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Chapter 4

Antibiotic-Treated SPF Mice as a Gnotobiotic Model

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Abstract

Decontamination of specific pathogen-free (SPF) mice of BALB/c line was accomplished by administration of amoxicillin *per os* potentiated with potassium clavulanate at a dose of 387.11 mg/kg body weight and ciprofloxacin administered s.c. at a dose of 18.87 mg/kg body weight every 12 h for 5 days. This resulted in a decreased viability of microorganisms in feces and the cecal content of mice and decreased counts of cultivable microorganisms in the feces, which by day 3 of study declined below the recovery level and to the reduction of animal microbiota to two detected cultivable species, namely *Escherichia coli* (GenBank KX086704) and *Enterococcus* sp. (GenBank KX086705). Convalescence of decontaminated animals under gnotobiotic conditions for 10 days prevented restoration of species diversity of mice microbiota and sufficed to return the metabolic, hematological and morphological values to the physiological range. It also restored the fermentative activity of the intestine to the level similar to that observed before antibiotic treatment. Animals subjected to this procedure can be used in further studies. As a result, we created a mouse gnoto model with reduced and controlled microbiota without alteration of the overall health status of the respective animals.

Keywords: amoxicillin-clavulanic acid, ciprofloxacin, mice, gnotobiotic, decontamination

1. Introduction

Autochthonous microbiota in the gastrointestinal tract (GIT) of mammals are a complex, dynamic, spatially and density diverse community of non-pathogenic micro-organisms. They are a metabolically active entity [1], playing an important role in affecting morphology of the intestine and thus also in its maturation and development, in forming a key barrier

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against pathogenic bacteria, affecting the immune system through modulation and providing essential products of its metabolism to the host. Accumulating evidence reveals that the gut microbiota plays a major role in promoting health, as a result of which it is often referred to as the "forgotten organ" [2, 3]. These microbiota are key factors in maintaining homeostasis, with functions affecting virtually every organ in the body, such as the regulation of bone mass [4], brain development and behavior [5–7], hepatic function [8], and aspects of adipose tissues [9] and the cardiovascular system [10].

In the several past decades, many animal models were used in the studies of dynamically and ecologically diverse community of micro-organisms in gastrointestinal tract (GIT). These micro-organisms are exactly those that help us to understand better the biological complexity of processes underlying their symbiotic relationships with the host. Extensive use of rodents in experiments is related to the fact that these animals can adjust easily to new conditions, multiply quickly, exhibit low nutritional needs and have low requirements on their environment [11]. Like human beings, conventional rodents harbor trillions of bacteria and viruses [12]. The uniformity of microbiota assumed previously in the controlled populations of inbred laboratory animals may not be so high. Some variations may be caused by differences in rearing and handling of animals, and others may result from factors that have not been identified as yet and may affect composition of the microbiota within populations and individuals over time. This should be taken into account when designing experiments involving laboratory animals and interpreting results of such experiments [13]. Despite the fact that only few studies were dealing with systematic comparison of microbiota of highly hygienically standardized mice to those kept in less strict environment, there is sufficient background that allows one to assume limited species complexity in highly microbiologically standardized animals [14, 15]. With increasing use of such rodents, it is reasonable to expect that microbiota of limited diversity alters the known responses of rodents within experimental settings [16]. Using a simplified approach, laboratory animals can be divided to conventional laboratory animals, i.e. those harboring various proportions of other live organisms, and gnotobiotic laboratory animals with accurately defined microbiota. The term germ-free (GF) (axenic) refers to an animal demonstrably free from microbes, including bacteria, viruses, fungi, protozoa, and parasites, throughout its lifetime [17, 18]. GF animals selectively colonized with one or more bacterial species are referred to as gnotobiotic [19, 20]. This term is derived from the Greek "gnotos", meaning known, and "bios" which means life [17, 21]. Gnotobiotic animals offer a wide range of advantages compared to other animal models when studying the physiology of the digestive tract. This involves particularly the study of mutual interaction of natural microflora and pathogens in the digestive tract and the mechanisms of probiotic effects of microorganisms [22]. Germ-free animal models have been used to explore host-microbiota interactions in entire fields, including lipid metabolism [9], cardiology [10], neurogastroenterology [5, 6, 23, 24], reproductive biology [25, 26], and bone homeostasis [4].

An alternative is a temporary gut sterilization, which may involve absolute or selective elimination of microflora [27, 28]. Some researchers [29, 30] described procedures based on oral administration of antibiotics that allowed them to achieve complete elimination of bacterial

flora of rats' digestive tract and to maintain its bacteria free status. In other studies, various cocktails of antibiotics sufficed to completely or selectively sterilize the gastrointestinal tracts of mice and rats [31–34]. Administration of oral antibiotic for the purpose of gut sterilization facilitated physiological studies of the nutritionally important relationship between the intestinal microflora and the host. However, when carrying such studies one must consider the extreme variability of such gut flora and thus expect considerable variations of the efficacy of antibiotics in gut sterilization between and within species. Therefore, it is necessary to test effectiveness of any antibiotic cocktail before its implementation [27]. Since the microflora of laboratory specific pathogen-free (SPF) mice is partially controlled and these animals do not come into contact with antimicrobial substances, they are the most suitable model for decontamination [35]. Due to the frequent testing, these animals do not serve as a reservoir of multiresistant or nosocomial micro-organisms [16]. By using antibiotics for decontamination of these animals, one can reduce considerably the number and species diversity of their microbiota.

Our study focused on obtaining an animal model with reduced and controlled microflora ensuring at the same time good health of these model animals.

2. Material and method

2.1. Isolator technology

The experiment was carried out in three germ free isolators (Velaz s.r.o., Prague, Czech Republic) using a gnototechnology described previously by Gancarčíková et al. [22]. A routine microbiological control of isolators was performed throughout the experimental study. Microbiological swabs were taken from gnotobiotic isolator walls, surface of animals and from their rectum. They were inoculated onto TSA agar (tryptic soy agar) with 5% ram's blood (BBL, Microbiology Systems, Cockeysville, USA).

2.2. Animals, housing and diet

The experiment was carried out on 66 specific pathogen-free (SPF) BALB/c female mice, (4 weeks old), obtained from Velaz s.r.o. (Prague, Czech Republic). All experimental procedures were approved by the Ethics Commission of the University of Veterinary Medicine and Pharmacy in Košice, Slovakia. The experimental protocol No. 1177/14–221 was approved by the State Veterinary and Food Administration of the Slovak Republic and the animals were handled and sacrificed in humane manner in compliance with the guidelines established by the relevant commission. All applicable institutional, national and international regulations for the care and use of experimental animals were observed. The conventional SPF mice were transported by air in special transport containers to the experimental facilities of the Laboratory of Gnotobiology, University of Veterinary Medicine and Pharmacy (UVMP) in Košice. After a thorough surface disinfection of the containers with peracetic acid, these were transferred to gnotobiotic isolators (Velaz s.r.o., Prague, Czech Republic). After subsequent

venting of peracetic acid vapors, the mice were transferred to three breeding polypropylene cages, 7–9 mice per cage. The following groups were formed: negative control C (n = 7); decontaminated/antibiotic- treated group DC (n = 9); decontaminated/antibiotic- treated and convalesced group DC + R (n = 8). All animals were fed *ad libitum* complex mixed feed for mice in system, a barrier feeding system ST-1 (Velaz s.r.o., Prague, Czech Republic), and had unlimited access to water kept in glass bottles. The diet contained (kg diet) crude protein 24%, crude fat 3.4%, crude fiber 4.4%, ash 6.8%, calcium 11 g, sodium 1.8 g, phosphorus 7.2 g, copper 20 mg and selenium 0.38 mg (vitamin A 28000 IU, vitamin D 2200 IU, vitamin E 100 mg). The mice were kept at temperatures maintained between 20 and 24°C, at relative humidity of 45–65%, under a 12-h light/dark regimen. Lignocel 3-4S (Velaz s.r.o., Prague, Czech Republic) bedding intended for barrier breeding system was used.

2.3. Antibiotic treatment of SPF mice

The experimental mice were administered amoxicillin and clavulanate potassium (Amoksiklav 2 × 457 mg/5 mL, Sandoz Pharmaceuticals, Ljubljana, Slovenia) perorally at a dose of 387.11 mg/kg body weight (0.2 mL of dilution) every 12 h during the first 5 days of the experiment.

Ciprofloxacin (Ciloxan 1 × 5 mL/15 mg, Alcon Cusí S.A., Barcelona, Spain) was administered subcutaneously at a dose of 18.87 mg/kg body weight (0.1 mL of dilution) every 12 h during the first 5 days of the experiment.

2.4. Sampling procedures

Health of the animals and consistency of feces were observed and recorded daily. Fresh fecal samples were collected on days 0, 1, 2, 3, 5 and 15 of the study. Blood samples for hematological and biochemical analysis were collected from anesthetized animals using retro-orbital technique. Anesthesia was induced with sodium pentobarbital at a dose of 86 mg/kg body weight. The mice were euthanized by cervical dislocation at the end of the study for the purpose of sample collection. During dissection, weight of internal organs (heart, liver, spleen, kidneys and lungs) was recorded and samples of feces, caecum and *lobus caudatus hepatis* from the liver were collected. Samples for microbiological examination, determination of percentage survivability by fluorescence-activated cell sorting (FACS) and fluorescence microscopy examination by visualization method with viability fluorescent quick test on a polycarbonate filter (VFQTOPF) were processed immediately, while samples for determination of production of organic acids were stored at −20°C until analysis. Samples from *lobus caudatus hepatis* intended for histological analysis were fixed in 4% solution of paraformaldehyde until analysis.

2.5. Microbiological analysis

2.5.1. Bacterial enumeration

For microbiological analysis, samples of feces and caecum were collected individually from each animal. The samples (1 g) were homogenized Stomacher Lab Blender 80 (Seward

Medical Limited, London, UK) with 9 mL of a sterile anaerobic diluent (0.4 g $NaHCO_3$, 0.05 g), L-cysteine-HCl, 1 mL resazurin (0.1%), 7.5 mL mineral solution I (0.6% K_2HPO_4), 7.5 mL mineral solution II (1.2% NaCl, 1.2% $(NH_4)_2SO_4$, 0.6% KH_2PO_4, 0.12% $CaCl_2$, 0.25% $MgSO_4$) and 84 mL distilled water (pH 6.8). A series of 10-fold dilutions (10^{-1} to 10^{-9}) were made under a CO_2 atmosphere. From appropriate dilutions, 0.1 mL aliquots were spread onto Trypticase soy blood agar (Oxoid Unipath, Ltd., Basingstoke, UK) with 10% sheep blood for total aerobes, Schaedler agar (BBL Microbiology systems, Cockeysville, USA) with 1% vitamin K1 - hemin solution for total anaerobes, and Man-Rogosa-Sharpe agar (MRS, Merck, Darmstadt, Germany) for lactic acid bacteria. Incubation of the inoculated media for anaerobic and lactic acid bacteria was carried out at 37°C for 3 days under anaerobic conditions (Gas Pak Plus, BBL). Plates for the enumeration of aerobic bacteria were incubated for 24 h at 37°C. Numbers of colony-forming units (CFU) were expressed as log CFU per gram of sample. The results were presented as arithmetical means ± standard deviation (SD).

2.5.2. Viability of microorganisms on fluorescence-activated cell sorting visualized with viability fluorescent quick test on a polycarbonate filter (VFQTOPF)

The samples of feces and cecal contents were diluted 1:100 in PBS (37°C; MP Biomedicals, France) and filtered through 70 μm and subsequently through 45 μm cell strainers (BD Falcon, NJ, USA). The prepared suspensions were stained with carboxyfluorescein diacetate (cFDA; Sigma) in final concentration of 25 μM and with propidium iodide (PI; Sigma) in final concentration of 45 μM at 37°C for 20 min. Flow cytometric analysis was performed employing a BD FACSCanto™ flow cytometer (Becton Dickinson Biosciences, USA) and BD FACS Diva™ software. The percentages of live and dead bacteria were evaluated based on presence of carboxyfluorescein (cF) (metabolized form of cFDA) detectable only in live bacteria, measured in FL-1 channel (530/30 nm) and the intensity of fluorescence was measured in FL-3 channel (695/40 nm) for propidium iodide (PI) which enters only damaged or dead bacteria [36]. Simultaneously, samples stained with cFDA and PI were analyzed by epifluorescence microscopy. Vacuum filtered samples were fixed on polycarbonate filters (Merck Millipore, Billerica, USA) and stained also with DAPI solution (1 mg of 4′,6-diamidino-2-phenylindole/mL). The filters were placed on microscopic slides and mounted with Vectashield Medium (Vector Laboratories, Peterborough, UK). The slides were examined under a Carl Zeiss Axio Observer Z1 epifluorescence microscope using filter sets 38HE, 64HE and Set 49 for detection of cF, PI and DAPI, respectively. Microphotography analysis was performed using Axio Vision Rel 4.8 software.

2.5.3. Determination of the minimum inhibitory concentration of antibiotics

The minimum inhibitory concentrations (MICs) of antibiotics against the tested strains were determined by Etest® strips for ciprofloxacin (AB bioMérieux, Marcy l'Étoile, France) and M.I.C. evaluator strips for amoxicillin and clavulanate potassium (Thermo Fisher Scientific, Basingstoke, UK). The results were read in accordance with the manufacturers' protocol, which is essentially identical for both strip products.

2.5.4. Phenotypical identification

Phenotypical identification of *Escherichia coli* was performed by means of a diagnostic kit ENTEROtest 24 N (Erba Lachema s. r. o., Brno, Czech Republic).

2.5.5. DNA identification

After microbiological cultivation on blood agar, DNAzol direct (Molecular Research Center Inc., Cincinnati, USA) was used to isolate DNA from bacterial colonies. The PCR reaction was performed with the help of primers 27F (5-AGAGTTTGATCMTGGCTCAG-3 and 1492R (5-CGGYTACCTTGTTACGACTT-3). The amplification protocol for PCR reaction was: 5-min at 94°C, 1 min at 94°C, 1 min at 55°C and 3 min at 72°C and a final at 72°C 10-min (TProfesional Basic, Biometra GmbH, Göttingen, Germany). PCR products were separated by electrophoresis on 0.7% agarose gel with the help of TAE buffer. The PCR amplicons were stained with GelRed™ (Biotium Inc., Hayward, USA) and visualized after the separation under UV light. Purification of PCR products was carried out by means of a kit NucleoSpin® Gel and PCR Clean-Up Kit (Mancherey-Nagel GmbH & Co. KG, Düren, Germany). The amplicons were submitted for sequence analysis to *E. coli* s.r.o. (Bratislava, Slovakia) and sequenced in both directions by either 27F or 1492R primers. The sequences were then analyzed by BLAST (compared with sequences available in the GenBank) and after the alignment and assembly processing by means of Genious 6.1.6 software they were submitted to the GenBank. The resultant sequences were published under GenBank accession numbers KX086704 and KX086705.

2.6. Blood and serum analysis

Hematological analysis was carried out using a BC-2008 VET automatic analyzer (Mindray, Shenzhen, China). An automated biochemical analyzer Ellipse (AMS, Rome, Italy) and standard kits (Dialab, Prague, Czech Republic) were used to determine concentrations of the following biochemical parameters: glucose; triglycerides; cholesterol; HDL-cholesterol; LDL-cholesterol; total protein; urea; albumin; creatinine; activities of enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Total activity of lactate dehydrogenase (CLDH) was determined spectrophotometrically (Alizé, Lisabio, France) and its isoenzymes (LDH-1: LDH-5) were determined by an electrophoretic method (Hydrasys, Lisses, France).

2.7. Short chain fatty acids (SCFAs) analysis

The produced organic acids were determined by isotachophoresis as described by Gancarčíková et al. [22]. After the collection, 0.5 g of feces and caecum contents were dissolved in 25 mL deionized water and 30 μL aliquots were used for analysis of short-chain fatty acids (SCFAs). The measurements were done on an Isotachophoretic analyzer ZKI 01 (Radioecological Institute, Košice, Slovakia). A leading electrolyte of the following composition was used in the pre-separatory capillary: 10^{-2} mol/L HCl + 2.2. 10^{-2} mol/L

ε-aminocaproic acid + 0.1% methylhydroxyethylcellulosic acid, pH = 4.3. A solution of 5. 10^{-3} mol/L caproic acid + 2. 10^{-2} mol/L histidine was used as a finishing electrolyte. This electrolytic system worked at 150 μA in the pre-separatory and at 40 μA in the analytic capillary.

2.8. Histology of the liver and kidneys

Liver samples from *lobus caudatus hepatis* and kidneys of mice were fixed in 4% paraformaldehyde in PBS (pH 7.2) (Amresco LLC, Solon, USA) for 72 h, washed for 5 h and paraffin blocks were prepared according to the standard procedure. Some paraffin sections (7 μm thick) were stained with Harrison's hematoxylin and eosin, and the tissue was mounted in Histochoice mounting fluid (Amresco LLC, Solon, USA). Tissue sections were examined using a light microscope (Olympus BX 51, Czech Republic) and Digital Analysis Imaging system "Analysis Docu" (Soft Imaging Systems 3.0, Prague, Czech Republic). A part of sections of livers and kidneys were used for fluorescent detection of late apoptosis seen as fragmented nuclei (blue color) and simultaneously for localization of neutral lipids (red color). Rehydrated sections were firstly stained with solution of Nile red (Sigma-Aldrich, USA) prepared in 75% glycerol in PBS at the concentration of 2 μg/mL for 2 h at 8°C. Following the washing step in PBS, sections were incubated with nuclei - staining solution prepared from Hoechst 33,342 (5 μg/mL) in PBS (Sigma-Aldrich, USA) for 2 h at 8°C. Then the washed slides were covered with mounting fluid (90% glycerol in PBS, 2.5% DABCO (Sigma-Aldrich, USA) and examined using fluorescent microscope Carl Zeiss Axio Observer Z1) and analyzed with Axio Vision Rel 4.8 software (Carl Zeiss Jena, Germany).

2.9. Statistics

Statistical evaluation of the results was performed using Statistic software GraphPad Prism 3.0 for Windows (GraphPad Software, San Diego, USA). One-way analysis of variance (ANOVA) was used, followed by a multiple comparison Tukey's test. Significance of differences between the groups of mice was tested using analysis of variance and unpaired Student's *t*-test. The significance level was set to $P < 0.05$. Most of the results are expressed as means ± SD (standard deviation).

3. Results

3.1. Clinical examination of animals

Laboratory SPF BALB/c mice were subjected to complex clinical examination during quarantine and at the end of the experiment. During experiment, all changes in clinical status were observed and recorded twice daily (8.00 and 15.00 h). The regular observation of overall health manifested by uptake of food, agility of animals and consistency of feces allowed us

to detect changes in consistency of feces from solid to pasty on day 3 of the experiment in 10 out of 17 animals treated with antibiotics. All SPF BALB/c mice were agile and their intake of food was unchanged.

3.2. Total body weight and relative weight of internal organs

On day 5 of the experiment, the total body weight of animals from experimental group DC (**Table 1**) was insignificantly lower by 0.23 g in comparison to negative control (C). Examination of internal organs showed a significant decrease in relative weight of the liver ($P < 0.05$) and spleen ($P < 0.01$) in decontaminated group (DC) in comparison with control group C. On day 15 of the experiment, group DC + R showed the highest relative weights of the heart, liver and spleen, approaching the weights of these organs in group C on day 5 of the experiment.

3.3. Hematology parameters

Total counts of leukocytes (WBC) and lymphocytes (Ly) in all investigated groups (**Table 2**) were in physiological ranges. However, the decontaminated group (DC) showed insignificantly lower counts of WBC (by 1.73 G/L) and lymphocytes (by 1.65 G/L) in comparison with control group C.

On day 5 of the experiment, group DC showed a significant increase ($P < 0.05$) in counts of monocytes (Mo), and their percentage value (Mo%) was also significantly increased ($P < 0.05$) in comparison with group not treated with antibiotics (C). Administration of antibiotics (ATB) affected also the number of granulocytes (Gran, Gran%). While the number of granulocytes (Gran) exceeded the physiological limit, it was only insignificantly higher compared to the control C (by 1.34 G/L). However, in case of percentage proportion of granulocytes (Gran%) the difference was significant ($P < 0.05$).

The changes in red blood components recorded in group DC after decontaminated with antibiotics (ATB) resembled those observed in white blood components in this group (**Table 2**). Increased counts exceeding the physiological range, although insignificantly different, were observed for erythrocyte counts (RBC), level of hemoglobin (HGB) and hematocrit

Group	The organ dimensions (g/kg)						Body weight (g)
	Heart	Liver	Spleen	Right kidney	Left kidney	Lungs	
C	5.49 ± 0.28	53.87 ± 2.6	4.40 ± 0.30	7.19 ± 0.34	7.16 ± 0.24	8.27 ± 0.67	16.13 ± 0.34
DC	5.07 ± 0.21	47.41 ± 0.68 ^{*C}	2.82 ± 0.13 ^{**C}	7.31 ± 0.30	7.33 ± 0.33	7.40 ± 0.33	15.90 ± 0.36
DC + R	5.96 ± 0.21	5.70 ± 1.49	4.71 ± 0.31	7.29 ± 0.29	7.16 ± 0.31	8.23 ± 0.27	17.33 ± 0.65

The results are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1. Body weight (g) and the organ dimensions (g/kg) of the BALB/c mice in control C (n = 7), treated with ATB for 5 days (DC group, n = 9) and then after 10 days without antibiotic treatment (DC + R group, n = 8).

Group	C	DC	DC + R	Ref BALB/c
WBC (G/L)	7.76 + 1.55	8.80 + 1.92	6.03 + 0.98	5.69–9.87
Ly (G/L)	6.08 + 1.31	5.18 + 1.09	4.43 + 0.65	3.60–7.29
Mo (G/L)	0.14 + 0.04	0.74 + 0.30^{*C}	0.15 + 0.04^{*DC}	0.34–0.70
Gran (G/L)	1.54 + 0.35	2.88 + 0.70	1.45 + 0.34	0.74 – 1.78
Ly (%)	77.64 ± 2.83	60.10 ± 5.06^{**C}	74.67 ± 1.98^{**DC}	55.06–73.44
Mo (%)	1.94 ± 0.20	7.62 ± 2.13^{**C}	2.75 ± 0.30^{**DC}	3.75–7.26
Gran (%)	20.42 ± 2.70	32.28 ± 3.34^{*C}	22.58 ± 1.77^{*DC}	10.46–18.94
RBC (T/L)	9.06 ± 1.17	10.77 ± 0.39	9.44 ± 1.16	8.16–9.98
HGB (g/L)	156.4 ± 19.85	189.00 ± 7.96	145.90 ± 13.52	124–154
HCT (%)	51.50 ± 7.01	61.00 ± 2.42	47.37 ± 4.20	43.50 – 55.4
MCV (fL)	56.60 ± 0.69	56.66 ± 0.45	55.88 ± 0.42	50.80 – 55.60
MCH (pg)	17.26 ± 0.28	17.46 ± 0.15	15.85 ± 0.96	13–15.5
MCHC (g/L)	306.4 ± 6.74	309.4 ± 1.03	297.5 ± 12.19	239–280
RDW (%)	14.78 ± 0.50	13.62 ± 0.43	13.76 ± 0.24	16.9–19.1

WBC white blood cells, *Ly* lymphocytes, *Mo* monocytes, *Gran* granulocytes, *RBC* red blood cells, *HGB* hemoglobin, *HCT* hematocrit, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *RDW* red blood cell distribution width, *Ref* reference range [91]. The results are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 2. Hematology parameters of the BALB/c mice in control C (n = 7), treated with ATB for 5 days (DC group, n = 9) and then after 10 days without antibiotic treatment (DC + R group, n = 8).

(HCT) in comparison with control (C). *On day 10* after termination of treatment with antibiotics, we recorded an insignificant decrease in counts of both leukocytes and lymphocytes in group DC + R. This group showed a significant reduction in counts of Mo, Gran% ($P < 0.05$) as well as in Mo% ($P < 0.01$) in comparison with group DC. A decreasing trend in the observed parameters in group DC + R following convalescence of animals and return of their levels to the physiological range was observed not only for the white components but also for red ones, represented by decrease in RBC, HGB and HCT. Mean cell volume (MCV) of erythrocytes, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were moderately increased in all groups and red blood cell distribution width (RDW) was moderately decreased in comparison to physiological range, but there were no significant differences between the groups.

3.4. Biochemical parameters

3.4.1. Nitrogen profile

Nitrogen profile (**Table 3**) represented by concentration of total proteins (TP) and albumin showed significant differences between groups DC and C on day 5 of the experiment. Despite

Group	C	DC	DC + R	Ref BALB/c
Total protein (g/L)	70.20 ± 1.65	89.98 ± 0.90^{**C}	66.60 + 5.78^{**DC}	60.8–73.0
Urea (mmol/L)	6.66 ± 0.63	6.68 ± 0.08	5.87 ± 0.16	5.70–7.14
Albumin (g/L)	33.98 ± 0.48	37.48 ± 0.57^{**C}	31.57 ± 0.82^{***DC}	31.0–37.0
Creatinine (μmol/L)	27.50 ± 0.96	24.50 ± 0.96	30.00 ± 0.58^{**DC}	up to 33.59
Glucose (mmol/L)	8.03 ± 0.17	6.35 ± 0.06^{**C}	8.03 ± 0.47^{**DC}	4.72–10.71
Triglyceride (mmol/L)	2.59 ± 0.05	2.26 ± 2.09^{*C}	2.96 ± 0.06$^{*C,\ ***DC}$	up to 3.42
Cholesterol (mmol/L)	2.62 ± 0.09	3.41 ± 0.03^{***C}	3.22 ± 0.16^{*C}	2.09–3.65
HDL cholesterol (mmol/L)	1.77 ± 0.06	1.76 ± 0.02	1.79 ± 0.02	up to 1.78
LDL cholesterol (mmol/L)	0.38 ± 0.01	0.75 ± 0.02^{***C}	0.57 ± 0.01^{***C,DC}	up to 0.38
DC	up to 0.38			
AST (μkat/L)	3.27 ± 0.18	3.64 ± 0.10	3.13 ± 1.57	2.67–3.05
ALT (μkat/L)	2.50 ± 0.24	3.26 ± 0.55	8.20 ± 1.63^{**C,DC}	0.68–2.89
ALP (μkat/L)	6.47 ± 0.30	5.48 ± 0.48	5.96 ± 0.45	1.83–6.23
LDH-Total (μkat/L)	58.4 ± 2.9	78.98 ± 9.81	64.83 ± 12.3	
LDH-1				
% z LDH-T	2.9 ± 1.1	1.55 ± 0.13	2.0 ± 0.15	
(μkat/L)	1.73 ± 0.73	1.22 ± 0.16	1.27 ± 0.18	
LDH-2				
% z LDH-T	2.6 ± 0.1	2.38 ± 0.15	3.0 ± 0.15	
(μkat/L)	1.53 ± 0.14	1.85 ± 0.16	1.98 ± 0.43	
LDH-3				
% z LDH-T	16.75 ± 3.15	14.35 ± 2.54	21.2 ± 1.25	
(μkat/L)	9.88 ± 2.33	10.98 ± 1.53	13.57 ± 2.16	
LDH-4				
% z LDH-T	9.25 ± 0.55	8.53 ± 0.24	10.97 ± 1.52	
(μkat/L)	5.39 ± 0.06	6.7 ± 0.74	7.44 ± 2.38	
LDH-5				
% z LDH-T	68.5 ± 3.8	73.2 ± 2.7	62.83 ± 0.8	
(μkat/L)	39.9 ± 0.24	58.24 ± 8.7	40.58 ± 7.4	

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase, *LDH-T* lactate dehydrogenase total, *Ref* reference range [91]. The results are expressed as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 3. Biochemical parameters in blood serum of the BALB/c mice in control C (n = 7), treated with ATB for 5 days (DC group, n = 9) and then after 10 days without antibiotic treatment (DC + R group, n = 8).

decreased exogenous intake of feed by animals of group DC, this group exhibited significantly higher concentration of both TP and albumin ($P < 0.01$). Their levels exceeded the upper physiological limit due to hemoconcentration and dehydration of the organism. While administration of antibiotics to mice of group DC did not affect significantly the level of urea in comparison with group C, serum creatinine in decontaminated mice decreased by 11%. *On day 10* after termination of treatment with antibiotics, group DC + R showed return of concentrations of TP and albumin back to the physiological range with significantly lower levels of TP ($P < 0.01$) and albumin ($P < 0.001$) in comparison with those recorded in group DC on day 5 of the experiment (**Table 3**). While on day 10 after termination of treatment with antibiotics the level of urea in group DC + R decreased, concentration of creatinine significantly increased ($P < 0.01$). After 10-day convalescence, all investigated parameters of nitrogen profile were in physiological range.

3.4.2. Energy and lipid profile

On day 5 of the experiment (**Table 3**), animals from decontaminated group DC showed significantly lower levels of glucose ($P < 0.01$) and triglycerides ($P < 0.05$) in comparison with group C, indicating reduced intake of feed, however, concentration of total cholesterol, which was in physiological range, was significantly higher ($P < 0.001$) in this group and indicated moderate irritation of intestinal mucosa. While the level of HDL-cholesterol was about the same in both investigated groups (C, DC), LDL-cholesterol was significantly higher ($P < 0.001$) in group DC and exceeded the physiological range. *On day 10* after termination of treatment with antibiotics, group DC + R showed an opposite trend in concentration of investigated parameters of energy and lipid profile of mice in comparison with group DC (**Table 3**). After 10-day convalescence, a significant increase in glucose ($P < 0.01$) and triglycerides ($P < 0.001$) was observed in group DC + R in comparison with group DC. At the same time, we recorded in this group a significant decrease ($P < 0.001$) in LDL-cholesterol; however, its concentration exceeded the physiological limit determined for mice of BALB/c line.

3.4.3. Enzymatic profile

While on days 5 and 15 of the experiment none of the investigated groups showed increased activity of enzyme ALP (**Table 3**), activities of enzymes AST and ALT were insignificantly increased in group DC in comparison with group C. ALT is a liver-specific enzyme and its increased activity indicates irritation or damage to the liver. Its increase is associated with damage to membrane of liver cells, even at the absence of their necrosis, and the enzyme is excreted at both reversible and irreversible damage to liver parenchyma. Increased activity up to 3-fold the reference level is considered a moderate increase. After *10-day* convalescence without treatment with antibiotics, an insignificantly lower activity of non-specific hepatic enzyme AST and significantly higher ($P < 0.01$) activity of enzyme ALT was observed in group DC + R in comparison with group C and decontaminated group DC, indicating irritation of the liver. In this case, ALT was released, however, without damage to hepatocytes. There was no alteration of AST, the activity of which was increased only slightly and thus the coefficient of hepatocyte damage was not decisive.

3.4.4. Activity of LDH-total and isoenzymes

LDH-T is a multi-organ cytosol enzyme that exists as 5 isoenzymes. It is released to circulation already at slight tissue damage. Observation of specific activity of total LDH (**Table 3**) and its isoenzymes in the serum of mice of the investigated groups (C, DC a DC + R) showed no significant differences.

On day 5 of the experiment, we observed an insignificant increase in activity of total LDH in decontaminated group DC, which was by 20.5 μkat/L higher in comparison with control group C. Determination of relative proportions of individual isoenzymes in decontaminated group DC revealed that besides increase in LDH-1, specific activities of isoenzymes LDH-(2, 3 and 4) were also increased; however, as far as their percentage proportion of total LDH was concerned, we observed a decrease in specific activities of all isoenzymes LDH-(1-4) in favor of increased activity of isoenzyme LDH-5, indicating irritation of hepatic tissue. The greatest although insignificant decrease in specific activity was observed in isoenzyme LDH-3, found in pulmonary parenchyma. Its activity was lower by 2.4% in comparison with the period without treatment with antibiotics. The activity of isoenzyme LDH-1, known as a heart enzyme, was lower by 1.35%, and activities of isoenzymes LDH-4, found in the kidneys and pancreas, and LDH-2, primarily associated with the reticuloendothelial system, were decreased by 0.72% and 0.22%, respectively. The most pronounced although insignificant increase in specific activity was observed in isoenzyme LDH-5, found in liver parenchyma and striated muscles. Its activity was higher by 18.34 μkat/L and percentage proportion of total LDH higher by 4.7% in comparison with control group C.

An insignificant decrease in total LDH was observed again in group DC + R after convalescence period. The activity of this enzyme was lower by 14.15 μkat/L in comparison with group DC. *On day 10* following the termination of treatment with antibiotics, group DC + R showed most pronounced but insignificant changes in activities of isoenzymes LDH-5, LDH-3 and LDH-4. While the activities of isoenzymes LDH-3,4 after convalescence (DC + R) showed an increase by 6.85% (LDH-3) and 2.44% (LDH-4) of total LDH, an opposite trend was observed for LDH-5. The isoenzyme associated with liver parenchyma and striated muscles (LDH-5) showed an insignificant decrease in specific activity down to the level determined before treatment with antibiotics (40.58 ± 7.4 μkat/L), which indicated reparation of hepatic tissue.

3.5. Microbiological parameters

3.5.1. Determination of counts of cultivable microorganisms in mice feces

Before the application of antibiotics (ATB), the plate counts (**Figure 1**) of microorganisms in feces in all groups of SPF mice (C, DC, DC + R) ranged between 8.15 and 9.19 log10 CFU/mL. Determination of plate counts 24 h after the antibiotic treatment showed a significant decrease by 4 logs (4.58 ± 0.31 log10 CFU/mL) after aerobic cultivation and by 3–4 logs

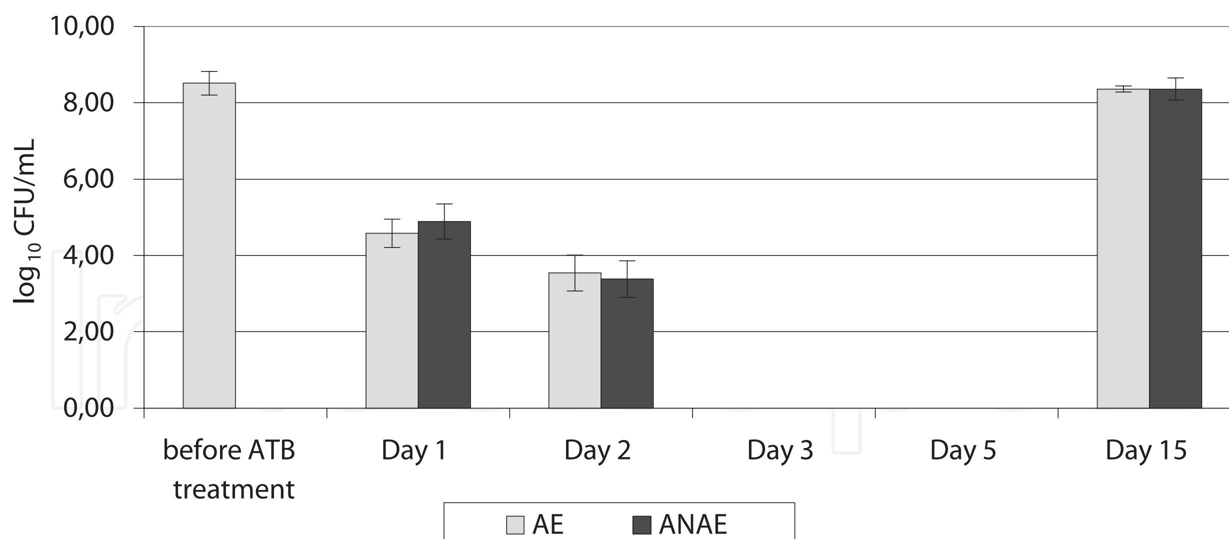

Figure 1. Plate counts of microorganisms in feces samples determined by cultivation on TSA agar. *AE* aerobic conditions, *ANAE* anaerobic conditions. The results are expressed as the means log 10 CFU/mL ± SEM.

(4.89 ± 0.46 log10 CFU/mL) after anaerobic cultivation when compared with the initial counts determined before antibiotic treatment (8.51 ± 0.31 log10 CFU/mL). Cultivation at 48 h from the beginning of antibiotic treatment revealed less pronounced decrease in plate counts of cultivable microorganisms. The counts were reduced by 1 log under aerobic conditions (3.54 ± 0.47 log10 CFU/mL) and by 1–2 logs when cultivated anaerobically (3.38 ± 0.48 log10 CFU/mL), in comparison with the counts determined at 24 h after the antibiotic treatment. The following investigations on days 3 and 5 of cultivation revealed absence of cultivable microorganisms in the feces (**Figure 1**). Determination of plate counts on day 10 after termination of antibiotic treatment showed recurrence of cultivable microorganisms in feces after both aerobic cultivation (8.36 ± 0.08 log10 CFU/mL) and anaerobic cultivation (8.36 ± 0.29 log10 CFU/mL).

3.5.2. Survivability of microorganisms in samples of feces and caecum content determined by FACS, visualized by means of VFQTOPF

Survivability of microorganisms in mice feces (BD FACS Canto flow cytometer, BD, USA) decreased significantly ($P < 0.01$) between days 1 (35.03 ± 2.43%) and 2 (28.33 ± 0.43%) of antibiotic treatment. The survival rates before the treatment reached 60.58 ± 5.28% (**Figure 2**). Survival rate of bacteria in the caecum on day 5 of treatment (**Figure 3**) was significantly lower ($P < 0.001$) in DC group (28.10 ± 1.56%) in comparison with control group C (76.77 ± 1.56%). Survival rate of microorganisms in the caecum of mice from group DC + R (kept in gnotobiotic isolators with microbiologically controlled environment) reached 75.47 ± 0.38% on day 10 after termination of antibiotic treatment. The viability fluorescent quick test on a polycarbonate filter (VFQTOPF) was also employed to detect survivability of microorganisms (**Figures 2** and **3**). It allowed visualization on the basis of color as the live bacteria stained green and dead bacteria turned red.

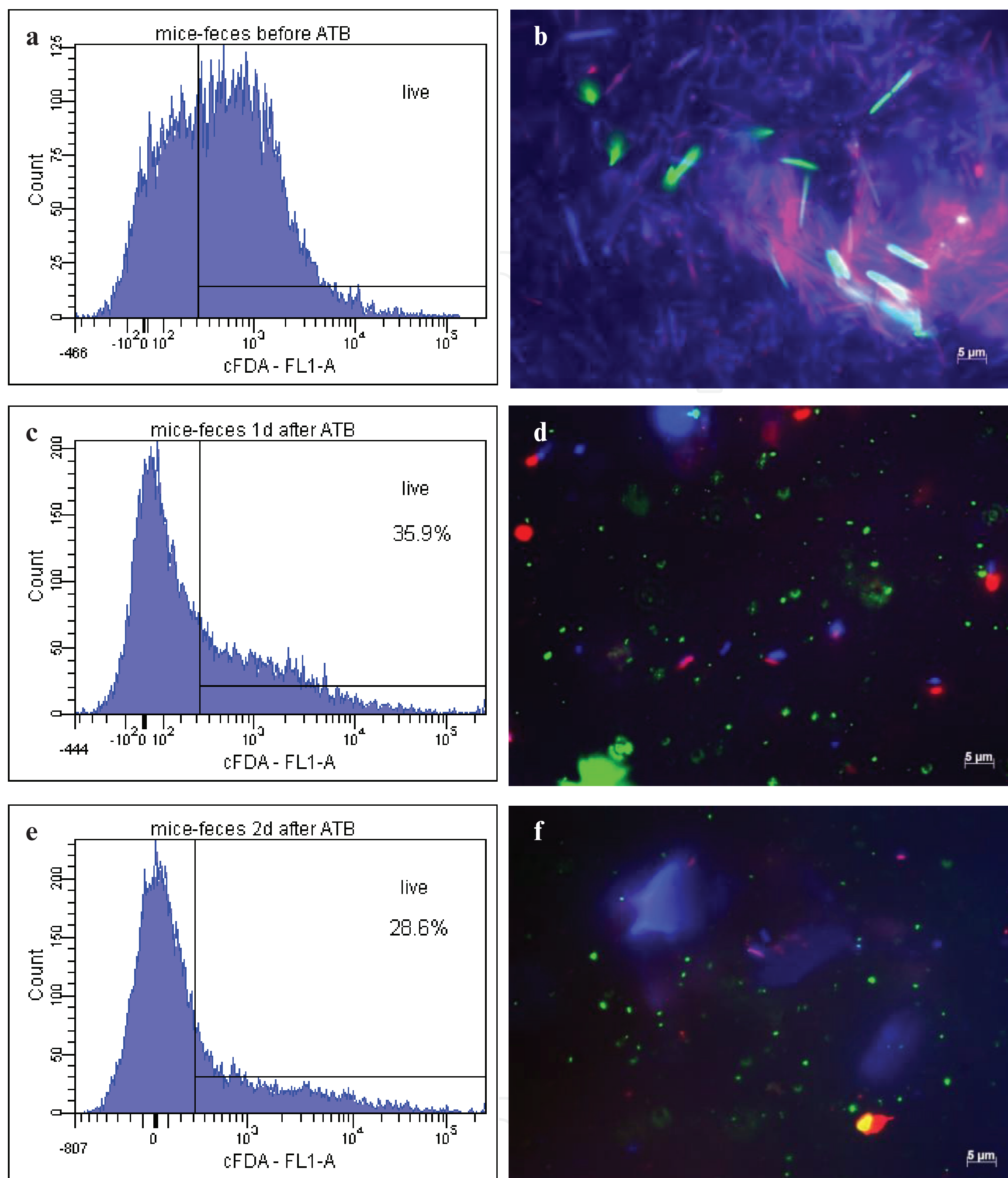

Figure 2. Viability of microorganisms in feces determined by FACS and visualized with VFQTOPF. Live bacteria are *green*. Dead bacteria are *red*. Barely active non-dead are *blue*. **a, b** Mice feces before antibiotic treatment. FACS analysis (**a**) and VFQTOPF visualization (**b**). **c, d** Mice feces on day 1 of the study. FACS analysis (**c**) and VFQTOPF visualization (**d**). **e, f** Mice feces analyzed on day 2. FACS analysis (**e**) and VFQTOPF visualization (**f**).

3.5.3. Cultivable bacteria detected in the study

At day 10 after termination of antibiotic treatment, the microbiota was reduced to two cultivable species. They were differentiated and identified on the basis of morphological, biochemical and genetic differences. The first species isolated from DC + R group was a Gram-negative

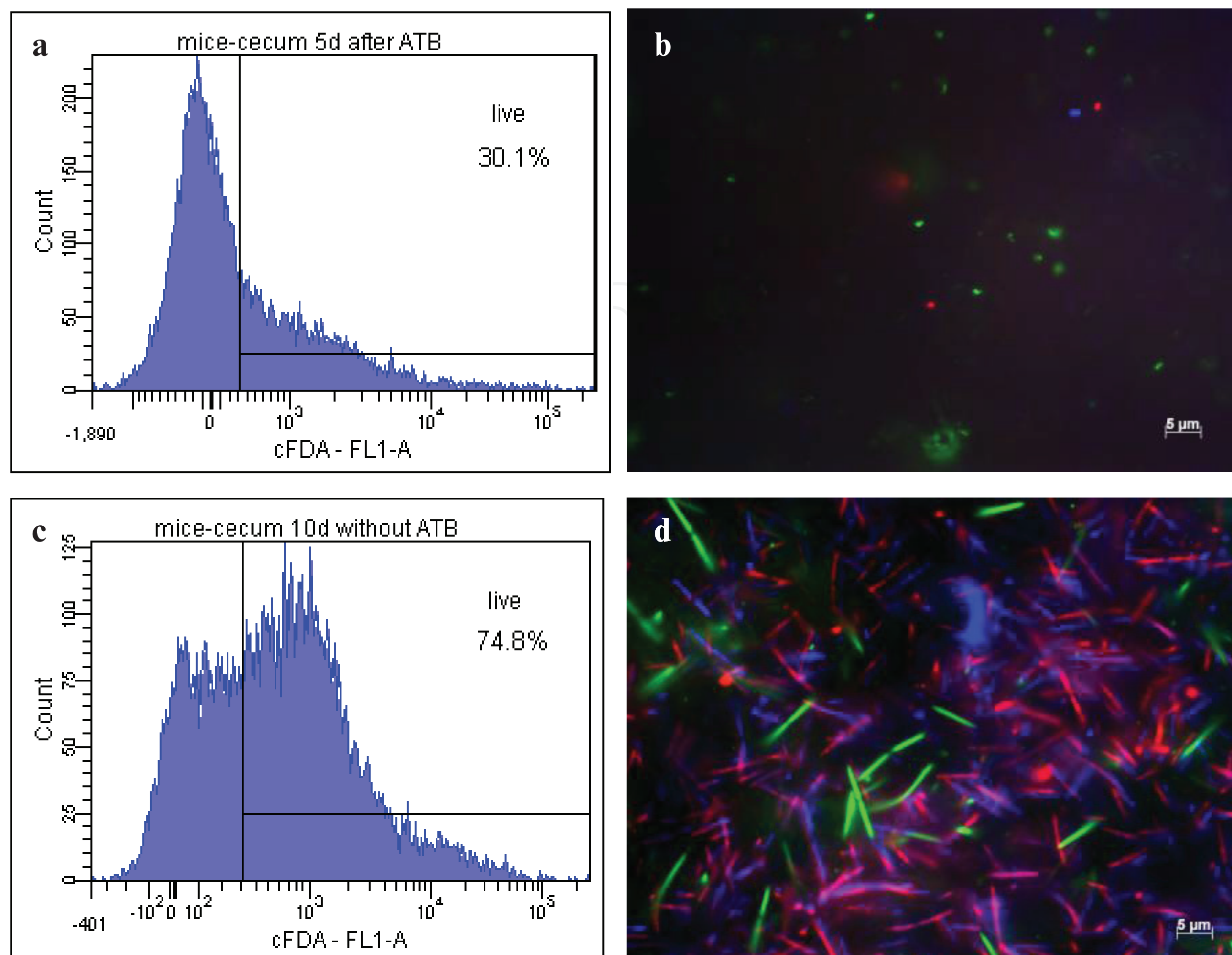

Figure 3. Viability of microorganisms in the caecum determined by FACS and visualized with VFQTOPF. Live bacteria are *green*. Dead bacteria are *red*. Barely active non-dead are *blue*. **a, b** Mice contents of caecum on day 5 of the study. FACS analysis (**a**) and VFQTOPF visualization (**b**). **c, d** Mice contents of caecum 10 days without ATB. FACS analysis (**c**) and VFQTOPF visualization (**d**).

rod-shaped bacterium. Determination of biochemical properties of this bacterium by means commercial ENTEROtest 24 N (Erba Lachema s.r.o., Brno, Czech Republic) showed that this involved species *E. coli* with accuracy ranging between 90.52 and 99.85%. Results of analysis of the DNA section corresponding to the 16S rRNA of bacteria by BLAST analysis and comparison of DNA templates showed that the best match was with *E. coli* (GenBank KU254762.1) species (**Figure 4**). The MIC determined by Etest® strips for ciprofloxacin (AB bioMérieux, Marcy l'Étoile, France) was 0.064 mg/L.

The second species isolated from DC + R group was a Gram-positive coccus. By analyzing the DNA section corresponding to 16S rRNA of bacteria by BLAST analysis and comparing it with DNA templates, the best match obtained indicated *Enterococcus* sp. (GenBank KT630829.1) (**Figure 5**). Determination of the MIC carried out by M.I.C. Evaluator strips for amoxicillin and potassium clavulanate (Thermo Fisher Scientific, Basingstoke, UK) showed that the MIC was equal to 0.25 mg/L.

Figure 4. Assembly of 16S rRNA sequences identified as *Escherichia coli* (GenBank: KU254762.1).

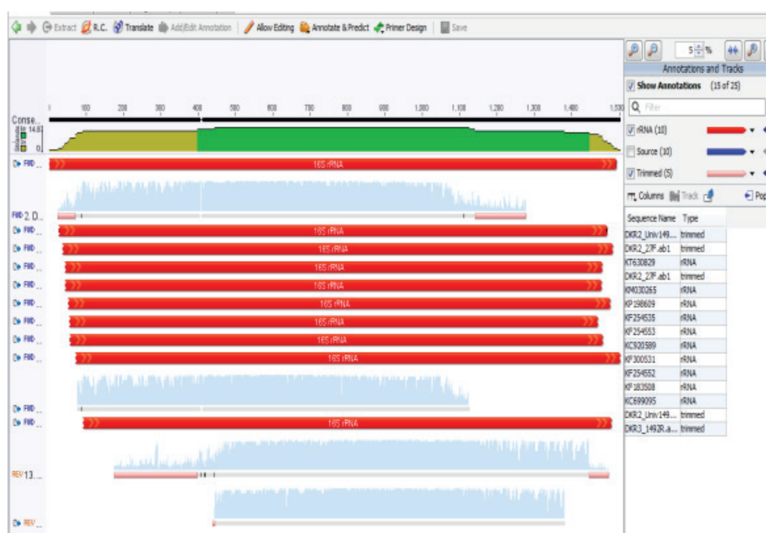

Figure 5. Assembly of 16S rRNA sequences identified as *Enterococcus* sp. (GenBank: KT630829.1).

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3.6. Production of SCFAs in feces and caecum

Production of organic acids (**Figure 6**) in the *caecum* of decontaminated DC group resulted in very low concentrations of these acids in comparison with control group C and group after convalescence (DC + R). The highest concentrations did not exceed the level of 27 mmol/L. *On day 5* of the experiment, examination of the caecum of decontaminated group DC showed a decrease in concentration of all investigated acids (**Figure 6**) with the exception of succinic acid in comparison with group not treated with antibiotics (C). The most pronounced decrease was observed in production of acetic and acetoacetic acids. The decrease in production and resulting concentrations of both acetic and acetoacetic acid was significant (26.97 ± 3.58 mmol/L, $P < 0.01$ and 12.69 ± 1.48 mmol/L,

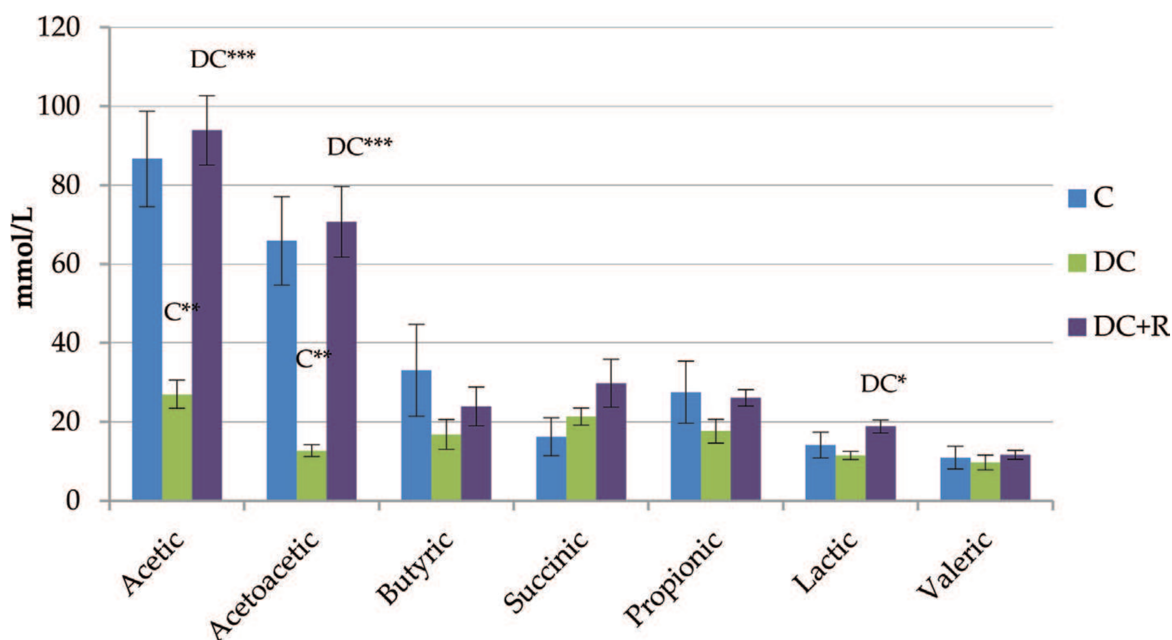

Figure 6. The caecum concentration of organic acids of the BALB/c mice in control C, treated with ATB for 5 days (DC group) and then after 10 days without antibiotic treatment (DC + R group). The results are expressed as the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

$P < 0.01$, respectively) in comparison with concentrations of these acids in control group C (86.65 ± 12.11 and 65.87 ± 11.20 mmol/L, respectively). After termination of treatment with antibiotics (ATB) and *10-day convalescence* period (DC + R), the concentrations of organic acids in cecal contents of mice (**Figure 6**) were higher with the exception of butyric and propionic acids in comparison with both DC group and control group C on day 5 of the experiment. A significant increase in concentration of acids ($P < 0.001$) after convalescence in comparison with 5-day period of treatment with ATB (DC) was recorded for acetic acid and acetoacetic acid (93.90 ± 8.76 and 70.69 ± 8.96 mmol/L, resp.) and in production of lactic acid (18.78 ± 1.66 mmol/L; $P < 0.05$).

Within the 5-day decontamination period, examination of *feces* of mice from group DC (**Figure 7**) showed the most pronounced significant decrease in concentration of *acetic acid* ($P < 0.01$) and *lactic acid* ($P < 0.05$) at 24 h after onset of treatment with ATB. The dynamics of concentration of acetic acid in group DC showed a similar course in the following days of decontamination (days 2–5) with concentrations varying around 40 mmol/L. The differences on days 2 and 5 of treatment with ATB were significant at levels $P < 0.01$ and $P < 0.001$, respectively, in comparison with concentrations before the treatment. In the same period, concentrations of lactic acid in group DC showed a gradual decrease with significant differences on day 2 ($P < 0.01$) and 5 ($P < 0.001$) of treatment, in comparison with concentrations before antibiotic treatment. The dynamics of concentrations of acetic and lactic acids (**Figure 7**) in group DC + R in the above period resembled that observed in group DC but the decrease in concentrations of acids at 24 h after onset of treatment with Antibiotics

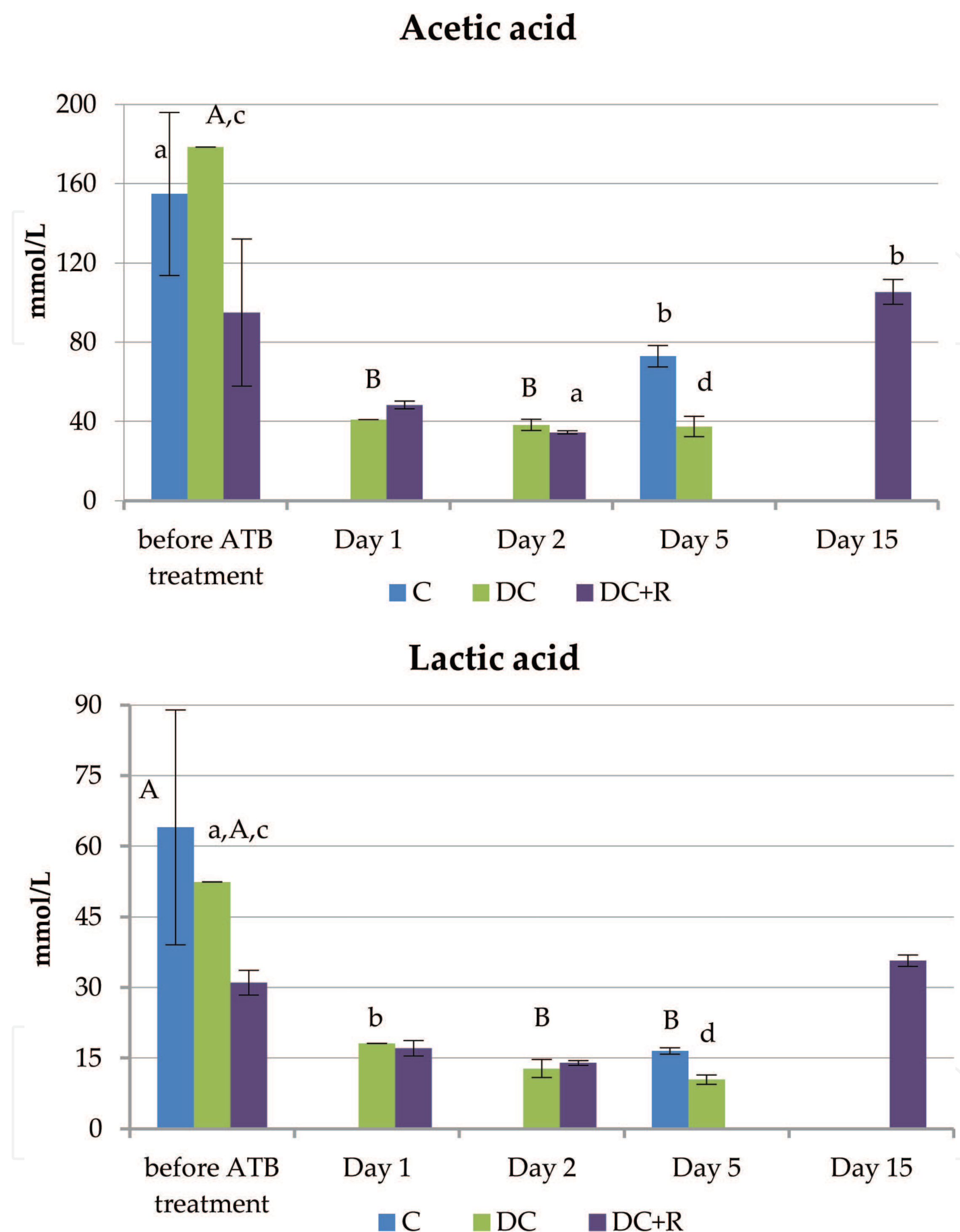

Figure 7. The fecal concentration of acetic and lactic acids of the BALB/c mice in control C, treated with ATB for 5 days (DC group) and then after 10 days without antibiotic (ATB) treatment (DC + R group). The results are expressed as the mean ± SD. $^{a,b}P < 0.05$, $^{A,B}P < 0.01$, $^{c,d}P < 0.001$ (statistical differences within groups).

(ATB) was less pronounced and insignificant. By day 2 of decontamination, both acids reached similar levels as those recorded in group DC (acetic acid 34.52 ± 0.79 mmol/L; lactic acid 3.96 ± 0.50 mmol/L). Concentrations of both acids lactic and acetic in group DC + R returned back to the level observed before treatment only after termination of treatment

with ATB and 10-day convalescence period. The increase in acetic acid was significant ($P < 0.05$; 105.4 ± 6.27 mmol/L) in comparison with day 2 of treatment with ATB.

Despite the fact that control mice (C) were not treated with ATB, they showed a significant decrease in concentrations of acetic and lactic acid ($P < 0.05$; $P < 0.01$) in comparison the level before treatment with ATB, probably as a result of their keeping in gnotobiotic (germ-free) environment and feeding with sterile food and water.

More pronounced although insignificant decrease in *propionic acid* (**Figure 8**) was recorded after 24-h treatment with ATB in feces of mice of both decontaminated groups (DC, DC + R). The level of propionic acid decreased from 54.97 ± 0.01 to 10.2 ± 0.01 mmol/L in group DC and from 60.94 ± 29.34 to 20.47 ± 1.68 mmol/L in group DC + R. In the following period (days 2 and 5 of treatment with ATB), the concentration of this acid in both decontaminated groups was very low and did not exceed 20 mmol/L in group DC and 24 mmol/L in group DC + R. The proportion of propionic acid (**Figure 8**) in feces of mice from group DC + R after convalescence period was insignificantly different (34.99 ± 5.92 mmol/L), and reached only 57.4% of the level determined before antibiotic treatment (60.94 ± 29.34 mmol/L).

Although the concentration of *succinic acid* (**Figure 8**) declined gradually in both decontaminated groups during the period of treatment (days 1–5), it was relatively high particularly at 24 h after treatment with ATB when it reached 34.97 ± 0.01 mmol/L in group DC and 31.14 ± 7.99 mmol/L in group DC + R. On day 5 of the experiment, we recorded in feces of control group C similar decreasing tendency of concentration of succinic acid as that observed for lactic, acetic and propionic acids. The level of succinic acid was significantly lower ($P < 0.05$) in comparison with that observed before the treatment with ATB. In group DC + R after convalescence, we recorded an insignificant increase in succinic acid to the level of 40.70 ± 3.46 mmol/L, which slightly exceeded its concentration from the period before decontamination (34.45 ± 9.13 mmol/L).

While the concentrations of acetic, lactic, succinic and propionic acids in groups DC and DC + R showed a decreasing tendency in the decontamination period (days 2–5) the concentrations of *acetoacetic acid* (**Figure 8**) exhibited an opposite trend. After 24 h of treatment with ATB, group DC showed an insignificant increase in acetoacetic acid from 71.95 ± 0.009 to 122.2 ± 0.01 mmol/L. In the same period, the second decontaminated group DC + R showed an opposite trend, i.e. insignificant decrease in the concentration of acetoacetic acid from 106.0 ± 9.04 to 79.67 ± 0.35 mmol/L. In the following period, concentration of acetoacetic acid decreased significantly in group DC ($P < 0.05$), however, its concentrations were still relatively high and reached the level of 54.73 ± 11.04 mmol/L by day 2 and 72.89 ± 12.50 mmol/L by day 5 of the treatment. Even more pronounced although insignificant increase was observed in group DC + R where concentration of acetoacetic acid reached 120.0 ± 20.04 mmol/L by day 2 of the experiment. High concentration of this acid persisted up to the convalescence period when it reached similar level (99.86 ± 7.106 mmol/ L) as that before treatment with ATB (106.0 ± 9.04 mmol/L).

Concentrations of *butyric acid* (**Figure 9**) in group DC were relatively even up to day 2 of the experiment, ranging from 30.34 to 36.98 mmol/L. By day 5 of the study, they decreased insignificantly down to 22.89 ± 1.51 mmol/L. Except for day 1 of treatment, group DC + R

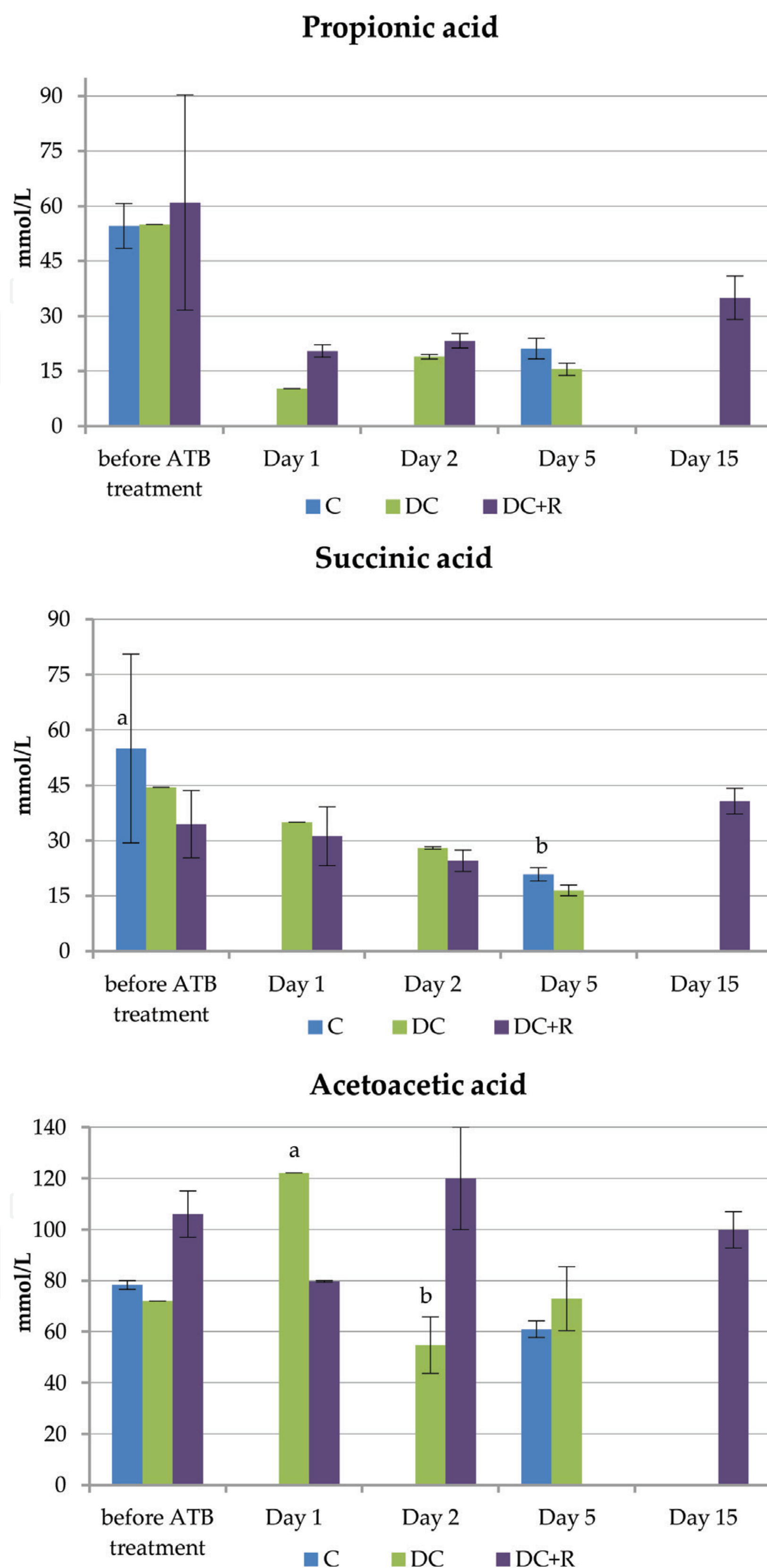

Figure 8. The fecal concentration of propionic, succinic and acetoacetic acids of the BALB/c mice in control C, treated with ATB for 5 days (DC group) and then after 10 days without antibiotic (ATB) treatment (DC + R group). The results are expressed as the mean ± SD. [a,b]$P < 0.05$ (statistical differences within groups).

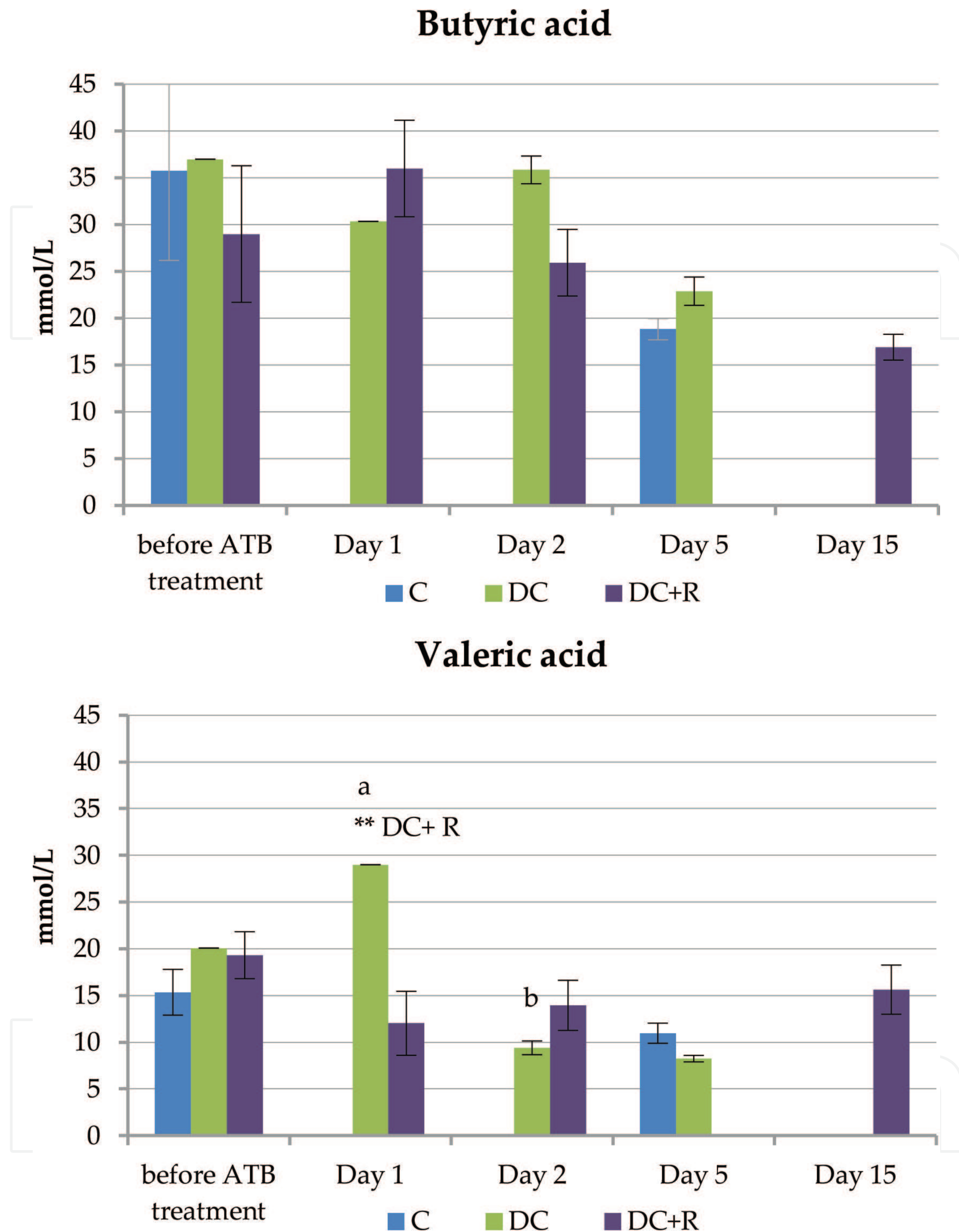

Figure 9. The fecal concentration of butyric and valeric acids of the BALB/c mice in control C, treated with ATB for 5 days (DC group) and then after 10 days without antibiotic (ATB) treatment (DC + R group). The results are expressed as the mean ± SD. $^{a,b}P < 0.05$ (statistical differences within groups). $^{**}P < 0.01$ (statistical differences between groups).

showed an insignificant increase in butyric acid, which persisted up to the end of the experiment. Concentration of this acid on day 15 of the study reached 58.5% of production recorded in the period before treatment with ATB.

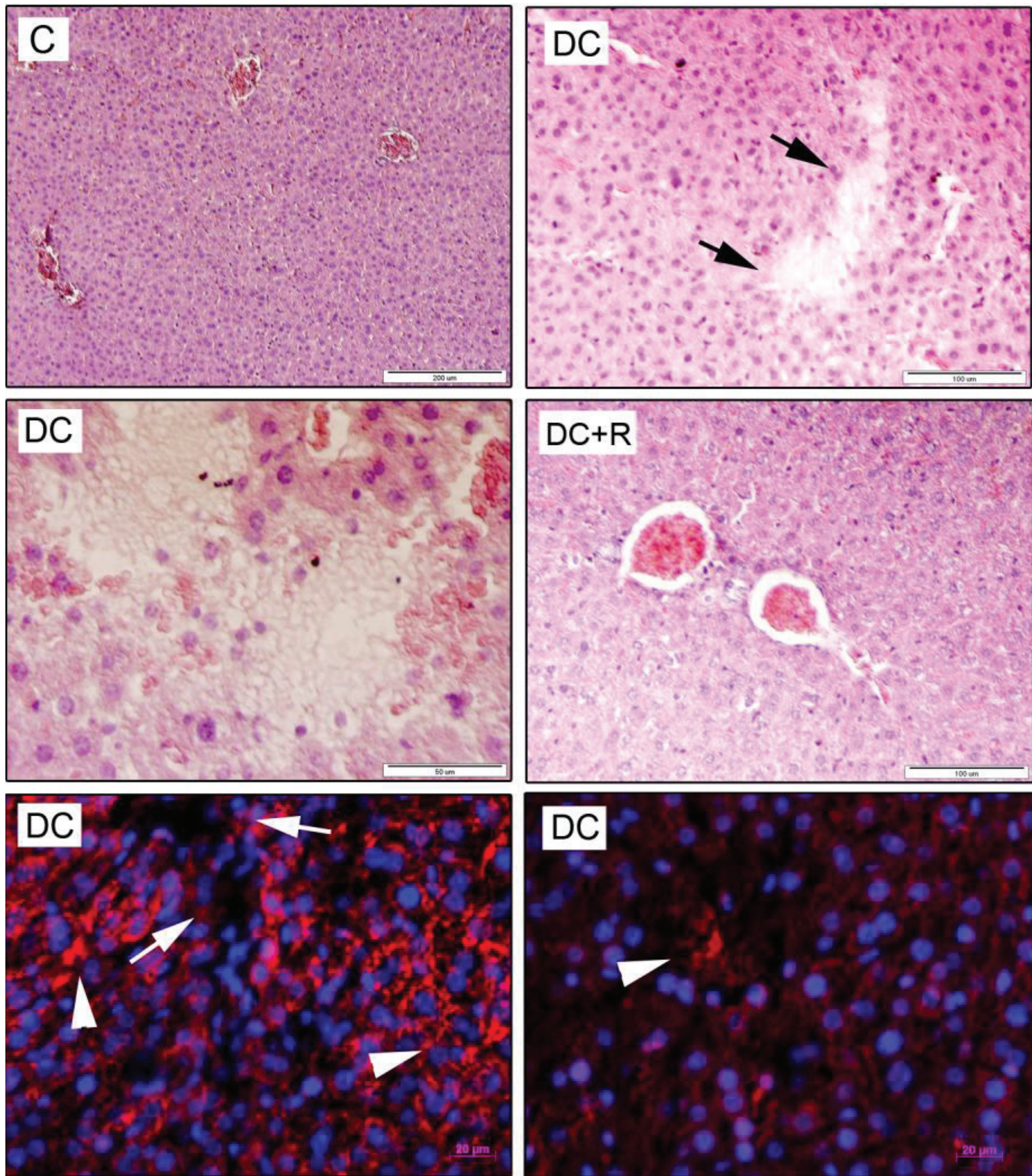

Figure 10. Representative microphotographs of the liver sections from control untreated mice (C), mice treated with antibiotics (group DC) and antibiotics-treated group after a period of recovery (DC + R). Upper panel formed of four images was prepared with light microscope on paraffin sections stained with hematoxylin/eosin. Lower panel formed of two images was prepared with fluorescent microscope on paraffin sections stained with Nile red (lipids showed in red) and Hoechst 33342 stains (nuclei showed in blue). In the livers from DC group, the sporadic occurrence of lesions (arrows) with advanced vacuolization containing a few, usually necrotic, hepatocytes and disrupted sinusoids (arrowheads) was observed. In this group, fluorescent stains demonstrated the presence of lipids droplets in some hepatocytes (arrowheads) and in the lesions (arrows) as well as the absence of fragmented apoptotic nuclei of hepatocytes and other cells. The representative microphotograph of the liver from DC + R group showed normal tissue morphology without any histopathological changes.

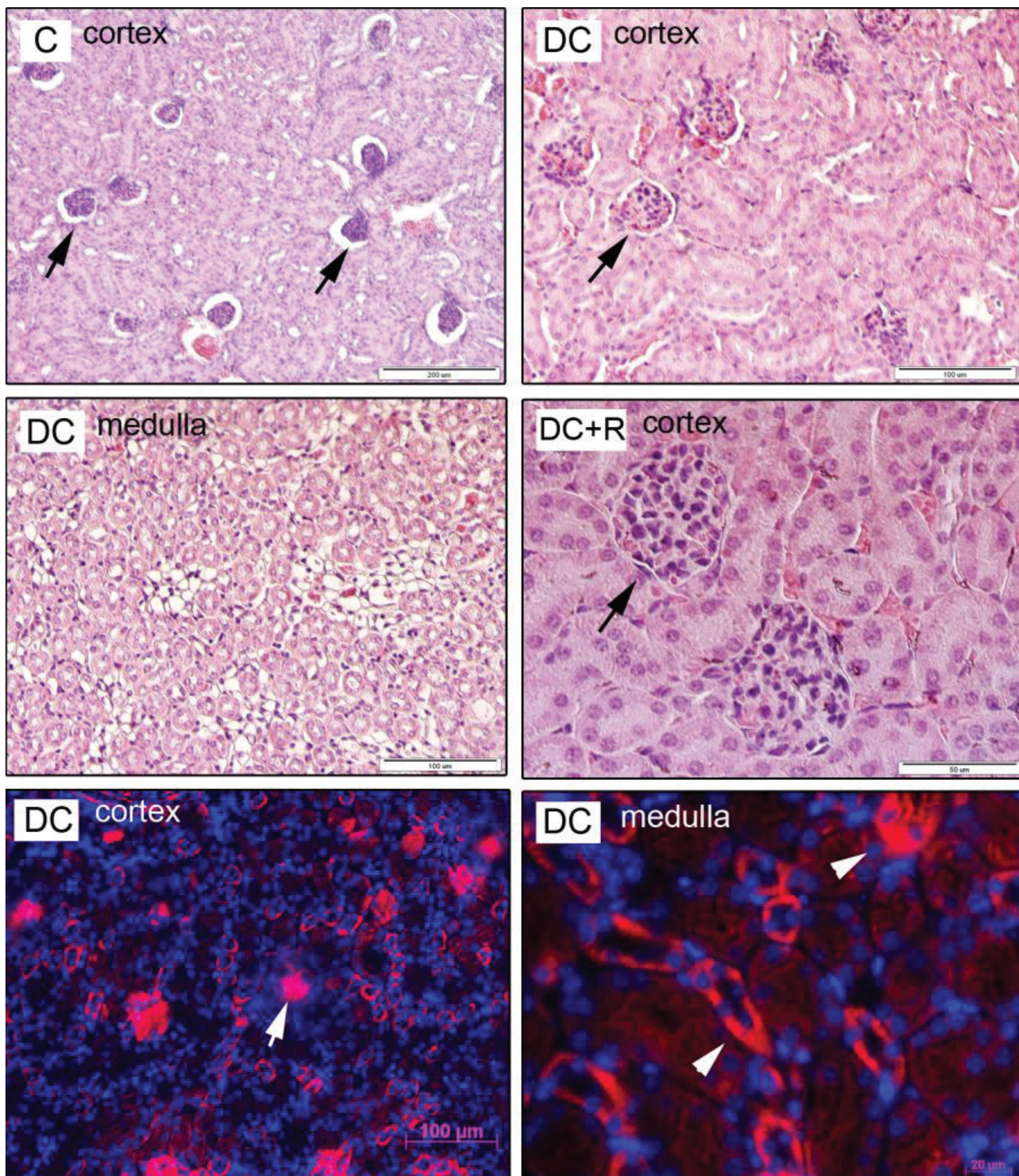

Figure 11. Representative microphotographs of the kidney sections from control untreated mice (C), mice treated with antibiotics (group DC) and antibiotics-treated group after a period of recovery (DC + R). Upper panel formed of four images was prepared with light microscope on paraffin sections stained with hematoxylin/eosin. Lower panel formed of two images was prepared with fluorescent microscope on paraffin sections stained with Nile red (lipids showed in red) and Hoechst 33342 stains (nuclei showed in blue). Normal morphology of the cortex of kidney from control group (C) showing multiple renal corpuscles consisting of the glomerulus and the surrounding capsule (arrows). In DC group, the overall morphology of cortex, appearance of these Bowman's capsules as well as morphology of central medullar part did not show any pathological alterations or damage to cells. A representative image of kidney´s cortex from DC + R group showed normal morphology. Images of DC group showing positive signal for neutral lipids droplets in the cortex (left, arrows) and in some of renal cells in medulla of kidneys (right, arrowheads). No apoptotic process in kidney cells was seen in either of examined groups.

The concentration of *valeric acid* in group DC (**Figure 9**) on day 1 of treatment with antibiotics (ATB) showed a similar increase as that observed for acetoacetic acid. The difference compared to group DC + R was significant ($P < 0.01$). Subsequently, a significant decrease ($P < 0.05$) in concentration of butyric acid was observed by day 2 of the experiment in comparison with day 1 of treatment with ATB. Similar increased concentrations of this acid persisted by day 5 after treatment with ATB. The group that convalesced after treatment (DC + R) showed an insignificant decrease in production of valeric acid after 24 h of treatment with ATB. In the subsequent period, the level of this acid did not exceed 16 mmol/L.

3.7. Histological examinations of livers and kidneys

The liver and kidney cells are highly sensitive to harmful effects of xenobiotics including antibiotics; therefore, we examined histomorphology of the livers and kidneys from control mice without treatment (C), from treated mice (DC) and from treated group of mice after a period of recovery (DC + R). Light microscopy revealed that liver sections from control mice showed normal liver architecture and hepatocytes were arranged in rows radiating out from central veins (**Figure 10**). In the livers from DC group, we observed sporadic occurrence of lesions (arrows) with advanced vacuolization containing a few, usually necrotic, hepatocytes and disrupted sinusoids (arrowheads). Such altered or loose liver parenchyma indicated an early metabolic injury to the cells. In this group, fluorescent staining specific for neutral lipids demonstrated the presence of lipids droplets in some hepatocytes (arrowheads) and in these lesions (arrows). However, we did not find the fragmented nuclei of hepatocytes and other cells indicating that treatment did not elicit apoptosis. The representative microphotograph of the liver from DC + R group showed normal tissue morphology without any histopathological changes.

The representative **Figure 11 (C)** of paraffin section after hematoxylin/eosin staining of cortex from untreated group demonstrates multiple renal corpuscles consisting of the glomerulus and the capsule around it (arrows). In DC group, the overall morphology of cortex and appearance of these Bowman's capsules did not show any pathological alterations or damage to cells. Central medullar part of kidneys from DC group had the same morphology as was observed on sections from control mice (not shown). A representative image of kidney's cortex from DC + R group (**Figure 11**) showed normal morphology. Using the fluorescent double staining methods we demonstrated the positive signal for neutral lipids droplets in the cortex (left, arrows) and in some of renal cells in medulla of kidneys (right, arrowheads) in all groups. No apoptotic process in kidney cells was seen in either of examined groups.

4. Discussion

Animal gut microbiota is a complex community of trillions of microbes colonizing the digestive tract of animals. This extensive community, comprising as many as 10^{12} colony-forming

units/mL in the colon, affects physiology of the gastrointestinal tract, the function of distant organs and susceptibility of animals to diseases [37]. Despite the enormous bacterial load carried by the gastrointestinal tract and the sheer variety of species present, an exquisite balance is maintained at almost all times. The combination of an efficient, self-repairing barrier, abundant mucus secretion, continuous luminal flow of contents and a vigorous yet finely regulated immune system is capable of keeping a massive foreign population contained within the limits of the mucosa [34]. This delicate equilibrium represents a well-balanced opposition of considerable forces. Alteration of this equilibrium is pivotal in the development of diseases of gastrointestinal tract.

Laboratory animals such as germ-free (GF) rodents have proved important for studying the effects of microbial mono- and poly-colonizations on host phenotype [38–40] and in the search for a mechanistic understanding of microbe-mediated changes in several disease models [41–45]. An alternative is temporary gut sterilization, which may involve absolute or selective elimination of microflora [27, 28]. The first studies devoted to decontamination of the digestive tract by ATB investigated successfulness of such decontamination and removal of microorganisms from the animal digestive tract. Results indicate that decontamination of mice [46], monkeys [47], dogs [48], Syrian hamsters [49] and pigs [50] with oral antibiotics is feasible. However, these studies did not investigate the effect of ATB on animal health. In human medicine, the beginnings of decontamination of digestive tract were related to prevention of septicemia in patients with granulopenia [51], in studies of burns therapy [52], acute pancreatitis [53], and later in acute stroke [54], critically ill patients [55] or esophageal resection [56] and prevention of acute graft-versus-host disease following allogeneic bone marrow transplantation [57]. Selective antibiotic treatment resulting in decontamination of the digestive tract was capable of preventing severe infections and reducing mortality rate in patients in the critical stages of diseases. Concern about development of bacterial resistance associated with the use of such decontamination and the absence of its influence on mortality, have not been confirmed [58]. The aim of SDD (Selective Digestive Decontamination) is to prevent or eradicate, if present, the oropharyngeal and intestinal abnormal carriage of potentially pathogenic microorganisms, such as Gram-negative aerobic microorganisms, methicillin-sensitive *Staphylococcus aureus* and yeasts [58, 59].

Various antibiotic cocktails have been shown to completely or selectively sterilize the gastrointestinal tracts of mice and rats [31, 32, 60]. Our study was aimed at decontamination of BALB/c SPF mice in a way that would not have adverse effect on their health. Similar to Johnson et al. [27], we strived to develop a non-invasive, relatively simple and inexpensive method of decontamination of the gut, testing for the sterility and maintaining controlled microbiota in model animals suitable for further experiments. In the study by Johnson et al. [27] animals were decontaminated and sterile environment in their gastrointestinal tract was maintained by enrofloxacin in Baytril 10% (Bayer, Germany) without barrier maintenance or using a laminar box. In other studies, the decontamination of gastrointestinal tract was carried out using ampicillin [61–63], bacitracin and neomycin [39], meropenem

[64, 65] and vancomycin [66] added to the drinking water. On the basis of our previous results [67], mice in our study were decontaminated with amoxicillin administered *per os*, potentiated with potassium clavulanate at a dose of 387.11 mg/kg in the form of preparation Amoksiklav (Sandoz, Slovenia) and subcutaneously administered ciprofloxacin at a dose of 18.87 mg/kg as a preparation Ciloxan (Alcon, Spain), while keeping the animals in strictly defined environment of gnotobiotic isolators. The administered doses were considerably lower than lethal doses (LD_{50}) of the selected ATB to mice. In mice the LD_{50} of amoxicillin potentiated with clavulanic acid was found to be 4526 mg/kg and of ciprofloxacin 5000 mg/kg. This means that the dosage of ATBs used in our study were lower 11.7-fold with amoxicillin and 265-fold with ciprofloxacin than the respective LD_{50} doses. In the case of ciprofloxacin, such a low dose was selected due to subcutaneous route of its administration and high nephrotoxicity associated with this ATB, which, however, was not manifested at the low dosage used in our study. While in our study we used a combination of *per os* and subcutaneous administration of the ATBs, the other studies used intragastric gavage [68–70], administration and withdrawal of antibiotics in drinking water [33, 71–73], or administration in food and water provided ad libitum [27]. The study by Donskey et al. [74] was also based on subcutaneous administration of ciprofloxacin.

Some research studies were conducted dealing with the comparison of antibiotic decontamination carried out on the basis of cultivation and studies based on commonly used antibiotic combinations. They included the clinical study E.O.R.T.C. [75], which investigated the effect of ATB selected on the basis of cultivation and compared it with the effect of combination of neomycin, cephaloridine, polymyxin (B or E) and nystatin or amphotericin B in granulocytopenic patients. Comparisons indicated good effectiveness of both methods and the differences were insignificant. However, it is worth mentioning that only non-absorbable ATB were used in the E.O.R.T.C. [75] study. In our study, we used the ATB selected on the basis of cultivation, as recommended by Johnson et al. [27] with the aim to eliminate the ATB with marked adverse impacts on animal health.

The length of antibiotic administration in the available studies differed. In our study, we administered ATB for 5 days. This was based on preliminary examinations and procedures carried out at our institution that showed null cultivation recovery of bacteria from feces on day 3 of antibiotic administration. Van der Waaij et al. [50] arrived to similar conclusions while the length of administration of ATB in other studies varied as follows: 4 days [27, 76], 7 days [71], 14 days [68], 21 days [77] or 28 days [73, 78–80]. While in our study the DC + R group of animals was kept under gnotobiotic conditions for 10 days following the antibiotic administration, in other studies, the mice convalescence period lasted from 14 days [71, 77] up to 5 weeks [68].

Following the 10-day convalescence period, the cultivable colonies obtained from feces and caecum content of SPF mice were tested biochemically and subjected to 16S ribosomal DNA (rDNA) sequencing that allowed us to identify *E. coli* and *Enterococcus* species. Puhl et al. [77] administered ATB for 21 days and after 14-day convalescence were able to identify by

sequencing only limited number of *Clostridium*-like and *Bacteroides* species. Ubeda et al. [71] detected an increased bacterial density 2 weeks after cessation of 7-day antibiotic treatment with ampicillin, vancomycin or combination of metronidazole, neomycin and vancomycin (MNV). They observed decreased frequencies of microbiota native to the *Bacteroidetes* phylum and the *Lactobacillaceae* family and increased frequencies of bacteria associated with the *Clostridium* and *Enterococcus* genera and *Enterobacteriaceae* family. In the study by Ubeda et al. [71], the effect of antibiotics on microbial density was investigated by quantitative PCR (qPCR) of bacterial 16S rRNA genes. The results showed that all three tested antibiotic regimens caused a decrease in the number of 16S rDNA copies in the ileum by a factor of approximately 100, whereas consistent reduction of bacterial density in the caecum was achieved only by ampicillin. Tenfold reduction in the quantity of 16S genes was observed in four of six mice treated with vancomycin and three of six mice that were administered MNV. Similar results were obtained by [81] by analyzing samples of feces after using a combination of four antibiotics (vancomycin, neomycin, metronidazole and amphotericin). By day 13, as many as 86% of the mice subjected to antibiotic treatment exhibited successful depletion of their cultivable aerobic and anaerobic fecal microbiota. The corresponding fraction determined on day 24 was 74%. Thus, a minimum of 100-fold reduction of cultivable aerobic Gram-negative rods and 106-fold reduction of cultivable aerobic Gram-positive cocci as well as cultivable anaerobic fecal bacteria was detected in depleted mice (1 CFU/mg feces). The authors observed significantly reduced copy number of 16S rRNA genes in feces of all mice subjected to the depletion protocol: all samples collected from mice treated with antibiotics displayed similar level of bacterial DNA that was, on average, more than 400-fold lower in comparison with untreated mice. In the study by Ge et al. [73], after 4 weeks of antibiotic treatment (ampicillin, neomycin sulfate, metronidazole, and vancomycin), the average number of operational taxonomic units (OTU) of mice decreased significantly from 383.4 ± 23.4 to 74.9 ± 3.1 ($P < 0.01$). The antibiotics resulted in changes in the composition of commensal bacteria examined by 16S rRNA analysis. At phylum level, only Proteobacteria accounted for more than 0.5% of all the microbiota in antibiotic-treated mice. Our study showed that a decrease in plate counts of microorganisms occurred 24 h after the antibiotic treatment. By this, time the bacterial counts decreased by 4 logs under aerobic conditions and by 3–4 logs at anaerobic cultivation. In the study by Ubeda et al. [71] bacterial density in the caecum increased after antibiotic cessation. This was in an agreement with the results of our study. Ubeda et al. [71] observed that the *Enterobacteriaceae* operational taxonomic units that predominated in antibiotic-treated mice were also present in the ileum wall of some of the untreated mice. This observation suggests that the ileum wall may be the source of the *Enterobacteriaceae* that had increased after antibiotic treatment. Stecher et al. [82] reported that the recovery of the normal microbiota, as measured at the phylum level, occurred 5 days after termination of treatment with streptomycin. Yuan et al. [68] demonstrated that neonatal amoxicillin treatment affected significantly the biodiversity of the murine intestinal *Lactobacillus* community and the impact was long lasting. In agreement with previous studies [71, 83], it seems reasonable to assume that some bacterial populations do not recover after antibiotic withdrawal.

The ciprofloxacin MIC against *E. coli* (GenBank KX086704), determined in our study by Etest® strips, was 0.064 mg/L. According to EUCAST [84], the MIC breakpoint for *E. coli* is ≤1.0 mg/L; therefore, we can assume absence of ciprofloxacin-resistant bacteria. This resembles observations of Bergan et al. [85] who studied the pharmacokinetics of ciprofloxacin in 12 volunteers, given 500 mg of ciprofloxacin orally twice a day for 5-days. In their study, counts of enterobacteria and enterococci in feces decreased markedly, whereas no marked changes were observed in anaerobic flora (anaerobic cocci, fusobacteria and bacteroids). Fourteen days after termination of drug treatment, the salivary and fecal microbiota returned to normal. The amoxicillin and potassium clavulanate MIC against our second cultured strain, specifically *Enterococcus* sp. (GenBank KX086705), determined by M.I.C. Evaluator strips was 0.25 mg/L. According to EUCAST breakpoint table for bacteria [84], the MIC breakpoint of amoxicillin-clavulanic acid for *Enterococcus* spp. is ≤2.0 mg/L. Although the bacteria recovered in our study were not resistant to the relevant antibiotics, they may have been inactivated as reported by Van der Waaij and Nord [86]. The ATB effective doses may be reduced to various degrees by enzymatic activity, or non-enzymatically by intestinal contents. Such reduction may be dependent on individual differences in microbiota and pharmacokinetic properties of the respective antibiotics [86].

Flow cytometry results obtained in our study showed a decrease in viability of microorganisms in feces. The differences were significant ($P < 0.01$) between days 1 (36.03%) and 2 (28.33%) following the antibiotic treatment and survival rates before the treatment (60.58%). Very similar method based on BacLight™ Live/Dead Viability Kit was used by Johnson et al. [27]. The authors investigated antibiotic inactivation by determination of bacterial viability in feces employing fluorescence staining of samples. Before antibiotic treatment, the mean proportion of live bacteria found in the feces of one mouse (expressed as a percentage of the total bacterial cells present) was 13.86%. By antibiotic treatment, this proportion was reduced to 0.17%. The corresponding values for the second mouse reached 13.37% before and 0.15% after the treatment. In both animals, the treatment with Baytril caused a significant reduction in viability of bacterial cells in feces to less than 1% of the originally determined values ($P < 0.05$ and $P < 0.05$).

Short chain fatty acids are the principal metabolites of intestinal fermentation and their concentrations in the digestive tract reflect the level of this fermentation. The most pronounced decrease in production of organic acids, particularly acetic, lactic and propionic acids in feces of both decontaminated groups (DC, DC + R), was recorded as soon as 24 h after starting with administration of ATB, which correlates with decreased plate counts of microorganisms in these groups by 4 logs after aerobic cultivation and 3–4 logs after anaerobic cultivation. Also during the following days of administration of ATB (days 2–5), low level of intestinal fermentation was detected in the feces of decontaminated mice. Eleven days lasting antibiotic treatment (ampicillin, bacitracin, meropenem, neomycin and vancomycin) caused marked changes in colon microbiota and gut dysbiosis was reflected in changed concentrations of several metabolites in the colon luminal contents [87]. The depletion of the SCFAs acetate, n-butyrate and propionate, the products of microbial fermentation of dietary fiber, agreed with results presented in other studies [88–90]. In our

study, we observed high concentrations of acetoacetic and butyric acids, the products of biodegradation of lipid tissue. Keto compounds that formed at physiological state of passive degradation of lipid stores became a substitute source of energy for normal functioning of the organism at the time of energy starvation. However, by day 15 of the study, the intestinal fermentation activity was restored in group DC + R and production of organic acids returned to the level before the treatment with ATB. An interesting observation was that concentration of organic acids decreased also in the feces of control group of mice C that was not treated with ATB. This decrease could be explained by the fact that these animals were fed sterile commercial feed, supplied sterile water in bottles and sterile bedding was replaced every day.

The macroscopic picture of all decontaminated mice was typical of germ-free animals, such as megacaecum condition and a significant decrease in relative weight of the liver ($P < 0.05$) and spleen ($P < 0.01$) in decontaminated group DC in comparison with control group C. Due to complete or partial absence of microbiota, we can find some typical morphological peculiarities in the digestion tract of germ-free and gnotobiotic animals. Distinctive features of germ-free rodents are considerably thinner intestinal mucosa and enormously enlarged caecum [20] the weight of which may be 10-fold greater than the physiological one. These morphological properties of the small intestine of germ-free animals are a consequence of the absence of both immunological stimuli induced by digested bacterial antigens and the potentiating influence of bacteria that affect the level of extrusion of cells from the tip of the villi.

The potential effect of antibiotic decontamination of mice on overall health of treated animals was investigated only in small number of relevant studies. Our study showed only a slight change in the blood picture of mice from DC group in comparison with group C. Moreover, after the convalescence, all parameters determined in group DC + R returned to the physiological range [91]. The mice from DC group showed increased levels of Mo ($P < 0.05$), as well as percentage proportion of Mo % ($P < 0.01$) and Gran % ($P < 0.05$). This may be associated with intense metabolic load on the liver during the ATB breakdown. Simultaneously, the levels of RBC, HGB and HCT were increased in DC group, possibly due to reduced water intake by decontaminated animals. As the liver is the main organ involved in detoxification of various xenobiotics introduced from the external environment, it plays the principal role also in the breakdown of ATB. For this reason, we selected this organ as a reliable indicator of overall health of the tested SPF mice. Our determinations focused on the activity of hepatic enzymes and histology of the liver parenchyma. We observed an increase in the activity of enzymes AST and ALT in DC group. Results obtained after 10-day convalescence period showed a significantly higher ($P < 0.01$) activity of ALT enzyme in this group exceeding twice the upper physiological limit [91]. Although no necrosis or reversible and irreversible damage to the liver parenchyma was observed, the increased level of ALT was associated with disturbances of liver cell membranes. An increase in ALT up to 3-fold the reference value [91] is referred to as moderate and higher than this level as marked. The decontaminated group of animals (DC) exhibited also increase in specific liver enzyme LDH-5. This enzyme catalyzes reversible conversion of lactate to pyruvate and is found in

the circulation already at minimum tissue damage. The above results correspond to histological findings in group DC, which showed structural alterations (presence of necrotizing hepatocytes, vacuolization and damage to sinusoids, multinuclear cells and lipid infiltration) in comparison with group C. However, after the convalescence period, the activity of isoenzyme LDH-5 returned to the level recorded before treatment with ATB. In our study, the activity of ALP enzyme showed no augmentation in any of the investigated groups. Marked elevation of serum ALP levels is characteristic for cholestatic hepatotoxicity [92]. Substances known to lead to this type of injury include amoxicillin/clavulanate and chlorpromazine. Cholestatic hepatotoxicity rarely progresses to the stage of chronic damage to the liver and gradual destruction of intrahepatic biliary tract [92]. In our study, we used amoxicillin and potassium clavulanate but no signs of development of cholestatic hepatotoxicity were not observed. Instead, mice from DC group showed signs of fatty liver, a reversible condition also known as fatty liver disease (FLD). Impaired metabolism of fatty acids results in accumulation of triglycerides that form nonmembrane-bound vacuoles in cells. These vacuoles may displace the nucleus from its usual location [93]. By day 10 of convalescence, the majority of hematological and biochemical parameters in group DC + R returned back to the physiological range. With respect to biochemical parameters, we observed an increased activity of enzyme ALT, hyperbilirubinemia and increased level of LDL-cholesterol indicating irritation of hepatic cells, however, the structure of liver tissue showed no marked changes. An interesting observation was that of isoenzyme LDH-3 in group convalesced for 10 days (DC + R) was increased by 20% in comparison with decontaminated group DC. As this is a pulmonary isoenzyme, its increased activity is associated with damage to pulmonary parenchyma. During the experiment, the mice from group DC + R were kept 3-fold longer in a gnotobiotic isolator with active ventilation and ventilation was probably the main cause of the change.

Animals obtained under this protocol can be used in our further studies such as nutritionally important relationship between the intestinal microflora and the host, interactions between microorganisms in the gut or modulation of metabolic and physiological parameters of host with selected probiotics.

In conclusion, decontamination of SPF BALB/c mice with combination of per oral administration of amoxicillin and clavulanate potassium and subcutaneous administration of ciprofloxacin every 12 h during 5 days reduced viability of microorganisms in feces and caecum content and resulted in absence of cultivable microorganisms in feces. After 10-day convalescence of antibiotic-treated SPF mice under gnotobiotic conditions the diversity of gut microbiota of mice was not recovered as it was reduced to only two detectable cultivable species, specifically to *E. coli* (GenBank KX086704) and *Enterococcus* sp. (GenBank KX086705), that returned to metabolic and morphological values within the physiological range. Finally, a mouse gnoto-model with reduced and controlled microflora was created without evident alteration of the overall health status. The animals obtained under this protocol can be used in further studies dealing with nutritionally important relationship between the intestinal microflora and the host, interactions between microorganisms in the gut, or modulation of metabolic and physiological parameters of the host using selected probiotics.

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