Influence of Different Probiotic Lactic Acid Bacteria on Microbiota and Metabolism of Rats with Dysbiosis

Elena ERMOLENKO, Ludmila GROMOVA, Yuri BORSCHEV, Anna VOEIKOVA, Alena KARASEVA, Konstantin ERMOLENKO, Andrei GRUZDKOV and Alexander SUVOROV

1 Department of Molecular Microbiology, Institute of Experimental Medicine, acad. Pavlov str. 12, Saint Petersburg 197376, Russia
2 Department of Physiology of Nutrition, Pavlov Institute of Physiology, Makarova emb. 6, Saint Petersburg 199034, Russia
3 Laboratory of Immunology, Pasteur Institute of Epidemiology and Microbiology, Mira str. 16, Saint Petersburg 197376, Russia

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Lactic acid bacteria (LAB) are often used for prevention and treatment of dysbiosis. However, the action of various strains of LAB on metabolism and digestion under these conditions are poorly understood. The purpose of this study was to investigate the influence of probiotic LAB on metabolism, digestion and microbiota in animals with dysbiosis. After administration of ampicillin and metronidazole male Wistar rats, were fed products containing Enterococcus faecium (E.f.), Lactobacillus fermentum (L.f.) or milk (control 1). Animals in control group 2 were fed milk, after water instead of antibiotics. Dyspeptic symptoms disappeared after administration of probiotic compared with control 1. At the end of the experiment, an increase in the content of enterococci and lactobacilli in the proximal part of the small intestine was found in the animals treated with E.f. and L.f., respectively. After the introduction of probiotic enterococci, the quantity of lactobacilli and bifidobacteria in the intestines of rats increased, and the content of Klebsiella spp. and Escherichia coli decreased in comparison with the control group 1 and the group fed lactobacilli. The activity of alkaline phosphatase and aspartate transaminase was greater in blood serum of rats with dysbiosis receiving milk and lactobacilli. Intestinal alkaline phosphatase activity increased in the epithelium and chyme in the jejunum of the animals treated with L. f. and in the chyme only in the animals treated with E. f. Thus, the specific effects of different strains of probiotic LAB on the microbiota, and on metabolism and digestion of various nutrients were demonstrated.

Key words: dysbiosis, lactobacilli, enterococci, metabolism

INTRODUCTION

Probiotics, live microorganisms with beneficial effects for the host, are widely applied in gastrointestinal and liver diseases [1–3]. Therapy with probiotic lactic acid bacteria (LAB) is based on reduction or elimination of the pathogens and toxins. In addition, probiotic LAB can modulate the immune defense mechanisms and influence metabolic processes and digestion via the normalization of altered gut flora [4–6]. On the other hand, it is known that LAB differ with regard to production of antimicrobial compounds [4, 7], the ability to colonize the intestinal mucosa [4, 8], resistance to the action of bile and pH [9], effects on the immune system [3, 10–12], metabolism of fats [13–15], carbohydrates [16–18] and minerals [19] and function of the mucus of the gastrointestinal tract of mammals [3, 12]. Despite the successful usage of probiotics for the treatment of dysbiosis in human and veterinary medicine [1, 5] the mechanism of action of various strains of LAB on microbiota, metabolism and digestion is poorly understood.

Based on available research and clinical data, it is believed there are several causes of intestinal dysbiosis: 1. putrefaction (the result of changes in diet); 2. fermentation dysbiosis resulting from inefficient host digestion; 3. deficiency dysbiosis, which is often caused by antibiotic exposure; 4. sensitization dysbiosis which is the result of abnormal immune responses caused by an alteration of the normal intestinal flora [20]; and 5. psychological and physical stress [21]. Regardless of the possible causes of a dysbiotic condition, in practical situations in human and veterinary medicine, it is most often caused by antibiotic treatment, which leads to a deficiency of normal intestinal flora and overgrowth of opportunistic bacteria [22].

We previously used an experimental model of intestinal dysbiosis [23] and showed that the short-term (for 3 days) consumption of ampicillin and metronidazole caused significant changes in intestinal microbiota. Microbiologically, an increase in the numbers of putative opportunistic bacteria and decrease in concentrations...
of *Bifidobacterium* spp., *Lactobacillus* spp., and *Enterococcus* spp. in the intestines were determined. During administration of these antibiotics, the symptoms of intestinal dysbiosis were observed, and the activity of certain liver enzymes and alkaline phosphatase were elevated in the blood serum.

The purpose of present study was to investigate the influence of two different probiotic LAB, *Lactobacillus fermentum* Z and *Enterococcus faecium* L3, on physical condition, intestinal microbiota and activity of alkaline phosphatase and biochemical parameters of blood serum in rats with dysbiosis caused by administration of antibiotics.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions**

Probiotic strains *Enterococcus faecium* L3 (*E.f*), originally isolated from milk fermented products and *Lactobacillus fermentum* Z (*L.f*), isolated from healthy human were used in this study. These strains have a long history of being used as probiotics in Russia and are included in different health-food products including Laminolact, Bakfir, BioBio and Bilaminolact. These two strains are patented in the Russian Federation (Nº 2220199 and Nº 2412239, respectively) and have been used for more than 10 years in therapies for and prevention of gastrointestinal, allergic and cancer diseases affiliated with dysbiotic conditions [24, 25].

*E. faecium* L3 was grown in tryptose broth (Difco, USA) and tryptose agar (Ferax, Germany) for 24 hours at 37°C aerobically. *L. fermentum* Z was grown in MRS broth M641 (Himedia Laboratories Pvt Ltd, Mumbai, India) and MRS agar (Difco, USA) for 48 hours at 37°C anaerobically.

Milk fermented products were prepared by growing *E. faecium* L3 or *L. fermentum* Z in milk (sterilized milk free of antibiotics and preservatives; protein 0.15 g/l, lipids 0.15 g/l, carbohydrates 0.47 g/l; processed at «Piskarevsky» milk Plant, Russia). Inoculums (1 ml 5.5x10⁸ CFU/ml) were added to the milk (50 ml), and bacterial cultures were incubated for 24 hours (*E. faecium* L3) or for 48 hours (*L. fermentum* Z) at 37°C aerobically.

**Animals and their living conditions**

We used male Wistar rats (weight 200–250 g, at the age of 6–7 weeks), obtained from the Rappolovo Animal Breeding Center, Russia. Rats were kept under similar conditions in separate cages. All the animals were treated according to the rules of Good Laboratory Practice; they were kept under the same temperature (18–22°C), light (for 12 hours), noise (up to 85 dB) and humidity (50–60%) conditions. They also received the same type of food (complete compound feeds for laboratory rats and mice, PK-120 sh. 1492, state industry standard R 50258–92 in pellets with a diameter of 14 mm, Russia). All the experiments with animals were performed in compliance with necessary ethical requirements, and the experiments were approved by the Ethics Committee of the Institute of Experimental Medicine, Saint Petersburg, Russia.

**Rat model of antibiotic-associated dysbiosis**

Experimental intestinal dysbacteriosis in rats was induced as previously described [23] by daily intragastric introduction of 75 mg/kg of body weight ampicillin (Organica, Russia) and 50 mg/kg of body weight metronidazole (Nycomed, Denmark) for three days.

**Design of the study**

Male Wistar rats (weight 200–250 g) were randomly divided into four groups with 12 animals in each group. After three days of antibiotic consumption, rats belonging to first experimental group (*E.f.*) were fed 0.5 mL of milk fermented product containing 5.5x10⁸ CFU/ml (pH 5.0) of *E. faecium* L3 intragastrically for 5 days. Rats from the second experimental group (*L.f.*) received 0.5 mL of milk fermented product containing 5.5x 10⁸ CFU/ml (pH 5.4) of *Lactobacillus fermentum* Z for 5 days. The first control group of rats (control 1) received 0.5 mL milk for 5 days after receiving the antibiotics. The second control group (control 2) of rats did not receive antibiotics or probiotics. They received distilled water for 3 days and then milk for 5 days. The characteristics of all the groups under study are presented in Table 1.

Physical activity, body weight, presence of dyspeptic symptoms, and consistency of the excrements were monitored throughout the entire experiment. Samples of feces for microbiological studies were selected on the first, third and eighth days of the study. At the end of the experiment, the samples of epithelium and chyme were taken from different segments of the gut (proximal part of jejunum - T1, distal part of jejunum - T2, ileum and colon) for the determination of the activity of intestinal alkaline phosphatase (iAP). Blood samples for biochemical analysis of the blood serum were also obtained at the end of the experiment.

**Microbiological studies**

Quantitative and qualitative contents of the intestinal microbiota were determined in different periods of the
experiment (before and after exposure to of antibiotics and at the end of the experiment). Changes in the gut microbiota (first, third and eighth days) were tested by bacteriological analysis of the fecal samples using a previously described method [26]. The time intervals between collection of samples and laboratory handling did not exceed 1 hour. The probes (1 g) were homogenized in 1 mL of phosphate buffered saline, PBS (8.00 g/l NaCl, 0.20 g/l KCl, 1.44 g/l Na₂HPO₄, 0.24 g/l KH₂PO₄, pH 7.4). Then the samples were diluted in 10⁻⁶ times employing method of serial dilutions. We monitored for the presence and quantity of bacteria that have been identified in our earlier studies as marker bacteria undergoing significant changes under the influence of metronidazole and ampicillin [23]. It was determined that bacteria belonging to the genera *Lactobacillus, Enterococcus, Bifidobacterium, Escherichia, Proteus* and *Klebsiella* underwent the most significant changes. The following selective and differential diagnostic culture media were used for the bacteriological studies: blood agar, mannitol salt agar, MacConkey’s agar, m-Enterococcus agar, MRS agar (Difco, USA) and Blaurock medium (Nutrient medium, Russia). After enumeration of the colonies on the agar plates, three to four colonies presenting different microscopic appearances were analyzed. These different morphotypes were isolated and submitted to microscopic examination. Microscopic examination were done by way of the gram stain procedure of pure cultures of bacteria. Biochemical tests were also carried out using the identification system kits (ERBA Lachema, Germany). More detailed identification was carried out by PCR studies employing species-specific DNA primers (Table 2).

At the end of the experiment, chyme samples were taken from the different parts of the gut. These samples were diluted in phosphate buffer and then plated on the selective diagnostic media and cultivated at 37°C aerobically or anaerobically. Chyme samples taken from rats of different groups were examined for the presence of *Lactobacillus spp.* and *Enterococcus spp.* by cultivation in MRS agar (Difco, USA) and m-Enterococcus agar (Difco, USA).

**Biochemical studies**

Blood samples were collected from the rats for analysis of biochemical parameters at the end of the experiment. Simultaneously, samples of chyme and mucosa (epithelium) were tested in order to determine the activity of iAP. Chyme samples were obtained from the proximal, medial and distal thirds of the small bowel (excluding duodenum), as well as from the colon. For this purpose, each section of the intestines was washed from the cavity with 30 mL of cold Ringer’s solution (pH 7.1–7.4). Samples of mucosa from the same sections were obtained by careful scraping with a spatula.

Intestinal mucosa and chyme were tested for the activity of iAP by employing p-nitrophenyl phosphate solution (0.6 mM) as a substrate and Ringer’s solution as a buffer (pH 7.4). Enzymatic activity of iAP was characterized as mol/min per segment of intestine. All
the samples were stored at –80°C until being tested. The probes of blood serum studied by employing an «Aeroset» biochemical analyzer (Abbot Laboratories, USA). The level of creatinine, glucose, protein, K, Na, Ca and Mg as well as the activities of aspartate transaminase (AST), alanine transaminase (ALT) and serum alkaline phosphatase (sAP), were determined.

Statistical methods
Statistical data processing was performed using the Student’s t-test. P values less than 0.05 were considered to be significant.

RESULTS

Changes in the physical condition of rats and weight of their intestinal mucosa
All the antibiotic-treated rats (E.f., L.f. and control 1) exhibited the following symptoms: diarrhea or constipation (lack of bowel movements for more than 6 hours) and changes of fecal consistency. It should be noted that in control group 1, the dysbiotic symptoms remained almost till the end of the experiment. Administration of E. faecium L3 or L. fermentum Z led to disappearance of dyspeptic symptoms in all groups of rats in contrast to control group 1. Flatulence was revealed after the autopsy on day 8 of the study in 10 of 12 rats of the control group 1. The feces of the animals in this group were of soft consistency.

At the end of the experiment, the masses of the mucosa (Fig. 1) in all intestinal segments of rats receiving E. faecium L3 was greater than in control group 2 and that in the colon was greater than in all other groups of animals. The masses of the mucosa of the entire intestine of rats receiving lactobacilli did not differ from those of the control group 2. However, the mass of the mucosa was smaller in segment T2 and the colon compared with the group of rats receiving enterococci and in segment T2 compared with control group 1.

Condition of the microbiota of the intestinal tract
After administration of antibiotics for 3 days, the following changes in gut microbiota of rats were identified: decrease (1–2 lg CFU/ml) in bacteria belonging to the genera Bifidobacterium, Lactobacillus, Escherichia coli and Enterococcus and appearance or increase (2–5.5 lg CFU/ml) in the quantity of putative opportunistic bacteria such as Proteus spp. and Klebsiella spp. It was shown that by the end of the experiment, only the experimental groups of rats that received probiotics showed recovery of the microbiota to normal in compared with control group 2. At the same time, opportunistic bacteria disappeared in groups L.f. and E.f. It should be noted that L. fermentum Z inhibited the growth of Klebsiella spp. and E. coli less efficiently than E. faecium L3 (Fig. 2, C-F). During the experiment, the content of lactobacilli and bifidobacteria in the feces of control group 2 was higher than in the other groups (Fig. 2, A-B). These parameters were higher after the administration of probiotic enterococci (group E.f.) than after administration of lactobacilli (group L.f.). This phenomenon might reflect the interspecies antagonism caused by the strain of L. fermentum Z.

At the end of the experiment, the quantitative content of lactobacilli and enterococci in the different intestinal segments of rats was determined (Fig. 3). The content of lactobacilli in the chyme of the T1 segment was the highest in the group treated with Lactobacillus sp. We were able to determine moderate increase in the E.f. group isolated from the colon (3A). Interestingly, the number of enterococci in the group taking the enterococcal probiotic did not change dramatically (3B). The only statistically valid increase was in the level of enterococci isolated from the T1 segment.

Activity of intestinal alkaline phosphatase
The activity of iAP in the epithelium of the proximal jejunum was significantly higher than in other intestinal segments but was not significantly different compared with the animals of the other groups (Fig. 4). The only exceptions were the samples of epithelium of the distal jejunum (T2) of rats treated with lactobacilli. The iAP activity in the epithelium and chyme of segment T2 and colon of rats of this group was higher than in control group 2. The strain of enterococci caused an increase in the activity of iAP only in the probes of chyme from the jejunum.
Fig. 2. Changes in quantitative characteristics of microorganisms (A-F) in the feces of rats with dysbiosis after administration of probiotics or milk

A, Lactobacillus spp., B, Bifidobacterium spp.
* p<0.05 when comparing control 2 with all other groups. ** p<0.05 when comparing group E.f. with groups L.f. and control 1. *** p<0.05 when comparing group E.f. with group L.f.

C, Enterococcus spp., D, Escherichia coli
* p<0.05 when comparing control 1 with other groups. ** p<0.05 when comparing group E.f. with other groups.
*** p<0.05 when comparing control 2 with other groups

E, Klebsiella spp., F, Proteus spp.
* p<0.05 when comparing control 1 with other groups. ** p<0.05 when comparing group L.f. with group E.f. and control 2. *** p<0.05 when comparing control group 2 with other groups
Biochemical parameters of blood serum of different groups of rats

After administration of ampicillin and metronidazole for 3 days, the activity of ALT, AST and sAP in the blood serum of rats was elevated (Table 3).

At the end of the experiment, the serum levels of creatinine, glucose, protein, K, Na, Ca and Mg and activities of sAP, AST and ALT were analyzed.

Study of biochemical parameters revealed differences between the groups of animals, which are presented in Table 3. Activity of aspartate transaminase, alanine transaminase and alkaline phosphatase in the blood serum of rats before and after administration of antibiotics (results of three experiments)

| Biochemical parameters of blood serum | Before administration of antibiotics (first day) | After administration of antibiotics (third day) |
|--------------------------------------|-----------------------------------------------|-----------------------------------------------|
| AST (U/L)                            | 197.43±6.08                                   | 510.6±71.0*                                   |
| ALT (U/L)                            | 48.14±1.75                                    | 95.6±11.5*                                    |
| sAP (U/L)                            | 434.6±26.3                                    | 450.2±78.9*                                   |

* p<0.05 when compared with the biochemical parameters on the first day

Table 3.
The aim of this study was to perform a comparative analysis of the effects provided by two probiotic strains, *E. faecium* L3 and *L. fermentum* Z, on the microbiota, physical condition and some metabolic functions of rats with intestinal dysbiosis, which is often associated with the usage of antibiotics [27, 28]. Dysbiosis was induced by introduction of antimicrobial agents (ampicillin and metronidazole) acting on a wide range of bacteria (Gram-positive, Gram-negative, anaerobic and aerobic bacteria). Clinical manifestations of dysbiosis were typical. After introduction of both probiotics, the physical condition of the animals returned to normal in contrast with the rats which received milk (control 1).

More interesting were the changes of intestinal microbiota. After consumption of antibiotics, we were able to determine a decrease in the number of lactobacilli, bifidobacteria and enterococci together with an increase in the number of bacteria belonging to genera *Pseudomonas*, *Proteus*, *Klebsiella*, *Staphylococcus* and *Clostridium* [23]. Similar results were obtained in other studies after administration of vancomycin, cephalosporins, aminoglycosides, ampicillin and metronidazole [26, 29–31]. Some authors also noticed a decrease in content of *Bacteroides* and increase in the number of *Firmicutes*, especially enterococci [27, 29]. The discrepancy in data apparently reflects peculiarities of the experimental schemes and spectrum of antibiotics used by different authors. Previously, an increase of the number of *Lactobacillus* spp. and *Bifidobacterium* spp. and a decrease in pathogenic *Clostridium* spp., *Enterococcus* spp. and Gram-negative bacteria were observed after administration of probiotic LAB for the treatment of dysbiosis [13, 32, 33]. In our studies, we revealed similar trends regarding the increase of useful Gram-positive bacteria. Moreover, we found specific characteristics of the impact of LAB strains on the microbiota. It was shown that *Enterococcus faecium* L3 expressed a much stronger influence on the microbiota than *Lactobacillus fermentum* Z. These probiotic enterococci demonstrated more pronounced lacto- and bifidogenic effects.

The use of enterococci led to significant decrease in the number of *Escherichia coli* and *Klebsiella* spp. relative to the groups of animals receiving the milk or lactobacilli. Probiotic lactobacilli inhibited the growth of *Proteus* spp. better than other bacteria. These effects can be partly explained by the strong antibacterial action of *E. faecium* L3 which produce bacteriocins A and B and other antimicrobial factors [34]. *L. fermentum* Z also inhibited putative opportunistic bacteria but not as strongly as an enterococcal probiotic [25]. It is possible that the increase of mass of the mucus found after consumption of *E. faecium* L3, in contrast with *L. fermentum* Z, reflected the specific cross talk between the probiotics and the host [5].

It should be noted that introduction of probiotic enterococci and lactobacilli caused a colonization of the proximal jejunum, in contrast to the control groups of animals. Previously, this effect was demonstrated by using strain *E. faecium* L5 (Ery<sup>+</sup> erythromycin-resistant derivative of strain L3) [23]. A similar manner of colonization of gastrointestinal tract of mice was demonstrated after using another probiotic LAB (*Lactococcus lactis* labeled with green fluorescent protein) [35].

Dysbiosis inevitably leads to the changes in metabolic functions. A correlation between the increase in the number of lactobacilli and enterococci and the iAP activity of chyme in the jejunum was found previously by the investigation of samples of the colon and jejunum of young rats [36]. In this study, the correlations between changes in the microbiota and activity of iAP could not be established. As shown in our preliminary studies, administration of ampicillin and metronidazole for three

Table 4. Activity of aspartate transaminase, alanine transaminase and alkaline phosphatase in the blood serum of rats from different groups (results of three experiments)

| Biochemical parameters of blood serum | Control 1 | *L. fermentum* Z (L.f.) | *E. faecium* L3 (E.f.) | Control 2 |
|--------------------------------------|-----------|------------------------|-----------------------|-----------|
| AST (U/L)                            | 217.0 ± 8.3 | 230.8 ± 21.2*          | 175.0 ± 10.7          | 163.0 ± 6.8 |
| sAP (U/L)                            | 417.1 ± 85.17 | 482.6 ± 74.49*        | 321.2 ± 186.81        | 360.8 ± 49.99 |

* p<0.05 when compared with groups L.f. and control 1
days led to a decrease in the total activity of iAP in the epithelium of the intestine and an increase in the activity of LAB of chyme in the small intestine and especially in the colon. In the present study, it was shown that the increased activity of iAP in the chyme of the small intestine after administration of both probiotics can be explained as a positive compensatory effect. This proposition is based on the fact that there is a positive correlation between the increase of iAP in the chyme of the cecum of young rats and their improved condition and increased weight. The mechanism of the total change in this parameter is difficult to evaluate because iAP is produced by leukocytes, enterocytes and bacterial cells. The production of iAP often depends on microbiota, concentration of bile acids and morpho-functional status of the liver. Also, it is important to consider the destruction of the enzyme under the action of proteases of different origin [28, 37]. However, some of the increase in iAP activity may also be caused by the increased solubilization of the enzyme in the cavity of the intestine due to the increase in the concentration of bile acids, which is due to changes in the bacterial flora [38].

The elevation of iAP in the chyme of the small intestine under the influence of probiotics can be explained either by the increase in the number of LAB in these segments or by the increase in the quantity of lipopolysaccharides, after destruction of Gram-negative bacteria by probiotics. It is also possible that the raise of activity of this enzyme is associated with a local reaction to the gut inflammation that is followed by an increased number of leukocytes and epithelial cells.

It was shown that the mucosal weight in the small intestine and in the colon was reduced after introduction of ampicillin and metronidazole [24]. A significant increase in mucosal weight after introduction of probiotic enterococci might be explained by the induction of anti-inflammatory cytokines as shown in previous studies [23].

The increase in activity of ALT, AST and sAP may be caused by both liver damage due to direct toxic effects of antibiotics on hepatocytes [38], as well as changes in the microbiota, leading to disturbances of the digestive system, including dysfunction of the liver and biliary tract [3]. Administration of *E. faecium* L3 led to normalization of liver enzymes AST and sAP in contrast to animals with dysbiosis treated with milk (control 1) and even probiotic lactobacilli. Apparently, the introduction of enterococci led to more rapid compensation functions of the hepatobiliary system than in the case of usage of lactobacilli.

In this paper, common and specific effects of the LAB and new criteria for comparison of the specific properties of LAB were identified. These specific properties are the influence of Gram-negative bacteria and the ability to compensate for disorders of the gastrointestinal tract and liver. Perhaps the study of individual properties of probiotic strains will increase the efficiency of treatment of patients with comorbidity and will help to avoid complications.

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