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Specific probiotic dietary supplementation leads to different effects during remission and relapse in murine chronic colitis

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Abstract

Although interest in using probiotics to prevent and treat intestinal diseases is increasing, the effects of specific probiotic strains still remain unclear. Here, we assess the therapeutic effects of two probiotic strains, *Lactobacillus rhamnosus* NutRes 1 and *Bifidobacterium breve* NutRes 204 on a dextran sodium sulphate (DSS)-induced chronic murine colitis model. The chronic colitis was induced by two DSS treatment cycles with a rest period of 10 days (the remission or resolution phase). The probiotic supplementation was started during the resolution phase, after the first DSS treatment cycle, and continued until the end of the experiment. In addition to clinical observations made during the experiment, cellular infiltration was measured along with mRNA expression of pro-inflammatory cytokines, T cell-associated cytokines, and Toll like receptors (TLR) in the inflamed colon after second DSS treatment cycle. *L. rhamnosus*, but not *B. breve*, rapidly and effectively improved the DSS-induced bloody diarrhoea during the resolution phase. However, a contradictory effect by both probiotic strains on the faecal condition was found after re-induction of colitis. The worsening of the faecal condition was accompanied by a reduced number of neutrophils and increased expression of interferon-γ in the colons of DSS-treated mice. Furthermore, an increased expression of TLR2, TLR6 and pro-inflammatory markers including chemokine (C-C motif) ligand 2, interleukin (IL)-1β, tumour necrosis factor α and IL-6 was found in DSS-treated mice with *L. rhamnosus* supplementation. These results indicate that therapeutic administration of specific probiotics might be beneficial during the resolution phase of colitis. However, caution should be taken as specific probiotic treatments reduce neutrophil influx, which may be the reason of exacerbation of chronic colitis.

Keywords: inflammatory bowel disease, probiotic therapy, chronic colitis, Toll-like receptors, neutrophils

1. Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of inflammatory bowel disease (IBD), which is a chronic inflammatory disorder of gastrointestinal tract. The disease course is characterised by series of relapses separated by remissions of varied durations, only a minority of the IBD patient undergoes a chronic, continuous disease course (Cosnes *et al.*, 2011). Although the aetiology remains largely unknown, the generally accepted hypothesis is that abnormal and hyperactive immune responses against components of the microbiota in a genetic susceptible hosts leads to chronic intestinal inflammation (Maloy and Powrie, 2011; Xavier and Podolsky, 2007). Recently, a new hypothesis has emerged that suggests that the underlying problem of CD is a failure of the innate immune response towards pathogens that penetrate the mucosal barrier, which leads to an impaired neutrophil infiltration and reduced bacterial clearance (Marks, 2011).

An overzealous immune response towards the intestinal microbiota is mediated via activation of pattern recognition receptors (PRRs), such as toll like receptors (TLRs), by bacterial expressed microbe-associated molecular patterns.

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Activation of TLRs leads to the recruitment of phagocytic leukocytes, predominantly neutrophils, to provide the first cellular immune defence against infections (Kolaczkowska and Kubes, 2013). To prevent local damage and spread of potential pathogens, an effective clearance of invading microbes by phagocytic cells is needed. The new hypothesis postulates that failure of microbial clearance mechanisms by diminished neutrophil chemotaxis and function results in persistent activation of immune system and drives subsequent T-cell mediated chronic inflammatory responses (Sewell *et al.*, 2009). The observations of disrupted neutrophil accumulation in CD patients (Marks *et al.*, 2006) and consequent impaired bacteria clearance (Smith *et al.*, 2009) supports this idea.

Probiotics are defined as 'live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002). The use of probiotics to treat intestinal diseases has received the attention of an increased number of researchers. Accumulated data indicates that probiotics restore the intestinal microbial balance and modulate the immune response (Ringel *et al.*, 2012). In addition, there is reasonable evidence supporting the applicability of specific probiotics in intestinal disease like IBD (Ringel *et al.*, 2012; Veerappan *et al.*, 2012). This was also demonstrated in our previous study using a preventive approach in a mouse model of colitis. *Bifidobacterium breve*, but not *Lactobacillus rhamnosus*, administration was found to have a beneficial effect on dextran sodium sulphate (DSS)-induced acute colitis (Zheng *et al.*, 2014). The aim of the current study was to investigate the applicability of therapeutic administration of the same specific bacterial strains in a chronic DSS-induced colitis model in mice.

2. Materials and methods

Animals

Female C57BL/6 mice were purchased from Charles River Laboratories (Maastricht, the Netherlands). All mice were used at 8-12 weeks of age and were housed under standard conditions in the animal facilities at Utrecht University. The animal experiments were approved by and were in accordance with the guidelines of the Dutch Experimental Animal Commission. The approval document is encoded with 2009.II.06.046.

Experimental colitis and administration of probiotics

Chronic colitis was induced by administration of two cycles of 1.5% DSS separated by a 10-day rest period. 1.5% DSS was added into the drinking water during each DSS treatment cycle for 5 days. The specific bacterial supplementation was started after the first DSS treatment cycle on day 6. 10^9 cfu/dose of *L. rhamnosus* NutRes 1 or *B. breve* NutRes 204 bacteria (Zheng *et al.*, 2014) were administrated by oral gavage every two days, starting the first day after the first DSS treatment cycle and continued till the end of the experiment (Figure 1). Colitis development was monitored by measuring the body weight and scoring of the faecal condition. The faecal condition assessments started at the end of the first DSS treatment cycle (day 5) and were continued till the end of the experiment (day 20).

The faecal condition score was determined from two parameters: stool consistency (0 = normal; 1 = soft with normal form; 2 = loss of form/diarrhoea) and faecal bleeding (0 = no blood; 2 = blood observation using Colo-rectal Test kit (Axon Lab AG, Reichenbach an der Fils, Germany), 4 = blood observation without test). The demonstrated body weight changes during the first DSS treatment cycle plus the resolution phase (day 0-15) and the second DSS treatment cycle (day 16-21) were normalised against the starting weight on the beginning of each DSS treatment cycle, on day 0 and day 15, respectively. The demonstrated faecal condition scores during the first DSS treatment cycle plus the resolution phase (day 0-15) and the second DSS treatment cycle (day 16-20) were normalised against the score on day 5 or day 15, respectively.

Histological evaluation of colon damage and immunohistochemical staining

After sacrificing the mice at day 21, colons (n=6) were collected for immunohistochemical staining and histological evaluation. The colon was opened longitudinally, washed in phosphate buffered saline (PBS) and placed on a piece of blotting paper. The half of each colon was fixed in 10% formalin for 24 h and embedded in paraffin-embedded as Swiss-rolls and sectioned (5 mm).

Two researchers assessed general inflammatory features blindly after staining sections with haematoxylin and eosin according the assessment system described before (Zheng *et al.*, 2013). Briefly, the histological assessments included four pathological criteria: the extent of cellular infiltration (0 = no infiltration; 1 = infiltration between the crypts; 2 = infiltration in the submucosa; 3 = infiltration in the muscularis externa; 4 = infiltration in entire tissue); the area of cellular infiltration in the region (0 = no infiltration; 1 = <25%; 2 = 25-50%; 3 = 50-75%; 4 = >75%); loss of crypts (0 = no damage; 1 = 30% shortening of crypts; 2 = 65% shorting of crypts; 3 = total loss of crypts; 4 = loss of entire epithelial

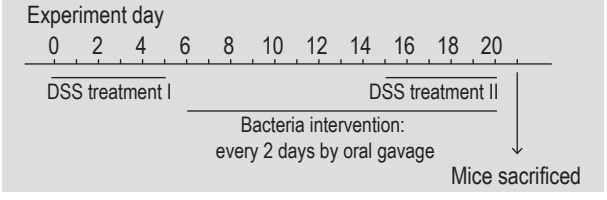

Figure 1. Schematic diagram of the study setup.

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layer); and the area of crypts loss in the region (0 = no crypt loss; 1 = <25%; 2 = 25-50%; 3 = 50-75%; 4 = >75%).

Immunohistochemistry was employed to determine Ly-6B.2+ cells (neutrophils and some activated macrophages). The sections were subjected to a heat-induced epitope retrieval step. Slides were washed with PBS and blocked with rabbit or goat serum before an overnight incubation (4 °C) with primary antibodies against Ly-6B.2 (AbD Serotec, Dusseldorf, Germany). For detection, biotinylated goat anti-rat (Dako, Glostrup, DK) secondary antibodies were administered followed by incubation with peroxidase-labelled streptavidin (Vectastain EliteABC kit, Vector, Burlingame, CA, USA). The peroxidase activity was visualised using the substrate DAB (Sigma, Gillingham, UK). The cell nuclei were visualised by a short incubation with Mayer's haematoxylin (Klinipath, Duiven, the Netherlands). Background staining was determined by substituting the primary antibody with a rat IgG isotype control (Abcam, Cambrige, UK). The number of Ly-6B.2+ cells was quantified by counting positive cells within the mucosal region throughout the whole colon.

Assessment of myeloperoxidase concentration in the colon tissue

After sacrificing the mice, colons (n=6) were collected for myeloperoxidase (MPO) concentration assessment, which is a marker for neutrophils. The colon was opened longitudinally and half of each colon was transferred into RIPA buffer (Thermo Scientific, Rockford, IL, USA) and homogenised using a Precellys®24-Dual homogeniser (Precellys, Villeurbanne, France). The homogenates were centrifuged at 14,000 rpm for 10 min at 4 °C and the MPO concentration in the supernatant was measured using an ELISA kit according to the manufacturer's protocol (Hycult Biotech, Uden, the Netherlands).

Real-time PCR

After sacrificing the mice, colons (n=6) were taken out for gene expression analysis. The colon was opened longitudinally and the proximal colon was distinct from the distal colon by its specific structure (bulges in the colon wall). The total RNA of the distal colon pieces was isolated using the RNAeasy kit (Qiagen, Germantown, MD, USA) and, subsequently, reverse transcribed into cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Real-time PCR quantification was performed using the iQ SYBR Green super mix kit (BioRad) with the CFX 96 Real-time system (BioRad) and the relative mRNA expression values were calculated using Bio-Rad CFX manager V1.6. The sequence of specific primers for *Tlr1, Tlr2, Tlr6* and the gene for the household protein ribosomal protein S13 (*Rps13*) are listed in Table 1. The primers for the cytokines: monocyte chemotactic protein-1 (*Ccl-2),* interleukin-1β (*Il-1β),* tumor necrosis factor-α (*Tnfα), Il6, Il4,* interferon gamma *(Ifnγ), Il17* and *Il10* were purchased from SABioscience (Frederick, MD, USA). The final data for the target samples were normalised against the internal control *Rps13.*

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.02 for windows (GraphPad Software, San Diego, CA, USA). *P*-values were calculated using either the two-way ANOVA followed by Bonferroni post-tests or, where needed, a Mann-Whitney test. The *P*-values were considered significant when <0.05.

3. Results

Faecal condition and body weight

In a previous study, we showed that, in a preventive setting, supplementation with *B. breve* ameliorates the development of acute colitis in mice (Zheng *et al.*, 2014). To study whether these specific bacterial strains, *L. rhamnosus* and *B. breve*, have similar effects in a chronic model of colitis, a therapeutic approach with these probiotics was employed in a murine chronic colitis model with two repeated DSS treatment cycles (Figure 1).

In healthy mice, supplementation with either *B. breve* or *L. rhamnosus* did not induce any clinical change as compared to mice without bacterial supplementation (data not shown). The first DSS treatment cycle led to decreased body weight

Table 1. qPCR primer sequences.

	Primer sequence 5'→3'	
	Forward primer	**Reverse primer**
Tlr1	GGTGTTAGGAGATGCTTATGGGG	GATGTTAGACAGTTCCAAACCGA
Tlr2	CCAGACACTGGGGGTAACATC	CGGATCGACTTTAGACTTTGGG
Tlr6	GACTCTCCCACAACAGGATACG	TCAGGTTGCCAAATTCCTTACAC
Rps13	GTCCGAAAGCACCTTGAGAG	AGCAGAGGCTGTGGATGACT

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(peaked at day 8) and gradually returned to baseline during the resolution phase. Therapeutic supplementation of the probiotics, starting after the first DSS cycle at day 6, had no effect on body weight change pattern in the resolution phase of mice suffering from DSS-induced acute colitis. DSS mice supplemented with *B. breve* seem to display an exacerbated weight loss compared to DSS control mice, however, this did not reach significance (Figure 2A). Faecal scores after the first DSS treatment had a maximal score on day 7, due to the appearance of bloody diarrhoea, and returned to baseline during the resolution phase. Supplementation with *L. rhamnosus,* but not *B. breve*, accelerated the restoration of faecal scores in DSS-treated mice and significantly reduced the occurrence of DSS-induced bloody diarrhoea on day 9, 10, 12, 13 and 15 of the resolution phase (Figure 2B).

The data of body weight change and the faecal score during the second cycle of DSS treatment were calculated relative to the data of starting date of second DSS treatment cycle on day 15. No significant differences in body weight were found between control and DSS-treated mice with or without probiotic supplementation except for day 21. On day 21, a significant DSS-induced decrease in body weight was observed (Figure 3A). Analysis of the tissue damage and cell infiltration in the colon indicated extensive cellular infiltration and crypt loss in mice with repeated DSS treatments. Supplementation with either *B. breve* or *L. rhamnosus* had no discernible effect as demonstrated in the graph and by the representative histology photos (Figure 3B, C). Assessment of the faecal score, however, revealed that DSS-treated mice supplemented with *L. rhamnosus* had significantly more pronounced bloody diarrhoea than DSS-treated mice without probiotics. Supplementation with *B. breve* tended to have a similar effect although this did not reach statistical significance (P=0.07) (Figure 3D).

Neutrophil infiltration in the colons of mice undergoing DSS-induced relapse

As neutrophils will be recruited to the targeted site upon infection or tissue damage (Chin and Parkos, 2006; Phillipson and Kubes, 2011), we examined both MPO expression and the amount of neutrophils in the colon. MPO is abundantly expressed in neutrophil granulocytes (Klebanoff, 2005) and is frequently used as marker of neutrophil infiltration, while Ly-6B.2 expression is particularly high on the surface of neutrophils and some inflammatory macrophages (Rosas *et al.*, 2010). Repeated treatment cycles of DSS, indeed, led to an increased MPO concentration and number of Ly-6B.2+ cells in the colon (Figure 4). Supplementation with the either *L. rhamnosus* or *B. breve* significantly reduced the enhanced MPO levels and the number of Ly-6B.2+ cells in the colon as shown in Figure 4.

Pro-inflammatory markers and interferon-γ expression in the colons of DSS-treated mice

To further investigate the effect of specific bacterial supplementation on the immune response in the colon, the expression of pro-inflammatory markers and T cell-associated cytokines was assessed. Figure 5 represents distal colon mRNA expression of chemokine (C-C motif) ligand 2 (CCL2), interleukin (IL)-1β, tumour necrosis factor alpha (TNF-α) and IL-6 and T cell associated cytokines IL-10 (Treg cell), IL-17 (Th17 cell), IL-4 (Th2) and interferon

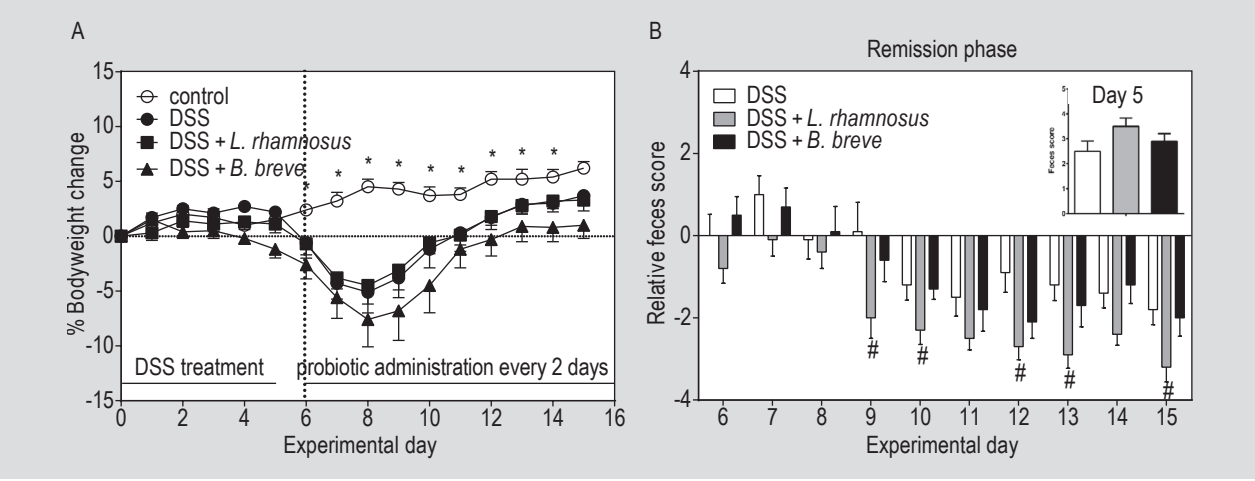

Figure 2. (A) The body weight changes of the control and DSS-treated mice with or without supplementation with *Bifidobacterium breve* or *Lactobacillus rhamnosus*, during the first DSS treatment cycle and the resolution phase are shown. (B) The faecal condition score change of DSS-treated mice during the resolution phase, calculated by normalising the results with the score on end of 1st DSS treatment cycle (day 5). The inserted graph represents the faeces score on day 5 (n=12 per group). All results are expressed as mean ± standard error of the mean. A '#' indicates the significant differences (P>0.05) between the groups with or without bacterial supplementation.

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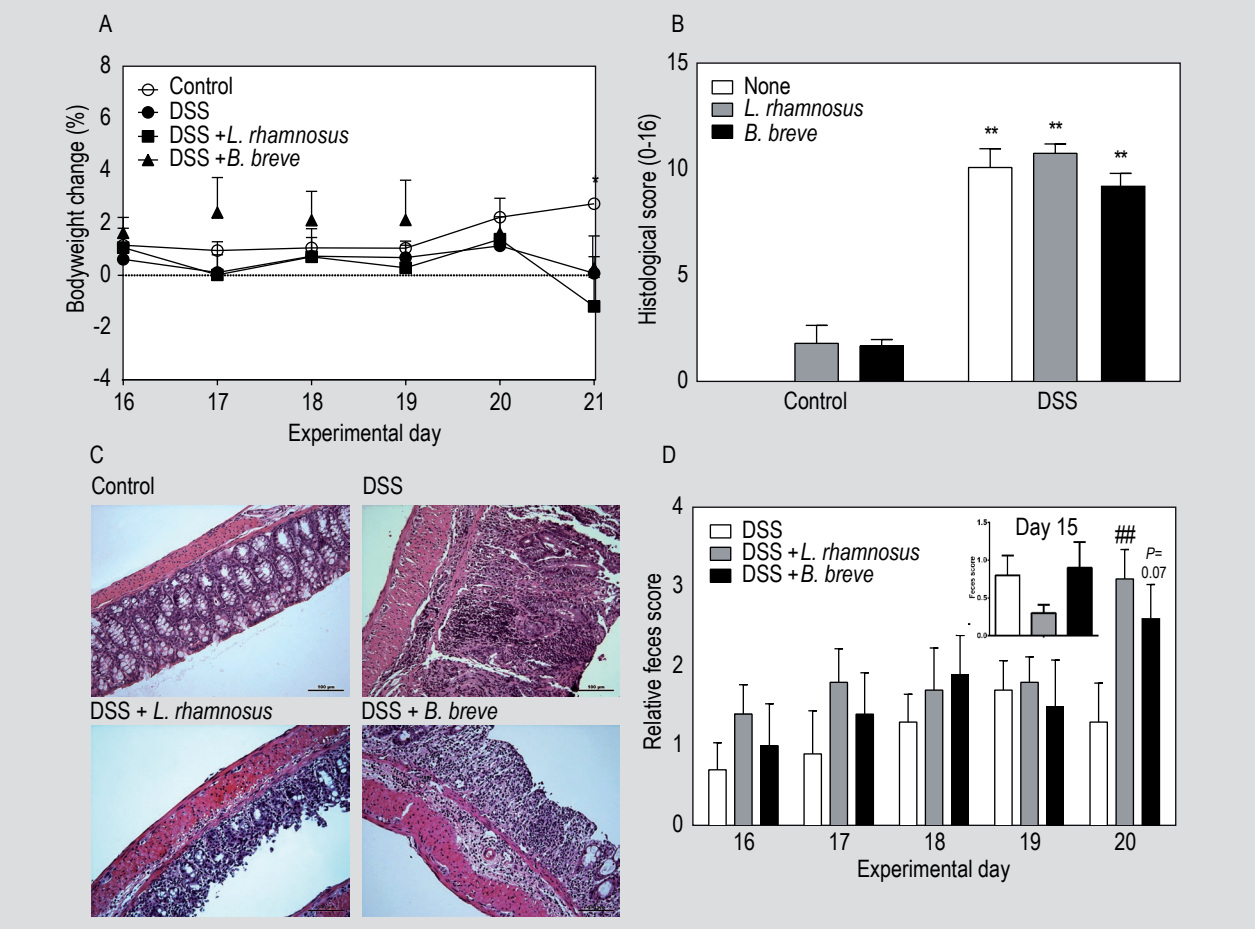

Figure 3. (A) The body weight changes of the control mice and DSS-treated mice with or without supplementation with *Bifidobacterium breve* or *Lactobacillus rhamnosus* during the second DSS treatment cycle. (B, C) Evaluation of the colons for tissue damage and cellular infiltration after sacrifice of the mice on day 21. (D) Faecal condition score of DSS-treated mice during the second DSS treatment cycle. The inserted graph represents the absolute faeces score on day 15 (n=12 per group). All results are expressed as mean ± standard error of the mean. Scores of (A) and (D) are calculated by normalising the results with the score on day 15. A '*' indicates a significant difference between the control and DSS treated groups, while the '#' indicates significant differences between the groups with or without bacteria supplementation. * = *P*<0.05; ## and ** = *P*<0.01.

gamma (IFN-γ) (Th1) in both healthy and DSS-treated mice with or without bacterial supplementation. In healthy mice, *IL-10* mRNA expression was significantly increased after *L. rhamnosus* supplementation (Figure 5A). Repeated DSS treatment significantly increased the mRNA expression of all examined cytokines in the colon of all treated mice regardless of probiotic supplementation as compared to healthy mice, except for IL-4 (Figure 5B). *Il4* expression was not affected by repeated DSS treatments or bacterial supplementation. Therapeutic supplementation of DSS-treated mice with *L. rhamnosus*, in contrast to *B. breve*, led to a significant increase in the expression of the pro-inflammatory markers CCL2, IL-1β, and IL-6. In addition, an increased expression of the Th1-assiociated cytokine IFN-γ was found in DSS-treated mice with both *L. rhamnosus* and *B. breve* supplementation as compared to mice with DSS treatment alone (Figure 5B).

mRNA expression of Tlr2 and Tlr6 in the colons of mice with repeated DSS treatment

To investigate whether a change in colonic expression of TLRs correlates with the observed phenotype in mice suffering from colitis, we determined mRNA expression of TLR1, TLR4, TLR5 and TLR9. No change in TLR expression was observed between diseased and healthy mice (data not shown). mRNA expression of TLR2 and TLR6 was higher in colonic tissue of repeated DSS-treated mice when compared to healthy mice, but this did not reach statistical significance. TLR1 and TLR6 both pair with TLR2 to form functional heterodimers (Farhat *et al.*, 2008). Although no significant differences for *TLR1* expression were found between the different mice groups, TLR2 and TLR6 expressions were significantly increased in DSS-treated mice with *L. rhamnosus* supplementation as compared to healthy mice with the same bacterial supplementation (Figure 6).

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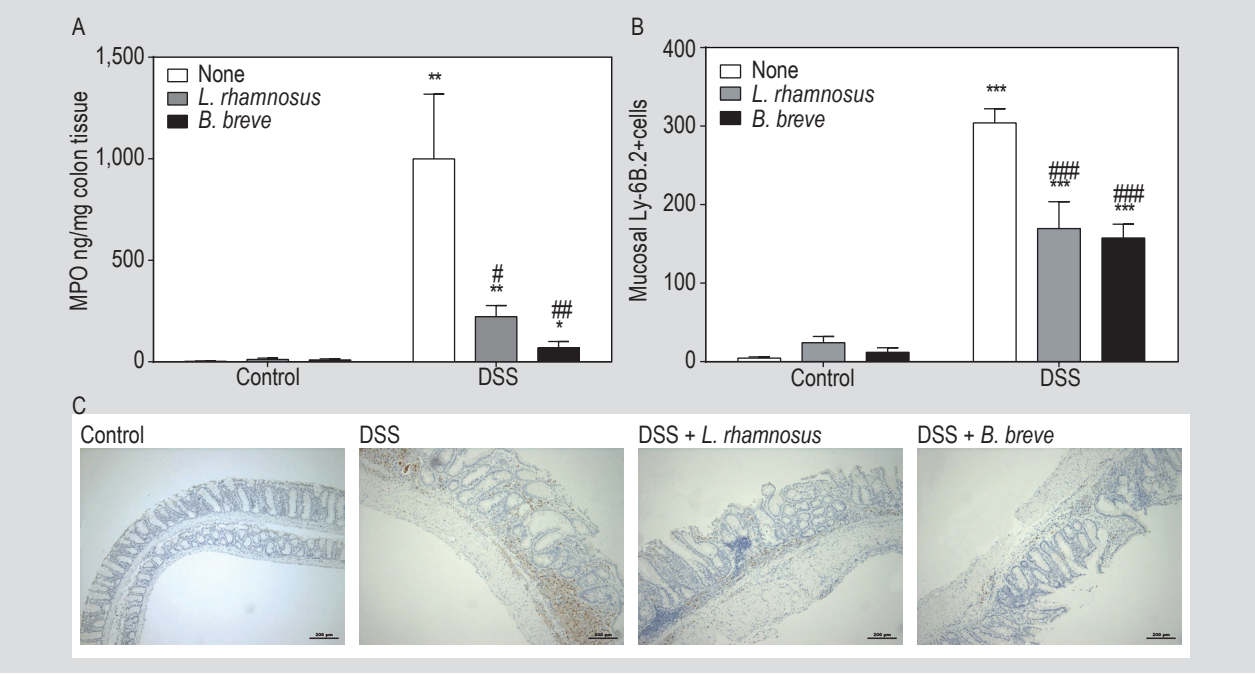

Figure 4. (A) Myeloperoxydase (MPO) concentration and (B) immunohistochemical quantification of neutrophils (n=6 per group) in colons of control mice and DSS-treated mice with or without supplementation with *Bifidobacterium breve* or *Lactobacillus rhamnosus* (day 21). (C) Neutrophil staining of representative images of the colon of control mice and DSS-treated mice. All results are expressed as mean ± standard error of the mean. A '*' indicates significant differences between the control and DSS-treated groups, while a '#' indicates significant differences between the groups with or without specific bacterial supplementation. * or ### *P*<0.001, ** or ## *P*<0.01, * or # *P*<0.05.**

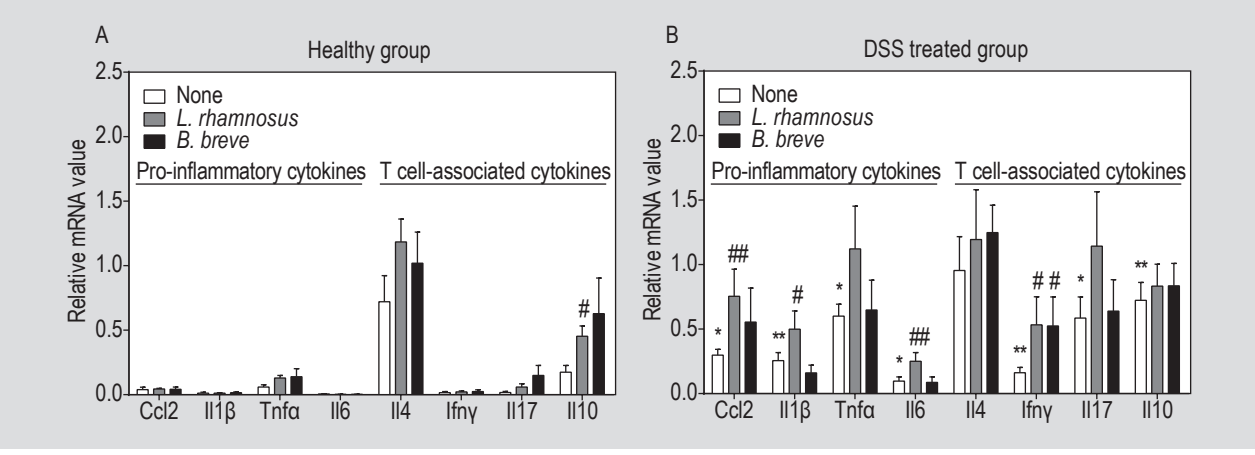

Figure 5. mRNA expression of pro-inflammatory cytokines (CCL2, IL-1β, IL-6 and TNF-α) and T cell-associated cytokines (IL-4, IFN-γ, IL-17 and IL-10) in the distal colons of (A) healthy and (B) DSS-treated mice with or without supplementation with *Bifidobacterium breve* or *Lactobacillus rhamnosus* (n=6 per group). All results are expressed as mean ± standard error of the mean. A '*' indicates a significant difference between the control and DSS-treated group, while a '#' indicates a significant difference between the group with or without specific bacterial supplementation. **, ## = *P*<0.01; *, # = *P*<0.05.

4. Discussion

There is growing recognition of the importance of the intestinal microbiota in modulation of the host immune response. Consequently, interest in strategies aimed to normalise the microbiota in order to restore immune homeostasis in intestinal disorders like IBD is also increasing. Evidence has shown that the administration of probiotics to patients suffering from IBD can restore the microbiota composition and induce beneficial effects (Ringel *et al.*, 2012; Vanderpool *et al.*, 2008). In patients suffering from UC, the clinical efficacy of probiotics was

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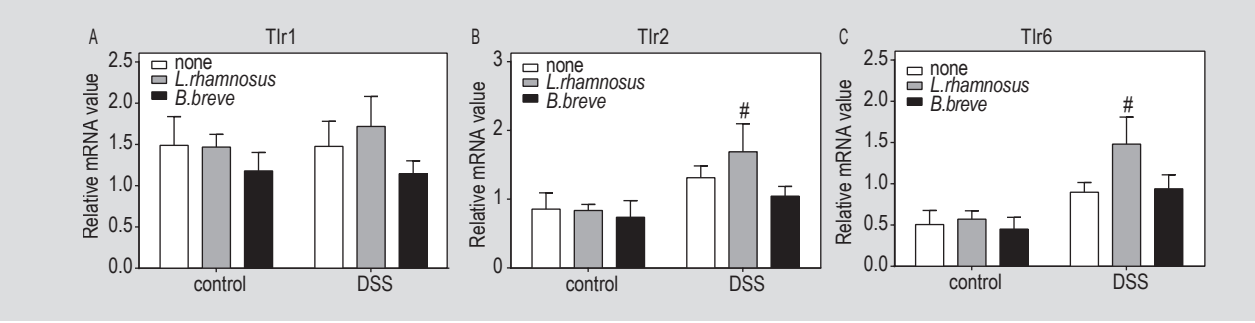

Figure 6. mRNA expression of (A) Toll-like receptor *(Tlr)* 1, (B) *Tlr2* and (C) *Tlr6* quantified in the distal colon of both healthy and DSS-treated mice with or without supplementation with *Bifidobacterium breve* or *Lactobacillus rhamnosus* (n=6 per group). All results are expressed as mean ± standard error of the mean. A '*' indicates a significant difference between the control and DSS-treated groups. ** *P*<0.01; * *P*<0.05.

demonstrated by administration of VSL#3 that resulted in a remission in adult patients with active UC (Sood *et al.*, 2009; Tursi *et al.*, 2010) and in children (Miele *et al.*, 2009). In addition, treatment with *L. rhamnosus GG* was effective in maintaining remission in patients suffering from UC (Zocco *et al.*, 2006). Moreover, several other probiotic strains such as *Escherichia coli nissle* 1917 and *Saccharomyces boulardii* have shown efficacy in treatment of UC (Guslandi *et al.*, 2003; Henker *et al.*, 2008). In the current study, supplementation with *L. rhamnosus*, but not *B. breve,* was found to accelerate and increase the improvement of the faecal condition (inhibition of DSS-induced bloody diarrhoea) in mice with colitis during the resolution phase. This result indicates that our *L. rhamnosus* strain has the potential to provide beneficial effects during recovery and the resolution phase of the disease.

However, this beneficial effect was not observed when the second cycle of DSS treatment started. In contrast, *L. rhamnosus* supplementation significantly increased the bloody diarrhoea after repeated DSS treatments. Supplementation with *B. breve* tended to have a similar effect, although it did not reach statistical significance. Further investigations demonstrated that this effect seen in probiotic supplemented mice was not mirrored by a worsened clinical score, indicating no change in DSS-induced epithelial cell damage or total cell influx. Instead, we observed an unexpected loss of neutrophils in the colon tissue. Neutrophil infiltration is usually considered a hallmark of inflammation, and increased amounts of neutrophils are associated with tissue damage and severe inflammation as observed in diseases like IBD (Chin and Parkos, 2006; Mantovani *et al.*, 2011). On the other hand, an alternative hypothesis for the pathogenesis of CD suggests that diminished neutrophil recruitment and consequent impaired bacterial clearance could contribute to chronic intestinal inflammation (Marks, 2011). In support for this hypothesis, CD patients were found to have a reduced neutrophil accumulation and showed an impaired clearance of bacteria from their tissues (Marks *et al.*, 2006; Smith *et al.*, 2009). In addition, a high similarity is found between patients with chronic granulomatous disease (CGD) and CD with as many as 55% of CGD patients meeting the criteria for CD. CGD is an inherited disorder that is characterised by a reduced ability of neutrophils to form reactive oxygen compounds necessary to kill ingested pathogens. This finding indicates that neutrophil mediated clearance of bacteria actively contributes to immune homeostasis in the colon (Marks *et al.*, 2009). Consistent with this hypothesis, we found probiotic supplementation to decrease neutrophil influx in the colon which was accompanied by an exacerbation of disease phenotype after the second DSS treatment.

Furthermore, it is postulated that inadequate clearance of bacteria leads to activation of the innate immune response. The subsequent increased release of pro-inflammatory cytokines triggers lymphocyte recruitment and directs the immune response towards a Th1 type response (Marks, 2011). In line with this hypothesis, supplementation with *L. rhamnosus* was observed to increase the mRNA expression of pro-inflammatory markers such as CCL2, IL-1β and IL-6 in the colon of DSS-treated mice. In addition, supplementation with both *L. rhamnosus* and *B. breve* increased the mRNA expression of the Th1-associated cytokine (IFN-γ*)* in the colons of mice suffering from an induced relapse. Taken together, it could be hypothesised that supplementation with *L. rhamnosus* worsens chronic colitis by reducing neutrophil recruitment and consequently inhibiting bacterial clearance. However, bacterial clearance was not measured in our studies indicating the need for further studies to show a causal relationship between the probiotic-induced reduction in neutrophil influx and subsequent effects on bacterial clearance.

An alternative explanation for the increased chronic disease phenotype observed upon supplementation with *L. rhamnosus* might lie with the increased expression of TLR2 and TLR6 in the distal part of the colon. Studies investigating the effect of activated TLR2/TLR6 receptors

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demonstrated contradictory results. On the one hand, TLR2/TLR6 stimulation of dendritic cells (DCs) *in-vitro* induces tolerogenic DCs and a type-1 regulatory T cell response (Depaolo *et al.*, 2008). On the other hand, a recent study performed by us has shown that TLR6 deficient mice were protected from DSS-induced colitis and that treatment with the TLR2/6 specific ligand, FSL-1, led to more severe colitis (Morgan *et al.*, 2014). In addition, 7 day long-term stimulation of isolated mesenteric lymph node cells with FSL-1 induces Th1 and Th17 cells which are associated with the pathogenesis of IBD (Morgan *et al.*, 2014). Lipotechoic acid (LTA) is a MAMP that has been identified as a ligand for TLR2/6. Thus, LTA contained within the cell wall of *L. rhamnosus* could activate TLR2/6 and trigger downstream signalling pathways (Claes *et al.*, 2012). Presence of *L. rhamnosus* LTA together with the enhanced colonic TLR2/6 expression in *L. rhamnosus* supplemented mice following the second DSS treatment, might lead to the induction of an inappropriate adaptive Th1/Th17 immune response and, as result, cause more severe and chronic colitis.

The effects of the two probiotic strains examined in this report have been previously investigated using a preventive approach in a mouse model of colitis. In this study, a preventive effect of *B. breve* and no effect of *L. rhamnosus* supplementation on disease phenotype was observed (Zheng *et al.*, 2014). In our current study we observed a beneficial effect of *L. rhamnosus,* but not *B. breve,* during the resolution phase and both probiotics worsened the faecal condition during the second DSS cycle. Although we are unable to exactly explain the differences found in these studies, one should note that the probiotics were administered when the mice were still healthy in the preventive study while in the current study probiotics were administered after the colitis had already been induced. DSS-induced colitis leads to structural changes in the colon including epithelial cell damage, a leaky gut as well as changes in immune components such as PRRs, T cells and cytokines (Zheng *et al.*, 2013). These data suggest that the same probiotic strains may carry out different effects in an intestinal environment that is recovering from a prior inflammation compared to effects in a healthy intestine.

In conclusion, we found that supplementation with *L. rhamnosus*, but not *B. breve*, affects the progression of DSS-induced colitis, and this is dependent on the status of the inflammation. During the resolution phase, *L. rhamnosus* supplementation enhanced the recovery of the colitis-associated faecal condition. However, bacterial supplementation during the second cycle of DSS, mimicking a relapse, exacerbated the DSS-induced bloody diarrhoea. Both *L. rhamnosus* and *B. breve* supplementation reduced the number of neutrophils in the colon of the mice suffering from DSS-induced colitis, accompanied by increased expression of Th1-associated cytokine, IFN-γ. In addition, *L. rhamnosus* supplementation further induced the expression of TLR2, TLR6 and pro-inflammatory cytokines in colon of mice suffering from DSS-induced colitis. These data provide evidence illustrating the importance of innate immunity during IBD development and suggests an important role for neutrophils during chronic intestinal inflammatory conditions. Our data warrant further research into the role of neutrophils in a chronic setting of experimental colitis. Moreover, further studies should address more in detail the differential effects of probiotics during remission and relapse. While our data show promise for specific probiotics in the remission phase, caution should be executed administering probiotics during relapse.

Conflict of interest

Jeroen van Bergehenegouwen, Paul Vos and Johan Garssen are employees of Nutricia Research, Utrecht, the Netherlands.

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