

Distinct effects of *Lactobacillus plantarum* KL30B and *Escherichia coli* 3A1 on the induction and development of acute and chronic inflammation

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Abstract

Objective: Enteric bacteria are involved in the pathogenesis of ulcerative colitis. In experimental colitis, a breakdown of the intestinal epithelial barrier results in inflow of various gut bacteria, induction of acute inflammation and finally, progression to chronic colitis.

Material and methods: In the present study we compared pro-inflammatory properties of two bacterial strains isolated from human microbiome, *Escherichia coli* 3A1 and *Lactobacillus plantarum* KL30B. The study was performed using two experimental models of acute inflammation: peritonitis in mice and trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats.

Results: Both bacterial strains induced massive neutrophil infiltration upon injection into sterile peritoneal cavity. However, peritoneal exudate cells stimulated *in vitro* with *E. coli* 3A1, produced far more nitric oxide, than those stimulated with *L. plantarum* KL30B. Interestingly, distinct effect on the development of TNBS-induced colitis was observed after oral administration of the tested bacteria. *Lactobacillus plantarum* KL30B evoked strong acute colitis. On the contrary, the administration of *E. coli* 3A1 resulted in a progression of colitis to chronicity.

Conclusions: Our results show that distinct effects of bacterial administration on the development of ongoing inflammation is strain specific and depends on the final effect of cross-talk between bacteria and cells of the innate immune system.

Key words: TNBS-induced colitis, *Escherichia coli*, *Lactobacillus plantarum*, inflammation, neutrophils.

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Introduction

Ulcerative colitis (UC), as well as Crohn's disease, belong to inflammatory bowel disease (IBD), chronic and spontaneously relapsing inflammatory disorders of unknown aetiology. Although the pathogenesis of IBD is still not fully understood, there is an increasing number of experimental data suggesting the role of various gut bacteria in the development (propagation/perpetuation) of chronic inflammation in IBD [1-3].

Among them, special attention has been paid to a possible role of *Escherichia coli* subtypes in pathogenesis of

IBD, especially UC, as *E. coli* are the predominant aerobic Gram-negative bacteria of the gut microbiome, and an increased number of *E. coli* in inflamed tissue in the gut of UC patients have been demonstrated [4]. Moreover, apart from various commensal strains, some diarrhoeagenic pathotypes of *E. coli* have been identified (e.g. shiga toxin-producing *E. coli*) [5]. They possess virulent properties such as adherence to the gut mucosa, production of enterotoxins and tissue invasion [6]. These pathogenic *E. coli* strains can cause acute diarrhoea, but with no documented role in the pathogenesis of IBD. So far there is no proof

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that any particular *E. coli* strain can cause ulcerative colitis. However, some pathogenic *E. coli* strains with pro-inflammatory properties may play a role in the exacerbation of UC [7, 8].

On the other hand, it is tempting to speculate that the increased number of non-pathogenic *E. coli* in inflamed tissue may have beneficial effect and may be involved in an amelioration and resolution of intestinal inflammation. Only recently, we have reported that *E. coli* strains isolated from patients with UC attenuate the detrimental Fenton's reaction by inhibiting the formation of hydroxyl radicals, important toxic agents of chronic inflammation [7]. It points to anti-inflammatory properties of these *E. coli* strains. Moreover, it has been demonstrated in clinical trials that non-pathogenic *E. coli* strain Nissle 1917 was as effective as 5-ASA (5-aminosalicylic acid: the primary anti-inflammatory drug used to treat IBD) in preventing relapse and maintenance of remission of ulcerative colitis [9]. All these data confirm dual role of *E. coli* in pathogenesis in UC and its probiotic potential. Indeed, *E. coli* strain Nissle 17 bacteria, along with various *Lactobacillus* strains (*L. plantarum*, *L. reuterii*, *L. fermentum*) are the most common probiotics used in the treatment of inflammatory bowel disease including UC [10-12].

The majority of data showing the preventive effect of probiotic bacteria on the development of UC was demonstrated in animal models of acute and chronic colitis [13-16]. However, further studies are necessary to explain in details their beneficial role in the regulation of acute and chronic inflammation and to estimate anti-inflammatory profile of the tested bacterial strains, as immunoregulatory properties of bacteria are strain-specific. The required anti-inflammatory properties of probiotics are revealed by catalase positive bacterial strains, such as *Lactobacillus plantarum* KL30B [3].

The aims of the present study were: 1) to compare the effect of administration of the selected strain of *E. coli* (3A1) and *L. plantarum* (KL30B) on the induction of acute peritonitis in mice; 2) to compare the effect of these bacteria on the development of TNBS-induced colitis in rats; 3) to compare their ability to stimulate the production of inflammatory mediators by *in vitro* activated murine peritoneal macrophages.

Material and methods

Mice

CBA/J male mice (8-12 weeks of age, 18-22 g) were maintained in the Animal Breeding Unit, Department of Immunology, Jagiellonian University Medical College. All mice were held in standard caging conditions with water and standard diet *ad libitum*. The authors were granted permission by the Local Ethics Committee to use mice in this study.

Rats

Wistar male rats (12-14 weeks of age, weighing approx. 300 g), were purchased from Animal Breeding Unit at the Faculty of Pharmacy Jagiellonian University Medical College and housed in the Animal Unit of Pathophysiology Department (Jagiellonian University Medical College). Animals were held in standard caging conditions with *ad libitum* access to water and standard rodent food (Labofeed, Keynia, Poland). The use of rats in this study and all experimental procedures were approved by the Local Ethics Committee.

Bacterial strains

Characterization and preparation of Escherichia coli strain

Escherichia coli 3A1 strain used in the experiments was selected from over 100 strains isolated from the colonic mucosa with inflammatory changes of patients with a diagnosis of Crohn's disease. The strain was selected basing on frequency analyses of the genes responsible for synthesis of proteins for acquisition of iron ions. The selected strain had the following genes encoding: *feoB* – high affinity ferrous iron transporter, *chuA* – heme uptake protein, *fepA*, *iroN*, *iutA*, *fhuA*, *fecA*, *fyu* – receptor proteins for siderophores: enterobactin, salmochelin, aerobactin, ferrichrome, rhizoferrin, yersiniabactin, respectively. Additionally, the selected strain of *E. coli* 3A1 produces two types of hydroperoxidases: catalase/peroxidase I (HPI) and hydroperoxidase II (HPII), encoded by *katG* and *katE*, respectively. The strain showed the highest level of catalase biosynthesis following the induction with iron.

For *in vivo* studies both living and killed *E. coli* 3A1 bacteria were used. For this purpose, the selected *E. coli* 3A1 strain was grown overnight at 37°C in MacConkey agar (Oxoid, Thermo Scientific, UK). This strain was inoculated in 10 ml of tryptic soy broth (TSB) (BD, USA) and incubated at 37°C for 3 h. After incubation, bacterial culture was microcentrifuged (5000 rpm, 10 min, 4°C), the supernatant was removed, and pellet was resuspended in 10 ml of phosphate buffered saline (PBS). This step was repeated three times. Subsequently, the sample was resuspended in 5 ml PBS. Bacterial density in the sample was determined by measuring optical density (JASCO Corporation Spectra Manager v.1.30.01) at a wavelength of 600 nm (OD₆₀₀). Inocula of the *E. coli* 3A1 bacteria had the OD reading 2.5 ± 0.02 which corresponds to 5 × 10⁸ colony forming units (CFU)/ml.

Bacterial cells were heat-killed by sterilization at 120°C for 20 min.

Characterization and preparation of Lactobacillus plantarum strain

Lactobacillus plantarum KL30B strain was selected from the gastrointestinal tract of a healthy human adult. It had unique antioxidant properties thanks to the production

of catalase and liberation it outside of the bacterial cells. The chemical decomposition of hydrogen peroxide to oxygen and water by fresh culture of *L. plantarum* KL30B was detected by colorimetric OxiSelect Hydrogen Peroxide Assay Kit (Cell Biolabs, USA).

The fresh culture of *L. plantarum* KL30B was prepared in 10 ml of MRS agar broth (Oxoid, Thermo Scientific, UK) at 37°C in anaerobic condition. After 48 h of incubation, the culture was centrifuged (3000 rpm, 15 min, 4°C). The pellet of *L. plantarum* KL30B was suspended in 1 ml of PBS (this step was repeated twice). The final density of live cultures of *L. plantarum* KL30B for oral administration to rats, was set at 0.5 MacFarland scale, which corresponded to 1×10^9 CFU/ml of PBS.

Experimental models

In vitro activation of murine macrophages

Peritoneal mouse macrophages (PEC) were induced by intraperitoneal injection of 3 ml of thioglycollate (3-4 mice). Four days later, cells accumulated in peritoneal cavity were harvested (washed out with PBS containing 1% heparin). After washing the cells 3 times in IMDM containing 5% FBS, 25 mM HEPES, 2 mM L-glutamine and 0.05 mg/ml gentamycin, they were seeded in 24-well plates at the density of 5×10^5 /well. Nonadherent cells were washed out 2-3 h later. Adherent cells (macrophages) were cultured in the presence of LPS (100 ng/ml) or bacteria (100 µl of 1×10^8 CFU/ml per well) either *E. coli* 3A1 (killed) or *L. plantarum* KL30B (live) or both *E. coli* 3A1 (killed) and *L. plantarum* KL30B (live) (100 µl of 1×10^8 CFU/ml each per well). Culture supernatants were collected 24 h later and saved for cytokine ELISA and NO assay.

Induction of acute peritonitis in mice

Acute peritonitis was induced by intraperitoneal injection of bacteria (100 µl/mouse of 1×10^8 CFU/ml) either *L. plantarum* KL30B (live or killed), killed *E. coli* 3A1 or a mixture of live *L. plantarum* KL30B and killed *E. coli* 3A1 (100 µl/mouse of 1×10^8 CFU/ml each). One group of mice received LPS (1 µg/mouse in 100 µl). Naïve untreated mice were used as a control. Each experimental group consisted of 5-6 mice. Eighteen hours later, peritoneal exudate cells were collected by washing out the peritoneal cavity with 5 ml of PBS containing 5 U/ml heparin (Polfa, Warsaw, Poland). Cells were centrifuged and red blood cells were lysed by osmotic shock with sterile distilled water. Equal volume of 2 × concentrated PBS was used immediately to restore osmolality. After washing, the cells were used for myeloperoxidase (MPO) assay and stained for the flow cytometry to assess the percentage of neutrophils.

TNBS-induced colitis in rats

Rats were randomly divided into five groups ($n = 5-8$ rats/group). Colitis was induced in all experimental groups

(1-5) by rectal administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) solution in 30% ethanol (Sigma-Aldrich, USA) (35 mg/rat) on day 0 and day 5. In addition, rats (groups 2-5) were orally administered with the tested bacterial strains (100 µl/rat of 1×10^9 CFU/ml), 3 times a day, for 11 consecutive days (day 2-12). Namely: group 2 received live *E. coli* 3A1, group 3 – killed *E. coli* 3A1, group 4 – live *L. plantarum* KL30B. Rats in group 5 received live *L. plantarum* KL30B on day 2, killed *E. coli* 3A1 on day 3 and then for consecutive 9 days (day 4-12) living *L. plantarum* KL30B together with killed *E. coli* 3A1. On day 12 (at the end of the experiment), rats were sacrificed by intraperitoneal administration of 100 mg/kg pentobarbital (Morbital, Biowet Pulawy, Poland). The rat colons were collected for further examination.

Histological evaluation of colitis

The selected fragments of intestine were fixed in 10% buffered formalin and sliced with a sharp blade into 3 mm long segments. These were routinely processed and paraffin embedded. Then, 2 µm thick sections were cut out from the paraffin blocks and stained with haematoxylin and eosin. The slides were assessed (by microscopic observation) and classified as acute or chronic lesions. The acute lesions were defined basing on the presence of mucosal infiltration by polymorphonuclears (neutrophils). Chronic lesions were defined basing on the presence of mononuclear inflammatory infiltrates (composed mainly of lymphocytes and macrophages) and granulation tissue. Ulcerations were classified as acute or chronic lesions depending on the type of infiltrate and presence of healing (granulation tissue).

In addition, we applied the following histological grading scoring system to classify intestinal inflammation (Score 0-3):

- 0 – No inflammation,
- 1 – Slight infiltration of cells in lamina propria (mild acute inflammation),
- 2 – Massive infiltration of neutrophils (moderate acute inflammatory lesion),
- 3 – Ulceration and a presence of granulation tissue (chronic type lesion).

Determination of nitric oxide production

The level of nitric oxide (NO) in the culture supernatant of peritoneal thioglycollate-induced macrophages was measured by the accumulation of nitrite as a stable end product according to a modified Griess method [17]. Cell culture supernatant (100 µl) was mixed with 14 mM 4,4'-diamino-diphenylsulphone (Dapsone; Sigma Aldrich, USA) in 2 M HCl (50 µl) and 0.1% N-1-naphtylenediamine dihydrochloride (50 µl) (POCH Gliwice, Poland). The absorbance of tested culture supernatants measured at 550 nm was compared with a sodium nitrate standard (NaNO₂) curve.

Measurement of myeloperoxidase enzymatic activity

Myeloperoxidase activity was measured according to the Bradley's method [18] with slight modifications. Shortly, intestinal tissue pieces (TNBS-induced colitis in rats) or cells collected from peritoneal cavity 18 h after injection of LPS or bacteria (acute peritonitis in mice) were placed in a 50 mM phosphate buffer, pH 6.0, containing 0.5% HTAB (hexadecyltrimethyl-ammonium bromide) (Sigma Aldrich, USA) and disrupted by three cycles of freezing and thawing. Tissue samples were additionally homogenized using Ultrasonic Homogenizer Sonopuls (Bandelin electronic GmbH, Germany). After centrifugation (4000 g, 15 min) supernatants were collected. Aliquots of tested supernatants (0.1 ml) were mixed with 2.9 ml phosphate buffer (50 mM) containing o-dianisidine dihydrochloride (0.167 mg/ml) and hydrogen peroxide (0.0005%) and incubated 20 min at room temperature. The absorbance was measured at 460 nm. Recombinant MPO (Sigma Aldrich, USA) was used as a standard. The activity of MPO was expressed in units. One unit of MPO activity was defined as that degrading 1 μ mol of H₂O₂ per minute at room temperature. The protein level in the tissues samples (intestine) was measured using Bicinchoninic Acid Protein Assay Kit (Sigma Aldrich, USA) and the MPO activity was expressed in units per mg protein.

Determination of cytokines by ELISA

Cytokines in cell culture supernatant were measured by sandwich ELISA. Microtiter plates (Costar EIA/RIA plates, Corning Incorporated, USA) were coated with cytokine specific antibody. Interleukin (IL)-10 and tumor necrosis factor α (TNF- α) were measured according to the manufacturer's instruction of OptEIA mouse IL-10 Set (BD Biosciences, USA) and mouse TNF- α ELISA Ready-Set-Go (eBioscience, USA). For IL-6 assay plates were coated with monoclonal rat anti-IL-6 antibodies (BD Biosciences, USA) followed by blocking with 3% low fat milk (IL-6). Recombinant IL-6 (eBioscience, USA) and cell culture supernatant was added into the plates followed by biotinylated rat anti-mouse IL-6 antibody (eBioscience, USA), and streptavidin-HRP (Vector Laboratories, USA). Hydrogen peroxide (Sigma Aldrich, USA) in the presence of chromogenic dye TMB (BioLegend, USA) was used to develop colorimetric reaction. The reaction was stopped with 2 M sulfuric acid (POCH Gliwice, Poland) and the optical density was measured at 450 (570) nm using the microtiter plate reader (PowerWaveX, Bio-Tek Instruments, Winooski, VT).

Flow cytometry

Peritoneal exudate cells collected 18 h after injection of LPS or bacteria were washed with PBS containing 2% FBS and 0.05% sodium azide. Cells were stained with bio-

tin conjugated anti-mouse Gr1 (Ly-6G/Ly-6C) monoclonal antibody (BioLegend, USA) followed by APC-Streptavidin (BD Biosciences). Cells were co-stained with PE-conjugated rat anti-mouse F4/80 monoclonal antibody (eBioscience, USA). Propidium iodide (PI) (BD Biosciences, USA) was added to the cells just before the analysis to exclude dead cells. Cells were analyzed in FACS Calibur (BD Biosciences) with CellQuestPro Software. Live (PI negative), FCS^{hi} cells were gated and analyzed for the expression of Gr1 (as a marker of neutrophils) and F4/80 (monocyte/macrophage marker).

Statistical analysis

Statistical significance of differences between groups was analysed using One-way ANOVA, followed, if significant, by a Tukey's test for post hoc comparison. Analyses were performed using Graphpad Prism v. 5.01 (GraphPad Software, Inc., USA). In some cases (experiments, as indicated), Student's *t*-test was used (Microsoft Excel), if appropriate. A *p*-value less than 0.05 were considered statistically significant.

Results

Effect of *E. coli* 3A1 and *L. plantarum* KL30B on the induction of acute peritonitis in mice

Escherichia coli 3A1 (killed) and *L. plantarum* KL30B (live or killed) were injected intraperitoneally to compare their pathogenicity and capability to induce local acute inflammation in a sterile tissue without concomitant participation of host microbiome bacteria. Mice were injected with killed *E. coli* bacteria only, and not treated with live *E. coli*, to avoid systemic infection and life-threatening severe peritonitis. As shown in Table 1, both tested bacterial strains induced a similar total number of infiltrating peritoneal exudate cells. LPS alone, as compared to killed *E. coli* A31, was a much weaker inducer of inflammatory cells. Moreover, phenotypic analysis of the cells present in peritoneal cavity 18 h after the injection of microbial agents showed the high content of neutrophils in all experimental groups, as measured by a percentage of Gr1⁺F4/80⁻ cells. The results were correlated with MPO activity of the tested cells (Tables 1, 2). The highest percentage of neutrophils was observed in mice treated with killed *E. coli* 3A1.

Effect of *E. coli* 3A1 and *L. plantarum* KL30B on the production of inflammatory mediators by *in vitro* stimulated murine peritoneal exudate cells

Unstimulated peritoneal exudate cells spontaneously produce negligible amounts of TNF- α and IL-6, and undetectable amounts of IL-10 and NO [19]. However, incubation of these cells *in vitro* with the tested microbial agents

Table 1. Cells infiltrating peritoneal cavity and MPO activity in acute peritonitis induced by LPS or tested bacterial strains

Group	MPO [$U \times 10^{-3}/10^6$ cells] ^b	The average cell number per mouse [$\times 10^6$] ^a
Control (naïve mice)	ND	2.3 ±1.4
LPS	181 ±49	1.4 ±1.3
<i>L. plantarum</i> KL30B (live)	194 ±37	4.48 ±0.5
<i>L. plantarum</i> KL30B (killed)	287 ±68	3.45 ±0.4
<i>E. coli</i> 3A1 (killed)	321 ±44	3.27 ±1.3
<i>E. coli</i> 3A1 (killed) + <i>L. plantarum</i> KL30B (live)	277 ±30	5.13 ±2.0

^aThe number of cells in the peritoneal wash-out represents the number of cells infiltrating peritoneal cavity after injection of LPS or tested bacterial strains. Data shown are the average number of cells per mouse.

^bMPO activity measured in cells infiltrating peritoneal cavity is expressed in units per million cells. Data shown are the mean (\pm SD) of three independent experiments.

Table 2. The phenotype of cells infiltrating peritoneal cavity upon induction of acute peritonitis with LPS or tested bacterial strains

Group	Gr1 ⁺ F4/80 ⁺ cells [%]
Control (naïve mice)	1.92 ±0.32
LPS	57.47 ±7.34
<i>L. plantarum</i> KL30B (live)	51.31 ±3.38
<i>L. plantarum</i> KL30B (killed)	60.16 ±0.87
<i>E. coli</i> 3A1 (killed)	73.02 ±5.30*
<i>E. coli</i> 3A1 (killed) + <i>L. plantarum</i> KL30B (live)	74.89 ±8.62

The percentage of cells expressing Gr1 (neutrophils) among cells infiltrating peritoneal cavity. Data shown are the mean (\pm SD) of three independent experiments. * $p < 0.05$ *L. plantarum* KL30B (killed) vs. *E. coli* 3A1 (killed) (Student's *t*-test).

(LPS, whole bacteria) resulted in a massive release of both pro- (TNF- α , IL-6, NO) and anti-inflammatory mediators (IL-10) (Fig. 1). All tested agents induced substantial secretion of IL-6 with no differences between groups. On the contrary, important differences in the production of IL-10 and TNF- α were observed in particular groups. Namely, the cells activated with *L. plantarum* KL30B primarily released TNF- α , while *E. coli* 3A1 preferentially stimulated IL-10 formation. Importantly, *E. coli* 3A1 shows strongest capacity to induce the production of NO. In some experiments the amount of NO released by the cells incubated with *E. coli* 3A1 was even $> 5\times$ higher than the amount of NO induced by *L. plantarum* KL30B. In addition, the high-

est production of NO was observed after the stimulation of the cells with both LPS and *E. coli* 3A1 (data not shown).

Comparative effect of oral administration of *E. coli* 3A1 and *L. plantarum* KL30B on the development/progression of TNBS-induced colitis in rats

Standard clinical evaluation of experimental chronic colitis in rats includes a control of body weight [20]. No weight loss was found in animals in our experimental model of colitis. Importantly, in contrast to mild clinical symptoms of colitis and lack of signs of anorexia, macroscopic and histological examinations of the colons showed common signs of inflammation such as hyperemia (Fig. 2), neutrophil infiltration and mucosa lesions (Fig. 3).

For the present study we have chosen the dose of TNBS which slightly damaged gut mucosa, after two administrations (see Material and methods), with the incidence of approximately 60%. In these rats, colon inflammation involved few neutrophils in the mucosa and was classified as mild acute inflammation (histology score = 1, MPO $< 100 U \times 10^{-3}/mg$).

As shown in Table 3, administration of either *E. coli* 3A1, *L. plantarum* KL30B or both bacteria resulted in the development of more severe colitis than that observed in the TNBS group. The incidence of colitis classified by histological examination in all experimental groups was similar (60-71%). In the *E. coli* 3A1 group, ulcerations with significantly lower level of MPO activity were present. Such lesions were classified as chronic inflammation (Fig. 4A). *Lactobacillus plantarum* KL30B administration was associated with a predominant neutrophil infiltration confirmed by a significantly higher level of MPO activity and the process was classified as acute inflammation (acute lesions) (Fig. 4B). In the group which received both bacteria, the observed lesions were characterised as acute (3 cases out of 7) or as a mixture of acute and chronic

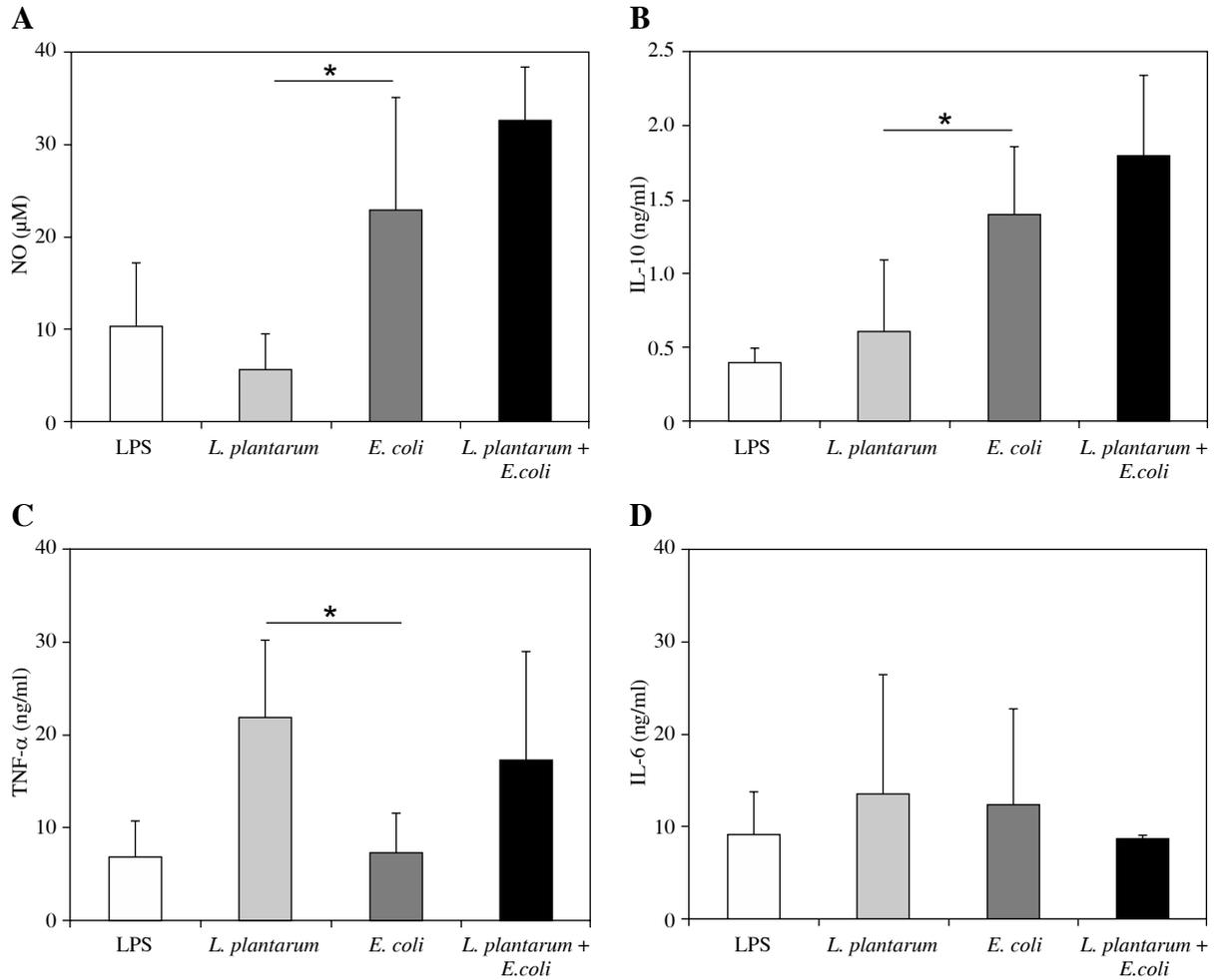


Fig. 1. The release of cytokines and NO from *in vitro* stimulated peritoneal exudate cells. The cells (> 90% macrophages) were stimulated *in vitro* with LPS (100 ng/ml), *L. plantarum* KL30B (live; 1×10^7 CFU/ml), *E. coli* 3A1 (killed; 1×10^7 CFU/ml) and *L. plantarum* KL30B (live) + *E. coli* 3A1 (killed) (1×10^7 CFU/ml each). The release into the cell culture supernatant: A) NO, B) IL-10, C) TNF- α and D) IL-6 is shown as a mean (\pm SD) of three independent experiments. * $p < 0.05$ *L. plantarum* KL30B (live) vs. *E. coli* 3A1 (killed)

lesions (2 out of 7), and this may be interpreted as early stage of transformation from acute to chronic type of inflammation with the more severe mucosa damage and the presence of ulceration and granulation tissue. In this group MPO activity was markedly lower than in the *L. plantarum* KL30B group but it was not statistically significant (Table 3).

Importantly, in our experimental models, acute colitis defined by a histological evaluation strongly correlated with a high level of MPO activity, the marker of neutrophils (Fig. 5).

Discussion

In the previous studies we have shown that probiotics (*lactobacilli*) as well as pathogenic bacteria (*E. coli*)

may exert either pro- or anti-inflammatory properties in a strain-specific manner [3, 19]. This observation is in agreement with a number of similar studies performed *in vitro* [21]. However, neither pro- nor anti-inflammatory activity of the bacterial strain tested *in vitro* is univocally correlated with either attenuation or exacerbation of inflammation *in vivo*, including ulcerative colitis. Namely, in severe systemic infection/inflammation a massive production of pro-inflammatory cytokines (e.g. TNF- α) contributes to organ failure. By contrast, in experimental models of locally induced inflammation (peritonitis, colitis), the strong activity of TNF- α has been found to be important for effective antibacterial host defence [22]. On the other hand, in several animal studies, IL-10, a prototypic anti-inflammatory cytokine, served both a protective and pathogenic role in various bacterial infections associated with acute inflamma-

tion [23]. Therefore, more detailed studies are necessary to compare various biological effects of potentially probiotic and pathogenic bacterial strains on the development and progression in animal models of inflammation.

In the present study we have compared the effect of selected strains of *E. coli* and *L. plantarum* on the development and propagation of acute inflammation. For this purpose, two human microbiotas, *L. plantarum* KL30B and *E. coli* 3A1, were isolated from healthy and inflamed colon, respectively. The primary criterion for such selection was the use of potentially probiotic, catalase positive *Lactobacillus* strain and comparing its properties with those of *E. coli*, the most common bacteria of gut microbiome with dual role in the pathogenesis of ulcerative colitis (IBD) [8, 24]. The study was performed using three experimen-



Fig. 2. The selected inflamed colon showing severe hyperaemia in TNBS-induced colitis

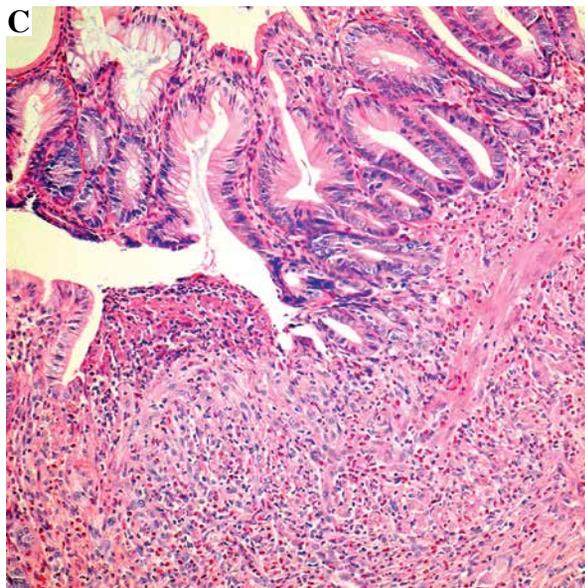
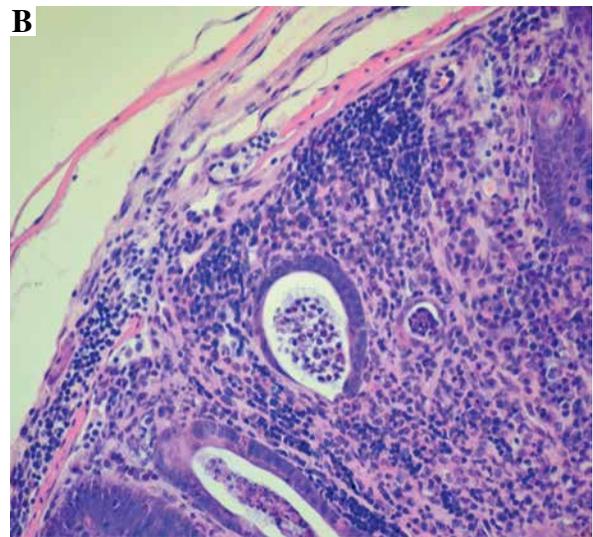


Fig. 3. Representative histological changes in rat colons stained with hematoxylin and eosin. A) A mild inflammatory infiltrate in the lamina propria; acute lesion; original magnification 400x. B) Extensive inflammatory infiltrate (neutrophils) in the lamina propria and formation of microabscesses in a crypt. The epithelial cells show reduced mucus production, increased nuclear-cytoplasmic ratio and nuclear hyperchromasia ('regenerative atypia'); original magnification 400x. C) Mucosal ulceration, extensive inflammatory infiltrate, tissue granulation and the beginning of fibrosis. The inflammatory infiltrate contains neutrophils featuring acute inflammation. The presence of tissue granulation and healing indicates chronic component; original magnification 200x

Table 3. The effect of *L. plantarum* KL30B and *E. coli* 3A1 administration on intestinal inflammation in TNBS-induced experimental colitis in rats

Group [n]	Incidence [%]	Histology score ^b	Type of colitis [n]	MPO [U × 10 ⁻³ /mg protein] ^a
TNBS control [7]	60	< 1	mild colitis [4]	77 ±9* [#]
TNBS + <i>E. coli</i> 3A1 (live) [5]	60	3.00 ±0.00	chronic [3]	98 ±22* ^{###}
TNBS + <i>E. coli</i> 3A1 (killed) [5]	60	2.67 ±0.33	acute [1] chronic [3]	255 ±104* ^{###}
TNBS + <i>L. plantarum</i> KL30B (live) [8]	62,5	2.00 ±0.28	acute [5]	1268 ±351
TNBS + <i>L. plantarum</i> KL30B (live) + <i>E. coli</i> 3A1 (killed) [7]	75	2.40 ±0.24	acute + chronic [2] acute [3]	392 ±46

^aMPO activity in inflamed colons, average ± SEM, **p* < 0.05, [#]control vs. *L. plantarum* KL30B (live); ^{##}*E. coli* 3A1 (live) vs. *L. plantarum* KL30B (live); ^{###}*E. coli* 3A1 (killed) vs. *L. plantarum* KL30B (live)

^bHistological score of inflamed colons, average ± SEM

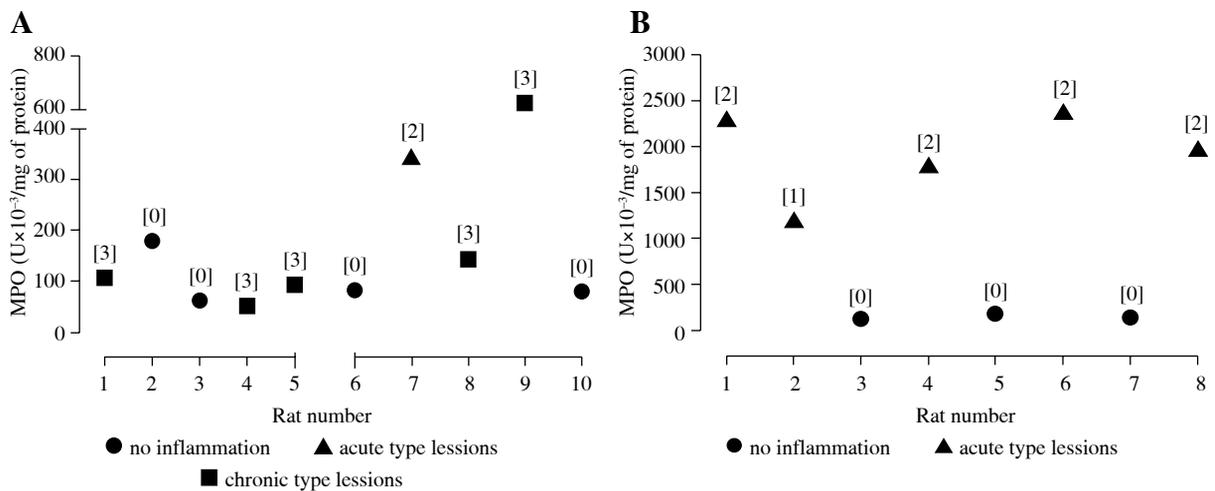


Fig. 4. MPO activity levels in mucosa fragments isolated from colons of rats treated with: A) *E. coli* 3A1 (live bacteria: rats no. 1-5; killed bacteria: rats no. 6-10) and B) live *L. plantarum* KL30B (rats 1-8). The histological grading score (see Methods) is shown in brackets [0-3]. *p* < 0.0001 MPO activity levels in inflamed colons *E. coli* 3A1 vs. *L. plantarum* KL30B treated rats

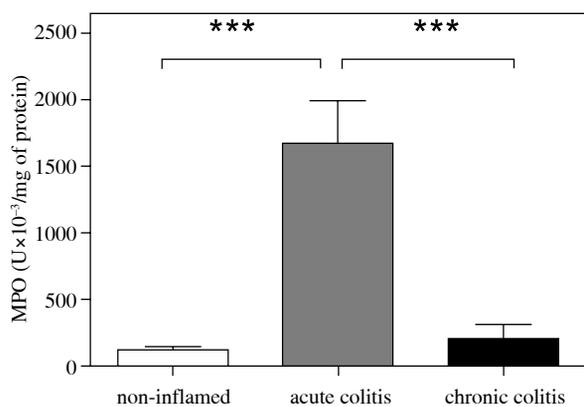


Fig. 5. MPO activity levels in mucosa fragments of rat colons with acute and chronic colitis. ****p* < 0.0001 acute colitis vs. non-inflamed colons and acute colitis versus chronic colitis

tal models: 1) acute peritonitis in mice; 2) TNBS-induced acute colitis in rats; 3) *in vitro* activation of murine exudate peritoneal macrophages to investigate the production of inflammatory mediators.

Acute bacterial peritonitis is an acute inflammation characterized by the presence of bacteria in normally sterile peritoneal cavity, and is associated with a high mortality rate. In humans, *E. coli* is found in more than 60% cases. In mice models of peritonitis even low number of live *E. coli* (~10⁵ bacteria/mouse) induces severe peritonitis with high mortality [22, 23]. In our experimental model, dead *E. coli* 3A1 (~10⁷ bacteria/mouse) showed stronger capability to induce acute peritonitis than that achieved by administration of live *L. plantarum* KL30B bacteria. Both tested strains showed comparable chemotactic activity as measured by a number of infiltrating peritoneal exudate cells. However, significantly higher percentage of neutrophils (Gr1⁺F4/80⁻ cells) correlated with higher ac-

tivity of MPO as observed in peritonitis induced by dead *E. coli* 3A1 than in peritonitis induced by live *L. plantarum* KL30B. Importantly, the pro-inflammatory properties of *E. coli* 3A1 were much stronger than LPS alone suggesting the presence of additional LPS-independent mechanism(s). In conclusion, the results presented above indicate that both tested bacterial strains may induce acute inflammation in bacteria-free tissues, such as peritoneal cavity, with much stronger virulence of *E. coli* 3A1. Moreover, macrophages collected from acute peritonitis and stimulated *in vitro* with *L. plantarum* KL30B produce different pattern of inflammatory mediators than those stimulated with *E. coli* 3A1. Namely, in our experimental set-up, *E. coli* induced massive production of NO, markedly higher than *L. plantarum* KL30B and LPS alone. Nitric oxide is toxic at high concentrations and is involved in damage of inflamed tissue [25, 26]. It may explain the detrimental role of *E. coli* pathogenic strains in local inflammation [5]. Unexpectedly, *E. coli* was also a stronger inducer of IL-10 than *L. plantarum*. It may suggest its anti-inflammatory potential. However, as mentioned above, a massive local production of IL-10 at early stages of acute inflammation may also result in inhibition of TNF- α and may be responsible for ineffective elimination of infectious agents [27]. Thus, from this point of view the observed profile of mediators induced by *L. plantarum* KL30B (TNF- α^{high} , IL-10 $^{\text{low}}$, NO $^{\text{moderate}}$) seems to correspond with a physiological response of innate immunity during bacterial infections [12, 24].

The next issue we addressed was whether *L. plantarum* KL30B and *E. coli* 3A1 showing different immunoregulatory potential in the development of peritonitis would also differentially affect the development, propagation or perpetuation of acute colitis in rats. We used TNBS-induced colitis terminated at day 12, after 2 cycles of TNBS administration. Our experimental model represents the model of acute colitis corresponding to early stages of ulcerative colitis. Other reports have demonstrated that acute colitis may turn into chronic colitis after 3-4 cycles of TNBS administration [28].

The mechanism of TNBS-induced colitis is not fully explained. Proposed mechanisms by which TNBS induces colitis may be: toxic effects on the epithelium followed by destruction of mucosal barrier and increased exposure to luminal bacteria, both endogenous microbiota and exogenous bacteria applied orally during probiotic therapies [3, 13, 29, 30]. However, gnotobiotic animals should be used in further studies to evaluate a direct effect of each of the administered bacterium. Nevertheless, recognition of any invading bacteria by TLRs in lamina propria of colon may induce acute inflammation characterized by a massive infiltration of neutrophils and phagocytosis of bacterial cells. Resulting inflammation has been shown to involve not only neutrophils but also macrophages and lymphocytes [8, 31]. Importantly, acute colitis, depending on various factors, may either terminate or progress to chronicity [7, 13].

Majority of experimental and clinical evidence coming from different laboratories suggests that neutrophils are responsible for mucosa injury and their migration across mucosal epithelia is correlated with oxidative stress in ulcerative colitis [32, 33]. For years neutrophils have been considered simple killer cells with concomitant detrimental effect on inflamed tissues including colon mucosa in ulcerative colitis [31]. However, a number of recent studies show that both inflammatory phagocytic cells (neutrophils and macrophages) as well as NO, their common toxic product, play a dual role in the development of experimental colitis [26, 34, 35].

It has been reported that neutrophils are not essential for the development of the major pathological features of colitis [36]. Moreover, only recently it has been shown that infiltrating neutrophils modulate the mucosal microenvironment to influence resolution of inflammation [37]. What would be the explanation of such opposite effects of neutrophils on initiation, resolution/progression and severity of acute inflammation? In our opinion, diverse functions of these cells are associated with different subsets of neutrophils, namely N1 and N2 cells. It is commonly accepted in cancer research that tumour environment may shape neutrophil and macrophage polarization to two distinct final populations. In general, N1 and M1 cells exert strong pro-inflammatory and defence properties while N2 and M2 cells (TAN – tumour associated neutrophils, TAM – tumour associated macrophages) are associated with propagation of tumours [38, 39]. As different strains of probiotic bacteria stimulate different sets of mediators released by inflammatory cells, it is tempting to speculate that bacteria can also polarise these cells (N1 vs. N2). This hypothesis is supported by the demonstration of polarization of M1 to M2-like macrophages by *L. plantarum* CLP-0611 [40].

Therefore, massive infiltration with neutrophils at a site of inflammation of unknown subtype (N1 vs. N2) after administration of bacteria cannot be univocally interpreted as a detrimental effect. The decisive is the final effect, either resolution of acute inflammation or progression to chronicity. The idea of probiotic therapy in IBD is to ameliorate colitis. For this purpose a number of various lactobacilli strains have been used with positive effects [11, 12, 30]. However, some immunostimulatory probiotic strains of lactobacilli amplified progression of colitis [41]. The present data clearly indicate that oral administration of *L. plantarum* KL30B induced massive infiltration of neutrophils correlated with high activity of MPO in inflamed mucosa in TNBS-induced acute colitis in rats. It indicates that *L. plantarum* KL30B has strong pro-inflammatory properties, stronger than *E. coli* 3A1, as confirmed by a histological evaluation and analysis of *in vitro* stimulation of TNF- α release by macrophages. On the contrary, *E. coli* 3A1, but not *L. plantarum* KL30B, progressed TNBS-acute colitis to chronicity and caused the severe mucosal damage. What would be the explanation for high

levels of MPO activity and mild mucosal lesions found in rats treated with *L. plantarum* KL30B? On one hand, MPO is responsible for the production of toxic hypochlorous acid (HOCl) and may be responsible for tissue damage [42, 43]. On the other hand, bactericidal properties of HOCl may limit transmigration of endogenous gut bacteria and attenuate the development of colitis. Moreover, it has been shown that MPO suppresses the induction of iNOS and therefore the production of deleterious nitric oxide [44].

In conclusion, our comparative studies of biological properties of *L. plantarum* KL30B and *E. coli* 3A1 show their distinct effect on the development and severity of acute peritonitis and colitis. We hypothesize that despite the strong pro-inflammatory properties of *L. plantarum* KL30B, its oral administration may have beneficial probiotic effect on the development and perpetuation of ulcerative colitis. However, to confirm our hypothesis concerning the protective role of neutrophil massive infiltration at the early stage of colitis, further studies with prolonged observation (up to 30 days) are necessary to explain the remote effect of *L. plantarum* KL30B resulting in either resolution of acute inflammation or in perpetuation of chronic colitis. Moreover, it is important to explain the impact of oral administration of probiotics on gut bacteria colonization and their transmigration across inflamed mucosal barrier.

Finally, we would like to stress that the final effect of bacterial therapies depends not only on the strain specific immunoregulatory properties but also on the route of administration and the fate of bacteria after homing in host tissues. Namely, the same bacterial strain may induce acute inflammatory response, may form biofilm in sterile tissues leading to chronic inflammatory response or it may cross-talk with host microbiome to attenuate ongoing inflammatory response. Therefore, it is important to remember that the response of innate immunity to bacteria administration/invasion may result in the induction of either acute or chronic inflammation or immune tolerance.

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