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ORIGINAL ARTICLE

Lactobacillus rhamnosus Affects Rat Peritoneal Cavity Cell Response to Stimulation with Gut Microbiota: Focus on the Host Innate Immunity

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Abstract— Gut microbiota contribute to shaping the immune repertoire of the host, whereas probiotics may exert beneficial effects by modulating immune responses. Having in mind the differences in both the composition of gut microbiota and the immune response between rats of Albino Oxford (AO) and Dark Agouti (DA) rat strains, we investigated if intraperitoneal (i.p.) injection of live *Lactobacillus rhamnosus* (LB) may influence peritoneal cavity cell response to *in vitro* treatments with selected microbiota in the rat strain-dependent manner. Peritoneal cavity cells from AO and DA rats were lavaged two (d2) and seven days (d7) following i.p. injection with LB and tested for NO, urea, and H_2O_2 release basally, or upon *in vitro* stimulation with autologous *E.coli* and *Enterococcus spp*. Whereas the single i.p. injection of LB nearly depleted resident macrophages and increased the proportion of small inflammatory macrophages and monocytes on d2 in both rat strains, greater proportion of $MHCII^{hi}CD163^{-}$ and $CCR7^{+}$ cells and increased NO/diminished H_2O_2 release in DA compared with AO rats suggest a more intense inflammatory priming by LB in this rat strain. Even though *E.coli*- and/or *Enterococcus spp.*-induced rise in H_2O_2 release *in vitro* was abrogated by LB in cells from both rat strains, LB prevented microbiota-induced increase in NO/urea ratio only in cells from AO and augmented it in cells from DA rats. Thus, the immunomodulatory properties may not be constant for particular probiotic bacteria, but shaped by innate immunity of the host.

KEY WORDS: Albino Oxford (AO) rats; Dark Agouti (DA) rats; *Lactobacillus rhamnosus*; Peritoneal cavity immune cells; *E. coli*; *Enterococcus spp.*

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INTRODUCTION

Gut microbiota dysbiosis and increased gut permeability are associated with increased risk for inflammatory and autoimmune diseases [1–3]. Even in the absence of infection, gut microbiota constituents are constantly turned over and either excreted or translocated across the gut mucosa into the circulation, which contributes to basal level of immune cell activation and facilitates a

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rapid response to pathogens [4]. Resident peritoneal cavity cells that guard the sterility of the peritoneal compartment against the microbial threat from the gut are in close contact with escaped intraluminal bacteria and support shaping the immune repertoire of the host [5].

We have recently reported that peritoneal cavity cells from rats of Dark Agouti (DA) rat strain displayed more vigorous priming and inflammatory response upon intraperitoneal (i.p.) stimulation with own commensal *E.coli* and *Enterococcus spp*., compared to the cells from rats of Albino Oxford (AO) rat strain [6]. These non-MHC compatible rat strains substantially differ in their susceptibility to the induction of autoimmune diseases, DA rats being significantly more susceptible to experimental autoimmune encephalomyelitis and adjuvant arthritis [7, 8]. There is higher variability of *Lactobacillus spp*. in AO rats, in both faecal and gut tissue samples [9], while DA rat samples contain other bacteria, like those from phylum *Firmicutes* (including family *Lachnospiraceae*) and phylum *Proteobacteria* (including *Undibacterium oligocarboniphilum* from order Burkholderiales) [10]. Transfer of AO gut microbiota even ameliorated EAE in DA rats [11], underlining the importance of commensal microbial constituents for host immune response. Since microbiota drives the differentiation of infiltrating monocytes to $MHCII^+$ resident peritoneal cavity macrophages [12], the differences in the composition of commensal microbiota between AO and DA rats probably contribute to differences between these rat strains in the response of macrophages to peritoneal cavity micro-environmental changes *in vivo* and treatments *in vitro* [13–16].

On the other hand, the use of probiotic bacteria with the intent to improve gut dysbiosis was shown to exert beneficial effects not only toward inflammatory bowel diseases, but also in blunting the signs of inflammatory and autoimmune diseases [17, 18]. Probiotics exercise complex activities beyond the gastrointestinal tract, including bacterial strain-specific immunomodulatory properties, and the diverse genus *Lactobacillus* stands out as particularly useful and safe. Oral gavage with lactobacilli may increase or decrease peritoneal cell phagocytic activity and IL-6, IL-12, and IFN-γ production, depending on particular *Lactobacillus* strain used [19, 20]. However, lactobacilli also express immunomodulatory properties following i.p. route of delivery by stimulating macrophage phagocytosis [21], MHCII expression [22] and lysosomal activity [23]. I.p. injection of lactobacilli eventually resulted in antimicrobial and disease-changing effects in both local and distant sites, i.e., inhibited growth of *Pseudomonas aeruginosa* in the peritoneal cavity [24], diminished *Trichinella spiralis* larvae burden in the intestine [25], and conveyed protection against *Listeria monocytogenes* or *Babesia microti* infection [26, 27]. Single i.p. injection of *Lactobacillus spp.* even mimicked the beneficial effect(s) of oral consumption of *Lactobacillus spp.* on inflammatory score during experimental colitis [28].

Hence, we wanted to extend our previous observations in AO and DA rat strains by modifying peritoneal cavity cells of these rats with a direct i.p. stimulation with *Lactobacillus rhamnosus* (LB) prior to treatment with selected microbiota *in vitro*. This particular probiotic strain possesses immunomodulatory properties, as it increases IFN-γ /IL-4 ratio in lymph node cells when used as adjuvant for ocular mucosal immunization in mouse [29]. It also improves the health of adult rats with colitis when applied during early postnatal period, most probably by increasing microbiota diversity and intestinal growth during early life [30]. Given that variations in the efficacy of probiotics may be a result of inter-individual differences in probiotic colonization [31] and that data regarding the efficacy of probiotics as a result of differences in host innate immunity are lacking, we were interested to investigate whether i.p. LB may affect peritoneal cavity cells and bias their response to subsequent microbial stimuli in a rat strain-dependent way. In the present study, we employed i.p. stimulation with LB in order to explore direct effect on phenotype and activity of cells *in situ,* and to circumvent changes in microbial composition and metabolic conditions or influence on other immune cells [32], which all accompanies the oral feeding with lactobacilli. For that purpose, peritoneal cavity cells from AO and DA rats were lavaged 2 and 7 days following i.p. injection of LB and then stimulated with autologous *E.coli* and *Enterococcus spp. in vitro.*

MATERIALS AND METHODS

Animals

Young (4–6-month-old) female rats of Albino Oxford (AO) and Dark Agouti (DA) rat strains were obtained from the local breeding colony (Immunology Research Center "Branislav Janković," Belgrade, Serbia). Rats were housed in a strain-matched manner in standard cages (3 rats/cage) in an environment in which temperature (22 ± 2 °C), humidity (40% to 70%), and light/dark

cycle (12:12 h) were automatically controlled. The rats had free access to conventional food pellets (Veterinarski zavod Subotica, Serbia) and tap water. Animals were euthanized by increasing dose of CO_2.

The experimental protocol and the procedures involving animals and their care were approved by Ministry of Agriculture and Environmental Protection (licence number 323–07-01,577/2016–05/14, issued on 02–25-2016), and were in accordance with the principles declared in Directive 2010/63/EU of the European Parliament and of the Council from 22 September 2010 on the protection of animals used for scientific purposes (revising Directive 86/609/EEC).

Isolation and Preparation of Bacteria

Lactobacillus rhamnosus (LB) was generously provided by Dr Vesna Cvetković, Institute of Virology, Vaccines and Sera "Torlak" (Belgrade, Serbia). LB was isolated from human gastrointestinal flora at Institute of Virology, Vaccines and Sera "Torlak," and has been characterized by 16S rRNA gene sequencing and subsequent sequence analysis (NCIMB Ltd, Aberdeen, UK; assigned strain name NCSQ 18,723), showing a sequence similarity of 99.41% when compared with the *Lactobacillus rhamnosus* species.

Commensal bacteria were isolated from stool specimens collected from AO and DA rats, and prepared as previously described [33]. Briefly, faecal samples were cultured using different agar plates (MacConcey agar medium, Endo agar medium, Institute "Torlak," Belgrade, Serbia) under the aerobic conditions, at 37 °C for 24 h and well-isolated colonies were identified using the Gram-staining and morphological characteristics, as well as the biochemical tests and a rapid identification system (ID-32A, API Biomerieux, Marcy l'Etoile, France). The Bile Esculin Agar medium (Institute "Torlak") was used for confirmation of the *Enterococcus* genus. Further identification of the species of enterococci was not obtained (MALDI-TOF analysis failed to provide identification using VITEK® MS V2.0 Knowledge Base, Biomerieux).

E. coli and *Enterococcus spp.*, identified as aerobic Gram (−) and Gram (+) bacteria, respectively, in both AO and DA rats, were grown aerobically for 24 h, harvested at stationary growth phase, washed with sterile phosphate-buffered saline (PBS) (1000 × g for 20 min), and resuspended in sterile PBS. The bacteria were killed by formalin (at 0.5% v/v final concentration), and the suspensions were held at 35 °C for more than 48 h. Cells were then washed with PBS to remove the formaldehyde, resuspended at the same density in sterile PBS/0.25% phenol and stored at 4 °C until use. The efficacy of killing of the bacteria in the suspensions was confirmed by sterility test. The estimated number of bacteria in the suspension was determined by reading the optical density at 580 nm and extrapolating the value using a standard curve.

Chemicals and Immunoconjugates

Lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA), phenylmethyl sulfonyl fluoride (PMSF), phenol red, α-isonitrosopropiophenone (ISPF), *o*-phenylene diamine (OPD), and RPMI-1640 medium (RPMI) were purchased from Sigma (St. Lewis, MO, USA). Foetal calf serum (FCS) was obtained from Gibco (Grand Island, NY, USA).

Monoclonal PE-conjugated mouse anti-rat CD163 (ED2-like) resident macrophage receptor (clone HIS 36), FITC-conjugated goat anti-rabbit IgG (GAR-FITC), FITC-conjugated goat anti-mouse (GAM-FITC), FITC-conjugated mouse anti-rat MHCII, alexa-fluor 488-conjugated mouse anti-rat Ki67, PerCP-conjugated streptavidin (Sav-PerCP), and appropriate IgG isotype controls were purchased from BD Biosciences Pharmingen (Mountain View, CA, USA). Biotin-conjugated mouse anti-rat CD68 (clone ED1) antibodies were obtained from Serotec (Oxford, UK). Polyclonal rabbit anti-rat C–C chemokine receptor type 7 (CCR7) antibody was obtained from Abcam (Cambridge, MA, USA); PE-conjugated mouse anti-rat granulocyte antibodies (clone HIS48) were purchased from eBioscience (San Diego, CA, USA). PE-conjugated mouse anti-rat CD43 (clone W3/13) was purchased from BioLegend (San Diego, CA, USA).

Intraperitoneal Treatment with *Lactobacillus rhamnosus* and General health Monitoring

AO and DA rats were treated with a single i.p. injection containing ~ 5×10^7 CFU LB in 300 µl of PBS, and peritoneal cavity cells were sampled by lavage 2 days (d2 LB) or 7 days (d7 LB) later. Pereyra and colleagues established that 5×10^7 of live lactobacilli was the maximal non-lethal quantity of injected bacteria [34].

Control rats were treated with a single i.p. injection of 300 µl of PBS, and peritoneal cells were isolated 2 days later. Preliminary experiments showed that the

number, phenotype, and functional activity were similar in peritoneal cells isolated either 2 or 7 days following i.p. injection of PBS.

Clinical appearance of rats challenged with live LB was monitored daily for 7 days. Subjective aspects of behaviour were recorded according to the General Health Score scale [35] ranged from S5 (bright eyed and alert, a smooth coat with a sheen, responds to stimulus, shows interest in environment) to S1 (nonreactive to stimulus, fur has a "bottle brush" appearance, hunched over preferring to sleep than react to environment, cold at touch, paws cold). Rats were weighed at the beginning of the experiment, and on days 2 and 7 following LB injection.

Isolation of Peritoneal Cavity Cells

Prior to peritoneal lavage, abdominal skin with fur was soaked with 70% alcohol and retracted to expose the intact peritoneal wall. Small incision was made along the midline with sterile scissors. A total of 10 ml of sterile ice-cold PBS pH 7.4 supplemented with 2% foetal calf serum (FCS) was injected and entire body gently shaked for 10 s by use of forceps. After exposing the peritoneal cavity, the fluid was gently aspirated. Individual cell suspensions were washed twice in PBS/2%FCS (250×g) at 4 °C and subjected to flow cytometry analysis and functional tests.

Flow Cytometric Analysis of Peritoneal Cavity Cells

Flow cytometric analysis of peritoneal cavity cells was performed immediately following their isolation. Individual cell aliquots (50 µl, 1×10^7 cells/ml) were immunolabeled and then analysed on a FACSVerse™ flow cytometer using BD FACSuite™ software (Becton Dickinson, Mountain View, CA, USA). Debris and dead cells were gated out on the basis of low FSC and low SSC. Cell doublets were excluded by displaying the FSC height values over the FSC area values.

For surface staining, peritoneal cells were immunolabeled with FITC-conjugated anti-MHCII and PE-conjugated anti-CD163, or with biotin-conjugated HIS48, and PE-conjugated anti-CD43 antibodies. For secondary staining, the latter cells were incubated (after washing in PBS/NaN3/2%FCS) with PerCP-conjugated streptavidin. The MHCII-FITC vs CD163-PE plot was used for identifying four relevant cell populations: $CD163^+MHCII^-$, $CD163^+MHCII^+$, $CD163^-MHCII^{low}$, and $CD163^-MHCII^{high}$. CD43-PE vs HIS48-SavPerCP revealed that $HIS48^+CD43^+$ cells may be clearly distinguished by SSC (monocytes are SSC^{low}, and granulocytes are SSC^{high}).

For intracellular staining, peritoneal cells were first labelled with unconjugated rabbit anti-CCR7 and PE-conjugated anti-CD163, and after washing, with FITC-conjugated anti-rabbit IgG. Cells were then fixed with 0.25% paraformaldehyde and permeabilized by 0.2% Tween 20, prior to the addition of biotin-conjugated anti-CD68. Anti-CD163 antibody labels the mature tissue resident macrophages, but not monocytes [36]. CD68 antigen is considered as pan-macrophage marker, but it labels dendritic cells as well [37]. All $CD163^+$ cells expressed CD68. For intracellular Ki67 staining, cells were first labelled with PE-conjugated anti-CD43, then fixed and permeabilized using the Fix-Perm buffer (otherwise used for Foxp3 staining, eBioscience, San Diego, CA, USA) after which the cells were labelled with alexa-fluor 488-conjugated anti-Ki67 and biotin-conjugated anti-CD68, followed by Sav-PerCP-conjugated streptavidin.

Functional Tests

Individual cell aliquots were adjusted to 1×10^6 cells/ml (for nitric oxide, urea, and cytokine production) or to 2.5×10^6 cells/ml (for hydrogen peroxide production) in RPMI/5%FCS. Cell aliquots were plated to tissue culture plates for 2 h at 37 °C/5% CO_2. The non-adherent cells were removed by warm sterile PBS, and adherent cells (highly enriched for macrophages) [38] were additionally incubated for 1 h (hydrogen peroxide production), 24 h (cytokine release), or for 48 h (nitric oxide and urea production). Optical densities (OD) were determined by Multiscan Ascent plate reader (Labsystems, Helsinki, Finland).

Hydrogen Peroxide (H_2O_2) Release Assay. H_2O_2 release was determined according to a method based on the HRPO-dependent oxidation of phenol red [39, 40]. Briefly, adherent cells were primed for peroxide production for 1 h at 37 °C with 100 µl of 25 nM PMA in phenol red solution (10 mM potassium phosphate buffer pH 7, 140 mM NaCl, 5.5 mM dextrose, 0.56 mM phenol red, and 19 U/ml of HRPO), in the absence or in the presence of 100 µl of 3×10^8 CFU/ml of *E.coli* or 3×10^8 CFU/ml of *Enterococcus spp*. Incubations were terminated with 10 µl of 0.5 M NaOH, and ODs were determined at 620 nm. The concentration of H_2O_2 in the samples

was calculated using standard concentrations of H_2O_2 (1–100 μM).

Cytokine Determination. Adherent cells were incubated for 24 h at 37 °C/5% CO_2 in RPMI/5%FCS, and culture supernatants were frozen at − 70 °C until assayed. Prior to performing ELISA, the thawed supernatants were centrifuged on 250 g, 15 min, + 4 °C. Commercially available ELISA kits for tumour necrosis factor (TNF)-α (Biolegend Inc., San Diego, CA, USA) and interleukin (IL)-10 (R&D Systems Inc., Minneapolis, MN, USA) were used according to the manufacturers' instructions.

Nitric Oxide (NO) and Urea Determination Assays. Adherent cells were incubated for 48 h at 37 °C/5% CO_2 in 100 μl of RPMI/5%FCS, in the absence or in the presence of 1 μg/ml LPS, or 3×10^8 CFU/ml of *E.coli* or 3×10^8 CFU/ml *Enterococcus spp*. Supernatants were collected and immediately analysed for nitrite concentration by a method based on the Griess reaction [41]. The concentration of nitrite in the samples was determined according to ODs measured at 405 nm and calculated using a $NaNO_2$ (1–40 μM) as a standard.

The plates were then centrifuged for 5 min at 100 g and rinsed with warm PBS. Cells were lysed with 0.1% Triton X-100 containing 2 mM PMSF and shaked for 30 min (200 cycles/minute). Enzyme arginase was activated by 10 mM $MnCl_2$/25 mM Tris–HCl, pH 7.5, for 10 min at 56 °C and 0.5 M arginine; pH 9.7 was added for next 22 h incubation at 37 °C. The reaction was stopped by acid mixture containing H_2SO_4, H_3PO_4, and H_2O (1:3:7). Arginase activity was indirectly determined by measuring the metabolite urea [42, 43]. The urea concentration was measured at 540 nm after adding ISPF dissolved in absolute ethanol, followed by heating at 95 °C for 45 min and calculated according to the standard curve obtained with known concentrations of a urea (25–1600 μM).

Statistical Analysis

GraphPad Prism 5 (GraphPad Software, San Diego, CA) was used for all calculations and statistical analysis. Statistical significance level was set at $p < 0.05$. Since the experimental design included variations in several factors, data were analysed by two-way ANOVA with factors rat strain (AO vs. DA) and treatment (PBS, d2 LB, and d7 LB). The effects of *in vitro* treatment with *E.coli* or *Enterococcus spp*. were analysed by one-way ANOVA. Bonferroni's multiple comparison post hoc test was performed.

RESULTS

The Health Concerns, Body Weight, the Number of Peritoneal Cavity Cells, and the Proportion of $CD163^+CCR7^+$ Cells Following Intraperitoneal Injection of LB

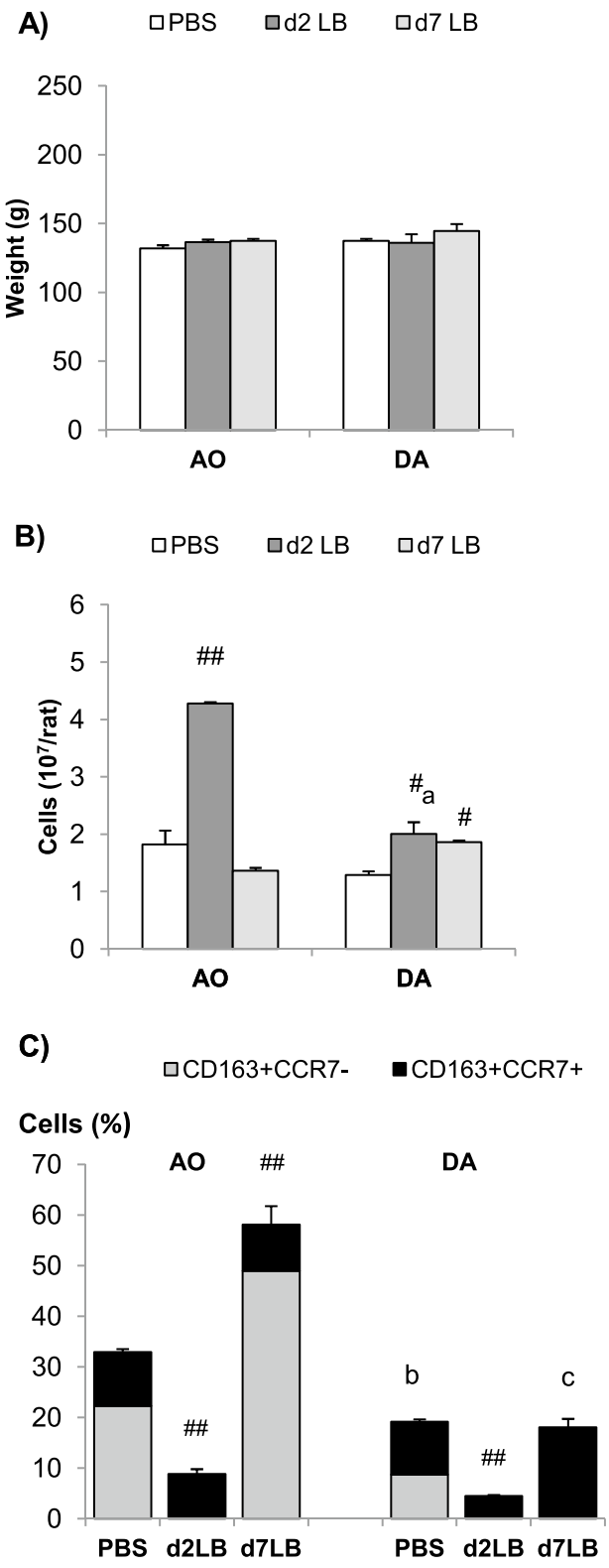
We did not record any deaths or discomfort of animals during this study. Bright eyed rats with a smooth coat and vigorous response to environmental stimuli during 7-day period after LB injection confirmed score 5 according to the General Health Score scale [35]. Moreover, single i.p. injection of 5×10^7 CFU of live LB did not affect body weight of any rat over the course of experiment (Fig. 1A) but increased the number of peritoneal cavity cells in both AO and DA rats, relative to strain-matched PBS-treated animals (Fig. 1B). Injection of LB substantially increased peritoneal cell number in AO rats only on d2 following injection, whereas it augmented cell number on both d2 and d7 after injection in DA rats (Fig. 1B). It is noteworthy that the number of peritoneal cavity cells obtained on d2 was significantly higher in AO relative to DA rats, while on d7 the number of peritoneal cells was slightly higher in DA relative to AO rats (Fig. 1B).

In accordance with our previous reports, the majority of peritoneal cavity cells and all $CD163^+$ macrophages expressed CD68, irrespective of rat strain and treatment (data not shown).

The proportion of all $CD163^+$ macrophages among peritoneal cavity cells was significantly higher in AO than in DA rats treated with PBS or on d7 following LB, but comparable between AO and DA rats on d2 (Fig. 1C). Even though $CD163^+$ macrophages of both rat strains expressed CCR7 antigen (Fig. 1C), only minute fraction of $CD163^+$ macrophages of AO rats treated with PBS and those treated with LB seven days before cell harvest expressed CCR7. Quite the opposite, in PBS-treated DA rats at least half of $CD163^+$ macrophages expressed CCR7 (Fig. 1C). It is worth noting that in samples obtained from both rat strains on d2, and in cells from DA rats on d7 following LB treatment, all peritoneal $CD163^+$ cells expressed CCR7 (Fig. 1C).

Phenotype of Peritoneal Cavity Cells Induced by Intraperitoneal Injection of LB

LB injection abruptly reduced the proportion of $CD68^+$ macrophages that express proliferative Ki67 marker on d2, which remained slightly diminished on d7


A)
PBS
d2 LB
d7 LB
Weight (g)
250
200
150
100
50
0
AO
DA
B)
PBS
d2 LB
d7 LB
Cells (10^7/rat)
6
5
4
3
2
1
0
##
#a
#
AO
DA
C)
CD163+CCR7-
CD163+CCR7+
Cells (%)
70
60
50
40
30
20
10
0
AO
##
DA
b
c
##
##
PBS
d2LB
d7LB
PBS
d2LB
d7LB

◂**Fig. 1** Body weight (**A**), the number of peritoneal cells (**B**), and the proportion of $CD163^+CCR7^+$ and $CD163^+CCR7^-$ cells (**C**) in rats of Albino Oxford (AO) and Dark Agouti (DA) rat strains following intraperitoneal treatment with phosphate buffer saline (PBS), or two (d2) and seven (d7) days following intraperitoneal treatment with *Lactobacillus rhamnosus* (LB). The number of rats was 3–6 per group. Statistically significant differences: $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. PBS; $^{a}p<0.05$, $^{b}p<0.01$, and $^{c}p<0.001$ vs. AO rats. Statistically significant differences in (C) refers to $CD163^+$ (including $CCR7^+$ and $CCR7^-$) cells.

when compared to cells from PBS-treated animals in both strains (Fig. 2B). Nevertheless, the ratio of $CD68^+Ki67^+$ cells was lower among peritoneal cavity cells from DA compared to AO rats, irrespective of treatment (Fig. 2B).

Treatment with LB diminished the proportion of classical ($CD163^+MHCII^-$) large resident macrophages in d2 samples and increased the proportion of small inflammatory $MHCII^{low}$-expressing $CD163^-$ macrophages (Fig. 3B) in AO rats. LB did not affect the proportion of other resident macrophage ($CD163^+MHCII^+$) and inflammatory macrophage/dendritic cell ($CD163^-MHCII^{hi}$) populations in these rats on d2 (Fig. 3B). In peritoneal cavity cells obtained on d7 following LB injection in AO rats, there was an increase in the proportion of resident (both $MHCII^-$ and $MHCII^+$)$CD163^+$ and inflammatory $CD163^-MHCII^{low}$ macrophages (Fig. 3B). In DA rats, treatment with LB diminished ratio of resident macrophages ($CD163^+MHCII^-$ and $CD163^+MHCII^+$) and increased

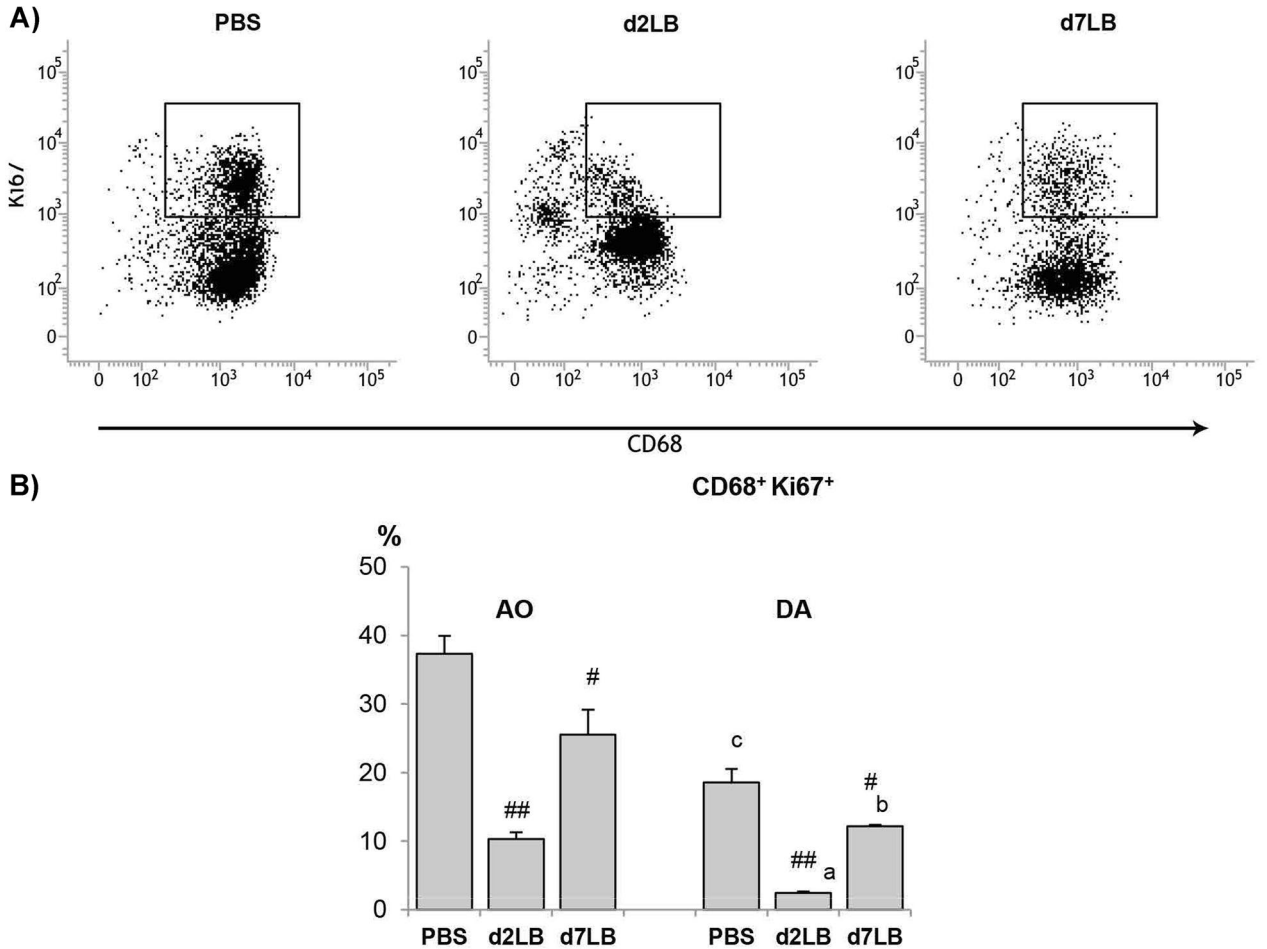


Fig. 2 Representative dot plots showing the Ki67 vs. CD68 immunofluorescence profile (**A**) and the proportion of $Ki67^+CD68^+$ cells (**B**) in rats of Albino Oxford (AO) and Dark Agouti (DA) rat strains following PBS, or two (d2) and seven (d7) days following intraperitoneal treatment with *Lactobacillus rhamnosus* (LB). The number of rats was 3–6 per group. Statistically significant differences: $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. PBS; $^{a}p<0.05$, $^{b}p<0.01$, and $^{c}p<0.001$ vs. AO rats.

the proportion of small inflammatory ($CD163^{-}MHCII^{low}$) macrophages, and inflammatory macrophage/dendritic cells ($CD163^{-}MHCII^{hi}$) on d2 (Fig. 3B). However, on d7, the proportion of both resident $CD163^{+}MHCII^{+}$ and small inflammatory ($CD163^{-}MHCII^{low}$) macrophages was diminished in comparison with PBS-treated DA rats (Fig. 3B). Rat strain differences were observed among PBS-treated rats, as greater proportion of $CD163^{+}MHCII^{+}$ and, especially, of $CD163^{-}MHCII^{low}$ cells, accompanied with diminished ratio of $CD163^{+}MHCII^{-}$ cells in DA compared to AO rats was noticed. Following LB injection, the proportion of $CD163^{-}MHCII^{hi}$ cells was greater on d2 in DA rats, whereas the proportion of resident $CD163^{+}$ macrophages (both $MHCII^{-}$and $MHCII^{+}$) was greater on d7 in AO rats (Fig. 3B).

Treatment with LB in both rat strains significantly enriched peritoneal cavity cell population by influx of SSC^{low} (less granular) $HIS48^{+}CD43^{+}$ monocytes on d2 (Fig. 4B, left), which accompanied decrease in the proportion of mature resident CD163-expressing macrophages (Fig. 1C). The proportion of SSC^{low} $HIS48^{+}CD43^{+}$ monocytes was higher in AO relative to DA rats (Fig. 4B, left). In further course of inflammation, decrease in the proportion of SSC^{low} $HIS48^{+}CD43^{+}$ monocytes on d7 was accompanied by the recovery of resident $CD163^{+}$ peritoneal macrophages in both rat strains (Fig. 1C). The ratio of peritoneal granulocytes (SSC^{hi} $HIS48^{+}CD43^{+}$ cells) did not change following LB injection in either of rat strains (Fig. 4B, right).

Release of Hydrogen Peroxide (H_2O_2), Cytokines, Nitric Oxide and Urea from Adherent Peritoneal Cavity Cells Following Intraperitoneal Injection of LB

In AO rats, single i.p. LB injection increased H_2O_2 release from peritoneal cells isolated on d2 and d7 (Fig. 5A) relative to cells from PBS-treated rats. However, in DA rats, LB injection decreased H_2O_2 release from cells isolated on d2, but increased it in those isolated on d7 (Fig. 5A). Adherent peritoneal cells from PBS-treated rats of AO and DA strain produced comparable quantity of H_2O_2, whereas cells from LB-treated AO rats produced superior amount of peroxide on d2 and d7 relative to cells from DA rats.

Adherent peritoneal cavity cells from both rat strains produced TNF-α (higher in PBS-treated AO than in PBS-treated DA rats) and IL-10 (higher in all groups of DA than in AO rats, Fig. 5B, C). LB injection diminished TNF-α release from cells obtained on d7 from AO rats, increased it in peritoneal cells obtained on d2 from DA rats, but did not affect IL-10 production in any way (Fig. 5B, C).

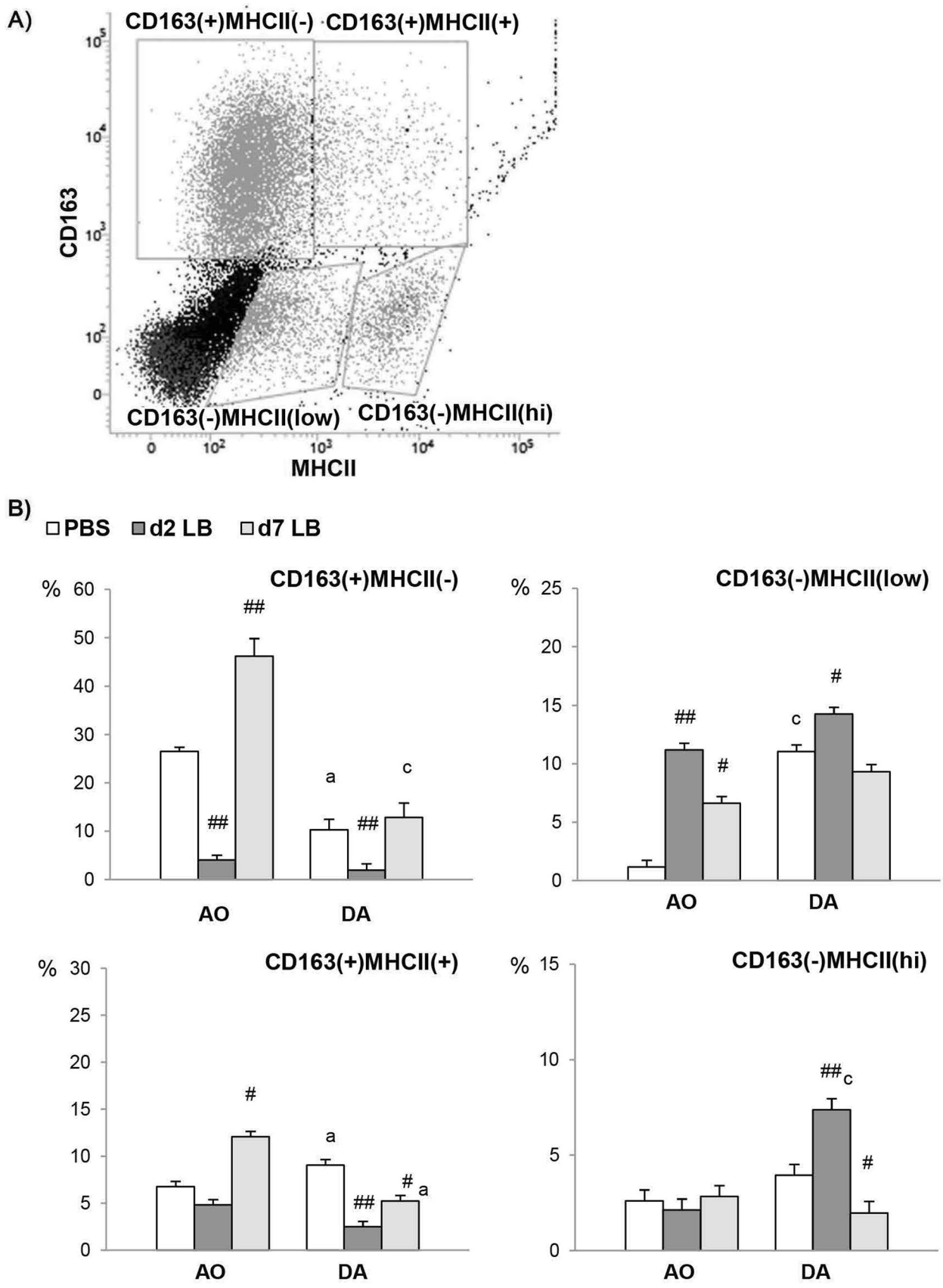
At the same time, injection of LB did not affect NO release from AO rats' peritoneal cells, but significantly decreased release of urea in cells isolated on d7 (Fig. 5D, E), which resulted in increased ratio of NO to urea (NO/Urea) in this rat strain (Fig. 5F). In DA rats, injection of LB augmented NO release from both d2 and d7 cells and decreased urea release on d7, which led to augmented NO/urea ratio on d2 and d7 following LB injection (Fig. 5D, E). Even though cells from LB-treated rats of DA strain produced superior amounts of NO compared to AO rats on d2, cells from all treatment groups of DA rats released higher amount of urea than the cells from AO rats (Fig. 5D, E). Consequently, NO/urea ratio was higher in d2 and d7 cells from AO than in respective groups of DA rats (Fig. 5F).

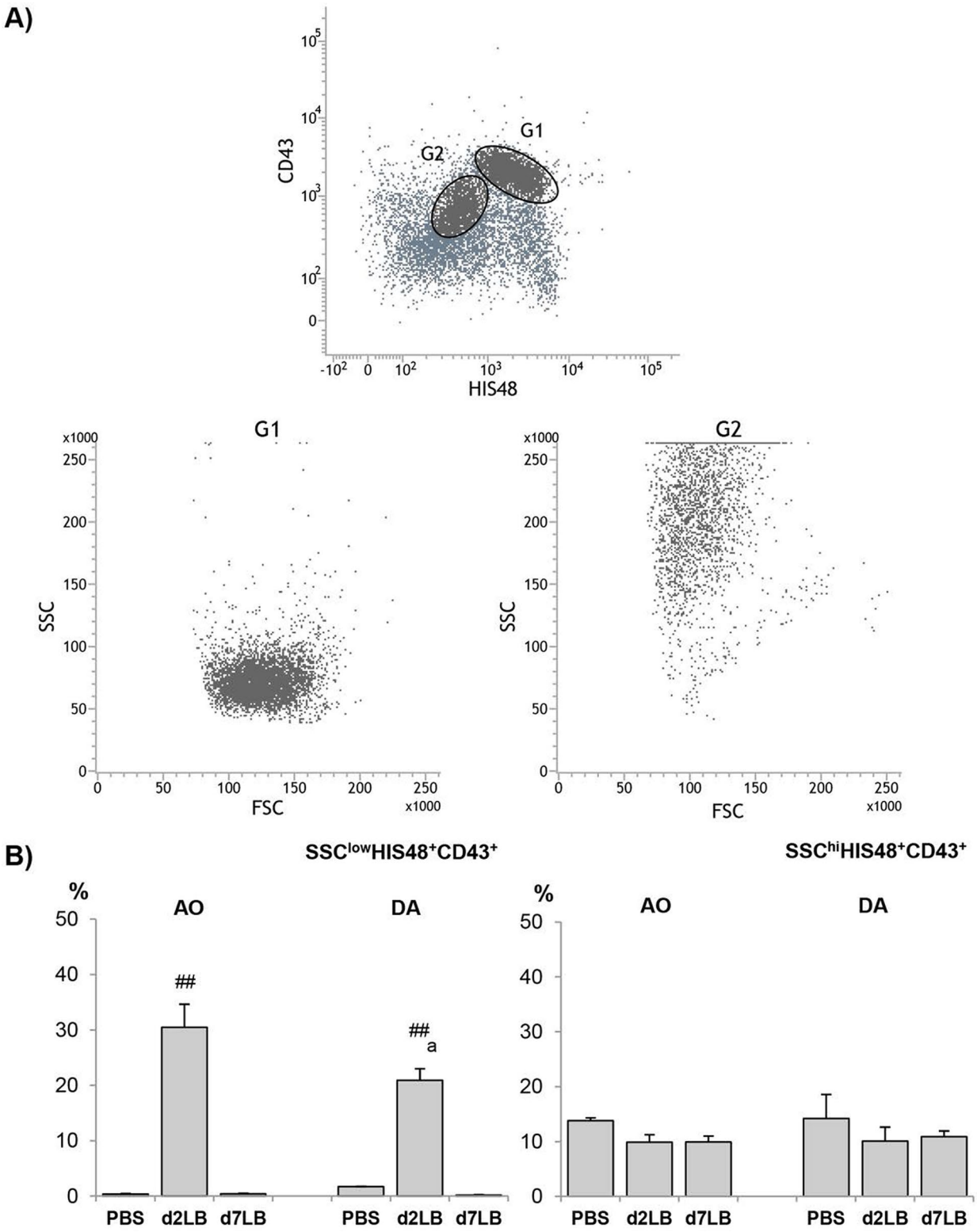
Fig. 3 Representative dot plot showing gating strategy for discrimination of MHCII vs. CD163 (**A**) and the proportion of $CD163^{+}MHCII^{-}$ (upper left), $CD163^{+}MHCII^{+}$ (lower left), $CD163^{-}MHCII^{low}$ (upper right), and $CD163^{-}MHCII^{hi}$ (lower right) cells (B) in rats of Albino Oxford (AO) and Dark Agouti (DA) rat strains following intraperitoneal treatment with phosphate buffer saline (PBS), or two (d2) and seven (d7) days following intraperitoneal treatment with *Lactobacillus rhamnosus* (LB). The number of rats was 3–6 per group. Statistically significant differences: $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. PBS; $^{a}p<0.05$, and $^{c}p<0.001$ vs. AO rats.

The Effect of *In Vitro* Stimulation with Selected Microbiota on Hydrogen Peroxide (H_2O_2), Nitric Oxide, and Urea Release from Adherent Peritoneal Cavity Cells Following Intraperitoneal Injection of LB

In vitro treatment with *E. Coli* or *Enterococcus spp.* significantly increased H_2O_2 release only in cells from PBS-treated but not by those from LB- treated AO rats (Fig. 6A). Actually, both bacteria suppressed H_2O_2 release from peritoneal cavity cells isolated from AO rats on d7 following LB injection (Fig. 6A). In DA rats *E. Coli* and *Enterococcus spp.* augmented H_2O_2 release by cells from PBS-treated and LB-injected rats obtained on d2 (Fig. 6A). However, *in vitro* treatment with *Enterococcus spp.* decreased H_2O_2 release from LB-treated DA rats' cells obtained on d7 (Fig. 6A).

In vitro treatment with LPS increased NO release by peritoneal cells from PBS-treated and LB-treated AO rats on d2, but stimulated release of urea in cells from all


A)
CD163(+)MHCII(-)
CD163(+)MHCII(+)
CD163
MHCII
CD163(-)MHCII(low)
CD163(-)MHCII(hi)
B)
PBS
d2 LB
d7 LB
CD163(+)MHCII(-)
CD163(-)MHCII(low)
CD163(+)MHCII(+)
CD163(-)MHCII(hi)
%
AO
DA


A)
CD43
HIS48
G1
G2
SSC
FSC
x1000
B)
SSC^low HIS48^+ CD43^+
SSC^hi HIS48^+ CD43^+
%
AO
DA
##
##a
PBS
d2LB
d7LB

◂**Fig. 4** Representative dot plots showing the CD43 vs. HIS48 immunofluorescence profile (**A**) and backgating to Forward Scatter (FSC, general size) vs. Side Scatter (SSC, granularity) profile showing discrimination between SSC^{low} (G1, left) and SSC^{hi} (G2, right) $HIS48^+CD43^+$ cells (**A**). The proportion of $SSC^{low}HIS48^+CD43^+$ (left) and $SSC^{hi}HIS48^+CD43^+$ (right) cells (**B**) in rats of Albino Oxford (AO) and Dark Agouti (DA) rat strains following intraperitoneal treatment with phosphate buffer saline (PBS), or two (d2) and seven (d7) days following intraperitoneal treatment with *Lactobacillus rhamnosus* (LB). The number of rats was 3–6 per group. Statistically significant differences: $^{\#\#}p < 0.01$ vs. PBS; $^{a}p < 0.05$, vs. AO rats.

groups of AO rats (Fig. 6B, C). This eventually resulted in NO/urea ratio comparable to that in RPMI-treated cells (Fig. 6D). On the contrary, treatment with *E.coli* stimulated NO in peritoneal cells from AO rats of all experimental groups, but exerted more complex effect on urea, as it diminished urea in cells from PBS-treated AO rats and had no effect on cells obtained on d2 and d7. In vitro treatment with *Enterococcus spp.* increased NO release in cells from LB-treated AO rats on d2, and slightly suppressed urea in cells from PBS-treated and LB-treated on d2 (Fig. 6B, C) Finally, both *E.coli* and *Enterococcus spp.* treatment increased NO/urea ratio in cells from PBS-treated and LB-treated rats on d2. Increasing effects of *E.coli* and *Enterococcus spp.* on NO/urea were not seen in cells obtained on d7 (Fig. 6D).

In cells from DA rats, *in vitro* treatment with LPS increased NO release in cells obtained from LB-treated animals on d2 but increased urea release in cells from LB-treated rats on d2 and d7 (Fig. 6B, C). Treatment with *E.coli* increased NO release in cells from all experimental groups of DA rats, but had no effect on urea production. However, *in vitro* treatment with *Enterococcus spp.* increased NO production only in d2 cell, and did not affect production of urea in any of the animals (Fig. 6B, C). Further analysis showed that only treatment *in vitro* with own *E.coli* was able to skew production toward NO instead of urea in cells from LB-treated DA rats on d2 and d7, while treatment with *Enterococcus spp.* did not affect NO/Urea ratio (Fig. 6D).

DISCUSSION

Our study showed that inflammatory reaction toward live LB, as well as the ability of LB to affect cell responses to microbiota stimulation *in vitro*, followed rat strain-specific pattern. Single intraperitoneal injection of LB provoked increase in peritoneal cell yield two days later in both rat strains, with characteristic decrease in the proportion of resident $CD163^+$ macrophages. This is in line with macrophage dissappearance reaction (MDR), representing the first response to inflammatory insult in serous cavities [44] in which resident peritoneal cavity macrophages robustly up-regulate the expression of adhesive molecules and coagulation pathway to form fibrin clots in omentum [45], becoming practically irretrievable from lavage. Microorganisms that access the peritoneal cavity through the digestive tract are in this way efficiently trapped in clots, even before they have a chance to be phagocytosed [45]. Contingent with inflammatory cascade reaction [46] an influx of inflammatory $CD43^+HIS48^+$ monocytes and a rise in the proportion of inflammatory $CD163^-MHCII^{low}$ macrophages were also observed on d2 in both rat strains. As Ki67 is a good indicator of proliferative potential and a measure of G_1 in macrophages [47], a diminished proportion of $CD68^+Ki67^+$ macrophages on d2 relative to control rats of both strains suggests that a low proportion of proliferating macrophages is sufficient to rebuild peritoneal cellular milieu in days following MDR [48]. The greater proliferative capacity in rats of AO rat strain both basally and following LB injection (d2 and d7), opposed to influx of inflammatory $CD163^-MHC^{hi}$ macrophages/dendritic cells on d2 only in rats of DA strain, confirms that the balance of proliferative expansion of resident macrophages versus recruitment of monocyte during infection may vary between inbred animal strains [49].

In accordance with lesser contribution of $CD163^+$ cells to $MHCII^+$ cell pool during inflammation [50], the ratio of $CD163^+$ macrophages within $MHCII^+$ cells, which was initially higher in AO than in DA rats, decreased on d2 in both rat strains (from ~ 68 to ~ 27% for AO, and from ~ 38 to ~ 10% for DA rats) and returned to basal levels at d7. The prevalence of resident $CD163^+$ mature macrophages in the peritoneal cavity of AO rats is in contrast to higher proportion of non-resident macrophages, especially those expressing MHCII, both basally and during inflammation, in rats of DA rat strain. Even though expression of CD163 is increased by anti-inflammatory cytokines IL-4/13 and IL-10 [51] and associated with immunomodulatory/tissue remodeling M2 profile [52], CD163-expressing macrophages also express M1 monocyte/macrophage marker CCR7 [53] that is up-regulated by pro-inflammatory cytokines IFNγ and GM-CSF [51]. Thus, the ratio of CCR7/CD163 gene expression in single cell or within cell population may indicate the prevalence of M1 or M2 response [54]. The

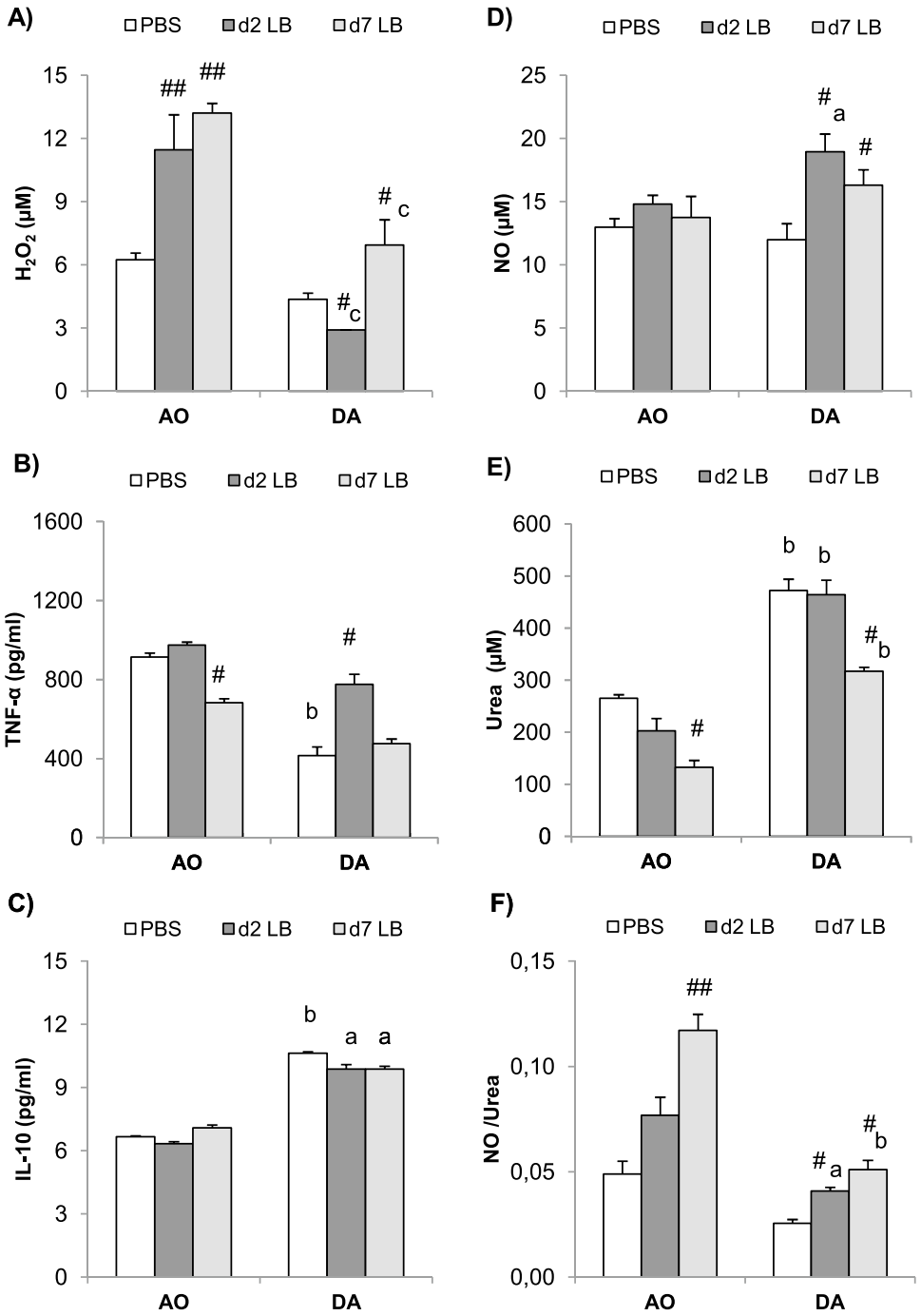


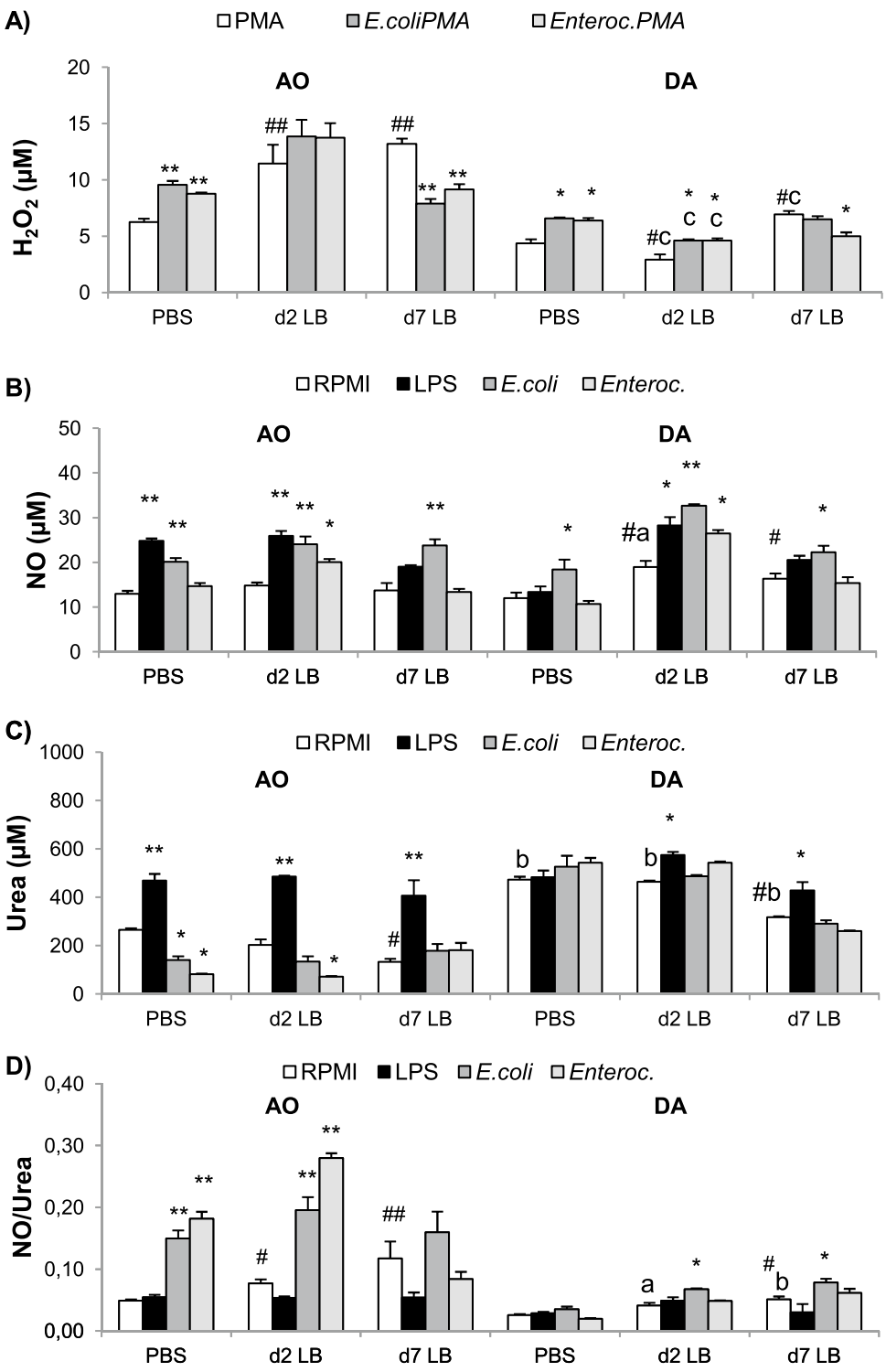
Fig. 5 The release of hydrogen peroxide (H_2O_2) (**A**), TNF-α (**B**), IL-10 (**C**), Nitric oxide (NO) (**D**), and Urea (**E**), and NO/Urea ratio (**F**), by adherent peritoneal cells from rats of Albino Oxford (AO) and Dark Agouti (DA) rat strains following intraperitoneal treatment with phosphate buffer saline (PBS), or two (d2) and seven (d7) days following intraperitoneal treatment with *Lactobacillus rhamnosus* (LB). The number of rats was 3–6 per group. Statistically significant differences: $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. PBS; $^{a}p < 0.05$, $^{b}p < 0.01$, and. $^{c}p < 0.001$ vs. AO rats.

greater proportion of CCR7-expressing $CD163^+$ macrophages in DA than in AO rats following PBS treatment and on d7 and, more importantly, the greater proportion of CCR7-expressing than CCR7-negative $CD163^+$ macrophages within $CD163^+$ macrophage pool in DA rats, additionally supports more intense priming of DA rat cells by local peritoneal millieu [6], both under basal conditions and during resolution following LB injection. The presence of CCR7 molecule on all $CD163^+$ macrophages on d2 following LB in rats of both rat strains creates an inflammatory milieu that is a prerequisite for later profibrotic environment and development of fibrosis during the chronic phase of inflammation [55]. It remains to be elucidated whether generally superior CCR7 expression in peritoneal cellular millieu of DA rats compared to rats of AO rat strain may explain generally higher production of wound-healing urea in macrophages from DA rats.

Peritoneal cavity cell production of cytotoxic molecules and cytokines also differed between rat strains and were differently affected by i.p. LB treatment. Interestingly, although high macrophage production of TNF-α was associated with susceptibility to autoimmune diseases in rats of DA strain [56], peritoneal cavity cells from autoimmune-resistant AO strain produced higher levels of TNF-α. Nevertheless, in the presence of pro-fibrotic/anti-inflammatory TGF-β, highly produced by cells of AO rats [57], TNF-α can induce peripheral $Foxp3^+$ T regulatory cells that may yield the most suppressive T cell phenotype that dampen the development of autoimmunity [58]. Besides, higher levels of H_2O_2 produced by cells of AO rats create an oxidative environment in the vicinity of immunological synapse, making it less favourable for development of autoreactive T cells in rats of this strain [59]. Conversely, significantly higher ability to produce IL-10 that was previously reported in central nervous system-infiltrating immune cells, cervical lymph node cells, and in peritoneal cavity inflammatory cells of DA compared to AO rats [16, 57, 60] was found to be important for susceptibility to *Trichinella spiralis* infection and for the recovery from EAE in DA rat strain [57, 60]. IL-10 prevents intraperitoneal recruitment of peripheral monocytes, but has no effect on recruitment of $MHCII^{hi}$ dendritic/macrophage-like cells during inflammation [61], which may explain modest increase in the proportion of $CD163^-MHCII^{low}$ monocytes and steep rise in the proportion of $CD163^-MHCII^{hi}$ inflammatory macrophages/dendritic cells on d2 in DA rats. Dendritic cells, in addition to high levels of superoxide oxidase which detoxifies superoxide radicals from monocyte/macrophage activation, also significantly express peroxiredoxin enzymes which are involved in the efficient removal of H_2O_2 [62]. Although lactobacilli are among the most potent inducers of reactive oxygen species both *in vitro* and *in vivo* [63], changes in the composition of peritoneal cells in DA rats on d2, i.e. influx of dendritic-like cells, may have contributed to diminished H_2O_2 production in this rat strain.

Previous reports show that direct stimulation with *L. rhamnosus GG* augments the TNF-α and IL-10 expression in monocyte-derived macrophages [64], whereas in our study, i.p. treatment with LB also increased TNF-α and NO release by cells obtained on d2 only in DA rats, but did not affect IL-10 or urea release in either rat strain on d2. It may be corroborated that LB, like some bacteria [65], induces migration and differentiation of $CCR2^+$ monocytes toward inflammatory TNF-α/iNOS-producing peritoneal dendritic cells. Inflammatory $CCR2^{hi}$ monocytes that are selectively recruited to the peritoneum during peritonitis also express CCR7 [66]. Hence, it would be interesting to explore if greater proportion of $CCR7^+$ cells and higher expression level of CCR7 molecules in cells of DA rats [6] may facilitate migration and putative differentiation of inflammatory monocytes to inflammatory dendritic cells in this rat strain.

In further course of experiment, it was noticed that the peritoneal cell yield and the ratio of $CD163^+$ cells in both rat strains on d7 after LB returned to values comparable to PBS-treated rats. However, cells obtained on d7 were functionally different compared to those from PBS-treated rats. Cells obtained on d7 retained some of the inflammatory features observed in cells on d2—increased capacity to release NO (DA) and H_2O_2 (AO), but these cells in AO rats produced less TNF-α, and those from DA rats released more H_2O_2, whereas cells from both strains on d7 had lower arginase activity relative to cells from PBS-treated animals. Hydrolysis of L-arginine by arginase to urea and L-ornithine limits availability of arginine for iNOS-mediated production of antiproliferative and cytotoxic NO [67], and several bacteria and parasites increase host arginase activity to circumvent NO-mediated killing [68]. Decrease in arginase activity favours NO and, together with enhanced H_2O_2 release by d7 cells, boosts antimicrobial potency of these cells. Cells from AO rats on d7 have greater antimictobial potency than those from DA rats. Thus, even though cell phenotype on d7 suggest cesation of LB-induced inflammatory response (i.e., resident $CD163^+$ cells repopulated the peritoneal cavity, in AO rats being more represented than in PBS-treated controls, and the proportion of


A)
PMA
E.coliPMA
Enteroc.PMA
AO
DA
H2O2 (μM)
PBS
d2 LB
d7 LB
B)
RPMI
LPS
E.coli
Enteroc.
NO (μM)
C)
Urea (μM)
D)
NO/Urea

◂**Fig. 6** The effect of *in vitro* stimulation with *E.coli* or *Enterococcus spp.* on release of hydrogen peroxide (H_2O_2) (**A**), nitric oxide (NO) (**B**), and urea (**C**), and on NO/Urea ratio (**D**), by adherent peritoneal cells isolated from rats of Albino Oxford (AO) and Dark Agouti (DA) rat strains following intraperitoneal treatment with phosphate buffer saline (PBS), or two (d2) and seven (d7) days following intraperitoneal treatment with *Lactobacillus rhamnosus* (LB). The number of rats was 3–6 per group. Statistically significant differences: $^{*}p<0.05$ and $^{**}p<0.01$ vs PMA (or RPMI); $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. PBS; $^{a}p<0.05$, $^{b}p<0.01$, and $^{c}p<0.001$ vs. AO rats.

$CD163^{-}MHCII^{low}$ inflammatory and $CD163^{-}MHCII^{hi}$ macrophages/dendritic cells in DA rats returned to basal level as in PBS-treated controls), the origin of these cells, i.e., proliferation or recruitment following MDR, and their experience of adhesion, extravasation, proliferation, and stimulation, is different to that of cells pre-inflammation. Previous reports showing that beneficial effect(s) of probiotic consumption may be mimicked by its single i.p. injection [28] must be interpeted cautiosly, as the flow of the events includes alterations in peritoneal cavity cell composition and activity that change over time. Moreover, these changes may vary between hosts, suggesting that immunomodulatory properties of probiotics depend on recipient's immune response. In addition to numerous data showing that, for example, variability in the effect of probiotic supplementation is a result of inter-individual differences in probiotic colonization [31], our results suggest that this variability may be also due to differences in host innate immunity, which corroborate the necessity of personalized use of probiotics and patient-based immunotherapy.

Finally, in further course of the study, we treated adherent peritoneal macrophages [38] isolated from PBS- or LB-treated AO and DA rats with autologous faecal formalin-killed Gram (−) and Gram (+) bacteria, and followed the changes in NO and H_2O_2 release, and arginase activity. Signature components of the cell wall of Gram (−) and Gram (+) bacteria, LPS, and lipoteichoic acid (LTA), respectively, may both induce oxidative stress and NO production [69, 70]. LPS, but not LTA, was shown to stimulate arginase activity [71, 72]. Here, *in vitro* treatment with autologous *E.coli* increased NO but suppressed (AO rats) or did not influence (DA rats) urea release, whereas commercially available LPS (from *E.coli* O111:B4) mostly increased both NO and urea. This points to additional LPS-independent mechanism(s) in the effect of *E.coli*, as previously suggested [73]. Alternatively, behaviour of bacterium-free LPS may be different from bacterium-bound LPS [74]. In AO rats, *E. coli*- and *Enterococcus spp.*-induced increase in H_2O_2 release (on d2 and d7) and cell polarization toward inflammatory M1 pattern (i.e., increase in NO/urea ratio on d7) were abrogated by previous i.p. treatment with LB. Quite the contrary, in DA rats, i.p. treatment with LB on d2 potentiated release of NO induced by both bacteria resulting in inflammatory M1 skewing by *E. coli* toward higher NO/urea ratio, but prevented increase in H_2O_2 release induced by *E. coli* and *Enterococcus spp. in vitro* in cells from d7. Other researchers reported that preincubation of epithelial cells with *L. rhamnosus* diminished *E. coli*-induced modulation of genes involved in inflammation [75]. Altogether, this implies that beneficial probiotic action(s) may include reconditioning of the pathogen sensing by the immune system [76].

Our results suggest that injection of LB had greater potential in AO than that in DA rats to prevent the functional changes induced by *in vitro* stimulation of peritoneal cavity cells with commensal microflora. Lactobacilli were shown to be potent immunomodulators in various models of inflammation and tumour, *in vivo* as well as *in vitro* [77]. However, animal substrain differences in the composition of gut microbiota, resulting from the propagation by different vendors, influence both the severity of tumour progression and the efficacy of anti-tumor therapy [78], which underlines the importance of the host genetics for the outcome of probiotic stimulation.

Host genetics is important for the rat strain-specific cell activation pattern during LB-induced inflammation, as well as for the outcome of the *in vitro* stimulation with *E. coli* and *Enterococcus spp.* [79]. Direct stimulation of peritoneal cavity cells by LB produces changes in cell phenotype that alter their response to stimulation with fecal bacteria. This may have either protective or deleterious effect on development of conditions associated with dysbiosis, and is strongly affected by animal strain.

CONCLUSION

Our results disclosed clear rat strain differences in (a) the peritoneal cavity cell response to stimulation with LB and (b) the ability of LB to affect cell responses to microbiota stimulation *in vitro*. In general, the final outcome of the peritoneal cell stimulation with autologous microbiota *in vitro* is mainly determined by rat strain-dependent profile of cell response to intraperitoneal stimulation with LB. In addition to the well-known importance of the probiotic strain for

inducing strain-specific immunomodulatory properties, it must be underlined that the ability of particular probiotic bacteria to alter the activity of immune cells and their response to commensals or pathogens is shaped by the interactions of host genetics and (inflammatory) cascade of the events produced following probiotic administration. These results may add to growing field of investigations that points to the importance of tailoring probiotics for personalized use.

AUTHOR CONTRIBUTION

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Veljko Blagojević, Ivana Ćuruvija, and Vesna Vujić, and study was supervised by Stanislava Stanojević. The first draft of the manuscript was written by Stanislava Stanojević and Veljko Blagojević, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of Data and Material All raw data are available upon request, but are not otherwise made public.

Code Availability Not applicable.

DECLARATIONS

Ethics Approval The experimental protocol and the procedures involving animals and their care were approved by Ministry of Agriculture and Environmental Protection (licence number 323–07-01577/2016–05/14, issued on 02–25-2016), and were in accordance with the principles declared in Directive 2010/63/EU of the European Parliament and of the Council from 22 September 2010 on the protection of animals used for scientific purposes (revising Di.rective 86/609/EEC).

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Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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