Yogurt starter cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* ameliorate symptoms and modulate the immune response in a mouse model of dextran sulfate sodium-induced colitis

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ABSTRACT

We investigated the yogurt starter cultures of *Lactobacillus bulgaricus* 151 and *Streptococcus thermophilus* MK-10 for their effect on the severity of experimental colitis, lymphocyte profile, and regulatory T-cell response. Colitis was induced in BALB/c mice via the administration of 3.5% dextran sulfate sodium salt (DSS) in drinking water for 6 d. Next, the mice were gavaged intragastrically with an active yogurt cultures (YC) mixture ($\sim 5 \times 10^9$ cfu/mouse per day) or saline (vehicle) for 8 d. Mice receiving DSS or saline alone served as positive and negative controls, respectively. The length of the colon, disease activity index, histological scores, myeloperoxidase activity, epithelium-associated microbes, short-chain fatty acid profile, total IgA antibody-forming cells, CD3⁺CD8⁺, CD3⁺CD4⁺, CD3⁺CD4⁺CD25⁺, CD3⁺CD4⁺CD25⁺Foxp3⁺ T-cell subsets, and cytokine profiles (IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- γ , and tumor necrosis factor) were examined after termination of the mice. Feeding mice with YC mixture reduced disease symptoms and modified intestinal microbiota and host inflammatory responsiveness to DSS. We observed limited weight loss and a decreased disease activity index score, lowered myeloperoxidase activity, and somewhat reduced damage of the intestine. The YC mixture upregulated the colon length, increased the amount and diversity of mucosa-associated microbes (enterobacteria, enterococci, and yeast), and decreased the concentration of putrefactive short-chain fatty acids in the cecal contents. It downregulated the input of cytotoxic CD3⁺CD8⁺ T cells and CD3⁺CD4⁺CD25⁺FoxP3⁺ regulatory T cells in Peyer's patches and enhanced CD3⁺CD4⁺CD25⁺ T cells in spleens and CD3⁺CD4⁺CD25⁺FoxP3⁺ cells in peripheral blood mononuclear cells. Simultaneously,

IgA antibody-forming cells were downregulated in mesenteric lymph nodes (MLN) and enhanced in spleens (SPL). The cultures mostly enhanced the production of cytokines tested in MLN and SPL, except for IL-6, which was downregulated in MLN. Interleukin-2 and IL-4 were the most upregulated in MLN, whereas IL-10, IL-4, IL-2, IFN- γ , and tumor necrosis factor were most upregulated in SPL. In serum, the YC mixture downregulated IFN- γ and clearly increased IL-2. Based on these results, we recognize the high anti-inflammatory and immunomodulatory potential of the *L. bulgaricus* 151 and *S. thermophilus* MK-10 set. The strains possess the ability to modulate the intestinal mucosal and systemic immune system toward both IgA production and induction of regulatory T cells, shifting Th1/Th2 balance.

Key words: yogurt culture, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, dextran sulfate sodium-induced colitis, regulatory T cell

INTRODUCTION

Yogurt has been recognized as a healthy food with therapeutic properties. Except for traditional products fermented with *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, many bio-yogurts have been reformulated to include other *Lactobacillus* and *Bifidobacterium* species, the presence of which may produce the claimed beneficial health effects. Thus far, the nutritional value of yogurt has been well recognized (Lourens-Hattingh and Viljoen, 2001). Numerous studies have also suggested the beneficial therapeutic effects of bacteria present in yogurt on health; however, these health benefits are often attributed to the ingestion of probiotic-containing yogurts (Granato et al., 2010; Wroblewska et al., 2011). Probiotics influence the host health and its immune system and possess the potential to control inflammation (Ghouri et al., 2014; Berg et al., 2015; Wasilewska and Wroblewska, 2018). Knowledge on the activity of yogurt cultures in the gut

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remains limited, presumably because *S. thermophilus* and *L. bulgaricus* do not belong to the indigenous gut microbiota and poorly survive passage through the gut (Elli et al., 2006).

The gut harbors well-balanced microbial communities in the mucosa, lumen, and feces. These microbes participate in metabolic, nutritional, physiological, and immunological processes in the body; hence, crosstalk exists to control their growth and preserve intestinal homeostasis, resulting in differential host responses to commensal and pathogenic microbes (Macpherson and Harris, 2004). Failure of these regulatory mechanisms or imbalance of the gut microbiota may result in an abnormal immune response and pathology such as inflammatory bowel disease (IBD).

Probiotic therapies have appeared to be effective in IBD management, especially in ulcerative colitis (Derwa et al., 2017; Wasilewska and Wroblewska, 2018). The efficacy of yogurt to improve IBD treatment has been poorly investigated, even with those containing probiotic cultures (Lorea Baroja et al., 2007; de Assis et al., 2016; Sheikhi et al., 2016). So far, despite the recognized nutritional and health benefits, traditional yogurt cultures in IBD were examined sporadically, and studies, although optimistic, have been conducted on single strains. Ito et al. (2008) found that *S. thermophilus* YIT 2001 with antioxidative activity revealed an improving effect on dextran sulfate sodium (DSS)-induced colitis. According to Takamura et al. (2011), *L. bulgaricus* OLL1181 could activate the aryl hydrocarbon receptor pathway, which plays an important role in the regulation of the inflammatory response. Ogita et al. (2011) described the anti-inflammatory effect of *S. thermophilus* ST28, which could result from its ability to suppress the excessive Th17 response in inflamed intestines.

Yogurt starter cultures are selected and combined into symbiotic sets; therefore, to assess their therapeutic activity, the combined strains (sets) should be tested because their physiological activity may be the result of the combined activity of both strains. *Streptococcus thermophilus* MK-10 and *L. bulgaricus* 151 are a compatible strain pair with mutual symbiosis and competition, resulting in balanced growth and synergism reinforcing their technological features and biochemical activity (Bielecka et al., 1994a,b; Bielecka and Majkowska, 2000; Bielecka et al., 2000).

Evidence exists that all-*trans* retinoic acid is required for establishing immune tolerance to dietary antigens in the upper gut, whereas microbial metabolites such as short-chain fatty acids (SCFA) can regulate microbiota-specific regulatory T cells (Tregs) in the colon (Smith et al., 2013). The Tregs, such as CD3⁺CD4⁺CD25⁺Foxp3⁺, and regulatory cytokines

play key roles in various immune response and protection against autoimmune diseases (Curotto de Lafaille and Lafaille, 2009; Buckner, 2010). The aim of our study was to identify whether yogurt starter cultures *S. thermophilus* MK-10 and *L. bulgaricus* 151 possess the capacity to moderate the symptoms of experimental colitis and alter the cytokine profile and induce the Treg response in a mouse model of DSS-induced colitis.

MATERIALS AND METHODS

Bacterial Strains

The *S. thermophilus* MK-10 and *L. bulgaricus* 151 strains used in the study originated from our own collection and were previously identified as starter cultures displaying synergistic effects during the manufacture of yogurt (Bielecka and Majkowska, 2000). For the experiment, *S. thermophilus* was cultured in M17 broth (Merck KGaA, Darmstadt, Germany) and *L. bulgaricus* was cultured in de Man, Rogosa, Sharpe broth (Merck KGaA) at 37°C until the stationary phase was achieved (the growth to about 5×10^8 cfu/mL and 1×10^9 cfu/mL, respectively, as was determined on M17 and de Man, Rogosa, Sharpe agar media). Next, the cultures were combined in the cell proportion they usually reach during yogurt manufacturing (i.e., 1:1.2, lactobacilli to streptococci, respectively), centrifuged ($5,000 \times g$, 15 min, 4°C), and the unwashed pellet was resuspended in sterile saline and further incubated at 37°C for 4 h. This culture [yogurt culture (YC) mixture] was used for experiments, as it contained about 2.5×10^{10} cfu/mL of bacterial cells and was made daily throughout the entire experimental period.

Mice and Experimental Design

Specific-pathogen-free BALB/c mice (female, 8 wk old) weighing 20 ± 2 g were used in the study. All mice were purchased from Animal House of the Mossakowski Medical Research Centre of the Polish Academy of Science in Warsaw (Poland). For the experiment, the animals were maintained in individual ventilated cages under controlled environmental conditions [room temperature ($22 \pm 2^\circ\text{C}$) with $50 \pm 10\%$ humidity and controlled 12-h light/dark cycle] and were fed total-pathogen-free 1324 maintenance diet (Altromin, Lage, Germany) and water ad libitum.

For the experiments, the mice were randomly assigned to 3 groups ($n = 12/\text{group}$) and were acclimated for 7 d. A natural enteric flora transfer step was then performed to reduce the variability in disease onset and severity. Dirty beddings were mixed and homogenized, and equal parts were spread over new litters.

Figure 1. Schema of the experimental design. DSS = dextran sulfate sodium salt; YC = yogurt cultures.

Colitis was induced in 2 groups by feeding the mice 3.5% dextran sulfate sodium salt (**DSS**; molecular weight = 36,000–40,000 Da; Sigma-Aldrich, Steinheim, Germany) in drinking water ad libitum for 6 d. At the end of d 6, all DSS-administered mice were returned to drinking plain water. Following the discontinuation of DSS, on d 6, supplementary feeding was started (Figure 1). The treatment colitis group (**DSS-YC** group) was administered 200 μ L/d per mouse of the YC mixture ($\sim 5 \times 10^9$ cfu per mouse) intragastrically for the next 8 d. The control colitis group (**DSS** group) served as the primary comparison with the YC intervention group and received saline intragastrically as a vehicle. The saline group comprised the healthy controls and received normal drinking water ad libitum (without DSS) throughout the entire study and 200 μ L/d per mouse of saline intragastrically during the intervention.

The mice were monitored daily for BW, stool consistency, and fecal blood, and the disease activity index (**DAI**) was calculated as described below. The mice were euthanized on d 14, after 8 d of interventional feeding. Immediately after euthanasia by carbon dioxide inhalation, the abdominal skin of the mouse was sprayed with 70% ethanol and blood was taken by cardiac puncture. Next, the spleens (**SPL**), mesenteric lymph nodes (**MLN**), colons, and Peyer's patches (**PP**) were carefully taken to avoid contamination for lymphocyte isolation or for histological, biochemical, or microbiological examinations. The length of the colon was measured as described below. All animal care and procedures were in accordance with institutional policies for animal health and well-being and were approved by the Institutional Animal Ethics Committee (Approval no 79/2011).

DAI

A DAI score was computed as the combined scores of the established scoring system (Kim et al., 2012) com-

prising percentage of BW loss (1–5% weight loss = 0 to >20% weight loss = 4), stool consistency (normal pellets = 0 to diarrhea = 4), and rectal bleeding (no rectal bleeding = 0 to visible gross bleeding blood around anus = 4). The percentage of BW loss was calculated to the initial value at the beginning of the experiment. Occult blood in feces was measured using the Hemocult test (Beckman Coulter Inc., Brea, CA).

Colon Length and Histological Evaluation

The length of the colon from the ileocecal junction to the anus was measured before it was quickly flushed using cold PBS (10 mM, pH 7.4) to remove feces and blood. The distal segment of the colon (~ 1.0 cm) of each mouse was fixed in 10% buffered formalin solution and embedded in paraffin blocks. Cross sections (4 μ m) were stained with hematoxylin and eosin and examined by an experienced histopathologist blinded to the intervention. Inflammation was assessed based on an established scoring system (Kim et al., 2012) comprising crypt architecture (normal = 0 to severe crypt distortion with loss of entire crypts = 3), degree of inflammatory cell infiltration (normal = 0 to dense inflammatory infiltrate = 3), muscle thickening (base of crypt sits on the muscularis mucosae = 0 to marked muscle thickening present = 3), goblet cell depletion (absent = 0 to present = 1), and crypt abscess (absent = 0 to present = 1). The histological damage score was computed as the sum of each individual score.

Myeloperoxidase Activity Assay

Colonic myeloperoxidase (**MPO**) activity was measured according to the method of Kim et al. (2012) with minor modifications. Briefly, the frozen middle segment of the colon (25–50 mg) was homogenized in ice-cold lysis buffer (50 mM potassium phosphate buffer containing 0.5% of hexadecyltrimethylammonium bromide,

pH 6). The obtained homogenate was centrifuged at $13,000 \times g$ for 10 min at 4°C to remove insoluble parts. Next, aliquots of the supernatant were dispensed in 96-well plates and mixed with the MPO assay buffer at a ratio 1:29. The MPO assay buffer contained 50 mM phosphate buffer (pH 6) diluted with distilled water at a ratio 1:9, *o*-dianisidine dihydrochloride (0.167 mg/mL), and 0.0006% of hydrogen peroxide. Three subsequent readings of absorbance at 450 nm were taken at 30-s intervals using a spectrophotometer. The MPO activity was measured in units of MPO per milligram tissue (U/mg tissue), where 1 U of MPO was defined as the amount needed to degrade 1 μ mol of H₂O₂/min at room temperature.

Epithelium-Associated Microbiota

Frozen proximal segments of the colons (~2.5 cm) were transferred to an anaerobic workstation filled with a 90% N₂, 5% CO₂, and 5% H₂ gas mixture (Bactron600 Anaerobic Chamber, Sheldon Manufacturing Inc., Cornelius, OR). Segments were cut longitudinally, washed 3 times serially in cold saline, weighed, homogenized, and serially diluted 10-fold with an anaerobic diluent. Dilutions were inoculated (100 μ L) into culture medium. Total anaerobes were enumerated on BBL Schaedler's agar enriched with vitamin K₁ and 5% sheep blood (BD, Sparks, MD), *Bifidobacterium* were counted on modified Garcke's agar (Wasilewska et al., 2008), and sulfite-reducing bacteria were counted on Difco Differential Reinforced Clostridial Agar (BD). The plates were left in the anaerobic chamber incubator at 37°C for 72 h. For the evaluation of aerobic and facultative anaerobic bacteria, aliquots were plated on appropriate agar medium under aerobic conditions and were incubated at 37°C for 48 h. *Enterobacteriaceae* were enumerated on Difco MacConkey's agar with crystal violet (BD). Lactobacilli were enumerated on MRS agar (Merck KGaA) plates overlaid with a second layer of medium. Enterococci were enumerated on Kanamycin Esculin Azide Agar (Merck KGaA), and yeasts were enumerated on Difco Sabouraud's Dextrose Agar with chloramphenicol (BD). The identity of representative colonies recovered from the selective medium was confirmed by conventional microbiological tests, including colony appearance (examined under a stereoscopic microscope SZX9; Olympus, Tokyo, Japan), cellular morphology (examined by phase contrast microscopy; Microphot FXA, Nikon, Tokyo, Japan), Gram staining, and biochemical patterns using API systems (BioMérieux, Marcy-l'Etoile, France). Genus affiliation of the grown bifidobacteria was also checked based on the presence of bifidobacteria-specific fructose-6-phospho-

ketolase (F6PPK-EC 4.1.2.22; Scardovi, 1986). Bacterial counts were expressed as the log of the number of colony-forming unit per gram of tissue.

SCFA

Samples of cecal contents preserved in 0.1% trifluoroacetic acid (1:1, vol/vol) and immediately frozen in liquid nitrogen and stored at -80°C were used for the analysis of SCFA. The analysis was performed using an Agilent 7890A gas chromatography system coupled with a flame ionization detector and a 7683B auto-injector (Agilent Technologies, Santa Clara, CA). The SCFA were separated using an SGE BP21 capillary column (30 m $\times$ 0.53 mm; film thickness = 0.5; SGE Analytical Science, Melbourne, Australia). Helium was supplied as the carrier gas at flow rate of 5 mL/min. The initial oven temperature was 85°C, raised to 180°C at 6°C/min, and held for 5 min. The temperature of the flame ionization detector and injection port were 290 and 250°C, respectively. Samples (0.5 μ L of clear supernatant) were injected in a split mode with a ratio of 10:1. The content of the individual compounds was measured using external standards of acetic, propionic, butyric, valeric, isovaleric, and lactic acid (Sigma-Aldrich, Saint Louis, MO).

IgA Antibody-Forming Cells ELISpot Assay

The lymphocytes were isolated from MLN and SPL and were added to anti-mouse IgA-coated 96-well plates (MultiScreen Filter Plates; Merck-Millipore, Cork, Ireland) at 10⁵ cells per well. After 24 h of incubation in a 5% CO₂ atmosphere at 37°C, the plates were washed 4 times with PBST (PBS with 0.1% Tween 20). Next, horseradish peroxidase-conjugated secondary antibodies (Sigma Aldrich) were added at a dilution of 1:1,000 to the plate and were left for the next 24 h at 4°C in a humidity chamber. After the final washing step, the color reaction was developed by using 3-amino-9-ethyl-carbazole (Moss Inc., Pasadena, MD). The plates were incubated for 15 to 60 min at room temperature and thereafter washed with tap water. The spots were read the next day using a stereo zoom microscope (SZX9, Olympus). The results were presented as the number of IgA antibody-forming cells (AFC) for 10⁵ cells.

Lymphocyte Isolation and Flow Cytometry Analysis

Lymphocytes were isolated from SPL, MLN, PP, and blood. Peripheral blood mononuclear cells were isolated using a density gradient technique. Anticoagu-

lated blood was subjected to Histopaque-1077 density gradient centrifugation (Sigma-Aldrich). The cells were collected from the interface, washed, and phenotyped. Splenic, MLN, and PP lymphocytes were isolated using the conventional method (Holmes et al., 2001; Zlotkowska et al., 2012). Briefly, each tissue was homogenized by the Dounce homogenizer, passed through a Nitex filter (80- μ m nylon mesh; Small Parts Inc., Miami, FL) to remove debris, and washed in IM medium [RPMI 1640 medium (Sigma-Aldrich) supplemented with 1 mM sodium pyruvate, 1 mM NEAA, and 10 U/mL penicillin-streptomycin]. Splenocytes were additionally lysed with red cell lysing buffer (Sigma-Aldrich) followed by the washing step. The washed cell pellets were suspended in 1 mL of CM medium (RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 1 mM NEAA, 10 U/mL penicillin-streptomycin, and 10% fetal bovine serum). The viability of cells was determined by Trypan blue staining.

For flow cytometry phenotyping, the lymphocytes were washed twice with FACS buffer (10 mM PBS supplemented with 2% fetal bovine serum) and then were stained with rat anti-mouse CD3-PerCP-Cy5.5 (clone 17A2), CD4-FITC (clone H129.19), CD8-APC (clone 53-6.7), and CD25-PE-Cy7 (clone PC61) monoclonal antibodies (BD Biosciences, San Diego, CA). After 15 min of incubation on ice, the cells were washed with FACS buffer, fixed with BD Cytofix Fixation Buffer (Cat. No. 554655) on ice for 30 min, and washed and permeabilized with BD Cytofix/Cytoperm Fixation and Permeabilization Solution (Cat. No. 554722) at room temperature (RT) for 30 min. After the washing step in FACS buffer, the cells were stained with rat anti-mouse Foxp3-PE monoclonal antibodies (clone MF23; BD Biosciences) at RT for 30 min. After final washing, all samples were subjected to flow cytometry analysis using a BD LSRFortessa system (BD Biosciences). All incubations were performed in the dark. At least 50,000 events were collected for each sample. The data were analyzed using Flow Jo software (Tree Star Inc., Ashland, OR).

Cytokine Measurements

Freshly isolated MLN or splenic lymphocytes (5×10^6 cells/mL) were cultured in CM medium in 48-well cell culture plates for 4 d in a total volume of 300 μ L per well. Thereafter, the supernatants were harvested by centrifugation ($16,900 \times g$, 10 min, 4°C) and stored until analysis at -80°C . The harvested blood, after coagulation at room temperature (30 min), was centrifuged at $13,500 \times g$ for 10 min at 4°C, and the serum was stored at -80°C until analysis.

Concentration of cytokines IL-10, IL-17A, IFN- γ , tumor necrosis factor (TNF), IL-6, IL-4, and IL-2 in the culture supernatants and in blood serum samples were measured using the mouse Th1/Th2/Th17 CBA kit (cat. no. 560485, BD Biosciences). Protocols were performed according to the manufacturer's instructions. Briefly, 50 μ L of sample was mixed with 50 μ L of the mixed capture beads and 50 μ L of the mouse detection reagent. After incubation at RT for 2 h in the dark, the samples were washed and suspended in 300 μ L of wash buffer and acquired on BD LSRFortessa system. At least 10,000 events were collected for each sample. The data were analyzed using the CBA analysis software (FCAP Array; BD Biosciences). The concentration of each cytokine was calculated using the corresponding standard curve.

Statistical Analysis

The values for all measurements were expressed as means $\pm$ SD. The *t*-test was used to evaluate differences between groups for parametric data and the Mann-Whitney test for nonparametric data. The results were considered to be statistically significant if $P < 0.05$.

RESULTS

S. Thermophilus MK10 and *L. Bulgaricus* 151 Ameliorate DSS-Induced Colitis

The DSS-induced colitis murine model is commonly used to examine the pathogenesis of IBD and test the efficiency of experimental therapies, including probiotics and clinical nutrition. In our study, feeding mice with DSS via drinking water led to colitis, macroscopically characterized by BW loss, changed stool consistency, and rectal bleeding. Figure 2 illustrates the BW changes in the tested groups during the study. Mice in the control saline group showed relatively constant BW. Only 2% body mass gain was noted throughout the experiment, whereas changes in the body mass of the remaining groups were significant. The DSS itself caused a sharp decrease in BW from d 5 to 9, when the highest weight loss (18%) was noted ($P < 0.05$ vs. saline group). Thereafter, gradual body mass gain (from 82 to 93% of the initial weight) occurred; however, the differences remained significant ($P < 0.05$ vs. saline group). The BW of DSS-administered mice fed the YC set was sharply reduced from d 5 to 7 ($P < 0.05$ vs. saline group); thereafter, BW increased from 87% of the initial mass at d 7 to 99% at the end of the experiment. From d 8 to the end of the experiment, the DSS-YC group differed in body mass from both the saline- and

Figure 2. Body weight changes among different groups of mice. Saline = healthy control group [without dextran sulfate sodium salt (DSS)], received saline during intervention; DSS = control colitis group, received saline during intervention; DSS-YC = interventional colitis group, received yogurt culture (YC) mixture during intervention. The data are expressed as the means $\pm$ SD. Statistical analysis was performed by Mann-Whitney test. * $P < 0.05$ in comparison with saline group; # $P < 0.05$ in comparison with DSS group.

DSS-treated mice ($P < 0.05$). The DAI score at d 9, when the highest BW loss was noted, was the highest in the DSS mice and differed from both the saline and DSS-YC groups ($P < 0.01$ and $P < 0.05$, respectively; Figure 3A). Administration of the YC mixture relieved the macroscopic severity of colitis and decreased the DAI score in acute phase at d 9 ($P < 0.05$ vs. DSS group). On d 14, we observed clinical improvement in both the DSS and DSS-YC groups; however, the recovery was better, although not statistically different, in the YC-treated animals (Figure 3B; $P = 0.061$ vs. DSS group). We found no difference in the DAI scores

between the saline and DSS-YC groups ($P = 0.174$), unlike the saline and DSS groups ($P < 0.01$).

After termination of the experiment, we scored the severity of colitis based on the shortening of the colon length and by histological and biochemical evaluation. Shortening of the colon is a typical marker of inflammation in DSS-induced colitis. The mice in the control colitis group have reduced colon lengths compared with the healthy group ($P < 0.01$; Figure 4A), whereas the colon lengths in saline and DSS-YC groups were similar and amounted 86.60 ± 5.50 and 85.60 ± 7.02 mm, respectively. Microscopic examination of histological sections of the colonic tissue showed integral epithelium and no inflammatory cell infiltration in the saline group, as well as increased histology score resulting from increased infiltration and changes in the mucosal architecture with signs of crypt damage, muscle thickening, and goblet cell depletion in the tissue of the control colitis mice (Figure 4B; $P < 0.01$ vs. saline group). Feeding mice with YC ameliorated the histological signs of DSS-induced colitis (Figure 4C). The histology score decreased from 5.33 ± 1.63 in DSS group to 2.83 ± 1.47 in DSS-YC-administered mice ($P < 0.05$). However, we still noted a difference in this assessment between saline and DSS-YC group ($P < 0.05$). Myeloperoxidase is a lysosomal protein that is most abundantly expressed in neutrophil granulocytes, where it is stored in azurophilic granules and released into the extracellular space during degranulation. Colonic MPO activity is a benchmark of neutrophil infiltration into inflamed tissue; thus, it is considered the hallmark of acute inflammation. Compared with the healthy group, the mice in the DSS colitis group showed increased MPO activity ($P < 0.01$), which was attenuated in the DSS-YC group ($P < 0.05$ vs. DSS group; Figure 4D).

Figure 3. Disease activity index (DAI) score in the saline, dextran sulfate sodium salt (DSS), and DSS-yogurt culture (YC) groups at d 9 (A) and 14 (B). Saline = healthy control group (without DSS), received saline during intervention; DSS = control colitis group, received saline during intervention; DSS-YC = interventional colitis group, received yogurt culture mixture during intervention. The data are expressed as the means $\pm$ SD. Statistical analysis was performed by Mann-Whitney test.

Figure 4. Evaluation of dextran sulfate sodium salt (DSS)-induced colitis in the saline, DSS and DSS-yogurt culture (YC) groups, as measured by (A) changes in colon length, (B) histology score, (D) myeloperoxidase (MPO) activity, and (E) changes in the spleen weight. (C) Examples of extreme changes in hematoxylin and eosin-stained colonic tissue section from healthy (saline) and colitis mice (DSS and DSS-YC). Saline showed normal crypt architecture and the absence of inflammation in colonic mucosa; DSS showed advanced lesions in control colitis mouse, widespread mucosal and submucosal alterations with severe crypt distortion and dense infiltrate of inflammatory cells [black arrow shows crypt distortion and gray (red) arrow shows infiltrate of inflammatory cells]; and DSS-YC showed visible restorative effect of YC-feeding. Saline = healthy control group (without DSS), received saline during intervention; DSS = control colitis group, received saline during intervention; DSS-YC = interventional colitis group, received yogurt culture mixture during intervention. The data are expressed as the means $\pm$ SD. Statistical analysis was performed by *t* test (A, D, E) and by Mann-Whitney test (B).

The mean values of spleen weight in the tested groups ranged from 74 ± 17.7 to 84 ± 14.4 mg (Figure 4E), and we do not have evidence to reject the hypothesis that the means are equal.

***S. thermophilus* MK10 and *L. bulgaricus* 151 Modulate Mucosa-Associated Microbiota Profile and Activity**

Table 1 illustrates the number of cultivable bacteria associated with the colonic epithelium of the tested mice. Strict anaerobes and lactobacilli dominated in the tested tissues. The mean counts for the groups were similar and ranged from 6.53 ± 0.28 to 6.95 ± 0.33 and from 6.30 ± 0.20 to 6.97 ± 0.12 log cfu/g tissue for total anaerobes and *Lactobacillus* spp., respectively; however, DSS administration reduced the *Bifidobacterium* population and enhanced *Enterococcus* ($P < 0.01$). The

amount of bifidobacteria in the tested samples was the highest in the control saline group and differed from both the DSS- and DSS-YC-receiving mice ($P < 0.01$ and $P < 0.05$, respectively). Enterococci were not detected in the tested tissue of healthy mice; however, they appeared in both DSS-receiving groups. Mice treated with the YC set possessed epithelium-attached *Enterobacteriaceae* and yeasts not found in the colonic samples of the remaining animals. We did not detect sulfite-reducing bacteria in any of the tested samples.

As shown in Table 2, the animals differed in the cecal concentration of SCFA. Administration of DSS caused an increase in the total SCFA and putrefactive short-chain fatty acids (PSCFA; $P < 0.05$). Particularly, acetic, propionic, valeric, and lactic acid concentrations were increased after DSS incorporation. Feeding mice YC lowered valeric acid and isovaleric acid and total PSCFA ($P < 0.01$) to below the level observed in the

Table 1. Bacterial counts in colon tissue (log cfu/g $\pm$ SD)^{1,2,3}

Item	Saline	DSS	DSS-YC
Total anaerobes	6.53 $\pm$ 0.28	6.72 $\pm$ 0.37	6.95 $\pm$ 0.33
<i>Lactobacillus</i> spp.	6.30 $\pm$ 0.20	6.44 $\pm$ 0.46	6.97 $\pm$ 0.12**
<i>Bifidobacterium</i> spp.	6.10 $\pm$ 0.28	4.89 $\pm$ 0.21**	5.24 $\pm$ 0.44*
<i>Enterobacteriaceae</i>	<0.93	<1.17	2.88 $\pm$ 0.13***###
<i>Enterococcus</i> spp.	<0.93	2.41 $\pm$ 0.33**	3.17 $\pm$ 0.23***#
Yeasts	<0.93	<1.17	3.07 $\pm$ 0.29***###

¹Saline = healthy control group [without dextran sulfate sodium salt (DSS)], received saline during intervention; DSS = control colitis group, received saline during intervention; DSS-YC = interventional colitis group, received yogurt culture mixture during intervention.

²The data are expressed as the means $\pm$ SD. Statistical analysis was performed by *t* test. When necessary, the detection limit was applied for the calculation. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 in comparison with saline group. #*P* < 0.05, ###*P* < 0.001 in comparison with DSS group.

³There was no sulfite-reducing bacteria on the tested tissues (the detection limit in the DSS-YC group was 0.88 log cfu/g).

saline group. The profile analysis of the major fatty acids (acetic, propionic, butyric, and lactic) did not reveal differences in the contribution of acetic, propionic, and lactic acids in the total SCFA between the groups. However, the percentage contributions of butyric acid in DSS-administered mice decreased as compared with healthy control (*P* < 0.05). Simultaneously, the ratio of propionic and lactic acids to total SCFA were slightly increased.

S. thermophilus MK10 and *L. bulgaricus* 151 Regulate the Regulatory T-cell Response

We performed the B cell ELISpot assay to check total IgA synthesis in the MLN and SPL of the tested mice. Treatment with DSS increased the level of IgA AFC

in MLN of the tested animals (*P* < 0.001; Figure 5A). Supplementing the diet with the YC set lowered IgA AFC (*P* < 0.001 vs. DSS group) in the MLN of DSS-fed mice; however, the level was still twice as high as in healthy mice (*P* = 0.055 vs. saline group). We found no difference in the amount of IgA AFC in the SPL of the control saline and DSS mice; however, feeding mice with the YC mixture increased IgA synthesis (Figure 5B; *P* < 0.05 compared with the saline group).

To further uncover how YC may protect against inflammation and colitis, we analyzed the T-cell population and cytokine profile. We acquired PP, MLN, SPL, and blood for lymphocyte isolation and phenotyping. The percentage of CD3⁺CD8⁺ cells was increased in PP and MLN in all DSS-administered mice (*P* < 0.01; Figure 6). However, feeding the DSS-treated animals

Table 2. Concentration and profile of short-chain fatty acids (SCFA) in the cecal digesta (mean $\pm$ SD)^{1,2}

Item	Saline	DSS	DSS-YC
SCFA, μ mol/g of digesta			
Acetic acid	94.08 $\pm$ 5.85	131.43 $\pm$ 21.54*	122.70 $\pm$ 19.35
Propionic acid	13.52 $\pm$ 2.63	23.67 $\pm$ 2.78**	22.83 $\pm$ 3.91*
Butyric acid	30.12 $\pm$ 2.72	34.37 $\pm$ 2.96	32.99 $\pm$ 3.53
Valeric acid	0.93 $\pm$ 0.15	1.60 $\pm$ 0.19**	0.51 $\pm$ 0.06*###
Isovaleric acid	1.12 $\pm$ 0.17	1.55 $\pm$ 0.24	0.76 $\pm$ 0.09*###
Lactic acid	7.69 $\pm$ 0.92	15.93 $\pm$ 2.16**	14.32 $\pm$ 2.03**
Total PSCFA ³	2.05 $\pm$ 0.30	3.15 $\pm$ 0.39*	1.27 $\pm$ 0.05*###
Total SCFA	147.46 $\pm$ 10.03	208.55 $\pm$ 24.65*	194.11 $\pm$ 27.94
Profile, % of total SCFA			
Acetic acid	63.88 $\pm$ 2.41	62.79 $\pm$ 3.04	63.14 $\pm$ 1.27
Propionic acid	9.13 $\pm$ 1.33	11.38 $\pm$ 1.01	11.74 $\pm$ 1.00
Butyric acid	20.42 $\pm$ 1.15	16.54 $\pm$ 1.01*	17.06 $\pm$ 0.81*
Lactic acid	5.20 $\pm$ 0.32	7.77 $\pm$ 1.91	7.39 $\pm$ 0.45

¹Saline = healthy control group [without dextran sulfate sodium salt (DSS)], received saline during intervention; DSS = control colitis group, received saline during intervention; DSS-YC = interventional colitis group, received yogurt culture mixture during intervention.

²The data are expressed as the means $\pm$ SD. Statistical analysis was performed by *t* test. **P* < 0.05, ***P* < 0.01 in comparison with saline group. ###*P* < 0.01, ####*P* < 0.001 in comparison with DSS group.

³PSCFA = putrefactive short-chain fatty acids (the sum of isovaleric and valeric acids).

Figure 5. Immunoglobulin A antibody-forming cells (AFC) in (A) mesenteric lymph nodes (MLN) and (B) spleens (SPL) of saline-, dextran sulfate sodium salt (DSS)-, and DSS-yogurt culture (YC)-treated mice. Saline = healthy control group (without DSS), received saline during intervention; DSS = control colitis group, received saline during intervention; DSS-YC = interventional colitis group, received yogurt culture mixture during intervention. The data are expressed as the means $\pm$ SD. Statistical analysis was performed by *t* test.

with the YC set lowered the level of CD3⁺CD8⁺ cells in PP ($P < 0.01$ vs. the DSS group). A similar trend in the PP occurred in the case of CD3⁺CD4⁺ cells; however, we found no evidence to reject the hypothesis that the mean values of CD3⁺CD4⁺ are equal in the MLN, SPL, and PBMC. Treatment with DSS did not influence the CD3⁺CD4⁺CD25⁺ population; however, this population was decreased in PP and increased in SPL after YC administration ($P < 0.05$ and $P < 0.01$ vs. both groups, respectively). The contribution of CD3⁺CD4⁺CD25⁺Foxp3⁺ Tregs in PP, in turn, was clearly the highest in the DSS group (5- and 28-fold vs. the saline and DSS-YC groups, respectively, $P < 0.01$) and lowest in the DSS-YC-treated mice. We observed a decrease in this population in the MLN of DSS-administered mice; however, it was greater in the DSS-YC group (7- to 9-fold vs. DSS and saline groups, respectively; $P < 0.01$). The differences almost disappeared in SPL, whereas in PBMC significant enlargement of the CD3⁺CD4⁺CD25⁺Foxp3⁺ population in DSS-YC mice was observed compared with both the saline and DSS groups (4- and 8-fold compared with the saline and DSS mice, respectively; $P < 0.01$).

S. Thermophilus MK10 and L. Bulgaricus 151 Modify Cytokine Secretion

We performed cytokine profiling to analyze the effect of YC on the production of physiologically relevant cytokine proteins (IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- γ , and TNF) and check the direction of T-cell differentiation and polarization (Th1, Th2, or Th17 response). In the serum, the DSS itself caused the enhancement of the concentration of IFN- γ (nearly 2-fold, $P < 0.05$

vs. saline group), and decreased the concentration of IL-10 (nearly 5-fold; $P < 0.01$; Figure 7A). Feeding DSS-administered mice the YC mixture downregulated IFN- γ and increased the concentration of IL-2 (almost 14-fold compared with both saline and DSS groups, $P < 0.01$).

To clarify the immunomodulatory effect of the YC mixture, we further examined cytokine production by MLN and SPL in vitro, after preincubation of the isolated lymphocytes in culture medium. As showed in Figure 7B and 7C, the tested animals differed in cytokine secretion by both MLN and SPL. Predominantly, cultures revealed increased levels of interleukin in the YC-administered mice compared with those in the saline and DSS groups. In MLN, however, IL-6 reached the highest level in the DSS group, 2-fold higher versus the saline group ($P < 0.05$; Figure 7B). Simultaneously increased level of IL-4 was noted in these mice (5-fold, $P < 0.05$). Out of the remaining cytokines tested, we found no significant difference in IL-6 concentration between the saline and DSS-YC groups; the level of IL-10 did not change whatsoever, whereas the levels of IL-17A, TNF, IFN- γ , IL-4, and IL-2 were the highest in the DSS-YC group and increased 4-, 6-, 5-, 10-, and 25-fold, respectively, against the saline group ($P < 0.05$). Likewise, in the SPL cultures, the highest secretion of cytokine proteins was noted in the DSS-YC group (Figure 7C). The tested cytokines, IL-10, IL-17A, TNF, IFN- γ , IL-6, IL-4, and IL-2, increased about 25-, 3-, 4-, 19-, 3-, 30-, and 8-fold, respectively, as compared with the saline group ($P < 0.05$). Administration of DSS alone increased IL-10 (2-fold) and IL-4 (10-fold) secretion and decreased the level of TNF (3-fold; $P < 0.05$ vs. saline group).

Figure 6. Distribution of T-cell (Tc) subsets in Peyer's patches (PP), mesenteric lymph nodes (MLN), spleens (SPL), and peripheral blood mononuclear cells (PBMC) of saline-, dextran sulfate sodium salt (DSS)-, and DSS-yogurt culture (YC)-treated mice. Saline = healthy control group (without DSS), received saline during intervention; DSS = control colitis group, received saline during intervention; DSS-YC = interventional colitis group, received yogurt culture mixture during intervention. The data are expressed as the means $\pm$ SD. Statistical analysis was performed by *t* test.

DISCUSSION

Feeding mice for a few days with DSS polymers in the drinking water induces a very reproducible acute

Figure 7. Cytokine profile in saline-, dextran sulfate sodium salt (DSS)-, and DSS-yogurt culture (YC)-treated mice measured in serum (A) or secreted by T cells isolated from mesenteric lymph nodes (MLN) and spleens (SPL) of the tested animals and preincubated for 4 d in vitro (B and C). Saline = healthy control group (without DSS), received saline during intervention; DSS = control colitis group, received saline during intervention; DSS-YC = interventional colitis group, received yogurt culture mixture during intervention. The data are expressed as the means $\pm$ SD. Statistical analysis was performed by *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in comparison with saline group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ in comparison with DSS group.

colitis characterized by weight loss, bloody diarrhea, and histological damage of the intestine (Kim et al., 2012; Rose et al., 2012). We demonstrated that the *S. thermophilus* MK-10 and *L. bulgaricus* 151 set attenuated the clinical symptoms of DSS-induced colitis in mice, such as reduced BW loss, decreased DAI and MPO activity, and upregulated colon length, compared with those in the control colitis mice. The mechanism by which DSS induces inflammation is unclear, as much of the acute inflammatory response induced by DSS likely arises from the nonspecific disruption or injury of the basolateral intercellular space of the colonic epithelium (Koboziev et al., 2011). We observed upregulation of colon length and measurably reduced damage of the mucosa caused by DSS in the YC-administered mice, what probably, to some extent, sustained the integrity of the epithelium (Figure 4B and 4C). Evidence exists that the intestinal bacteria and probiotics may change the expression and distribution of tight junction proteins or alleviate oxidative stress and thus strengthen the intestinal barrier; for example, the nonpathogenic *Escherichia coli* Nissle 1917 strain, which appeared to be safe and effective in maintenance of clinical remission of ulcerative colitis in humans (Ukena et al., 2007; Wasilewska et al., 2018). Putt et al. (2017) described the improving effect, dependent on digestion, of standard yogurt on barrier integrity in the Caco-2 cell model. Oxidative stress can lead to damage of the mucosa and bacterial invasion, and further to inappropriate activation of immune signaling pathways and inflammation. We observed lowered activity of MPO, a protein released into the phagosome of neutrophils during the degranulation process, which when overexpressed can damage normal tissue and contribute to inflammation. Ito et al. (2008) tested 2 *S. thermophilus* strains and found that only one, *S. thermophilus* YIT 2001, displaying high antioxidative activity, prevented oxidative injury to the colonic mucosa and improved the disease activity index in DSS-induced colitis in mice. We observed accelerated healing in the YC-administered mice, but the DSS-induced injury patterns and changes within the colonic mucosa and muscular compartment contributed to colonic dysregulation, resulting in immunological and microbiological alterations.

Little information exists concerning the structure and function of bacterial biofilms in the large intestine. One of the risk factors for IBD development is that the mucus layer overlaying the epithelium becomes more permeable to bacteria and bacterial products (Johansson et al., 2014). The inner colon mucus layer overlaying the epithelium poses a barrier separating the body from the external environment and is impenetrable to bacteria in the healthy gut. Defects in this system may trigger inflammation and extensive bacterial contact

that may consequently cause leakage into the tissue. Particularly, during dysbiosis, some pathogenic bacteria can pass across the epithelium and penetrate the host's body. An intense load of microbes in turn can pose a burden on the immune system. In our study, the total amount of the intestine-associated microbiota did not change significantly; however, appreciable variations occurred in the number of cells of the tested bacterial groups. The DSS itself considerably decreased bifidobacteria and enlarged enterococci, whereas feeding mice with YC enhanced not only tissue-associated enterococci but also *Enterobacteriaceae* and yeasts not detected in the remaining groups. We do not know if it was an increase in the amount of microbes bound to the colonic epithelium or the ones that penetrated the epithelial barrier and pass into intestinal tissue or both; however, we observed upregulation of colon length with decreased MPO activity and lowered crypt distortion. Thus, in this case, the microbes associated with the colonic intestine seem to have a rather supportive and not detrimental effect. We found no microbes translocated across the intestinal barrier of the DSS-YC mice in blood, MLN, and other organs (E. Wasilewska, unpublished data). Bailey et al. (2017) showed that *S. thermophilus* NCIMB 41856 delayed onset of colitis and reduced clinical signs of disease in a DSS-colitis mouse model. Those authors did not observe a difference in the amount of microbes attached to the colonic epithelium between DSS and DSS+*S. thermophilus* treated mice, but the *S. thermophilus* NCIMB 41856 strain reduced amount of microbes crossing mucosal and cellular epithelial barrier.

Composition of the epithelium-associated microbiota communities is probably determined by a variety of host factors, such as immunity and IgA and defensins secretion, mucus composition, turnover rate of the epithelial cells, intestine motility, availability of adhesion sites, or colonization resistance mediated by composition of the luminal gut microbiota. Normal resident bacterial communities in the healthy gut are highly diversified, with predominance of *Firmicutes* and *Bacteroidetes* and low concentration of *Enterobacteriaceae*. Dysbiosis in IBD patients is characterized by decreased biodiversity and expansion of *Enterobacteriaceae* and certain *Clostridium* subsets (Lane et al., 2017; Wasilewska and Wroblewska, 2018). Banerjee et al. (2009) showed that *Lactobacillus delbrueckii* ssp. *bulgaricus* B-30892 reduced colonization and *Clostridium difficile* mediated cytotoxicity in Caco-2 model. Injured mucosa and enhanced exfoliation of the inflamed cells expose new binding sites for luminal microbiota and create chance for rearrangements in intestinal biofilm structure, depending probably on a variety of other circumstances. Feeding

mice a YC mixture promoted members of epithelium-associated *Firmicutes* (lactobacilli, bifidobacteria, and enterococci, to varying degrees) but also enterobacteria and yeasts. All of these microbes possess high binding affinity for epithelial cell membranes (Boudeau et al., 2003; Swidsinski et al., 2008; Wasilewska et al., 2010; Christoffersen et al., 2012; Sengupta et al., 2013; Richardson et al., 2018). Enterococci increased in both DSS groups; however, enterobacteria and yeasts increased only in the mice administered YC mixture. We do not know if the effect is stable and positive in the long run, but we can speculate that in the case in question the attached cells are beneficial for the host and exert a protective effect due to observed clinical symptoms. The YC tested could act antagonistically and suppress opportunistic pathogenic microbes, as these strains were specially selected based on their antagonistic effect toward some pathogenic *E. coli* and *Staphylococcus aureus* strains (Bielecka et al., 1994a,b). On the other hand, bacteria adjacent to the epithelium could protect against the adhesion and invasion of resident luminal pathogens (Boudeau et al., 2003). Moreover, the cell-attached bacteria may be quickly exfoliated together with damaged cells or may persist on the epithelium for some time if the cell is, for example, in an intensified repair process, as was probably the case of DSS-YC mice. Nonpathogenic *Enterococcus* strains may produce antimicrobials (e.g., enterocin) and block adhesion sites for pathogens, such as enterotoxigenic *E. coli*; similarly, yeast may exert protective effect. *Saccharomyces boulardii* (subtype of *Saccharomyces cerevisiae*) also possess the therapeutic potential for ulcerative colitis and even Crohn's disease management, especially when combined with baseline medical therapy (Wasilewska and Wroblewska, 2018). On the other hand, excessive proliferation of *S. boulardii* can cause candidiasis and even sepsis. Antibodies against *S. cerevisiae* are detectable in the serum of patients with Crohn's disease and have been proposed as one of the serological markers for Crohn's disease diagnosis (Bertin et al., 2013).

We measured the concentration of SCFA to evaluate changes in bacterial metabolic activity in the gut; SCFA are main bacterial metabolites with anti-inflammatory properties that play a role in protecting intestinal integrity. Many studies have indicated that SCFA exhibit a wide range of physiological function, including T-cell polarization and induction (Smith et al., 2013; Levy et al., 2016). The role of butyrate as a major energy source for epithelial cells, regulating their gene expression, proliferation, differentiation, and apoptosis is well recognized (Topping and Clifton, 2001; Kim et al., 2014). Di Sabatino et al. (2005) showed the high anti-inflammatory effect of butyrate in experimental colitis

and Crohn's disease lamina propria cells *in vitro*. Our cultures did not change butyrate concentration, but they generally downregulated SCFA in mice administered DSS, especially the concentrations of putrefactive valeric and isovaleric acids and total PSCFA. Valeric, isovaleric, and isobutyric acids are primarily produced from the catabolism of proteins, particularly from branched AA fermentation (Cardona et al., 2005). Cardona et al. (2005) found a strong correlation between isobutyric and isovaleric acids in the gut of different species irrespective of age, diet, and living conditions, probably due to the microbial breakdown of intestinal sloughed cells. The observed increased amounts of isovaleric and valeric acids in the control colitis group may result from enhanced exfoliation of the inflamed epithelium cells and intensified bacterial proteolytic activity that, in turn, may exacerbate inflammation. One of the suggested mechanisms that tie an intestinal dysbiosis to the pathophysiology of IBD involves the release of enteric bacterial proteases that interact with protease-activated receptors on epithelial cells, resulting in intestinal barrier dysfunction and exposure of the enteric immune system to luminal antigens (Carroll and Maharshak, 2013). Thus, the observed decline of putrefactive acids in the DSS-YC mice seems to confirm protective effect of the tested cultures on the inflammatory process.

An intestinal barrier separates the gut lumen from the inner host and consists of mechanical (mucus, epithelial layer), humoral (defensins, IgA), immunological (lymphocytes, innate immune cells), and muscular and neurological elements that prevent the entry of pathogenic microorganisms and toxic luminal substances into the body (Bischoff et al., 2014). Mucosal surfaces and the mucosa-associated lymphoid tissues are in continuous contact and crosstalk with gut microbiota. Thus, they function to distinguish between commensal and pathogenic microbes to control the latter. Many probiotics and prebiotics were shown to modulate the intestinal mucosal and systemic immune system (Ashraf and Shah, 2014). Except for the amelioration of clinical symptoms, we investigated the effect of the tested YC on the immune system and regulatory T-cell response. Using flow cytometry, we analyzed T-cell populations in PP, MLN, SPL and PBMC, as well as the Th1/Th2/Th17 cytokine profile secreted by MLN and SPL and in the serum of the tested mice; we also assessed IgA AFC in MLN and SPL to check the activity of B cells. The animals fed DSS showed significantly higher levels of helper CD4⁺ T cells in PP and cytotoxic CD8⁺ T cells in PP and MLN, most probably due to intensified activity of antigen-presenting cells and further activation of cytotoxic CD8⁺ and naïve helper CD4⁺ T lymphocytes.

Such activated T cells secrete cytokines (IFN- γ , IL-2, IL-4, and others) that activate their differentiation into functional Th1, Th2, Th17, and Treg subsets or effector Tc cells, such as macrophages. Cytotoxic T cells kill abnormal or infected cells and, once activated, proliferate with the help of many regulatory cytokines. In MLN, we observed increased levels of IL-6 and IL-4 in the DSS group and increased levels of IL-2, IL-4, IFN- γ , IL-17A, and TNF in mice administered YC. Some of the increases may be driven from the apoptotic or cytotoxic action of activated effector CD8⁺ T cells, and others from activated Th0 cells (Chávez-Galán et al., 2009; Shrikant et al., 2010; Hamada et al., 2013). Interleukin-6 is a proinflammatory cytokine that, among other traits, stimulates B lymphocytes proliferation, whereas IL-4 stimulates macrophages differentiation as well as a Th2 response and differentiation of B lymphocytes into plasma cells secreting antibodies, both probably increased amount of IgA-AFC in the DSS group. The administration of the YC mixture downregulated CD8⁺ cells in PP, probably due to better gut microbiota balance and less extensive inflammation-mediated damage in the colonic tissue, but also due to generally enhanced immune response and healing. Gobbato et al. (2008) demonstrated the anti-inflammatory effect of yogurt in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, which was related to an increase in the number of the IgA⁺ cells, a decrease in CD8⁺ population, and an increased apoptosis of the infiltrative cells in the colonic tissue, which essentially accelerated regeneration of the colonic epithelium. However, mice in Gobbato et al. (2008) were fed yogurt before and after TNBS administration, and although an increased number of IgA-producing cells in the large intestine was observed, the effect was visible in the course of TNBS administration and not in the course of yogurt feeding. Our studies revealed increase in the IgA AFC in SPL and, by extension, this seems to confirm the stimulating effect of YC on IgA secretion in mice with colitis.

Treatment with YC significantly increased the level of CD3⁺CD4⁺CD25⁺ cells in splenocytes. Natural CD4⁺CD25⁺ Tregs are critical to maintain immune homeostasis (Ng et al., 2001; Barnes and Powrie, 2009), as they possess anti-inflammatory properties. Thus, an increase in Tregs can aid in the control of clinical manifestations. Regulatory T cells work through cytokine intermediaries such as TGF- β and IL-10, both of which mostly inhibit the immune system, and via a direct cell to cell contact (Glick et al., 2013). We observed the increased input of CD3⁺CD4⁺CD25⁺ cells combined with increased IL-10 secretion in splenic lymphocytes of mice administered DSS-YC. The IL-10 produced by Tregs controls inflammatory responses to self and nonself an-

tigens (Vieira et al., 2004). Evidence exists that SCFA play a role in the regulating the gut-associated lymphoid tissue (Smith et al., 2013; Morrison and Preston, 2016). Microbiota-specific Tregs in the colon can be regulated by SCFA that signal through the G protein-coupled receptor GPR43 (Maslowski et al., 2009). Generally, intestinal homeostasis is maintained by regulatory T-cell populations consisting of 2 major $CD4^+$ cell subsets, $Foxp3^+$ and Tr1-like cells, that produce IL-10 (Barnes and Powrie, 2009). The increased IL-10 production correlates with the ability to reduce the inflammatory response associated with colitis (Glocker et al., 2011). del Carmen et al. (2015) enhanced the *S. thermophilus* therapeutic activity in an acute colitis model through incorporation into the strain of the therapeutic cDNA delivery plasmid, enhancing IL-10 production.

To identify the developmental capacity of $CD4^+CD25^+$ cells, we analyzed the levels of $CD4^+CD25^+Foxp3^+$ T cells. Adaptive $Foxp3^+CD4^+$ regulatory T cells are driven by the need to maintain a noninflammatory environment in the gut, to suppress the immune response to allergens, and to decrease chronic inflammation (Curotto de Lafaille and Lafaille, 2009). The DSS itself did not change the frequency of $CD3^+CD4^+CD25^+$ in the tested samples, but significantly increased the frequency of $CD3^+CD4^+CD25^+Foxp3^+$ in PP, which was probably due to an intensified load of antigens nearby the intestine that pose a burden on the immune system and necessity of enhanced regulation by antigen presenting cells. The effect was different in MLN, where DSS administration did not influence the level of $CD3^+CD4^+CD25^+Foxp3^+$ Tregs; however, we found a significant increase in IL-6 concentration and cytotoxic $CD8^+$ T cells and IgA AFC appeared. Interleukin-6 is an important mediator of fever and the acute phase response, and also supports the growth of B cells and acts antagonistically to regulatory cells. Evidence exists that proinflammatory cytokine IL-6 plays a crucial role in the uncontrolled intestinal inflammatory process (Atreya and Neurath, 2005). Opposite DSS, the YC mixture significantly decreased $CD3^+CD4^+CD25^+Foxp3^+$ Tregs in MLN. It also suppressed pro-inflammatory IL-6 secretion, clearly enhanced IL-2 and IL-4 regulatory cytokines, and increased IL-17A, IFN- γ , and TNF secretion, thus generally enhancing immune activity and healing process by modulating Th1/Th2/Th17 cells response. In turn, the lowered IgA production took place in DSS-YC mice, likely due to the therapeutic effect of the administered bacteria resulting in decreased inflammation. However, ingestion of *S. thermophilus* MK-10 and *L. bulgaricus* 151 increased the IgA AFC in SPL of DSS-YC-treated mice, concurrent with a significant

elevation of $CD3^+CD4^+CD25^+$ cells, which are critical in healing and in the maintenance of self-tolerance and the prevention of autoimmune disease (Ng et al., 2001; Barnes and Powrie, 2009). We observed no such effect in the MLN of DSS-treated mice, where YC downregulated the expression of IgA AFC, although the level remained high. In SPL, the YC mixture downregulated the expression of $CD3^+CD4^+CD25^+Foxp3^+$ cells, likely due to the therapeutic effect of the administered bacteria.

The differentiation, migration, and maintenance of intestinal Tregs are controlled by specific signals from the local environment; in particular, intestinal microbiota provides antigens and immunomodulatory molecules that modulate intestinal Tregs expression (Tanoue et al., 2016). Evidence exists that propionate administration increased the expression of $Foxp3$ and IL-10 in colonic Tregs (Smith et al., 2013). We observed the increased propionic acid concentration in both DSS and DSS-YC groups; however, only the DSS group showed increased $Foxp3^+$ Tregs in PP, probably due intensified barrier dysfunction and direct exposure to environmental antigens. Simultaneously, a significant increase in IgA AFC was observed in the control DSS mice. The tested YC mixture improved microbial balance and downregulated inflammatory cytokine secretion and the Th2 response in MLN and enhanced Th1/Th2/Th17 polarization in SPL. In particular, the expression of regulatory IL-10, IL-2, IL-4, IFN- γ , and TNF in SPL seem to be enlarged by the YC mixture. Tumor necrosis factor- α is well known for its proinflammatory activity in various diseases, including IBD; however, in our study, the mean serum TNF levels were similar for all groups. In addition, we found no spleen enlargement in the tested mice (Figure 4E). Evidence exists that TNF suppresses the pathogenesis of acute intestinal inflammation by promoting local steroidogenesis (Noti et al., 2011). Naito et al. (2003) found significantly enhanced DSS-induced inflammation in $TNF-\alpha^{-/-}$ mice compared with that in $TNF-\alpha^{+/+}$ mice. The regulatory effect of the *S. thermophilus* MK-10 and *L. bulgaricus* 151 set on cytokine expression was also visible in serum; however, the lowered level of IL-10 was observed in all DSS administered mice but the level of proinflammatory IFN- γ was downregulated and the levels of the remaining cytokines were similar for saline- and YC-administered mice, except for the enhanced amount of regulatory IL-2. Interleukin-2 plays an important role in the development and expansion of effector T cells and is critical for the establishment and maintenance of immune tolerance, as it regulates $Foxp3$ expression in $CD4^+CD25^+$ regulatory T cells (Zorn et al., 2006).

CONCLUSIONS

Our data provide evidence that *S. thermophilus* MK-10 and *L. bulgaricus* 151 exert therapeutic effects on DSS-induced colitis in mice. We demonstrated that the strains improved mucosal healing. A significant increase in BW and colon length and a decrease in the DAI scores and MPO activity were observed in DSS mice after feeding with the tested YC. The mechanism of healing could be activated through direct and indirect effects on the gut microbiota balance and activity and epithelium-attached microbes, causing changes in local and systemic immunity. The observed therapeutic effect of *S. thermophilus* MK-10 and *L. bulgaricus* was mediated through modulation of T-helper-cell subset activities (Th1/Th2/Th17) and Tregs response, by improving colonic healing and downregulation of CD3⁺CD4⁺CD25⁺Foxp3⁺ T cells in the intestine but enhancement in peripheral lymphoid organs.

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