International Journal of Pharmaceutics 527 (2017) 126–134

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Research Paper

Synbiotic loaded chitosan-Ca-alginate microparticles reduces inflammation in the TNBS model of rat colitis

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ARTICLE INFO

Article history:
Received 8 March 2017
Received in revised form 19 May 2017
Accepted 22 May 2017
Available online 25 May 2017

Keywords:
Lactobacillus casei 01
Oligofructose-enriched inulin
Trinitrobenzenesulphonic acid
Rat colitis
Chitosan-Ca-alginate microparticles

ABSTRACT

New therapeutic strategies against inflammatory bowel disease (IBD) consider the usage of probiotics, prebiotics and synbiotics as beneficial for the intestinal microbial balance. Limitations of such an approach are addressed into difference in survival, persistence, colonization and variable effects among different probiotic strains, lack in understanding of probiotic mechanisms of action, as well the complex etiology of IBD. The anti-inflammatory activity of *Lactobacillus casei* 01 (*L. casei* 01) was assessed in trinitrobenzenesulphonic (TNBS) acid model of rat colitis when the probiotic was used alone and/or in combination with oligofructose-enriched inulin (Synergy 1), and as synbiotic (*L. casei* 01 + Synergy 1) loaded chitosan-Ca-alginate microparticles; all suspended in ayran. The results from the probiotic/synbiotic treatments (8.5–8.9 log CFU g^{-1} *L. casei* 01 and 1.5% Synergy 1) have shown reduction in the colonic damage and increased lactobacilli counts in feces. Lactobacilli translocation to sterile extra-intestinal organs demonstrated acceptable safety of the probiotic strain used. The best effect at reducing inflammation and lesions associated with a significant decline in myeloperoxidase (MPO) activity was observed in rats that received synbiotic microparticles. This finding suggests colon targeted delivery of the probiotics/synbiotics, as an advantageous approach in prevention and treatment of IBD.

1. Introduction

IBD, with its distinct chronic inflammatory relapsing forms Crohn's disease and ulcerative colitis, has long been a global healthcare problem with an increasing incidence. The most accepted hypothesis of IBD pathogenesis is that an aberrant immune response against the gut microbiota is triggered by environmental factors in a genetically susceptible host (Cammarota et al., 2015; Matsuoka and Kanai, 2015). In normal conditions, stimulation of the mucosal immune system by gut microbiota determines a state of "low-grade physiological inflammation", where the mucosal immune system is continually activated in response to commensals and/or towards pathogens. While in these conditions, the gut microbiota shows insignificant temporal change, it is unstable in IBD patients, even in the patients in remission (Minihane et al., 2015). In this condition, normal anaerobic bacteria are decreased and the diversity of the gut microbiota is reduced (Andoh et al., 2012). In addition, the composition of the gut microbiota is changed by the medications used to treat IBD (Andrews et al., 2011).

A number of studies has shown that the therapies correcting dysbiosis, including fecal microbiota transplantation (Pigneur and Sokol, 2016) and probiotics (Korada et al., 2016), are promising in IBD. Immunomodulatory/probiotic properties of lactic acid bacteria (LAB) are well-known and a lots of studies in animal (Foligné et al., 2016) and human subjects (Shen et al., 2014) confirm their capability to restore the intestinal microbial balance and to suppress the inappropriate and continuing inflammatory response to commensal microbes. Anti-inflammatory activities of different *Lactobacillus* strains have been confirmed in animal models of colitis, including *Lactococcus lactis* subsp. *lactis* S-SU2 (Kawahara et al., 2015), *Lactobacillus salivarius* ssp. *salivarius* (Peran et al., 2005), *Lactobacillus acidophilus* L10 (Peran et al., 2007) and *Lactobacillus plantarum* 21 (Satish Kumar et al., 2015). In addition, beneficial effects of the species *Lactobacillus casei* in rat colitis

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http://dx.doi.org/10.1016/j.ijpharm.2017.05.049
0378-5173/

models have also been reported for the strains *L. casei* Lbs2 (Kumar Thakur et al., 2016), *L. casei* DN-114 001 (Llopis et al., 2005) and *L. casei* L26 (Peran et al., 2007).

However, the anti-inflammatory potential of each probiotic strain must be a distinctive issue, as the effects of probiotics are strain specific (Lee et al., 2013). Another property that should be considered is the safety of the strains. When the intestinal barrier is compromised, as in IBD, there is a risk for translocation of viable probiotic cells across the gut, leading to serious side effects (Verna and Lucak, 2010). In addition, minimal count of 10^6 and 10^8 viable probiotic cells per mL intestinal content in the small bowel and colon, respectively, is required to provide an effective colonization and beneficial health effects (Bertazzoni Minelli and Benini, 2008). Having in regard that as much as 60% of probiotics are killed in the gastric environment prior to reaching the intestine, protecting probiotic bacteria from the harsh gastric environment is an imperative. To overcome this challenge, there are different approaches which have been studied over the last decade, including conventional and non-conventional formulations for probiotic delivery (Govender et al., 2014). Among these approaches, microparticulate probiotic delivery systems have been gaining an immense importance for local targeting of probiotic to colon, in addition to their capability to improve probiotic stability during industrial processes (Cook et al., 2012). Polymers composing probiotic microparticles are often pH selective, allowing protection against the harsh gastric conditions and subsequent probiotic release in the alkali media of the intestinal system. Herein, bio-adhesive properties of the probiotic delivery system are one of the key factors enabling prolonged residence time of the probiotic in the intestine and thus more effective colonization (Solanki and Shah, 2016).

Combination of probiotics with suitable prebiotics may additionally improve the survival, implantation, and growth of newly added probiotic strains or promote growth of existing strains of beneficial bacteria in the colon. When reaching the colon, prebiotics are fermented by anaerobic bacteria, producing short-chain fatty acids (SCFA) and CO_2 and H_2. As a result, intraluminal pH drops, favoring the increase of bifidobacteria, lactobacilli and nonpathogenic *E. coli* and decrease of Bacteroidaceae. The fermentation of carbohydrates also leads to the production of acetic, propionic and butyric acids that are involved in several colon-specific and systemic pathways. Of these, butyrate is of great importance to the metabolism of the colonocyte, exerting anti-inflammatory action by reducing the expression of TNF-α-related cytokines and upregulating IL-10, possibly by inhibition of the nuclear translocation of NF-κB (Säemann et al., 2000; Segain et al., 2000). Fructooligosaccharides, inulin, oligofructose, lactulose, and galactooligosaccharides have all been identified as prebiotics due to characteristics such as resistance to gastric acidity and hydrolysis by mammalian enzymes. They are fermented by gastrointestinal microflora to further selectively stimulate the growth and activity of beneficial microorganisms (Tan et al., 2014; Viladomiu et al., 2013). Oral inulin consumption stimulated butyrate production and increased the number of bifidobacteria and lactobacilli in the colon reducing the severity of distal colitis induced by DSS in rats (Videla et al., 2001), while administration of oligofructose in combination with *Bifidobacterium infantis* improved DSS-induced acute colitis in rats (Osman et al., 2006).

Having all these in regard, in a previous study we have prepared an optimal formulation of chitosan-Ca-alginate microparticles in which *Lactobacillus casei* 01 and oligofructose-enriched inulin were encapsulated using a spray-drying method associated with polymer complexation and cross-linking with calcium (Petreska Ivanovska et al., 2014, 2015). Cationic microparticles were prepared (zeta-potential, $+19.04 \pm 0.3$ mV), with a spherical, but also flattened disk shape, narrow size distribution (d_{50} 8.6 ± 0.3 μm), and number of viable cells of *L. casei* 01 after preparation 11.3 ± 0.13 log CFU g^{-1}. The aim of the present study was to examine the anti-inflammatory potential of the encapsulated synbiotic and to compare it with the anti-inflammatory effects of the probiotic alone and in combination with the prebiotic, in TNBS model of colitis. In addition, the safety of the probiotic strain used was assessed, testing the risk for lactobacilli translocation to the mesenteric lymph nodes (MLNs), spleen, kidney and liver. To our knowledge, it is the first study investigating the anti-inflammatory capacity of the encapsulated probiotic and/or synbiotic in rat model of colitis.

2. Materials and methods

2.1. Preparation of synbiotic chitosan-Ca-alginate microparticles

Synbiotic chitosan-Ca-alginate microparticles were prepared as previously described (Petreska Ivanovska et al., 2014), using *L. casei* 01 (Chr. Hansen, Hoersholm, Denmark) as a probiotic and oligofructose-enriched inulin (Orafti® Synergy 1, Orafti-Rue L. Maréchal, Tienen, Belgium), a mixture of oligofructose (DP 2-8) and long-chain inulin fraction (DP 10-60), as a prebiotic. In short, a probiotic suspension was activated in MRS broth (Merck, KGaA, Darmstadt, Germany) overnight at 37 °C, to a cell load ca. 11-12 log CFU mL^{-1}. Afterwards, the cells were stirred in alginate solution (4% w/w) containing Synergy 1 (1.5% w/w) and the mixture was infused into a spray-dryer (Büchi Mini Spray Dryer B-290, Switzerland) at inlet temperature of 120 °C, outlet temperature of 58 ± 3 °C, flow rate of 6 mL min^{-1}, nozzle diameter 0.7 mm, aspirator pressure 90% and atomizer pressure 600 Nlh^{-1}. Polyelectrolyte complexation and cross-linking were performed in a solution of chitosan (0.5% w/w) with deacetylation degree ≥ 85% and low viscosity 342 (viscosity of 10 mg g^{-1} solution in acetic acid 20–100 mPa s, M_w 150 kDa) (France Chitine, Marseille, France) and $CaCl_2$ (5% w/w) (Merck, KGaA, Darmstadt, Germany) in 1% v/v acetic acid under continuous stirring. The next day, the obtained microparticles were freeze-dried (−50 °C, 0.07 mbar, 24 h, Free-Zone Freeze Dry System, Labconco, Kansas City, USA).

An optimal formulation for preparation of probiotic/synbiotic microparticles was selected using a polynomial regression model at 2nd level considering physico-chemical (particle size, zeta-potential, Ca-content) and functional properties (viability of the probiotic during microencapsulation and in simulated gastrointestinal conditions) (Petreska Ivanovska et al., 2014). In this study, the synbiotic loaded microparticles showing good mucoadhesion *in vitro* (Petreska Ivanovska et al., 2015) were used to reduce the colitic damage in rats induced by TNBS (Picrylsulfonic acid solution, 5% m/v, Sigma-Aldrich, Co., St. Louis, USA).

2.2. Anti-inflammatory properties of probiotic/synbiotic formulations

2.2.1. Induction of TNBS colitis

All animal experiments were performed in accordance with the OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98) 17, as revised in 1997) and national legislation, which is harmonized with the Directive 2010/63/EU on the protection of animals used for scientific purposes. Female Wistar rats (180–250 g, 10–14 weeks old) were obtained from the national accredited supplier (Laboratory Animal Service of the University "Ss Cyril and Methodius", Faculty of Natural Sciences and Mathematics, Skopje, Republic of Macedonia). Divided randomly into groups of six, they were settled in cages under controlled environment at a room temperature of 25 °C, humidity 55 ± 5%, 12/12 h light/dark cycle and *ad libitum* access to standard pelleted food and water.

TNBS-induced model of rat colitis was adopted from Morris et al. (1989). Rats treated intra-rectally with TNBS share similar characteristics to people with IBD (often lose weight, present with bloody diarrhea, and exhibit mucosal, submucosal and transmural inflammation) (Brenna et al., 2013), making the TNBS-colitis a relevant model in the preclinical testing of various compounds in terms of their anti-inflammatory effects (Antoniou et al., 2016). Having in regard the adjuvant role of pro- and prebiotics in IBD, in this study, mild colitis was induced using lower TNBS doses than commonly applied (100 to 150 mg kg^{-1}). Experimental colitis was induced intra-rectally to the anaesthetized rats at 8 cm proximal to the anus, with a solution of TNBS in 50% ethanol. TNBS solution (0.25 mL per kg body weight equivalent to 10 mg kg^{-1} TNBS) was administered to six groups of rats (three treated and three control groups). Additionally, in four colitic groups (two treated and two control groups), an experimental colitis was induced by 0.25 mL of TNBS solution per kg body weight containing 30 mg kg^{-1} TNBS after an overnight fast of the rats. The rats from the non-colitic groups (three in total) were treated with 0.25 mL 0.85% saline intra-rectally, instead of TNBS. After 7 days of continuous probiotic/synbiotic treatment, the rats were euthanized. During the whole experiment, no reduction of body weight over 20%, nor mortality was observed.

2.2.2. Experimental design

To evaluate the anti-inflammatory potential of probiotic/synbiotic formulations, the synbiotic chitosan-Ca-alginate microparticles, non-encapsulated probiotic and synbiotic were suspended in commercially available ayran (Ayran, Zdravje, Macedonia) to prepare functional drinks. To three colitic groups of rats (Group I; 10 mg kg^{-1} TNBS), 1 mL ayran containing non-encapsulated probiotic (I-1, n = 6), synbiotic (I-2, n = 6) or synbiotic microparticles (I-3, n = 6), probiotic load 8.5–8.9 log CFU per mL of the food product, were administered orally, once daily for 14 days prior to induction of colitis, and 7 days thereafter. At the same time, three TNBS-control groups (Group II; 10 mg kg^{-1} TNBS) received drinking water (II-1, n = 6), plain ayran (II-2, n = 6) or ayran with blank chitosan-Ca-alginate microparticles (II-3, n = 6). Non-colitic control groups received drinking water (III-1, n = 6) and plain ayran (III-2, n = 6).

The effect of administration frequency was evaluated in the second phase of the experiment. For that aim, two groups of colitic rats (Group IV; 30 mg kg^{-1} TNBS) received ayran containing synbiotic microparticles once (IV-1, n = 6) and twice daily (IV-2, n = 6), while the colitic control groups (Group V) plain ayran (V-1, n = 6) and ayran containing blank microparticles (V-2, n = 6). Non-colitic control group receiving plain ayran was also included in the protocol (III-3, n = 6).

2.2.3. Assessment of colonic damage and inflammation scoring

Water and food intake, animal body weight, and clinical signs of colitis such as occurrence of diarrhea or bleeding were recorded daily throughout the experiment. From the euthanized rats, the distal part of the colon was removed aseptically and longitudinally cut. Each colon specimen was rinsed with phosphate buffer (pH = 6.8), cleaned of fat and mesentery, and blotted on filter paper. The length and weight of each colon extracted was scored and expressed as weight/length ratio (mg cm^{-1}). Macroscopic assessment of the colonic damage was performed on a basis of previously defined scoring system (Santiago et al., 2007). These criteria include presence of adhesions (0, 1, and 2, for absent, minor, and mild, respectively), diarrhea (0 for absent and 1 for present), colon thickness (mm), and ulceration (0 for no damage, 1 for localized hyperemia with minimal ulceration, 2 for presence of one or two regions with ulcers 1–2 cm, and 3 for major ulcers >2 cm). For microscopic analysis, specimens that corresponded to the macroscopically visible colon inflammation were fixed in 10% v/v formalin, and embedded in paraffin. Colon specimens were also taken from the non-colitic groups. Afterwards, 3–5 mm selected cross-sectional segments were stained with hematoxylin-eosin for light microscopy (Optical Microscope, Nikon Eclipse E600, Lucia 4.21 G, Nikon Co., Japan), and analyzed by an experienced pathologist (T. R.). Colonic damage was scored depending on the presence of focal epithelial edema and necrosis, mucosal ulceration and necrosis of the villa, necrosis with neutrophil infiltration in submucosal tissue, and severe necrosis with extensive inflammatory infiltrate and hemorrhage.

Clinical activity score system ranging from 0 (healthy) to 4 (maximal activity of colitis) was based on selected parameters such as weight loss, stool consistency, and rectal bleeding (Lamprecht et al., 2001). Well-formed feces without rectal bleeding and no weight loss was assigned with 0 activity, weight loss from 1 to 5% indicated level 1 activity, level 2 was given when weight loss ranged from 5 to 10%, associated with rectal bleeding and/or semi-formed feces, level 3 activity assumed weight loss from 10 to 20%, while weight loss >20%, accompanied with liquid stool and excessive bleeding, was assigned as level 4 activity.

2.2.4. Myeloperoxidase assay

The extent of the infiltration of inflammatory cells in the colon was examined by the assessment of myeloperoxidase activity, as described by Krawisz et al. (1984). Frozen colon specimens were homogenized (Polytron homogenizer, Kinematica, Eschbach, Germany) with 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in 50 mM phosphate buffer, pH = 6 on ice. The homogenate was sonicated for 10 s, freeze-thawed three times, and centrifuged at 12 000 rpm for 5 min. To 100 μL of the supernatant, 0.167 mg mL^{-1} *o*-dianisidine hydrochloride (Sigma-Aldrich, Co., St. Louis, USA), and 0.0005% hydrogen peroxide were added. Change in absorbance at 460 nm at regular time intervals of 3 min was recorded. One unit of MPO activity is defined as the amount required to degrade 1 μmol of hydrogen peroxide per minute at 25 °C. The results are expressed as MPO units per mg of wet colon tissue.

2.3. Microbiological studies

2.3.1. Total lactobacilli enumeration

Enumeration of total lactic acid producing bacteria in fecal samples, taken from the rats in predetermined time intervals, was performed to evaluate the influence of the probiotic/synbiotic treatment on rat microflora. Fecal samples were homogenized, serially diluted in 0.85% saline, and plated on Man Rogosa Sharp Agar (MRS), a selective media for lactobacilli growth. The dishes were incubated under anaerobic conditions at 37 °C for 72 h, and the numbers of colonies were expressed as the log CFU g^{-1} of fecal material.

2.3.2. Bacterial translocation

The risk for bacterial translocation (*L. casei* 01 and/or other lactobacilli) in MLNs, spleen, hepatic lobes, and kidneys was examined in aseptically removed samples from the respective rat tissue. Tissues were cleaned from fat, weighed, and dipped in Brain Heart Infusion broth (Oxoid Ltd., Hampshire, UK) supplemented with 20% glycerol. Serial dilutions of tissue homogenates were plated in Petri dishes containing MRS agar, and incubated as described in Section 2.3.1. The results were reported as absence of lactobacilli or quantification of their colonies (log CFU g^{-1} of tissue).

2.4. Statistical analysis

The data are presented as mean ± SEM of three independent determinations. Statistical significance between different groups of rats was calculated by non-parametric Mann-Whitney test, and differences were considered significant at a $P < 0.05$.

3. Result and discussion

3.1. Macroscopic and biochemical assessment of probiotic/synbiotic effects in mild colitis

Histopathological, immunological and clinical features of the inflammation in human IBD are highly correlated to the TNBS model of rat colitis, which is a well-established reproducible model of intestinal inflammation. It is also a useful animal model in the development and evaluation of therapeutic approaches, allowing investigation on epithelial and barrier damage in IBD and on the impact of microbiota on inflammation process and repair mechanisms (Nell et al., 2010). In accordance with the existing clinical evidence on protective effects of probiotics in chronic intestinal inflammation (Cammarota et al., 2015; Viladomiu et al., 2013), the colitic rats were treated by probiotic/synbiotic for 14 days before induction of colitis and 7 days thereafter.

According to the literature data (Morris et al., 1989; Peran et al., 2007), TNBS-induced inflammation is apparent 7 days after the TNBS administration and this was evident in the actual study when the rats were sacrificed and histopathological and biochemical assessment of colonic samples was performed. The stool of TNBS-control rats (II-1 and II-2) was sticky and mellow, and increased values of colonic tissue edema indicators (the colon weight/colon length ratio and colonic weight/body weight ratio) and biochemical marker of inflammation (MPO activity) were also observed (Table 1). The data presented in Table 1 demonstrate that the ayran used as a medium for administration of the probiotic/synbiotic to rats did not affect the TNBS-induced changes significantly ($P > 0.05$), *i.e.* no significant differences between the rats that received drinking water (II-1) and plain ayran (II-2) were observed. Interestingly, in the control rats that received blank chitosan-Ca-alginate microparticles (II-3), compared to both TNBS-control groups (II-1 and II-2), significant reduction in colon weight/colon length ratio, MPO activity and total damage score ($P < 0.05$) (Table 1) was observed. However, the reduction of these parameters in the rats that received synbiotic microparticles (I-3) was more significant comparing to the rats that received blank microparticles (II-3) ($P < 0.05$; Table 1), indicating synergistic anti-inflammatory effect of the biopolymers and synbiotic. This effect can probably be attributed mainly to chitosan, for which suppression of certain anaerobic bacteria growth in isolated human colon and modification of colonic microflora composition have been reported (Šimůnek et al., 2012). Furthermore, depending on the concentration used, chitosan has been shown to exhibit antimicrobial effect against pathogenic bacteria usually involved in the development of intestinal inflammation (Goy et al., 2016). For preparation of *L. casei* 01 loaded microparticles, chitosan was applied as an outer coating to increase probiotic protection because of its cationic nature *i.e.* ability to buffer gastric acid (Cook et al., 2012).

Chemically induced inflammation in the rats that received probiotic/synbiotic treatment (Group I) was associated with mellowed stool and reduction of body weight, ranging from 5% to 10%, but without signs of apparent diarrhea (data not shown). Higher values for colon weight/colon length ratio, colonic weight/body weight ratio, and macroscopic score for total damage/clinical activity of colitis when non-encapsulated probiotic (I-1) was administered, pointed to reduction of colon inflammation to a less extent in comparison with the non-encapsulated synbiotic (I-2). However, the difference between these two groups in the MPO activity, as the most sensitive biochemical marker of the colon inflammation, was insignificant ($P > 0.05$) (Table 1). The treatment based on synbiotic microparticles (I-3), non-encapsulated probiotic (I-1), and non-encapsulated synbiotic (I-2) revealed ≈ 3.5-fold, 1.6-fold, and 1.8-fold decrease in MPO activity, respectively, compared to TNBS-control rats (II-2) (Table 1). Synbiotic microparticles displayed the most significant anti-inflammatory effect in the colitic rats, confirmed by significant reduction in MPO activity,

Table 1
Effects of probiotic/synbiotic treatments on parameters of colon inflammation in rats.

Experimental groups	CW/BD (mg/mg)	CW/CL (mg/cm)	MPO activity (U/g)	Total damage/colitis activity score
Non-colitic rats				
III-1	$0.007 \pm 0.0007^{a,b}$	$87.44 \pm 8.89^{a,b}$	$15.47 \pm 1^{a,b}$	$0^{a,b}$
III-2	0.0072 ± 0.0007^{a}	88.70 ± 8.44^{a}	15.55 ± 1.9^{a}	0^{a}
III-3	0.0071 ± 0.0005^{a}	90.65 ± 9.75^{a}	15.95 ± 1.8^{a}	0^{a}
Colitic rats (10 mg kg^{-1} TNBS)				
II-1	0.011 ± 0.0011	148.66 ± 15.33	66.19 ± 6.2	2.9 ± 0.03
II-2	0.0098 ± 0.0009	137.54 ± 14.44	60.63 ± 6	2.85 ± 0.03
II-3	0.009 ± 0.0009^{f}	$109.06 \pm 11.11^{a,f}$	$47.34 \pm 4.65^{a,f}$	$2.5 \pm 0.22^{a,f}$
I-1	0.01 ± 0.0009	112.96 ± 11.33	34.45 ± 4^{a}	2.3 ± 0.22^{a}
I-2	$0.0083 \pm 0.0009^{b,e}$	$100.85 \pm 10.44^{a,b}$	30.79 ± 4.1^{a}	$2.1 \pm 0.02^{a,e}$
I-3	$0.0072 \pm 0.0007^{a,b,c}$	$89.21 \pm 6.67^{a,b,c}$	$18.45 \pm 1.8^{a,b,c,d}$	$1.8 \pm 0.02^{a,c,d}$
Colitic rats (30 mg kg^{-1} TNBS)				
V-1	0.012 ± 0.0008	149.49 ± 15.25	74.89 ± 7.18	3 ± 0.3
V-2	$0.0097 \pm 0.0007^{a,g,h}$	$136.11 \pm 12^{g,h}$	$56.27 \pm 5.18^{a,g,h}$	$2.7 \pm 0.3^{g,h}$
IV-1	$0.0079 \pm 0.0005^{a,b}$	$104.1 \pm 10^{a,b}$	28.85 ± 2.71^{a}	2 ± 0.21^{a}
IV-2	$0.0075 \pm 0.0006^{a,b}$	$105.73 \pm 10.5^{a,b}$	24.24 ± 2.47^{a}	2 ± 0.2^{a}

CW/BW–colon weight/body weight ratio; CW/CL–colon weight/colon length ratio. Colitic rats receiving ayran supplemented with: non-encapsulated probiotic (I-1); non-encapsulated synbiotic (I-2); synbiotic microparticles (I-3); synbiotic microparticles once daily (IV-1); synbiotic microparticles twice daily (IV-2). Colitic control rats receiving: drinking water (II-1); plain ayran (II-2–first experiment; V-1–second experiment); ayran with blank microparticles (II-3–first experiment; V-2–second experiment). Non-colitic control rats receiving: drinking water (III-1); plain ayran (III-2–first experiment; III-3–second experiment). Data were evaluated by non-parametric Mann-Whitney test and significant difference was considered at $P < 0.05$: $^{a}P < 0.05$ *vs.* II-1 and/or II-2 (first experiment) and V-1 (second experiment); $^{b}P > 0.05$ *vs.* III-1 and/or III-2 (first experiment) and III-3 (second experiment); $^{c,d}P < 0.05$ I-3 *vs.* I-1 and I-2, respectively; $^{e}P < 0.05$ I-2 *vs.* I-1; $^{f}P < 0.05$ II-3 *vs.* I-3; $^{g,h}P < 0.05$ V-2 *vs.* IV-1 and IV-2, respectively; $^{i}P < 0.05$ IV-1 *vs.* IV-2; $^{j}P < 0.05$ II-1 *vs.* II-2 and III-1 *vs.* III-2, respectively.

colon weight/body weight ratio, and total damage/clinical activity score based on weight loss, stool consistency, and rectal bleeding (Table 1).

3.2. Histology evaluation of probiotic/synbiotic effects in mild colitis

Microscopic analysis of the colonic samples from the TNBS-control group that received plain ayran (II-2) has shown disruption of the normal colon architecture, with hemorrhage and ulcerations on mucosa and sub-mucosa, accompanied by extensive inflammatory infiltrate and congested blood vessels (Fig. 1b). Histological assessment of colon samples showed that the treatment of rats with non-encapsulated probiotic (I-1) and synbiotic (I-2) was able to ameliorate colon inflammation, but limited regions of ulceration and subepithelial polymorph nuclear infiltration were visible (Fig. 1c). The reduction of inflammation was associated with decreased leukocyte infiltration in these groups. Significant improvement in the integrity of the mucosa tissue with dilated blood vessels and intestinal glands and a reduction of the inflammatory cells in the submucosal layer was observed in rats receiving synbiotic microparticles (I-3) (Fig. 1d). Histological characterization was in conjunction with the biochemical results for MPO activity, showing no significant difference between the non-colitic rats that received plain ayran (III-2) and rats treated by synbiotic microparticles (I-3) (Fig. 1a and d, respectively) ($P > 0.05$) (Table 1).

According to Lee et al. (2013), anti-inflammatory potential of any probiotic strain must be investigated in a particular study to reveal relevant findings. In this study, *L. casei* 01 alone or in combination with oligofructose-enriched inulin demonstrated anti-inflammatory activity in TNBS-induced rat colitis. Taking into account the overall score, based on macroscopic, biochemical and histologic examinations, synbiotic microparticles have shown significantly higher effectiveness in reduction of inflammation

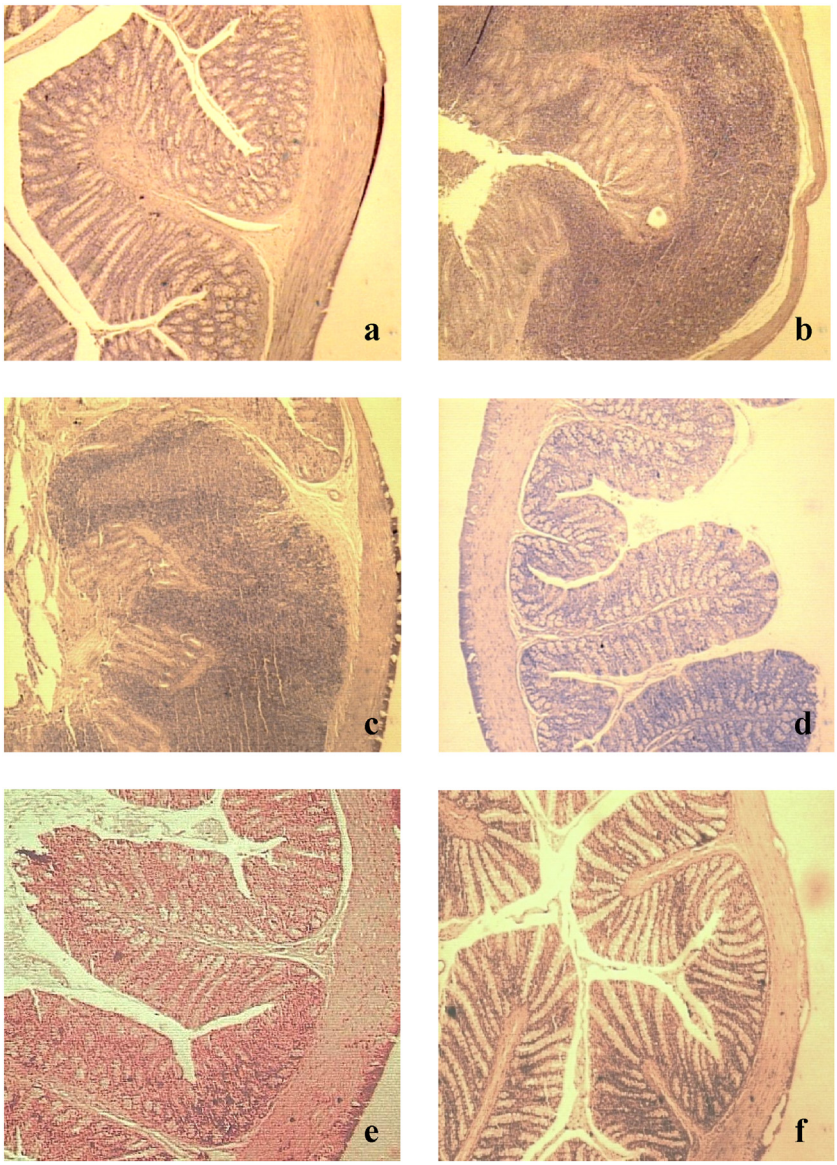

Fig 1. Histopathological changes of rat colon segments: a) non-colitic rats receiving plain ayran (III-2); b) control rats treated with 10 mg kg^{-1} TNBS and receiving plain ayran (II-2); c) rats treated with 10 mg kg^{-1} TNBS and receiving non-encapsulated probiotic/synbiotic (I-1 and I-2); d) rats treated with 10 mg kg^{-1} TNBS and receiving synbiotic microparticles (I-3); e) rats treated with 30 mg kg^{-1} TNBS and receiving synbiotic microparticles once daily (IV-1); f) rats treated with 30 mg kg^{-1} TNBS and receiving synbiotic microparticles twice daily (IV-2).

parameters, probably due to the improved viability and bioavailability of the probiotic cells. These data are partly consistent with the published literature data on the protective effect of probiotics in IBD. For example, *L. casei* Shirota did not prevent the DSS-induced colitis when tested as a prophylactic agent 10 days before the induction, but improved the clinical condition of mice significantly when administered simultaneously with the colitis induction and/or 10 days thereafter (Herias et al., 2005). Two *L. casei strains*, Lbs2 and Lbs6 alleviated macroscopic and histopathological features of TNBS colitis in probiotic-fed mice, while the suppression of pro-inflammatory cytokines (TNF-alpha, IL-6) secretion was dominantly generated by Lbs2 (Kumar Thakur et al., 2016). The strain *L. casei* LAFTI® L26, administered to rats for 3 weeks, starting 2 weeks before TNBS instillation, has shown anti-inflammatory activity mainly as a result of decreased colonic COX-2 expression (Peran et al., 2007).

3.3. Anti-inflammatory effects of the synbiotic microparticles in moderate colitis

To evaluate the anti-inflammatory potential of synbiotic microparticles in moderate colitis and to investigate the optimal treatment frequency, macroscopic, microscopic and biochemical features of the colitis induced with 30 mg kg^{-1} TNBS were assessed in rats to which microparticles once (IV-1) or twice daily (IV-2) were administered. In the colitic-control rats that received plain ayran (V-1), significant weight loss (≈10%) and diarrhea was observed, but without severe rectal bleeding. The beneficial effect of the synbiotic microparticles administered once or twice daily was apparent macroscopically by significant reductions of colon weight/colon length ratio and colonic weight/body weight ratio, and biochemically confirmed by lowered colonic MPO activity (Table 1). Compared to the TNBS-control rats (V-1), decrease in colon weight/body weight ratio and MPO activity in the rats that received blank microparticles (V-2) was observed. However, the anti-inflammatory activity, evidenced by all investigated parameters in the rats that received synbiotic microparticles once daily (IV-1) and twice daily (IV-2), was significantly enhanced ($P < 0.05$) compared to the TNBS-control rats that received blank microparticles (V-2) (Table 1), suggesting that *L. casei* 01 and Synergy 1 are the main carriers of the anti-inflammatory effect. The differences in the reduction of MPO activity and in other parameters of inflammation between the rats treated by synbiotic microparticles once daily (IV-1) and twice daily (IV-2) were insignificant ($P > 0.05$) (Table 1). In addition, histopathology assessment showed recovery process associated with dilated blood vessels in submucosal layer and dilated intestinal glands in both groups, regardless of the administration frequency (Fig. 1e and f). Comprising the overall results, one can conclude that the probiotic microparticles prepared have potential to deliver

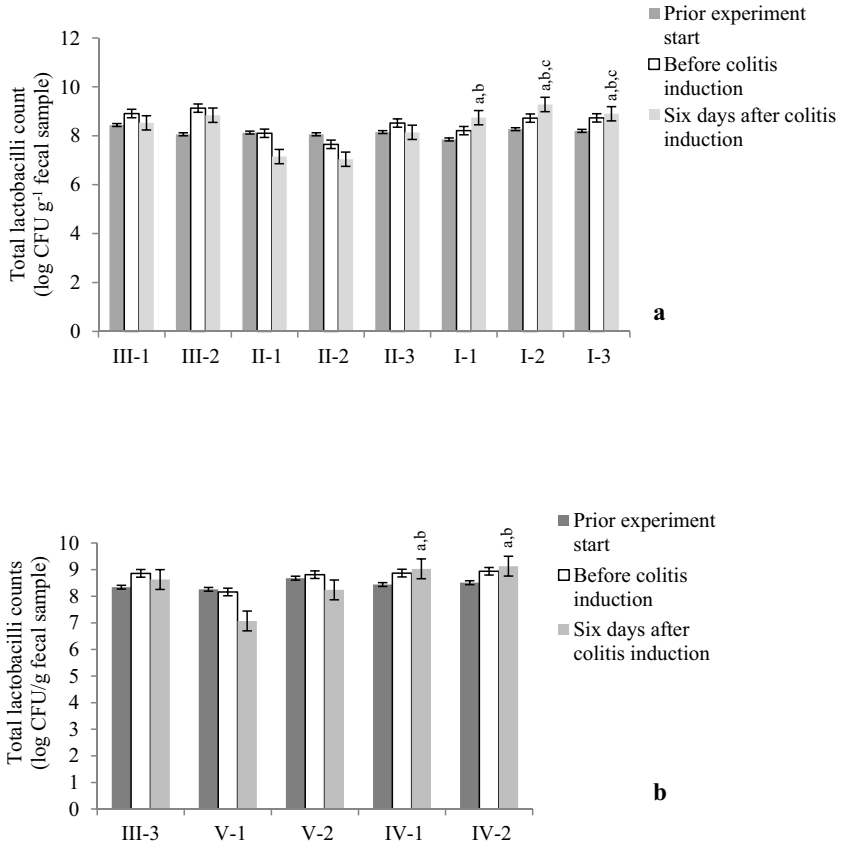

Fig. 2. Effects of probiotic/synbiotic treatment on total lactobacilli count in rat colitis. a) *TNBS was administered at a dosage of* 10 mg kg^{-1} *(first experimental phase)* on rats receiving: drinking water (II-1); plain ayran (II-2); blank microparticles (II-3); non-encapsulated *L. casei* 01 (I-1); non-encapsulated synbiotic (I-2); synbiotic microparticles (I-3). [a,b,c]Significant increase in lactobacilli numbers ($P < 0.05$) in probiotic/synbiotic treated rats *vs.* control colitic groups II-1, II-2 and II-3, respectively. b) *TNBS was administered at a dosage of* 30 mg kg^{-1} *(second experimental phase)* on rats receiving: synbiotic microparticles once daily (IV-1); synbiotic microparticles twice daily (IV-2); plain ayran (V-1); blank microparticles (V-2). Two non-colitic groups of rats, receiving drinking water (III-1) and plain ayran (III-2), were included in the first experimental phase and one group of rats receiving plain ayran in the second phase of the experiment (III-3). [a,b]Significant increase in lactobacilli numbers ($P < 0.05$) after probiotic/synbiotic treatment *vs.* control colitic groups V-1 and V-2, respectively. The difference in lactobacilli count between groups IV–I and IV-2 was insignificant ($P > 0.05$). Non-encapsulated probiotic/synbiotic and blank/synbiotic microparticles were administered to rats suspended in ayran. Data are expressed as mean ± SEM (n = 3).

sufficient number of viable probiotic cells into the lower intestinal parts, preserving their viability through the upper region of GIT. Prolonged residence time of the synbiotic microparticles that allows advantageous colonization of the lower intestine is probably the reason for similar anti-inflammatory effects when the probiotic microparticles with different frequencies are administered.

3.4. Effect of probiotic/synbiotic treatment on total lactobacilli count

Lactobacilli persistence in rat fecal samples was examined before the beginning of the treatment and colitis induction, and before sacrificing the rats. Regardless of the TNBS dose used, lactobacilli count decreased for approximately 1 log in TNBS-control rats that received drinking water (II-1) or plain ayran (II-2 and V-1) (Fig. 2a and b). This was also observed by Peran et al. (2005), who reported significant reduction of lactobacilli count in the colonic lumen as a result of colonic damage induced by TNBS. In the rats treated with 10 mg kg^{-1} TNBS that received probiotic or synbiotic, both non-encapsulated (I-1 and I-2, respectively), and synbiotic microparticles (I-3), the number of lactobacilli was increased for 0.7-1 log at the end of the treatment, showing ability to restore the lactobacilli microflora (Fig. 2a). Lactobacilli counts in rats that received probiotic/synbiotic were returned to normal values observed in healthy rats, which might suggest that *L. casei* 01 alone or in combination with oligofructose-enriched inulin stimulated the growth of lactobacilli.

The inflammatory process in the colonic tissue was reported to be related to alterations of the ratio lactic acid bacteria and pathogenic bacteria, mainly entherobacteria and coliforms (Peran et al., 2005, 2007). Therefore, an increase in lactobacilli counts may help to the host microbiota in re-establishing a favorable relationship with intestinal mucosal cells and a balance between pro- and anti-inflammatory factors (Cammarota et al., 2015). The probiotic treatment has potential to achieve this aim, although inconsistent literature data exist. *L. salivarius* ssp. *salivarius* (5×10^8 CFU/rat/day) increased the counts of lactobacilli species in colonic content of probiotic treated rats (Peran et al., 2005). However, the difference was insignificant when compared to the TNBS-control rats and non-colitic rats. *B. lactis* given orally to rats (5×10^8 CFU suspended in 0.5 mL of skimmed milk) during 3 weeks improved the ratio bifidobacteria/pathogenic bacteria, while *L. acidophilus* and *L. casei* contributed in a restoration of lactobacilli (Peran et al., 2007). Herias et al. (2005) has observed an increase in the total

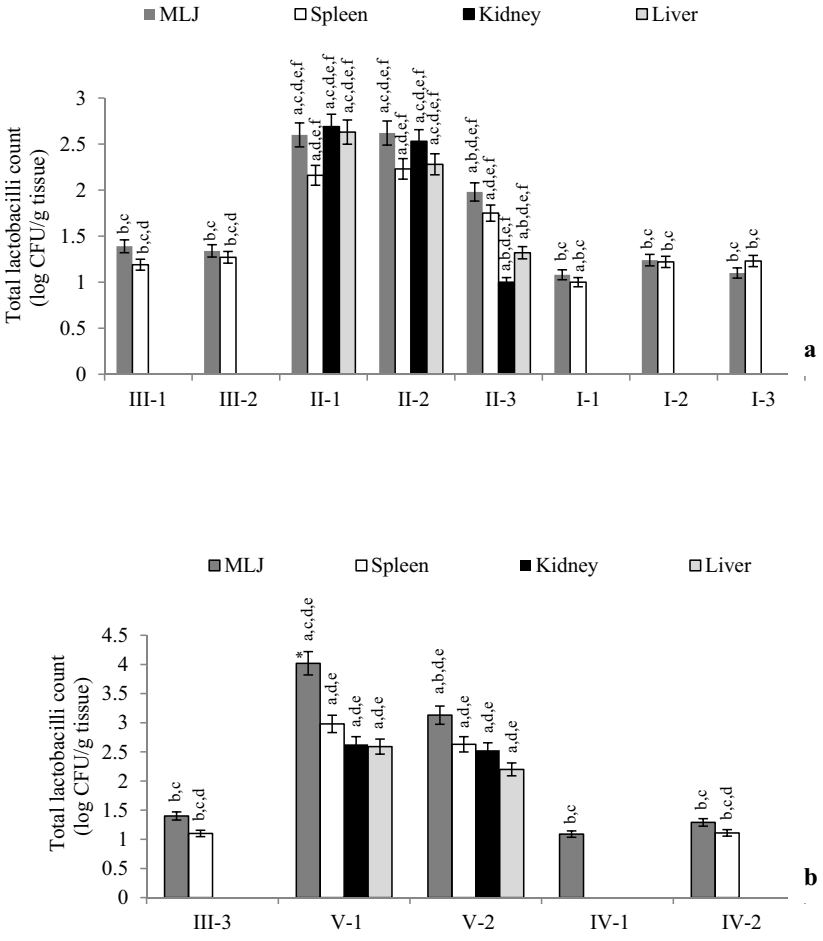

Fig. 3. Quantitative recovery of lactobacilli translocated to extra-intestinal organs in rat colitis. a) *TNBS was administered at a dosage of* 10 mg kg^{-1} *(first experimental phase)* on rats receiving: drinking water (II-1); plain ayran (II-2); blank microparticles (II-3); non-encapsulated *L. casei* 01 (I-1); non-encapsulated synbiotic (I-2); synbiotic microparticles (I-3). ^{a,b,c,d,e,f}Significantly different ($P < 0.05$) from group III (III-1 and III-2), group II (II-1and II-2), II-3, I-1, I-2 and I-3, respectively. b) *TNBS was administered at a dosage of* 30 mg kg^{-1} *(second experimental phase)* on rats receiving: synbiotic microparticles once daily (IV-1); synbiotic microparticles twice daily (IV-2); plain ayran (V-1); blank microparticles (V-2). Two non-colitic groups of rats, receiving drinking water (III-1) and plain ayran (III-2), were included in the first experimental phase and one group of rats received plain ayran in the second phase of the experiment (III-3). ^{a,b,c,d,e}Significantly different ($P < 0.05$) from group III-3, V-1, V-2, IV-1 and IV-2, respectively. $^{*}P < 0.05$ V-1 *vs.* II-2. Non-encapsulated probiotic/synbiotic and blank/synbiotic microparticles were administered to rats suspended in ayran. Data are expressed as mean ± SEM (n = 3).

number of entherobacteria for 2–3 logs 7 days after DSS-induced colitis in mice, but with no difference between the DSS-control groups and mice intragastrically intubated with 10^8 *L. casei* Shirota. In addition, the role of prebiotics in promotion of the intestinal LAB growth may also contribute to attenuation of inflammation. In this regard, the beneficial effect of oligofructose and inulin in reducing the severity of DSS-induced colitis in rats has been documented (Osman et al., 2006; Videla et al., 2001).

In this study, after induction of colitis using 30 mg kg^{-1} TNBS, high numbers of lactobacilli were found in both groups of rats receiving synbiotic microparticles once (IV-1) or twice daily (IV-2), opposite to the decline in rats receiving plain ayran (V-1) and blank microparticles (V-2) (Fig. 2b). The synbiotic microparticles applied twice daily (IV-2) did not promote further increase in lactobacilli counts, confirming the biochemical and histological findings that displayed no significant difference in inflammation reduction with increase in treatment frequency. Mild and/or moderate colitis was associated with significant reductions of lactobacilli counts in fecal samples, which were recovered after the probiotic/synbiotic treatment. Since lactobacilli content in the fecal samples of probiotic/synbiotic treated rats was returned to the level found in healthy rats, restoration of colonic lactobacilli microflora could be one of the mechanisms that contribute to attenuation of this chemically induced inflammation in the rats.

3.5. Safety assessment of lactobacilli in TNBS-treated rats

Considering the risk for translocation of pathogenic or commensal bacteria to sterile body tissues when intestinal barrier is disturbed and intestinal permeability increased, safety assessment of the probiotics applied in inflammatory conditions is strongly recommended (Daniel et al., 2006). Basal translocation level of lactobacilli typical for healthy subjects was observed in non-colitic rats (III-1, III-2 and III-3) (Fig. 3a and b). Colon inflammation resulted in lactobacilli translocation at a higher level in TNBS-control rats. Translocation level in rats treated with 30 mg kg^{-1} TNBS (V-1) *vs.* the group treated with 10 mg kg^{-1} TNBS (II-2) was significantly higher only in MLNs ($P < 0.05$) (Fig. 3a and b), while no significant difference was found in other tissues (spleen, kidney and liver) ($P > 0.05$) (Fig. 3a and b). Administration of the probiotic alone (I-1), combined with the prebiotic (I-2) and synbiotic microparticles (I-3) in rats treated with 10 mg kg^{-1} TNBS, resulted in lower numbers of lactobacilli recovered from MLNs, insignificantly different from the numbers quantified in healthy rats ($P > 0.05$) (Fig. 3a). In rats treated with 30 mg kg^{-1} TNBS, a non-significant difference in lactobacilli numbers translocated to MLNs and spleen compared to healthy rats was also observed in the group treated by synbiotic microparticles twice a day (IV-2) ($P > 0.05$) (Fig. 3b). On the other hand, a single day administration of synbiotic microparticles (IV-1) revealed absence of lactobacilli in culture from spleen (Fig. 3b), showing advantageous safety profile of this treatment, besides the beneficial effect in reducing the inflammation. Lactobacilli growth was not detected in the cultures of liver and kidneys extracted from all rats that received probiotic or synbiotic in non-encapsulated and encapsulated form.

In summary, our results displayed low numbers of translocated lactobacilli in rats treated by probiotic/synbiotic under conditions of mild (10 mg kg^{-1} TNBS) and moderate (30 mg kg^{-1} TNBS) colitis. Considering the basal translocation level of endogenous microflora characteristic in physiological conditions (Daniel et al., 2006), the probiotic strain *L. casei* 01 did not stimulate the passage of endogenous lactobacilli across the inflamed mucosa to the bloodstream. The data obtained are partly consistent with the literature data related to the probiotic safety concerns. In the study of Daniel et al. (2006), from all orally administered probiotic strains in TNBS-treated mice, *L. salivarius* Ls-33, *L. plantarum* Lp-115 and *L. acidophilus* NCFMO, only the first one contributed to a significant reduction of translocation of the endogenous microflora to extra-intestinal organs. In the study, an evaluation of the safety profile of probiotic strains used in strong and very strong colitis was performed (120–150 mg kg^{-1} TNBS). Llopis et al. (2005) identified attenuation of mucosal injury and inflammatory response associated with partial prevention in bacterial translocation to spleen, liver and MLNs, when *L. casei* DN-114 001 was administered to rats rendered colitic by intraluminal instillation of 3 mL TNBS solution (45 mg mL^{-1} in 10% ethanol). Similarly, Osman et al. (2006) reported reduction in translocation of aerobes, anaerobes, and entherobacteria to MLNs and liver due to administration of *Bifidobacterium infantis* strains, with and without a combination of oligofructose and inulin in DSS-treated rats.

4. Conclusion

In conclusion, the anti-inflammatory potential of *L. casei* 01 encapsulated in synbiotic chitosan-Ca-alginate microparticles suspended in ayran was higher than the effects observed with the non-encapsulated probiotic *L. casei* 01 and prebiotic oligofructose-enriched inulin. Synbiotic microparticles administered once a day provided significant beneficial effect, partly due to the favorable properties of the formulation to improve the viability and activity of the probiotic used. Probiotic and synbiotic treatment promoted the lactobacilli growth in colitic rats, with the latter being more significant. In addition, *L. casei* 01 may be regarded as safe probiotic strain for administration in conditions of mild to moderate inflammation. This study supports the usage of encapsulated probiotic and/or synbiotic-based treatments as a step forward in a conceptualization of successful therapeutic perspective for IBD.

Acknowledgements

This research was financially supported by the Ministry of Education and Science of the Republic of Macedonia (Project No. 13-3583/1). The authors would like to express gratitude to Orafti Beneo Group, IMCD (UK) and Chr. Hansen (Hoersholm, Denmark) for the donation of Orafti® Synergy 1 (L. Maréchal, Tienen, Belgium), sodium alginate (Protanal 10/60 LS, FMC BioPolymer, USA) and *Lactobacillus casei* 01, respectively.

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