or of caecum contents (collected from animals infected with *C. difficile* and treated with potential probiotics) has been evaluated upon cell lines using classic label-based, endpoint methods (Banerjee et al. 2009; Trejo et al. 2010, 2013; Valdes-Varela et al. 2016a). However, label-free technologies are currently been available and being used in drug development processes, which are noninvasive techniques that allow the continuous (real-time)

Table 2 Summary of some in vitro models used to study potential probiotics against *Clostridium difficile*

In vitro experimental models			References
Microbial cultivation	*Vs.* probiotic	Co-cultures of *C. difficile* with probiotic candidates	Trejo et al. (2010), Best et al. (2012), Kolling et al. (2012), Lee et al. (2013), Schoster et al. (2013), Kondepudi et al. (2014), Yun et al. (2014), Ambalam et al. (2015), Andersen et al. (2016), Spinler et al. (2016), and Rätsep et al. (2017)
	Vs. microbiota/ probiotic	Static batch system	Tejero-Sarinena et al. (2013)
		Semicontinuous system	Le Lay et al. (2015)
		"Colonic" model	Forssten et al. (2015)
Intestinal cell lines	Adhesion/ exclusion	HT29-MTX cell	Zivkovic et al. (2015)
		Immobilized intestinal mucus	Collado et al. (2005), Banerjee et al. (2009), and Ferreira et al. (2011)
	Cytotoxicity	Label-based endpoint methods	Banerjee et al. (2009), Trejo et al. (2010, 2013), and Valdes-Varela et al. (2016a)
		Label-free, RTCA (real-time cell analyzer) method	Valdes et al. (2015) and Valdes-Varela et al. (2016a, b)

monitoring of the status of live cells (Xi et al. 2008). Indeed the label-free, impedance-based RTCA (real-time cell analyzer) technology has been applied to develop methods allowing the clinical diagnosis of toxigenic *C. difficile* in different biological samples (Yu et al. 2015). Recently, this RTCA technology was also used in our group to develop a model to test the cytotoxicity of *C. difficile* supernatants upon the intestinal epithelial cell lines HT29 and Caco-2 (Valdes et al. 2015). Moreover, this model was used to search for potential probiotic strains able to counteract the toxic effect of *C. difficile* supernatants upon HT29 (Valdes-Varela et al. 2016a) as well as to evaluate the toxicity of *C. difficile* co-cultured with some of these probiotics (Valdes-Varela et al. 2016b).

On the other hand, several models have been used to assess the ability of probiotic candidates to modify the adhesion of *C. difficile* to the intestinal mucosa, such as those using immobilized (human) intestinal mucus which showed a good correlation with data obtained with a enterocyte-like (Caco-2) model (Collado et al. 2005; Banerjee et al. 2009; Ferreira et al. 2011). The ability of potential probiotic strains to inhibit the adhesion of *C. difficile* has also been evaluated using intestinal cell lines, such as HT29-MTX which is a derivative from HT29 (adapted to methotrexate) thus synthesizing higher amounts of mucus (Zivkovic et al. 2015). A study has suggested that this cell model may be more suitable for studying cell-pathogen interactions, as well as effectiveness of antimicrobial treatments, as compared to Caco-2 or HT29 models which do not have goblet cells or do not constitutively secrete mucus, respectively (Gagnon et al. 2013).

In a step forward, several authors have evaluated the protective effect of selected probiotic candidates against CDI in animal models (Best et al. 2012; Kolling et al. 2012; Trejo et al. 2013; Kondepudi et al. 2014; Yun et al. 2014; Andersen et al. 2016; Arruda et al. 2016; Spinler et al. 2016; Rätsep et al. 2017). This infection has been studied in different models, including mice, hamsters, rats, rabbits, hares, guinea pigs, prairie dogs, quails, foals, piglets, and monkeys. Moreover, zebrafish embryos have been described as suitable models for identification of in vivo targets of *C. difficile* toxins and evaluation of novel candidate therapeutics; zebrafish possess many of the major organs present in humans, and due to the transparency of the embryo, damage by toxins can be visualized by standard light microscopy (Best et al. 2012). Each of the *C. difficile* animal models has inherent advantages and disadvantages. The hamster model has been widely used to study pseudomembranous colitis in human because of extreme sensitivity to infection following antibiotic administration, using clindamycin as agent of choice; however, this model does not represent the usual course and

spectrum of CDI in humans. Recently, new mouse and piglet CDI models have been developed which appear to mimic many of the disease symptoms observed in humans (Sun et al. 2011; Best et al. 2012; Hutton et al. 2014).

4 Mechanisms of Probiotic Action

As pointed in previous sections, probiotics are gaining more and more interest as preventive and co-adjuvant therapies for treatment of antibiotic-associated dysbiosis. However, their modes of action are poorly understood and vary between probiotic microorganisms. Indeed, the effects of any probiotic are strain-specific, and therefore, beneficial effects cannot be extrapolated to other species or strains (Hickson 2011). It has been described that probiotics could have diverse positive actions on the host by (1) modulating the intestinal microbiota and inhibiting pathogenic microorganisms at the intestinal luminal environment, (2) enhancing of intestinal barrier function at the intestinal epithelium, and (3) modulating the immune response, among others (Ng et al. 2009). Several mechanisms have been proposed for explaining the potential role of probiotics against *C. difficile*. Some of these effects, such as the production of antimicrobial factors (Corr et al. 2007), competitive inhibition of the pathogen (Collado et al. 2005), and the ability to degrade and to reduce the toxicity of *C. difficile* (Castagliuolo et al. 1999; Valdes-Varela et al. 2016a), could be of help not only in the prevention but also in the treatment of CDI.

4.1 Microbial Antagonism: Interaction Probiotics vs. *C. difficile*

The restoration of intestinal microbiota after dysbiosis, caused by any etiological agent, is the main way of action of any treatment against intestinal pathogens including *C. difficile* (Gareau et al. 2010; Reid et al. 2011). This was evidenced, for example, in an in vivo study with a murine CDI model of antibiotic-induced dysbiosis, in which the gut microbiota was restored after treatment with a multi-strain probiotic supplement (*Lactobacillus plantarum* F44, *Lactobacillus paracasei* F8, *Bifidobacterium breve* 46, *Bifidobacterium animalis* subsp. *lactis* 8:8) (Kondepudi et al. 2014). There are several mechanisms by which probiotics can help the restoration of the intestinal microbiota, some of them being related to typical bacterial antagonism (Ng et al. 2009); however, little is known about those mechanisms acting specifically in the context of CDI (Parkes et al. 2009; Ollech et al. 2016).

Some probiotic strains are able to compete with pathogenic bacteria for the adhesion sites, that is, competitive exclusion, thus providing a "physical" barrier that increases the colonization resistance (Fig. 2a). In vitro studies showed the ability of selected *Bifidobacterium* and *Lactobacillus* strains to modify the adhesion of *C. difficile* to intestinal epithelial cells or intestinal mucus, the effect being strain-dependent (Collado et al. 2005; Zivkovic et al. 2015). A reduction from 60% to 3% in the adhesion of *C. difficile* to gingival epithelial cell cultures (obtained from healthy horses) was reported when *Lactobacillus reuteri* Lr1 was added; additionally, it was detected that this strain was able to co-aggregate with the pathogen (Dicks et al. 2015). In this regard, it has been suggested that the aggregation capability between lactobacilli and *C. difficile* could be a way to reduce the adhesion of the pathogen to the intestinal mucosa (Ferreira et al. 2011). *S. boulardii* is also able to reduce the adhesion of *C. difficile* to epithelial cells, and the same effect was detected using extracts obtained from the cell wall of this yeast (Tasteyre et al. 2002). Similarly, it has been proved that cell-free supernatants obtained from *Lactobacillus delbrueckii* ssp. *bulgaricus* B-30892 (Banerjee et al. 2009) and different bifidobacterial strains (Trejo et al. 2006) were able to reduce the adhesion of *C. difficile* to intestinal epithelial Caco-2 cells. Different treatments of the bifidobacterial supernatants showed that the factors related to the anti-clostridial adhesion

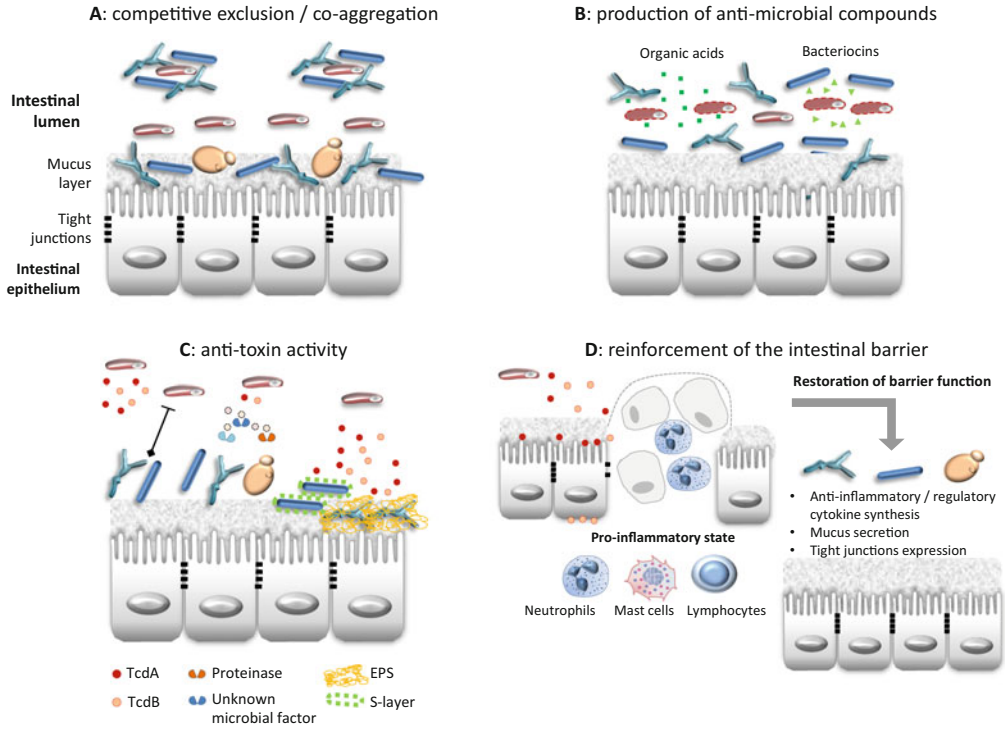

Fig. 2 Potential mechanisms of action proposed for probiotics against *C. difficile*. (**a**) Competitive exclusion/co-aggregation. (**b**) Production of antimicrobial compounds. (**c**) Anti-toxin activity. (**d**) Reinforcement of the intestinal barrier

were not heat-resistant and nonrelated with acids (active at neutral pH) and were not affected by proteinases, but its nature remains unknown (Trejo et al. 2006). Indirect evidence suggests that exopolysaccharides covering the surface of some probiotics could be involved in the inhibition of the binding capability of some pathogens, including *C. difficile*, by probiotics (Ruas-Madiedo et al. 2006). Thus, altogether, these studies suggest that different surface molecules and/or secreted factors might be implicated in the interference of probiotics against *C. difficile* adhesion to the intestinal mucosa.

Another mechanism of probiotic action is the inhibition of the pathogen growth through the competition for the limiting nutritional sources and/or by the production of antimicrobial factors, such as organic acids and bacteriocins (Fig. 2b). In a study carried out with a CDI animal model, it was shown that mice treated with *Streptococcus thermophilus* LMD-9 exhibited less pathology and lower detectable toxin levels in cecal contents, compared with untreated controls; an inverse correlation was observed between the levels of luminal lactate and the abundance of *C. difficile*, suggesting that the anti-clostridial effect was due to the production of this organic acid (Kolling et al. 2012). Similarly, the lactic acid synthesized by *Lactobacillus acidophilus* GP1B had an inhibitory effect on *C. difficile* growth in a CDI mouse model, which may be related to a reduction in pH as a result of organic acids produced by the probiotic bacterium (Yun et al. 2014). Several in vitro studies have investigated the activity of probiotics to inhibit *C. difficile* growth; using a fecal, pH-controlled (between 6.7 and 6.9), anaerobic batch model, it was found that *Lactobacillus casei* NCIMB30185 and *B. breve* NCIMB30180 were able to reduce the numbers of *C. difficile* in this complex

microbial ecosystem (Tejero-Sarinena et al. 2013). Co-cultivation of *C. difficile* with cell-free supernatants from different commercial probiotics highlighted that the mechanism of inhibition was pH-dependent; thus, the production of organic acids, mainly lactic and acetic acids, is the inhibition factor controlling the growth of *C. difficile* (Schoster et al. 2013). In another in vitro study, the co-incubation of *C. difficile* with *L. rhamnosus* LR5, *Lactococcus lactis* SL3, *B. breve* BR3, and *B. animalis* subsp. *lactis* BL3 demonstrated their potential to decrease *C. difficile* numbers, mainly mediated by the organic acid production. However, among those strains, SL3 appeared to have the strongest activity which seems to be pH-independent and likely could be mediated through the action of a bacteriocin (Lee et al. 2013). Similar pH-dependent and pH-independent effects against *C. difficile* were also reported using cell-free supernatants from other commercially available probiotics (Fredua-Agyeman et al. 2017). With respect to the competition for nutrients, some studies have been carried out using "synbiotic" combinations, which are mixtures of probiotics and prebiotic substrates that (theoretically) will improve the performance of probiotics or other beneficial microbes in the gut. In a mice (C57BI/6) model of CDI, the feeding with a synbiotic formulation, consisting of four strains (*L. plantarum* F44, *L. paracasei* F8, *B. breve* 46, *B. animalis* subsp. *lactis* 8:8) and three prebiotics (galacto-oligosaccharides, isomalto-oligosaccharides, and resistant starch), conferred protection against this pathogen (Kondepudi et al. 2014). Some studies have suggested that the growth inhibition of *C. difficile* by probiotics is strain but also carbon source specific. Ambalam et al. reported the ability of cell-free supernatants from *L. paracasei* F8 and *L. plantarum* F44 to inhibit the growth of *C. difficile* strains when they grew on glucose, due to the production of organic acids and heat-stable antimicrobial proteins, while the effect was only pH-dependent when growing on prebiotics (Ambalam et al. 2015). Our workgroup recently analyzed the influence of carbon sources upon *C. difficile* growth and toxicity when co-cultured with *Bifidobacterium longum* IPLA20022 or *B. breve* IPLA20006 in the presence of short-chain fructo-oligosaccharides (scFOS) or inulin. The use of scFOS reduced the growth of the pathogen, as well as the toxicity of the co-culture supernatants, which was not observed with inulin (Valdes-Varela et al. 2016b).

4.2 Probiotics Against *C. difficile* Toxin Activity

The toxins produced by *C. difficile* are responsible for the clinical profile of the CDI. Therefore, therapeutic agents that reduce toxin-induced damage could be valuable tools to alleviate the severity of symptoms and to improve the course of the disease. Some authors have reported that probiotics are able to reduce the activity of *C. difficile* toxins but, in most cases, the specific mechanisms of action by which probiotics exert the protective effect in this infection are unknown (Fig. 2c). In a hamster model of enterocolitis induced by *C. difficile*, *Bifidobacterium bifidum* CIDCA5310 protected the animals, and avoided mortality, when compared with the control (infected) group; besides, the supernatants obtained from caecum contents were less toxics upon Vero (cells from monkey's kidney) cultures in animals fed with the bifidobacteria, suggesting that this strain is able to in vivo counteract the effect of clostridial toxins (Trejo et al. 2013).

Co-culture of toxigenic strains of *C. difficile* with different strains of bifidobacteria and lactobacilli leads to a reduction of the cytotoxic effects of spent culture supernatants on cultured Vero cells, which correlates with a diminution of clostridial toxins present in these supernatants (Trejo et al. 2010). However, the growth of clostridial strains in BHI medium with different concentrations of cell-free supernatants from bifidobacteria or lactobacilli cultures did not decrease the toxic effect of pathogens; taking into account these results, authors hypothesized that co-culture of clostridia with lactobacilli or bifidobacteria leads to the modification of the environment, thus leading to the repression of toxin synthesis/secretion pathway. Similarly, a cell extract from *L. acidophilus* GP1B was able

to decrease the pathogenicity of *C. difficile* by inhibiting quorum sensing signaling, probably by lowering the expression of quorum sensing-regulated toxin genes (Yun et al. 2014).

On the other hand, it was observed that some microorganisms release metabolites that are able to inhibit the harmful effects of toxins. A bacterial cell-free supernatant obtained from *L. delbrueckii* subsp. *bulgaricus* LDB B-30892 reduced cytotoxic effects of *C. difficile* ATCC9689 upon the human intestinal epithelial cell line Caco-2- (Banerjee et al. 2009). Banerjee et al. (2009) suggested that bioactive components, of unknown nature, were released by this strain which were the probable causative agents of inhibition of the clostridial toxins. Similarly, bacterial cell-free supernatants obtained from *L. lactis* CIDCA8221 contained heat-sensitive metabolites, higher than 10 kDa, that were not affected by treatment with different proteases or protease inhibitors, which were able to inhibit cytotoxic effects of *C. difficile* toxins upon epithelial Vero cells (Bolla et al. 2013). These results suggest that the protective effect of *L. lactis* CIDCA8221 supernatant could be owing to a non-covalent interaction between molecules present in the lactococcal supernatant and toxins. In this regard, surface components of the bacterial cell envelope, such as exopolysaccharides which can be released to the environment, have been proposed to in vitro inhibit the adverse effect of pathogenic toxins (Ruas-Madiedo et al. 2010). A study showed the ability of the outermost (proteinaceous) S-layer from *Lactobacillus kefir* strains to inhibit the damage induced by supernatants obtained from *C. difficile* upon Vero cells; the protective effect was not affected by inhibitors of proteases or heat treatment, while pre-incubation with specific anti-S-layer antibodies reduced the inhibitory effect of these proteins (Carasi et al. 2012). From this study, it was concluded that the capability for reducing the toxigenic effect of *C. difficile* could be attributed to an interaction between its toxins and the *L. kefir* S-layer protein (Carasi et al. 2012). Recently, our workgroup analyzed the capability of *Bifidobacterium* and *Lactobacillus* strains to reduce the toxic effect of supernatants obtained from *C. difficile* LMG21717 ($TcdA^+$, $TcdB^+$) culture upon the human intestinal epithelial cell line HT29. For this purpose, the probiotic candidates were incubated together with a toxigenic supernatant of *C. difficile*, and the analyzed strains from *B. longum* and *B. breve* species were able to reduce the toxic effect of the pathogen; more specifically, the strain *B. longum* IPLA20022, in a viable state, showed the highest ability to reduce the levels of both clostridial toxins and to counteract the cytotoxic effect upon HT29 (Valdes-Varela et al. 2016a). Furthermore, the incubation of supernatant from *B. longum* IPLA20022 with the toxigenic *C. difficile* supernatant showed similar effect on the cell line than that obtained with the bifidobacterial biomass. The treatment of the clostridial supernatant with this probiotic strain prevented the rounding of HT29 cells, detected in cells treated only with *C. difficile* supernatant, thus keeping a monolayer structure resembling that of the control (nontreated HT29) (Fig. 3). Taking into account these results, we hypothesize that the adsorption of toxins to the bifidobacterial surface and the secretion of molecules able to reduce the cytotoxic effect by degrading the toxins are both probable mechanisms of action (Valdes-Varela et al. 2016a). In this regard, 20 years ago, it had been reported that *S. boulardii* inhibited *C. difficile* TcdA effects in the rat ileum by releasing a 54kDa serine protease which hydrolyzed toxin A and its intestinal receptor (Castagliuolo et al. 1996); this could be the mechanism behind the effectiveness of this yeast in both the prevention and the treatment of antibiotic-associated colitis in humans (Castagliuolo et al. 1999). More recently, it was observed that a protease secreted by *Bacillus clausii* O/C is able to inhibit the cytotoxic effect of *C. difficile*; thus this enzyme could be involved in the protective effect of this bacilli in antibiotic-associated diarrhea (Ripert et al. 2016). A similar phenomenon may be taking place with the abovementioned *Bifidobacterium* strains (Valdes-Varela et al. 2016a).

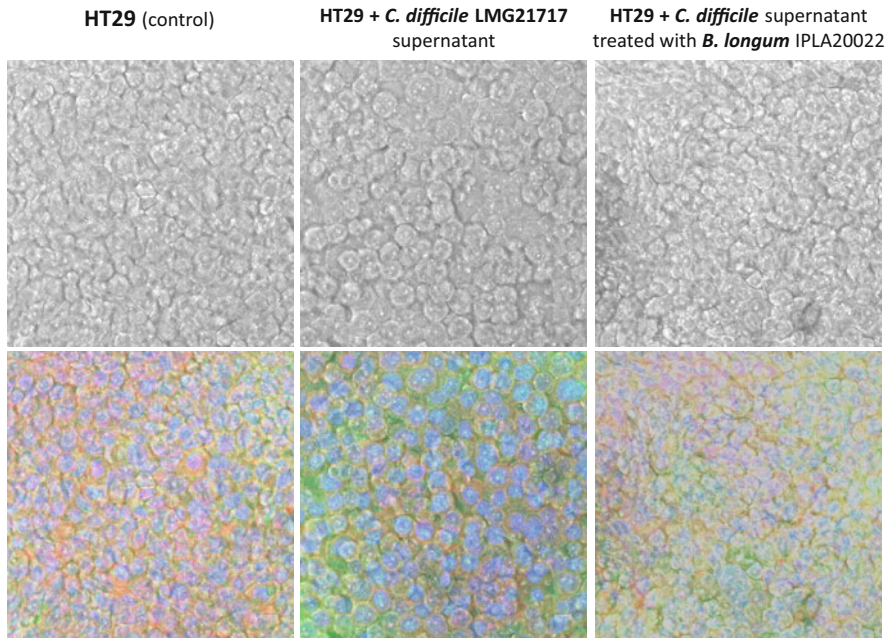

Fig. 3 CSLM (Leica TCSAOBS SP8 X confocal microscopy) images obtained, after 20 h incubation, for HT29 cells submitted to different treatments. (**a**) Panel shows transmission (visible) images and (**b**) panel shows Z-projection snapshots resulting from a combination of the transmission image with the "blue" image, captured with the violet laser diode (excited at 405 nm, showing DAPI-stained nucleus); the "red" image, captured with the white laser (excited at 578 nm, showing phalloidind-alexafluor-568-stained F-actin); and the "green" image resulting from the autofluorescence emitted by the intracellular components of HT29. The 63×/1.4 oil objective was used; bars 10 μm. Individual images of stained nucleus and/or F-actin were included in the reference Valdes-Varela et al. (2016a)

4.3 Other Mechanisms of Action

The intestinal barrier function given, among other factors, by the presence of an intact intestinal epithelium enabling the absorption of nutrients and the exclusion of harmful substances can be compromised by the activity of enteric pathogens including *C. difficile* (Barreau and Hugot 2014). In fact, internalized clostridial toxins induce changes in the F-actin cytoskeleton and a breakdown of the tight junctions, thus contributing to the disruption of the epithelial barrier function; the increase in the permeability of this barrier ends with an inflammatory process due to the infiltration of neutrophils, production of chemokines and pro-inflammatory cytokines, and activation of mast cells and lymphocytes, among other events (Voth and Ballard 2005; Rupnik et al. 2009; Abt et al. 2016). Thus some probiotics have been claimed to be able to reinforce the intestinal barrier function, although there is not much information in the context of CDI (Fig. 2d). In a hamster model of CDI, the oral administration of live *S. boulardii* five days before the infection significantly reduced cecal tissue damage, NF-κB phosphorylation, and TNFα protein expression caused by different *C. difficile* ribotypes, thus indicating that this probiotic can prevent intestinal damage and inflammation (Koon et al. 2016). In fact, after a literature search conducted by Stier and Bischoff (2016), they found that mechanisms of *S. boulardii* action involve not only a direct effect on the pathogen or its toxins but also impact on the innate and adaptive immune response of the host induced after CDI. Regarding probiotic bacteria, it has been shown that *L. rhamnosus* L34 and *L. casei* L39 are able to modulate, by

different ways, the inflammation caused by *C. difficile*, thus making suitable the use of these vancomycin-resistant lactobacilli for treating CDI (Boonma et al. 2014). In our research group, we have detected that lactobacilli strains are able to increase the synthesis of interleukin (IL)-8 and mucins by HT29-MTX monolayers challenged with *C. difficile*, thus helping to the reinforcement of the innate immune defense (Zivkovic et al. 2015). More recently, a combination of *Lactobacillus helveticus* BGRA43, *Lactobacillus fermentum* BGHI14, and *S. thermophilus* BGVLJ1–44 was in vitro tested against *C. difficile* in a Caco-2 model, and results showed an increase in the release of transforming growth factor (TGF)-β, thus resulting in a promising probiotic candidate to be further evaluated against CDI (Golic et al. 2017).

Finally, recombinant lactobacilli, although they cannot be considered as probiotics, could be suitable vehicles for the in situ production and delivery of therapeutic molecules in the intestine. In a recent study, the basis for an oral antitoxin strategy based on engineered *Lactobacillus* strains expressing TcdB-neutralizing antibody fragments in the gastrointestinal tract was explored; the results showed that only lactobacilli displaying the anti-TcdB variable domain of the heavy chain antibody can inhibit the cytotoxic effect of TcdB in the gastrointestinal tract of a hamster model (Andersen et al. 2016).

5 Conclusion and Future Trends

The search for probiotics with anti-*C. difficile* activity has been an active area of research for more than two decades. However, in spite of the abundance of in vitro studies, the in vivo evidence is less conclusive. The role of probiotics in preventing antibiotic-associated diarrhea is well established by several clinical intervention studies and meta-analyses. Good evidence is also available regarding the benefit of certain probiotics in the prevention of specific *C. difficile* diarrhea, being still necessary to define the best conditions for maximizing the efficacy. However, the studies on the use of probiotics in the treatment of CDI are still scarce; this is in spite of the several potential mechanisms of action that would be of interest in the case of *C. difficile* infection. Among them, the ability of certain strains to inhibit the growth of *C. difficile*, or to promote the restoration of the normal gut microbiota, represents two very direct potentially beneficial mechanisms of action. Moreover, specific probiotic strains have been found to be able to reduce the toxicity of this pathogen and/or to degrade the produced toxins. This inhibition of *C. difficile* toxicity may constitute an interesting strategy for the treatment of CDI by probiotics: first by eliminating the toxins from the intestine and second by the promotion of the microbiota restoration by the use of selected probiotic strains with both properties.

The existing clinical interest of CDI together with the successful application of FMT allows foreseeing that the interest in the use for probiotic therapies, likely using defined combinations of strains, will continue rising during the next years. In this regard, the development of products, based on the combination of strains with different properties and anti-*C. difficile* mechanisms of action, promises to allow the development of highly efficacy products for both prevention and treatment of CDI.

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