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Gelatin tannate and tyndallized probiotics: a novel approach for treatment of diarrhea

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Abstract. – OBJECTIVE: Intestinal permeability impairment is implicated in many gastrointestinal (GI) diseases. Chronic diarrhea, defined as the presence of diarrhea for more than 3 weeks in adults and 2 weeks in children, requires a different diagnostic and therapeutic work-up than acute diarrhea. Gelatin tannate, by reducing the clinical activity of acute colitis and the proinflammatory effects of lipopolysaccharide (LPS), is emerging as a mucosal barrier protector.

MATERIALS AND METHODS: New therapeutic strategies focusing on the physiological function of the intestinal barrier, may offer an innovative approach for the clinical improvement of highly debilitating chronic GI diseases. We review the available data on the role of gelatin tannate and tyndallized probiotics in the treatment of diarrhea.

RESULTS: Gelatin tannate and tyndallized probiotics can be used to re-establish the physiological functions of the gut barrier, as well as for preventing dysbiosis. There is evidence that due to their particular properties, gelatin tannate and tyndallized probiotics are highly effective in the treatment of acute gastroenteritis and may be especially indicated in the management of moderate and prolonged diarrhea.

CONCLUSIONS: Gelatin tannate and tyndallized probiotics may be effective in the management of chronic diarrhea. Further clinical trials are necessary to further explore their effects in clinical practice.

Key Words:
Gelatin tannate, Tyndallized probiotics, Microbiota, Gut barrier, Gastrointestinal disorders, Diarrhea.

Introduction

The gastrointestinal (GI) tract constitutes one of the largest sites of exposure to the outside environment. Therefore, the role of the gut barrier is crucial[1,2]. The gut barrier is a functional unit organized as a multi-layer system. It is divided into two main parts: a physical barrier and a functional inner barrier that differentiates between pathogens and commensal microorganisms[3]. A component of the gut barrier is the resident microbiota, while the subsequent barrier is the mucus layer[1,4,5]. The inner part of the gut barrier is represented by a complex network of immune cells known as the gut-associated lymphoid tissue (GALT). These structures are essential for the overall activity of gut barrier. Their impairment allows the passage of the luminal contents into the underlying tissues and hence into the bloodstream (leaky gut), resulting in the activation of the immune response and turn gut inflammation. This alteration in permeability is the basis for the pathogenesis of many GI diseases[1,6,7].

The Gut Barrier

The gut microbiota exerts some functions: it acts as a physical barrier and is involved in synthesis, immune stimulation, metabolism of nutrients, and metabolism of drugs and toxins[4,8-13]. Several lines of evidence suggest that the intestinal microbiota plays a key role in the pathophysiology

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of irritable bowel syndrome (IBS) including: the development of symptoms fulfilling the criteria for IBS in about 10% of individuals experiencing acute infectious gastroenteritis (so-called post-infectious IBS, PI-IBS), the presence (in a subgroup of patients with IBS) of circulating antibodies against flagellin and increased fecal levels of human β-defensin-2, the qualitative and quantitative changes in the intestinal bacterial composition and, finally, the improvement of symptoms after modulation of gut microbiota with probiotics and non-absorbable antibiotics[14].

The mucus is the first physical barrier that bacteria encounter within the digestive tract and it acts as a 'shield' for the epithelium, defending it from harmful microorganisms and antigens. It is composed of two layers: an inner layer firmly attached to the epithelial cells and an outer layer that is less sticky[15]. Both layers are organized around the highly glycosylated mucin MUC2, which forms an amorphous polymer-like cover and is secreted by goblet cells. The MUC2 mucin is a molecule that has been preserved through evolution[16]. This compartmentalization is essential for intestinal homeostasis within a highly colonized colon[17,18]. The importance of the mucosal barrier has been extensively demonstrated in MUC2 deficient animals[19-21]. The intestinal epithelium is organized in a monolayer of cells, and is formed by five different cell types: enterocytes, endocrine cells, M cells, G cells and Paneth cells. The enterocytes are the most common and act as a physical barrier to block the translocation of luminal contents to the inner tissues. They are connected by inter-cellular structures called tight junctions (TJ)[22]. An impairment of this balance increases the mucosal permeability leading to the activation of the adaptive immune response and the inflammatory state[23-29].

Gut Barrier Leaking and Related Diseases

There is a body of scientific data showing a significant association between gastro- and extra-intestinal diseases and permeability alteration. In some cases, such as in IBS and celiac disease, the alternations in permeability may be the primary pathogenic cause. In others, such as in liver cirrhosis, it produces the translocation of microbial antigens into the entero-hepatic circulation with the consequent exacerbation of liver fibrosis and portal hypertension, and a further increase in permeability[1]. Whether the alteration of the barrier function is an epiphenomenon, an early event, or an essential step in the pathogenesis of these diseases, remains to be clarified. However, it is well known that an increase in intestinal permeability contributes to the exacerbation of these pathologies. In many cases, the same drugs that lead to disease resolution, even temporarily, also lead to a recovery of the permeability and restoration of physiological intestinal homeostasis[19]. For example, in IBS patients, *in vivo* and *in vitro* studies demonstrate a significantly higher intestinal permeability, in comparison with healthy subjects, associated with changes in TJ expression. This increased mucosal permeability causes the passage of endoluminal antigens into the deeper layers and the activation of the immune response leading to the low-grade inflammation demonstrated at least in a subgroup of patients with IBS. In particularly activated mast cells release into the intestinal milieu some bioactive mediators that affect intestinal motor function and visceral sensitivity[7,30-33]. Therefore, in this context new therapeutic strategies focused on the restoration of the physiological function of the intestinal barrier may offer an innovative therapeutic approach for these highly debilitating chronic diseases.

Focus on Diarrhea

Diarrhea is the passage of increased amounts of loose or watery stools. Normal stools are usually solid because the small intestine and colon are highly effectives in absorbing nutrients, fluid and salts from the liquid, upper gut contents. Diarrhea occurs when these processes are impaired. It can be acute (short-term, less than 7 days), prolonged (a form of acute diarrhea lasting more than 7 days) and chronic (long-term) if symptoms persist more than 2 to 4 weeks, depending on age-appropriate definitions. Diarrhea is also classified as mild, moderate and severe on the basis of symptom severity and dehydration reflects the severity of symptoms. Most people, especially children, are affected by diarrhea at some point in their life. In fact, the incidence of diarrhea ranges from 0.5 to 2 episodes per child per year in children younger than 3 years old in Europe[34]. Although it is a mild disease in most European countries, acute diarrhea leads to a high number of hospital admissions and is associated with high socioeconomic costs. Rotavirus is the most severe enteric pathogen of diarrhea in children. Fever, abdominal pain and vomiting are common symptoms of a rotaviral infection[34]. Moreover, acute diarrhea is the third most common cause of death in children under 5 years old in developing countries, immediately after perinatal conditions and acute respiratory

Table I. Clinical dehydration scale.

Characteristic	Score 0	Score 1	Score 2
General appearance	Normal	Thirsty, restless or sleepy Irritable when touched	Drowsy, limp and cold May be comatose
Eyes	Normal	Slightly sunken	Deeply sunken
Tongue	Moist	Sticky	Dry
Tears	Present	Decreased	Absent tears

infections[35]. Both living in certain regions (Indian sub-continent, Africa, and Latin America) and pediatric age are the most important risk factors. Other risk factors include immune suppression, the use of proton pump inhibitors and antibiotics[36]. According to the World Health Organization (WHO), the recommended treatment for acute diarrhea is oral rehydration with antibiotics and anti-parasitic drugs prescribed only in specific cases. The clinical assessment of the degree of dehydration should be used to guide therapy in the management of pediatric patients with acute gastroenteritis. To overcome the limited accuracy of individual clinical signs, the WHO recommends using a combination of different clinical signs to identify severe dehydration[37-39] (Table I and Table II).

Chronic diarrhea is defined as a condition characterized by the presence of diarrhea for more than 3 weeks in adults and 2 weeks in children and it requires a different diagnostic and therapeutic work-up than acute diarrhea[40,41]. In developing countries, chronic diarrhea is frequently caused by bacterial, mycobacterial, and parasitic infections, although functional disorders such as malabsorption, and inflammatory bowel disease (IBD) are also common. In developed countries, common causes of chronic diarrhea are IBS, IBD, celiac disease, malabsorption syndromes, including lactose intolerance and small intestinal bacterial overgrowth, or chronic infections, particularly in immune-compromised patients[42]. However, there is a substantial number of subjects (particularly children) with acute gastroenteritis, in whom symptoms run a course longer than expected. In one study, one out of 5 children with community-acquired diarrhea, had symptoms for a prolonged period[43]. Prolonged diarrhea, defined, as an episode of diarrhea longer than 7 days, is associated with reduced growth and increased risk of persistent diarrhea[44]. Prolonged diarrhea is due to a longer and more serious infection, such as those caused by rotavirus and adenovirus or it may be the result of multiple host-related conditions.

All of these conditions have been associated directly or indirectly with gut microbiota alterations (or dysbiosis). In order to classify the involvement of gut microbiota in chronic diarrheal disorders,

Table II. Gorelick scale for dehydration (used in children between 1 month and 5 years).

Clinical features	No or minimal dehydration=0	Moderate to severe dehydration=1
General appearance	Alert	Restless, lethargic, unconscious
Capillary refill	Normal	Prolonged or minimal
Tears	Present	Absent
Mucous membrane	Moist	Dry, very dry
Eyes	Normal	Sunken; deeply sunken
Breathing	Present	Deep; deep and rapid
Quality of pulses	Normal	Thready; weak or impalpable
Skin elasticity	Instant recoil	Recoil slowly; recoil > 2 s
Heart rate	Normal	Tachycardia
Urine output	Normal	Reduced; not passed in many hours

Scoring:
- 4 point scale (italics);
- ≥2 Clinical Signs (4 pt) ≥5% body weight from baseline;
- ≥3 Clinical Signs (4 pt) ≥10% body weight from baseline;
- 10 point scale (all signs/symptoms);
- ≥3 Clinical Signs ≥5% body weight from baseline;
- ≥7 Clinical Signs ≥10% body weight from baseline.

we have divided them into three groups: gut microbiota alteration in the presence of enteric pathogens; gut microbiota alteration in the presence of intestinal inflammation and damage; gut microbiota alteration as the major determinant of disease without signs of intestinal inflammation or damage.

In the first group, the main determinants are represented by pathogens, whose mechanisms of damage have been extensively studied. In particular, rotavirus is the leading cause of acute diarrhea in infants and children and is associated with a substantial clinical and economic burden[34]. It is also well-known that many bacterial species act by the production of toxins, including *Escherichia coli*, *Shigella dysenteriae* (with the so-called 'shiga-toxin'); *Clostridium perfringens* (*C. perfringens* enterotoxin, α-toxin, β-toxin and epsilon-toxin); *Clostridium difficile* (toxins A and B and the binary toxin), *Staphylococcus aureus* (α-hemolysin), *Bacillus cereus* (cytotoxin K and hemolysin BL); *Aeromonas hydrophila* (aerolysin, heat labile cytotoxins and heat stable cytotoxins). These toxins can cause diarrhea by different mechanisms including the direct action of toxins on intestinal epithelial ion channels, indirect interaction with ion transporters, cytotoxic or hemolytic or pro-inflammatory action resulting in intestinal mucosal integrity loss with reduction of normal absorptive capacity. Other studies highlight how bacterial toxins or other related substances interact with other cell types deeper within the intestinal mucosa, including neuronal cells and other mesenchymal cells, modifying their function[45]. It is possible that pathogens can cause alterations within other populations of organisms making up the gut microbiota, which in turn lead to changes in intestinal homeostasis[46-48]. The second group includes chronic disorders with multifactorial pathogenesis, such as IBD, where it remains unclear whether gut microbiota alterations are the cause or the consequence of the disease.

The last group includes diseases without major signs of intestinal inflammation or damage, for example IBS. Prospective studies have shown that 3-36% of GI infections, causing an alteration in the intestinal ecosystem, lead to IBS symptoms (PI-IBS). Risk factors for PI-IBS include the virulence of the pathogen, female sex, younger age, a longer duration of the initial illness and the use of antibiotics during acute infectious episode. Interesting results were obtained from a long-term prospective, controlled, culture-proven, follow-up study in 1811 patients, predominantly children, involved in an outbreak of *Salmonella enteritidis* group D in Bologna, Italy. This study showed that acute gastroenteritis is a risk factor for the development of IBS 16 years after the acute illness if the infection was acquired during childhood, but not in adulthood[49].

Gelatin Tannate

Among the mucosal protectors, gelatin tannate is emerging as one of the most promising intestinal barrier modulators[50]. It is a combination of tannic acid (penta-m-digallolyl glucose) and gelatine, and may act by creating a protective film, forming bonds with the mucin thereby protecting the gut from the aggressive penetration of commensal bacteria (barrier protector)[51]. Gelatin tannate is commonly used as an intestinal fecal output regulator[52]. It passes unaltered through the stomach and once in the intestine it may exert its action by restoring the physiological barrier function[53,54]. Furthermore, results of recent studies[55] show that at the intestinal mucosal level, gelatin tannate acts in a non-dissociated form as a muco-adhesive film, with a protective effect on the intestinal barrier (gut barrier enhancer) and an indirect anti-inflammatory effect.

The effect of gelatin tannate in dextran sodium sulfate (DSS)-induced murine colitis has been recently studied[19-21]. DSS-induced colitis represents a T-cell independent, chemically induced model of epithelial damage and acute inflammation, primarily driven by innate immune responses. DSS directly affects gut epithelial cells of the basal crypts, disturbing the integrity of the mucosal barrier[56]. It exerts its action by causing a fast alteration in the mucus permeability and a disruption of the mucus biophysical structure. Thus, it allows bacteria to enter and penetrate the inner mucus layer. Several studies[57-62] have focused on the clinical improvement in acute DSS-induced colitis using probiotics and antibiotics in order to modulate the commensal microflora. On the other hand, this model is a useful way to evaluate those factors that can modulate the anatomical and functional health of the gut barrier, by acting not only on the gut microbiota, but also by restoring the mucus layer. In this model gelatin tannate significantly reduced the clinical activity of acute colitis compared to placebo. In particular, confocal microscopy showed the polymeric protective layer that gelatin tannate forms on the ulcerated mucosa, thus preventing the activation of the immune response, modulating the micro-flora composition, restoring the mucus layer and, consequently,

gut permeability[51]. Furthermore, gelatin tannate can reduce the pro-inflammatory effects of LPS in human intestinal epithelial cells[63]. It inhibits the intercellular adhesion molecule-1 (ICAM-1) expression in LPS-stimulated Caco-2 cells[63]. ICAM-1 is induced on a wide variety of cells by inflammatory stimuli such as LPS. Together with this, adding gelatin tannate at different concentrations induces a dose-dependent inhibition of IL-8 and TNF-α released by LPS-stimulated Caco-2 cells[63]. Finally, tannic acid is a polyphenolic compound, which includes gallic acid that has been shown have a potent antioxidant activity. It can be speculated that the observed evidence could also depend on this important characteristic of gelatin tannate. Bueno et al[55] also investigated the effect of gelatin tannate on the mucosal TJs. Preliminary findings demonstrated that gelatin tannate, by forming a protective biofilm, could prevent the junction leaking both in basal conditions and after an insult induced by bacteria (*in vitro*) and LPS (*in vivo*). These effects could not be replicated by either tannic acid or gelatine, suggesting that gelatin tannate is the active form able to prevent gut leakiness and subsequent inflammation[55]. In this scenario, gelatin tannate working as a mucosal barrier protector with its chemical structure appears to supply mucus function impairment and reestablish the gut barrier permeability and homeostasis, by acting as a gut barrier enhancer and modulating gut microbiota. Overall, these data suggest that not only microbiota modulation, but also the recovering of gut mucus layer in the course of acute colitis and diarrhea, can decrease the severity of clinical disease in mice. The translational therapeutic implications of these concepts are clear and open new horizons to novel, targeted, and more effective options for patients with colitis, acute diarrhea and impaired gut permeability[51]. Following these findings the safety and efficacy of gelatin tannate were evaluated in a randomized, parallel, double-blind, placebo-controlled study. Gelatine tannate showed a good safety profile and no adverse events were reported in either active treatment or placebo arms. Moreover, gelatine tannate was significantly more effective than placebo - adult patients treated with gelatine tannate had significantly less watery stools and less abdominal pain compared to patients treated with placebo[64]. Carretero et al[65] observed a significant decrease in the number of stools and an improvement in the consistency of stools in children between 3 months and 12 years with acute diarrhea[65].

The role of Probiotics and Tyndallized Probiotics

Probiotics are microorganisms that when administered in adequate amounts, confer health benefit to the host, especially by improving intestinal microbial balance[66]. They exert beneficial effects on intestinal epithelial cells in some ways. For example, some strains block pathogen entry into the epithelial cell by providing a functional and physical barrier: probiotics can increase the mucus barrier by releasing mucin granules from goblet cells, maintaining intestinal permeability by increasing the intercellular integrity of apical TJs, for example by up-regulating the expression of zonula-occludens 1 (a TJ protein) or by preventing TJ protein redistribution thereby stopping the passage of molecules into the lamina propria[67-69]. Probiotic bacteria can antagonize pathogenic bacteria by reducing luminal pH[70], inhibiting bacterial adherence and translocation, or producing antibacterial substances like defensins and bacteriocins. The inhibitory activity of these bacteriocins varies - some inhibit *Lactobacillus* or related Gram-positive bacteria, and some are active against a much wider range of Gram-positive and Gram-negative bacteria and yeasts[71]. Probiotics are also involved in the modulation of the immune response and inflammation. Probiotic bacteria can shape the mucosal immune system toward a non-inflammatory, tolerogenic pattern through the induction of T cells with regulatory properties. Probiotics can down-regulate the Th1 response and inhibit the production of pro-inflammatory cytokines, such as IL-12, TNF-α, and IFN-α by dendritic cells or increase the production of antiinflammatory cytokines, including IL10 and TGFβ. Selected probiotics act directly on the enterocyte inducing an ion proabsorptive effect[72].

There are two types of biological response modifiers: live probiotics, able to produce active metabolites and the tyndallized probiotics. Some different live probiotics have been evaluated in the prevention and treatment of diarrhea in adults and children, including the nonpathogenic yeast *Saccharomyces boulardii* and multiple lactic-acid fermenting bacteria. *S. boulardii* is efficacious in preventing post-antibiotics diarrhea when compared with placebo in patients treated with certain antibiotics such as the β-lactams[73,74]. *Lactobacillus GG* was used by Vanderhoof et al[75] in the treatment of children with antibiotic-associated diarrhea, demonstrating a significant reduction in the duration of diarrhea but not its frequency[75] and led to a restoration of disrupted intestinal microbiota of children with cystic fibrosis[76]. *Lacto-*

bacillus rhamnosus at a dose of $2x10^{10}$ CFU/ml once daily reduced the incidence of diarrhea in children treated with antibiotics[77]. A mixture containing *Lactobacillus casei* DN-114 001 (*L casei imunitas*) (1.0×10^8 cfu/ml), *Streptococcus thermophiles* (1.0×10^8 cfu/ml) and *Lactobacillus bulgaricus* (1.0×10^7 cfu/ml) reduced the risk of diarrhea (also *C. difficilis* diarrhea) in hospitalized patients treated with antibiotics[78].

Safety concerns regarding the extensive use of live microbial cells have increased interest in inactivated bacteria, as they could eliminate shelf-life problems and reduce the risks of microbial translocation and infection[79]. The tyndallization procedure sterilizes probiotics and blocks active metabolite production. Tyndallized probiotics are unable to reproduce, to inherit antibiotic resistance genes or to cause sepsis. Some *in vitro* and *in vivo* studies have investigated the potential of sterilized strains. A strain mix preparation containing a specific human strain of heat stabilized *L. acidophilus* (Strain LB) for the management of acute diarrhea and IBS was tested in a randomized controlled trial[80]. Moreover, it has been shown that *L acidophilus* (strain LB*)* showed antibacterial activity in cellular and animal models[81,82]. Following these findings, lyophilized, heat-killed *Lactobacillus acidophilus LB* were tested *vs.* placebo, in the treatment of acute diarrhea in Thai children as an adjunct to oral rehydration therapy. Heat killed *Lactobacillus* were shown to be stable and safe without alteration of clinical efficacy[83]. In fact, the addition of *L. acidophilus LB* to oral rehydration therapy was effective in the treatment of children with acute diarrhea by decreasing the duration of diarrhea[84]. Furthermore, previous studies have shown that selected strains of *Lactobacillus* are effective in rotavirus-induced diarrhea. However, only a few reports have documented their efficacy against non-rotavirus diarrhea. Heat-killed *L. acidophilus LB* together with its culture medium produced clinically significant benefits in the management of children with non-rotavirus, established diarrhea, by antagonizing the increased paracellular permeability in intestinal Caco-2/TC7 cells[85]. Finally, an *in vitro* study has demonstrated an anti-*H. pylori* effect of *L. acidophilus*, suggesting that the inactivated *L. acidophilus* could also be effective in increasing eradication rates of a standard anti-*H. pylori* therapy[86]. This result may be due to an inhibitory effect on the *H. pylori* attachment to gastric epithelial cell lines[87-89].

Tyndallized probiotics have been demonstrated to positively affect human health. They represent a new generation of safer and more stable products. In this context, some direct interactions with the host can be mediated by bacterial cells independently of their viability, thus contributing to restoring the optimal conditions for the endogenous bacterial flora, and the gut health. Interestingly, a growing body of evidence suggests that some of the benefits derived from probiotics are more likely to be due to the presence of metabolites or dead probiotic cells than to the probiotics themselves[90].

Gelatin Tannate and Tyndallized Probiotics

Gelatin tannate and tyndallized probiotics are the first combinations of gelatine tannate and a mixture of inactivated tyndallized probiotics including *Lactobacilli, Bifidobacterium, Streptococcus.* It can be used to reestablish the physiological functions of the gut barrier, as well as preventing and alleviating dysbiosis. It is indicated for the prevention and treatment of GI symptoms including diarrhea and abdominal bloating, caused by infectious gastroenteritis, antibiotic treatments and chemotherapy. Indeed, it is able to reestablish intestinal physiology by promoting gut microbiota interplay and homeostasis and contributing to increasing host health. It has a rapid onset of action, being effective within the first 12 hours.

Probiotics included in gelatin tannate and tyndallized strains undergo heat treatment (tyndallization) that is able to inactivate them and block their reproduction. Tyndallization is in essence a sterilization technique that uses repetitive cycles of humid heat. Tyndallized probiotics are processed in their medium, which contains bacterial wall and their products released during their death. However, tyndallized probiotics can maintain their immunological effect for the gut barrier[91]. In fact, they can adhere to the intestinal wall, increasing their protective activity and exerting a bacterial interference or competition with potentially pathogenic bacteria. Moreover, they possess a synergic effect with gelatine tannate against pathogenic bacteria (*E.coli*)[92]. Tyndallized probiotics have several advantages versus live strains that have some important disadvantages – they can constitute high-risk factors for immunosuppressed patients and elderly patients, they can promote resistance to antibiotics and can lead to sepsis in preterm children. Moreover, the use of tyndallized probiotics overcomes the reduced shelf life problems.

Among gelatin tannate and tyndallized strains, *Lactobacillus acidophilus*, as shown above, is effective in treating acute gastroenteritis. Gelatin tannate and tyndallized probiotics have been demonstrated to positively interact with the mucous proteins of a well-established *in vitro* model that recreates the intestinal mucosa (HT29-MTX) after pre-inoculation with *E. coli*. Results obtained through permeability tests showed that gelatine tannate combined with probiotics, protects intestinal cells from *E. coli* infection by inhibiting the adhesion and internalization of bacteria, preventing the increase of tight junction permeability and modulating cytokine gene expression[92].

Conclusions

Gelatin tannate and tyndallized probiotics may be highly effective in the treatment of acute gastroenteritis and are particularly indicated in the management of moderate and prolonged diarrhea, Interestingly, up to now there have been no specific drugs for condition that requires specific targeted treatment. Gelatin tannate and tyndallized probiotics could be useful in preventing and treating antibiotic-associated diarrhea and also diarrhea in immunosuppressed children (leukemic patients or patients treated with chemotherapy). Further clinical trials will need to explore these clinical applications with the end goal of promoting human health.

Statement of Interests

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