Research Article
Volume 7:7,2021

Journal of Antimicrobial Agents

ISSN: 2472-1212 Open Access

Immune-mediated Effects of Lactitol in Animals with Antibiotic-induced Dysbiosis after Infection with Clostridium Difficile

A. A. Sergeev[1*], N. A. Mnafki[1], E. A. Ryabenko[1], A. A. Nikitina[1], C. Shammas[2], E. Nikolaou[2], A.V. Dikovskiy[2], T.I. Kombarova[3], O. V. Korobova[3], E. A. Ganina[3], I. P. Mitsevits[3], A. I. Borzilov[3], M. V. Khramov[3], and L. C. Zacharia[4]

[1]Department of R and D, MC-Development LLC, Moscow, Russia

[2]Department of Genetics, AVVA Pharmaceuticals LLC, Limassol, Cyprus

[3]Department of Microbiology and Biotechnology, Federal Budget Institution of Science, Obolensk, Russia

[4]Department of Genetics, University of Nicosia, Nicosia, Cyprus

Abstract

The research objective was to study the effect of dysbiosis in mice and hamsters caused by the treatment with combination of amoxicillin trihydrate and potassium clavulanate on the colonization with Clostridium difficile (C. difficile) and course of C. difficile infection (CDI), to estimate the effect of the prebiotic lactitol when was administered with antibiotic for prevention and therapy of the CDI. CDI is a current public health problem. The combination of amoxicillin and clavulanic acid is considered an antibiotic with high risk of adverse CDI. Meanwhile, prebiotics as a substrate for bacteria, are able to normalize the composition of the microbiota damaged by antibiotics and could prevent CDI. It was found that lactitol in animals led to a significant 10-fold decrease in excretion of C. difficile from mice feces, and 2.5-fold decrease in feces of infected hamsters, compared with animals who weren't administered prebiotic. Besides, in mice with CDI, when lactitol was administered, a 53% decrease in the response of lymphocytes and 1.5-fold increase in level of immunoglobulin G was observed 10 days after infection in comparison with animals without lactitol administration. In hamsters, lactitol normalized ratio between granulocytes and lymphocytes at first day after infection. Analysis of the mice intestinal microbiota by 16s rRNA sequencing showed positive trends in a shift of the microbiota profile for the bacterial families Lactobacilliaceae, Erysipelotrichaceae. Thus, we demonstrated that of co-administration lactitol with Amoxicillin and Clavulanate reduce development of CDI, and maintain the immune system of animals to increase effectiveness of host response.

Keywords: Clostridium difficile • Amoxicillin-clavulanic acid • Dysbiosis • Animals • Lactitol • Immune cells

Introduction

C. difficile is a Gram-positive, anaerobic, spore-forming, and toxin-producing pathogenic bacillus. The bacterium is ubiquitous in the environment and can be transmitted to humans by the fecal-oral route. Potential reservoirs of C. difficile are asymptomatic carriers, patients, contaminated environmental objects, and animal feces. There is a high frequency of colonization with C. difficile in newborns, from 15% to 70%. Currently, the infection caused by C. difficile (CDI) is considered a significant medical nosocomial infection. In humans, CDI can be asymptomatic and may present itself with moderate to severe diarrhea. Sometimes the disease takes the form of pseudomembranous colitis, toxic megacolon, or sepsis. The cause of C. difficile diseases is usually associated with using antibiotics but not in all cases. About 124,000 cases of CDI are registered in European countries every year. The combination of amoxicillin and clavulanic acid is one of the drugs widely used in modern medicine, and its use is considered by physicians to increase the risk of developing CDI. It is known that the pathogenic effect of C. difficile is realized by exotoxins A and B produced by this bacterium, that cause serious injuries to the intestinal mucosa and, in some cases, death of patients. According to research data, amoxicillin and clavulanic acid have shown to enhance the proliferation of C. difficile and the production of its toxins [1].

Studying the pathogenesis of CDI is an important element in understanding C. difficile-induced diseases and ways of managing them. Models of this infection have been developed using animals such as mice, rats, hamsters, rabbits, pigs, etc. Importantly, hamsters and mice are most often used in studies, and are usually pretreated with various antibiotics in order to induce negative changes in the

***Address to correspondence:** A. A. Sergeev, R and D, MC-Development LLC, Moscow, Russia, Tel: 89035944129; E-mail: artsergeev@mail.ru*

Received: 06 October, 2021; **Accepted:** 20 October, 2021; **Published:** 27 October, 2021

composition of the intestinal microbiota and increase sensitivity of animals to *C.* difficile. Hamsters are the most susceptible animals to *C.* difficile. After challenging with *C.* difficile, they develop a pronounced clinical picture of the disease with a fatal outcome. Mice are also susceptible to *C.* difficile. Under some conditions, they also develop a pronounced clinical picture of the disease with a fatal outcome, however, these animals show greater resistance to the pathogen compared to hamsters. More recently, mice are used as model animals for studying the mechanisms of colonization, carriage, and spread of C. difficile.

Prebiotics are nutrients that are not absorbed by the intestines of humans and animals but are metabolized by the microbiota with the formation of useful molecules for the body. Being substrates for many beneficial bacteria, prebiotics stimulate their growth and improve the microbiome composition. Thus, it was shown that the use of lactitol led to an increase in the number of beneficial Bifidobacterium and Lactobacilli in human and animals, elevated the level of propionate, butyrate, immunoglobulin A and shifted the pH in the intestine to the acidic side, that are all important positive effects for the body. Lactitol (E-966) is a sugar alcohol, which is obtained by the hydrogenation of lactose. This disaccharide is normally not absorbed in the gastrointestinal tract, is selectively fermented by saccharolytic bacteria and stimulates the growth and/or activity of a number of bacteria in the intestine. In general, lactose-derived prebiotics such as lactitol, are of unique physico- and bio-chemical importance with great health benefits to the human organism. Higher lactitol intakes can induce laxative effect that it is medically used as laxative agent. Studies have shown that lactitol promotes the growth of bifidobacteria and lactobacilli. On the other hand, it has also been shown to decrease counts of proteolytic bacteria, such as Bacteroides, Clostridium, coliforms, Eubacterium, Enterobacteria, and Enterococci. 13 Based on the aforesaid, the main objective of this research was to study the effect of dysbiosis caused by treatment with a combination of amoxicillin trihydrate (Am) and potassium clavulanate (Cl) on the infection with the strain ATCC-43255 of *C.* difficile in mice and hamsters. In addition, we aimed to determine the parameters of the pathogenic colonization in their bodies, and estimate the effect of the prebiotic lactitol on the course of the clostridial infection in animals when it was co-administered orally with antibiotic [2].

In the present study, we examined the effect of a combination of Am and Cl in different doses on the sensitivity of mice and hamsters to *C.* difficile after long-term use (within 10 days). Besides, the immune responses of animals and the role of the microbiota in the development of CDI were studied, and we have made an attempt to reduce the severity of the negative impact of *C.* difficile through the effect of the prebiotic lactitol on the microbiota.

Results

The impact of antibiotic-induced dysbiosis on the colonization of intestine with *C.* difficile in mice

The ability of *C.* difficile to cause disease in mice was assessed by intragastric (i/g) infection with a spore culture of these bacteria. Specific Pathogen-Free (SPF) C57BL/6 mice were infected with spores of the strain ATCC-43255 of *C.* difficile at a dose of 1.0 × 104 Colony Forming Units (CFU), followed by an assessment of clinical signs of the disease and other characteristics of the infection. 15 days after infection (a.i.), animals did not show any visible clinical signs of the disease, but vegetative forms of C. difficile were found in their fecal samples 8 and 15 days a.i. in 100% and 66% of animals, respectively. The data are presented in Table 1.

It is known that antibiotics, due to their antimicrobial effect, are capable of changing the composition of the microbiota in the gastrointestinal tract of humans and animals, destroying certain taxonomic groups of microorganisms that are most sensitive to them. 14 The target organ of C. difficile is the intestine, which, as a result of the antibiotic impact, may become more susceptible to colonization by this pathogen due to a decrease in the number of bacteria of the microbiota, which normally inhibit settlement and growth of C. difficile.

In order to reproduce antibiotic-induced dysbiosis, mice were pretreated with the antibiotic (Am and Cl) for 10 days, and then 1 day later they were infected by i/g administration with spores at different doses of the C. difficile for the subsequent assessment of the development of their infection (as described in Material and Methods). During the entire period of observation of the infected animals (15 days a.i.), no visible and significant specific clinical signs of diseases to these animals with infections of the gastrointestinal tract (GIT) were found. However, a significant increase in the concentration of vegetative forms of C. difficile 8 days a.i. was detected in the fecal samples of mice, which was dependent on the infecting dose. The data on the content of C. difficile in the feces of infected mice, depending on the infecting dose are presented in Table 1.

C57BL/6 mice were susceptible to colonization by *C.* difficile in their GIT, despite the absence of visible clinical signs of the disease. Bacterial analysis of feces from mice infected with 1.0 × 104 CFU of *C.* difficile (AmCl/CD 104) showed a significant increase (more than 40 times) in the proliferation of the pathogen in GIT of animals 8 days a.i. in the group that received the antibiotic versus the group that did not receive the drug (Intact/CD 104) ($p<0.05$).

Group of Animals	The proportion of mice with CD colonization of the gastrointestinal tract and the number of colonies in their feces at different periods after infection:			
	8 days		15 days	
	Number of animal carriers of CD*	The average concentration of CD, M ± SD lg CFU/g	Number of animal carriers of CD*	The average concentration of CD, M ± SD lg CFU/g
AmCl/CD, dose 1×10^1 CFU	6/6	3.29 ± 0.08# 2/6		2.50 ± 0.08
AmCl/CD, dose 1×10^2 CFU	6/6	3.67 ± 0.52#$ 3/6		3.87 ± 1.39
AmCl/CD, dose 1×10^3 CFU	6/6	4.08 ± 0.10#$ 5/6		3.95 ± 1.82
AmCl/CD, dose 1×10^4 CFU	6/6	4.22 ± 0.12#$ 5/6		2.41 ± 0.48
Intact/CD, dose 1×10^4 CFU	6/6	2.60 ± 0.56	4/6	2.00 ± 0.00

Footnote: CD -the strain ATCC-43255 of *C. difficile*; AmCl/CD-animals were intragastrically (i/g) administered the antibiotic (1170 mg/kg/day of amoxicillin trihydrate, and 167.2 mg/kg/day of potassium clavulanate) for 10 days, then after 1 day they were i/g infected with spores of CD in different doses; Intact/CD -intact animals were i/g infected with CD spores at the indicated dose; * - in the numerator-the number of mice with CD, in the denominator – the number of examined animals in the group; M ± SD- the mean and its standard deviation for the number of animals with detected CD; #-the concentration is significantly higher than in Intact/CD in multiple comparison Post Hoc test with Bonferroni correction p<0.05; $-concentration is significantly higher than in group No. 1 in multiple comparison Post Hoc test with Bonferroni correction p<0.05; lg CFU/g-decimal logarithm of the number of colony forming units in a gram of feces.

Table 1. Data on the content of Clostridium difficile in the feces of C57BL/6 mice 8 and 15 days after intragastric infection.

However, 15 days a.i. there were no significant differences in the concentrations of C. difficile in the feces of mice from the studied groups. The dose of infection influenced the titers of C. difficile in animal feces. Thus, when animals were infected with 10 CFU of C. difficile (AmCl/CD 101), a significantly lower concentration of these bacteria were found in the feces in comparison with the groups infected with 100, 1000, and 10000 CFU's of C. difficile (AmCl/CD 102-104). At the same time, the number of C. difficile in the feces of mice from groups AmCl/CD 102-104 did not significantly differ from each other, but had a tendency to increase in direction to the highest dose. It is interesting to note that colonization of the GIT in mice by C. difficile occurred in 100% of cases even at the lowest infectious dose (10 CFU), which indicates a high sensitivity of animals to the bacteria (50% colonization dose (CD) is less than 10 CFU's). At the same time, a decrease in the number of animals with C. difficile in feces and a decrease in the level of infection with C. difficile 15 days a.i., in comparison with 8 days, shows the ability of animals to gradually independently eliminate bacteria from their body [3].

Administration of the antibiotic to mice demonstrated a change in the ability of C. difficile to colonize and grow in their GIT. Considering the expected impact of the antibiotic on the mouse microbiota, we studied the changes in the parameters of the abundance and biodiversity of taxonomic groups of bacteria in animal feces by sequencing 16S rRNA. The data from this study demonstrates significant differences in the parameters of absolute diversity and alpha diversity of fecal microbiota in mice. The data are presented in Table 2.

Group of animals, (n=10)	Indicators of the diversity of the microbiota, assessed by 16S rRNA sequencing of bacteria from animal feces, at different intervals from the start of the study.			
	Absolute diversity		Shannon Index	
	Day 10	Day 21	Day 10	Day 21
AmCl/CD	11.977*#	28.527*#	0.218*#	2.583*#
Intact	48.98	46.783	3.739	3.784
Intact/CD	48.96	47.563	3.774	3.307*

Footnote: CD-the strain ATCC-43255 of Clostridium difficile; AmCl/CD group -animals were administered intragastrically (i/g) with 1170 mg/kg/day of amoxicillin trihydrate and 167.2 mg/kg/day of potassium clavulanate (AB) for 10 days, then 1 day later they were infected i/g with spores of CD (at a dose of 2.0 × 104 CFU); Intact/CD group-animals were infected with 5 × 104 CFU of *C. difficile*; Intact group-uninfected animals; * -p<0.05 compared with the intact group; # -p<0.05 compared with the Intact/CD group (p is the level of significance of differences (p-value), corrected by the Benjamini-Hochberg method).

Table 2. Data on the parameters of the diversity of fecal microbiota in different groups of C57BL/6 mice that received and did not receive the antibiotic or Clostridium difficile at different intervals, assessed by 16S rRNA sequencing of bacteria.

Significant differences were noted in the index of absolute diversity and Shannon index in the microbiota from feces of animals treated with the antibiotic 10 and 21 days after the start of its administration (AmCl/CD) in comparison with the microbiota of intact animals (Intact/CD). Thus, a decrease in the biodiversity index of microorganisms in feces was discovered in group of animals AmCl/CD 10 days (a decrease in the absolute diversity by 4.1, and for the Shannon index by 17.2 and 17.3 times) and 21 days (a decrease in absolute diversity by 1.6 and 1.7, and for the Shannon index by 1.3 and 1.5 times) after the start of the administration of the antibiotic in comparison with the groups Intact/CD, and those not infected with C. difficile (Intact). Indices of microbiota diversity in the groups of animals Intact/CD (infected with 1.0 × 104 CFU of C. difficile) and Intact were similar. Nonetheless, infection with C. difficile of mice Intact/CD led to a significant change in bacterial diversity by 1.1 times according to the Shannon index (p<0.05) in fecal samples 21 days after the start of the study (the date of the first administration of the antibiotic to animals in other groups) in comparison with Intact.

The microbiota profile of the mice from Intact group was represented by the families: Clostridiales unknown family, S24-7, Ruminococcaceae, Helicobacteraceae, Rikenellaceae, Lactobacillaceae, etc. At the same time, the list of the most abundant genera included unclassified representatives of Clostridiales, S24-7 unknown genus, Lactobacillus, Prevotella, Oscilospira, Lachnospiraceae unknown genus, Ruminococcaceae unknow genus, Rikenellaceae unknown genus, etc., at the species level -unclassified representatives of Clostridiales and S24-7, genera Lactobacillus, [Prevotella], Oscillospira, Ruminococcaceae, Flexispira, Odoribacter, etc. Microbiota profile is shown in Figure 1.

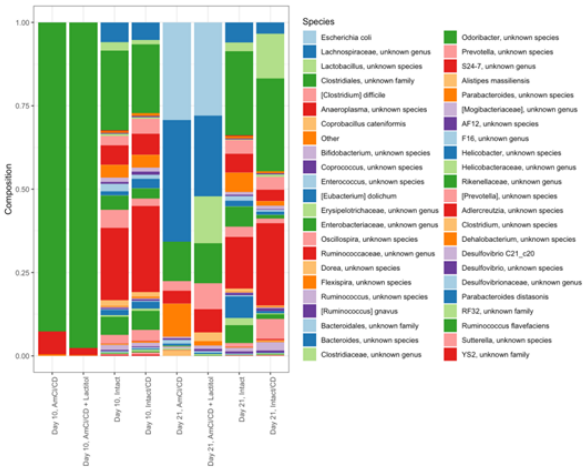

Figure 1. Data on changes in the composition of the fecal microbiota of C57BL/6 mice treated with Lactitol in a prophylactic regimen, obtained 10 and 21 days after infection with Clostridium difficile (CD) (n = 10 in each group).

Analysis of changes in the abundance of individual families, genera, and species of the microbiota 21 days after the start of the study showed significant differences between Intact and Intact/CD groups ($p<0.05$). Specifically, we observed an increase in the abundance of the genera S24-7 unknown genus, Adlercreutzia, Lactobacillus and the families Coriobacteriaceae and Bacteroidales [Paraprevotellaceae], and a decrease in the family Bacteroidales [Odoribacteraceae] in the animals infected with C. difficile. In the group of mice AmCl/CD, dramatic changes in the intestinal microbiota profile were observed. Ten (10) days after the start of the study, the abundance of most families, genera, and species of bacteria significantly decreased ($p <0.05$) compared with the group Intact, and the residual detection of the intestinal microbiota was presented mainly by unclassified bacteria Clostridiales and Anaeroplasma. Comparing Intact/CD and AmCl/CD groups, it can be noted that the microbiota profile of the infected animals from Intact/CD is much closer to Intact group than to AmCl/CD group on the 21st day of the study. Comparative statistical analysis showed a significantly ($p<0.05$) higher abundance of the microbiota in AmCl/CD group in comparison with Intact/CD group (Enterobacteriaceae, Anaeroplasmataceae, Peptostreptococcaceae, Lachnospiraceae, Paenibacillaceae, Bifidobacteriaceae), and a significant decrease ($p<0.05$) in the proportion of Bacteroidales [Odoribacteraceae], Desulfovibrionaceae, Lactobacillaceae, Bacteroidales S24-7, Bacteroidales [Paraprevotellaceae] at the family level.

At the genera level, a significant increase ($p <0.05$) in the abundance of Escherichia, Lachnospiracea unknown genus, Peptostreptococcaceae [Clostridium], Anaeroplasma, Coprococcus, Clostridium, Paenibacillus, Clostridiaceae unknown genus, Enterobacteriacea unknown genus, Bifidobacterium, Enterococcus, and a decrease ($p<0.05$) in Desulfovibrio, Oscillospira, Ruminococcaceae unknown genus, S24-7 unknown genus, [Paraprevotellaceae] [Prevotella], was observed. At the species level a significant ($p<0.05$) increase in the abundance of [Clostridium] difficile, Escherichia coli, Anaeroplasma unknown species, Clostridium unknown species, Coprococcus unknown species, Bifidobacterium unknown species, Enterococcus unknown species, Coprobacillus cateniformis against the background of a decrease ($p<0.05$) of the abundance of Oscillospira unknown species, [Prevotella] unknown species was observed Data are shown in Figure 1.

The effects of lactitol in mice with antibiotic-induced dysbiosis infected with *C. difficile*

It is known that prebiotics, such as lactitol, are able to have a beneficial effect on GIT environment and its beneficial for microbiota, being a food substrate for many bacteria.13 In order to assess the possible positive effect of lactitol on the colonization ability of C. difficile in the intestine, mice were pretreated i/g with AmCl, were then infected with C. difficile strain ATCC-43255 at a dose of 2.0 × 104 CFU (AmCl/CD 2×104), and were also pretreated with lactitol for 21 days from the start of antibiotic administration in a prophylactic scheme (AmCl+Lactitol/CD 2×104). Infection with C. difficile did not lead to appearance of any clinical signs of disease in mice in the groups, which coincided with the results of the aforementioned study. At the same time, 7 and 10 days a.i. a significant decrease in the C. difficile count was observed (detected in samples of intestinal contents) in animals from AmCl+Lactitol/CD 2×104 group compared to the group AmCl/CD 2×104. The results of this study are presented in Table 3.

Mice from the AmCl+Lactitol/CD 2×104 group had a significantly lower content of C. difficile ($p<0.05$) (approximately by 9-10 times) in their intestinal content compared with the group of AmCl/CD 2×104, which were pretreated with the antibiotic without lactitol both 7 and 10 days a.i. Analysis of the composition of the microbiota by sequencing showed indicative differences between AmCl/CD 2×104 and AmCl+Lactitol/CD 2×104 groups ($p<0.05$ without correction, but $p> 0.05$ after correction for multiple comparisons), which, when using lactitol, manifested itself as an increase in the abundance of bacterial families Erysipelotrichaceae, Lactobacillaceae and a decrease in Clostridiaceae, Paenibacillaceae, as well as in reduced abundance of bacterial genera Paenibacillus, Clostridium, Clostridiaceae unknown genus, Ruminococcus, and Clostridium unknown species, Ruminococcus unknown species. wheras significant differences in the abundance of [Clostridium] difficile were not achieved between the groups. At the same time, in AmCl/CD 2×104 and AmCl+Lactitol/CD 2×104 groups, there was a significant increase in the abundance of the bacterial family Lachnospiraceae and an increase in the genus Bifidobacterium compared to the Intact/CD 2×104 group (Figure 1).

Group of animals, (n =4 - 6)	Comparison data of CD concentrations (lg CFU/g) in the intestinal contents of mice collected at different time intervals after infection:					
	7 days			10 days		
	M	I95	p.value M		I95	p-value
AmCl/CD	7.1	0.26	-	*6.94	0.16	
AmCl + Lactitol/CD	6.12#$	0.03	0	5.75#$	0.43	≤ 0.014
Intact/CD	*4.61#	0.3	0	**2.12#	0.25	0

Footnote: CD – the strain ATCC-43255 of C. difficile; AmCl/CD – animals were administered intragastrically (i/g) with 1170 mg/kg/day of amoxicillin trihydrate and 167.2 mg/kg/day of potassium clavulanate (AB) for 10 days, then 1 day later they were infected with spores of CD at a dose of 2.0 × 104 CFU; AmCl+Lactitol/CD – animals were administered i/g with AB for 10 days, then 1 day later they were i/g infected with spores of CD at a dose of 2.0 × 104 CFU, while lactitol was administered in a prophylactic regimen at a dose of 3480 mg/kg/day from 0 up to 21 days after the start of the antibiotic administration; Intact/CD –animals were i/g administered with spores of CD at a dose of 2.0 × 104 CFU; p-value – the level of significance of intergroup differences in the multiple comparison Post Hoc test with Bonferroni correction; M – mean value; I95 –95% confidence interval for M; * – when calculating M, data from 5 animals with identified CD were used; ** – when calculating M, data from 4 animals with identified CD were used; # – the concentration is significantly lower than in the AmCl/CD group; $ – the concentration is significantly higher than in the Intact/CD group; lg CFU/g – decimal logarithm of the number of colony-forming units in a gram of feces.

Table 3. Data on the effects of different regimens of lactitol administration on the accumulation levels of Clostridium difficile in the intestinal contents of C57BL/6 mice infected intragastrically.

When analyzing the microbiota profiles by the hierarchical clustering method (Figure 1D) by bacterial families, it can be noted that AmCl+Lactitol/CD 2×104 group 21 days after the start of the antibiotic and lactitol administration is clustered closer to the Intact group than the AmCl/CD 2×104 group, partly owing to an increase in the abundance of the families Lactobaillaceae, Erysipelotrachaceae,

and a decrease in the family Clostridiaceae. However, the fecal microbiota profiles of animals from the groups that received the antibiotic followed by infection with C. difficile are largely close to each other compared with the microbiota of animals that did not receive the antibiotic. The fecal microbiota profiles of animals from Intact and Intact/CD 2×104 groups share a lot of similarities and are clustered together both 10 and 21 days after the start of the study. Despite the absence of visible clinical signs of the disease in all mice infected with C. difficile, and a different ability of this pathogen to accumulate in the intestines of animals, including lactitol usage, we discovered significant shifts in the concentrations of some immune blood cells of mice from different groups. The results of the concentrations of leukocytes, lymphocytes, and granulocytes in the blood of infected mice are shown in Figure 2.

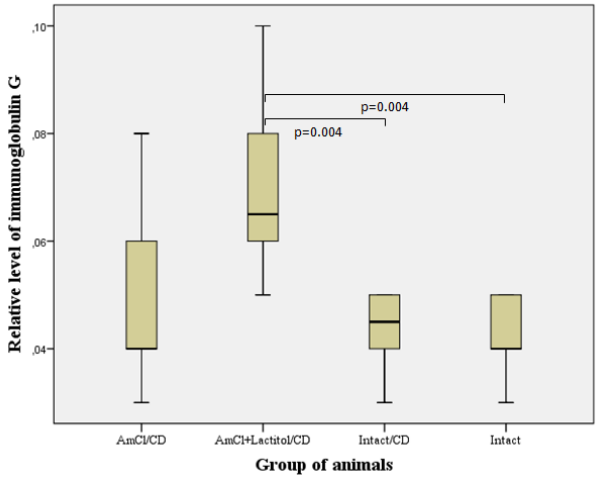

Figure 2. Data on the relative content (proportion) of nonspecific immunoglobulin G to the level of total protein in the blood of C57BL/6 mice 10 days after infection with Clostridium difficile.

The content of lymphocytes in the blood of mice in the AmCl +Lactitol/CD 2×104 group that received lactitol in a prophylactic regimen 10 days a.i. with C. difficile was significantly lower by 53%and 45% ($p<0.05$) than in the AmCl/CD 2×104 or Intact/CD 2×104 groups, respectively. Furthermore, in the AmCl+Lactitol/CD 2×104 group, a significant 42% lower content of leukocytes ($p<0.05$) was observed compared to Intact/CD 2×104 group. No significant changes in the concentration of granulocytes and other blood cells were found in the compared groups of animals (data not shown). Changes in the concentration of full fraction of lymphocytes in the blood of mice were also accompanied by a difference in the content of IgG (the main product expressed by B lymphocytes) in their blood, as evidenced in the different groups of animals, 10 days a.i. The results estimating the proportion of IgG in total protein in the blood in the studied groups of mice. As it can be seen in Figure 3, the proportion of nonspecific IgG in the total protein significantly increased on average by about 1.5 times ($p<0.05$) in the AmCl +Lactitol/CD 2×104 group, compared with groups Intact and Intact/CD 2×104. At the same time, the proportion of IgG did not differ in other groups and did not depend on the antibiotic administration, or infection with C. difficile. Despite the fact that no significant difference were found between the groups AmCl +Lactitol/CD 2×104 and AmCl/CD 2×104, there was a trend towards higher IgG levels in the lactitol group.

Infected mice from different groups also had differences in the pathological signs of the disease observed in tissue sections from different parts of the intestine. Thus, microscopic examination of tissue sections of the intestine, obtained 10 days a.i. with *C.* difficile, made it possible to establish that pathological changes in the intestinal mucosa of mice were observed only in samples from the cecum, while no visible changes were found in the small intestine and colon (data not shown). Micrographs of sections of the cecum taken from mice from different groups 10 days a.i. with *C.* difficile are shown in Figure 3.

The effects of lactitol in hamsters with antibiotic-associated diarrhea caused by *C. difficile*

It is known from the literature that hamsters treated with antibiotics are highly susceptible to *C.* difficile and develop a clinical picture of antibiotic-associated diarrhea.8,9 The results of our own research showed that a 10-day administration of a mixture of Am and Cl to hamsters in the dose ranging from 5.33 and 0.76 mg/kg/day to 26.67 and 3.77 mg/kg/day, respectively, leads to a different degree of suppression of the number of bacteria in the intestine, and a different average daily time of death of animals after infection with *C.* difficile. Thus, when hamsters are administered with a low dose (5.33 Am and 0.76 Cl mg/kg/day) a significant decrease in the number of Lactobacterium spp, as well as other types of bacteria (Bifidobacterium spp., E. coli, Proteus spp., Enterococcus spp., Staphylococcus spp.) were not observed, while when using a high dose (26.67 Am and 3.77 Cl mg/kg/day) there was a significant 1000-fold decrease in the concentration of the aforementioned bacteria that were not detected in fecal samples of animals by microbiological method (data not shown).

Further, to establish the probable role of Lactobacterium spp. and other bacteria in the development of antibiotic-associated diarrhea in hamsters, a low dose of the antibiotic was used, which did not inhibit the number of these bacteria. Moreover, the study included the prebiotic lactitol to assess its possible positive effect when used at a dose of 2116 mg/kg/day in mixed therapeutic and prophylactic regimen (i.e., immediately after the start of the antibiotic administration-prophylactic and within manifestation of clinical signs-therapeutic). The administration of the antibiotic and lactitol did not lead to the death of the animals or the appearance of any clinical manifestations m until the moment of infection with *C.* difficile. After i/g infection with *C.* difficile at a dose of 1.0 × 104 CFU, the animals in the groups that received the antibiotic died one day a.i. (Table 4)(AmCl/CD). At the same time, in hamsters, by the end of the first day a.i., depression developed (the animals were inactive and stooped), their coats were messy. In all animals, the fur around the anus and the root of the tail was polluted with liquid feces, bloated abdomens were observed, and skin turgor was reduced. The clinical picture showed the development of an acute clostridial infection with diarrhea.

The administration of lactitol to hamsters in mixed scheme of therapeutic and prophylactic administration (i.e., immediately after the start of the antibiotic administration - prophylactic and within manifestation of clinical signs - therapeutic) (AmCl+Lactitol/CD group) significantly increased the life expectancy of these animals by 33% ($p<0.05$). For all control animals that received only water

(Intact), no deaths were observed up and until day 14 (until the end of the study). Evaluation of the effects of lactitol on the colonization of hamster intestines by C. difficile made it possible to establish that at a day a.i. (at a dose of 2.0 × 104 CFU) of the animals from the AmCl/CD group, the average concentration of pathogen cells in feces was significantly higher by 2.5 times ($p<0.05$) compared with animals from the AmCl+Lactitol/CD group. A culture of the pathogen was isolated from all hamsters The results showing the concentration of *C.* difficile in animal feces are presented in Table 4.

Group of animals,	The number of C. difficile cells (lg CFU/g) in the feces of animals from different groups and the significance of intergroup differences	
(n=6)	M ± SD	P
AmCl/CD	6.64 ± 0.11	-
AmCl+Lactitol/CD	6.21 ± 0.20	0.0017

Footnote: CD – the strain ATCC-43255 of C. difficile; Group 1 -animals were administered intragastrically (i/g) with 5.33 mg/kg/day of amoxicillin trihydrate and 0.76 mg/kg/day of potassium clavulanate (AB) for 10 days, then, after 2 days, they were infected with CD at a dose of 2.0 × 104 CFU; Group 2 -animals were administered i/g with AB for 10 days, and after 2 days, they were infected with spores of CD at a dose of 2.0 × 104 CFU, lactitol was used at a dose of 2116 mg/kg/day during the time of participation of the animal in the experiment until its death; M -the mean value; SD -the standard deviation of the mean; p-the level of significance of difference from the AmCl/CD group, when compared by the two-sided Student's t-test; lg CFU/g -decimal logarithm of the number of colony-forming units in a gram of feces.

Table 4. Data on determining the concentrations of Clostridium difficile in feces of golden hamsters one day after intragastric infection.

In addition to the effect of lactitol on an increase in the average day of death among the hamsters and a decrease in the concentration of C. difficile bacteria in their feces obtained a day a.i., changes in the quantitative composition of some immune blood cells were revealed. The results of the comparative assessment of the concentrations of such cells in animals from different groups infected with C. difficile are shown in Figure 3. Clinical analysis of the blood of hamsters, taken a day a.i. with C. difficile, showed a significantly lower concentration of lymphocytes by 4.8 and 3.8 times ($p<0.05$), and a significant increase of the number of granulocytes by 3.9 and 2.8 times ($p <0.05$) in the AmCl/CD group, compared to the intact group (Intact), as well as to AmCl+Lactitol/CD) group, respectively. Animals in the AmCl +Lactitol/CD group and in the Intact group had similar concentrations of lymphocytes and granulocytes. At the same time, the concentration of leukocytes and monocytes between all groups of hamsters did not differ significantly.

The effects of lactitol in hamsters infected with C. difficile was also carried out according to histological studies of cecum sections. Necropsy of euthanized animals was performed one day after i/g infection. In hamsters that received high dose of the antibiotic for 10 days and then infected with C. difficile, notable histopathological changes were observed in the cecum. In the mucous membrane, there were regions with distinctly enlarged, plethoric capillaries. Swelling of the mucosal stroma was noted. There was a desquamation of some cells of the epithelial layer in the apical part of the crypts. In the cell infiltrate, along with plasmocytes and lymphocytes, a small number of polymorphonuclear leukocytes was found. At the same time, in the lamina propria of the intestinal mucosa of hamsters from the group that received additional lactitol in the scheme of therapeutic and prophylactic usage, infiltration mainly represented by plasma cells and lymphocytes was revealed, and there were also separate regions in the mucosa with stromal infiltration due to polymorphonuclear leukocytes. The epithelial layer retained its integrity. A large number of atrophied epithelial cells were found in the intestinal lumen. The histopathological view of the cecum sections is similar in all the studied animals in this group. Degenerative changes in the epithelium of the cecum of hamsters from this group were less pronounced than in animals from the group that received only the antibiotic before infection.

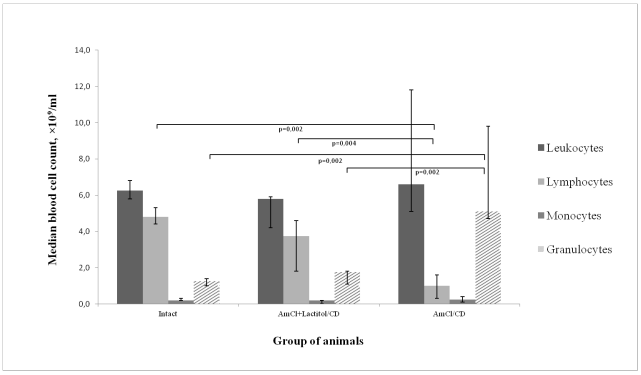

Figure 3. Micrographs of histological sections of the cecum of intact golden hamsters one day after infection with C. difficile, 20x magnification.

Discussion

Despite the fact that much remains to be studied regarding clostridial infection, it is well known that *C.* difficile colonize the human gastrointestinal tract transmitting through the fecal-oral route. Furthermore, although *C.* difficile is an anaerobic microorganism, its spores can survive under aerobic conditions for months or even years. When spores enter the stomach, being resistant to its acidic contents, they penetrate into the intestines, maintaining their viability, where, under anaerobic conditions, they form vegetative bacterial forms and multiply, including with the formation of spore forms, which are released into the environment with feces and spread. In humans, *C.* difficile may not cause any signs of the disease, it is a so-called asymptomatic carriage. In other cases, these bacteria provoke the development of pathologies from moderate and self-recovering to severe and life-threatening, such as pseudomembranous colitis, toxic megacolon, sepsis, or even causing the death of patients [4]. CDI is associated with the accumulation of the bacteria in the intestine producing toxins that induce inflammation of the intestinal mucosa with damage to colonocytes. The virulence factors of C. difficile include the main glycosylating exotoxins A and B, which cause clinical symptoms of the disease by binding to receptors on intestinal cells. Another toxin found in some *C.* difficile strains is binary toxin transferase, which is also associated with high mortality in patients. Toxin-induced inflammation of the mucous membrane in the large intestine, results in massive fluid loss, clinically manifesting itself as diarrhea. Nonetheless, the reasons for asymptomatic cases of *C.* difficile carriage, including highly toxigenic strains, have not yet been precisely established.

Mice and hamsters are the most common animal models used to study *C.* difficile infection. They have different sensitivity to this

pathogen, especially because of the alteration of the composition of their microbiota by antibiotics. In contrast to hamsters, severe disease is not always possible to initiate in mice. For example, long-term administration of a special cocktail of antibiotics (kanamycin, gentamicin, colistin, metronidazole, and vancomycin) are able to reproduce the clinical picture of CDI. According to other researchers, when using such a cocktail, there is a significant shift in the composition of the microbiota in mice, which ensures the clinical manifestation of C. difficile infection. At the same time, long-term use of vancomycin alone for 7 days did not lead to the development of a severe form of the disease in mice, despite a significant shift in the composition of the microbiota, but was accompanied by the formation of a special form of infection, in which mice became super-shedder of *C.* difficile.

In turn, during this study absence of clinical signs of the disease were also observed. Thus, in mice treated for 10 days with the mixture of Am and Cl before i/g infection by spores of C. difficile AmCl/CD, intestinal colonization and excretion of vegetative forms of bacteria with feces in concentrations corresponding to the shedders of these bacteria (to 106 CFU/g) were recorded. Besides, there were signs that this pathogen gradually was released from the gastrointestinal tract of animals, and was manifested as a decrease in the concentration of C. difficile excreted with feces and a gradual decrease in the proportion of animal shedders. Noteworthy is the ability of the strain ATCC-43255 used to colonize the intestines of mice, including those that did not receive an antibiotic; however, the ability to isolate vegetative forms of C. difficile from the feces of such mice was significantly lower than in animals that were given antibiotics. In general, mice were found to be highly susceptible to colonizing their intestines, as indicated by the estimated 50% colonization dose of the pathogen which was less than 10 CFU. We tested a new method, which has not been presented in the literature before, for inducing microbiota disorders in mice using a combination of Am and Cl, which was given to animals for 10 days before infection i/g. This method led to a similar effect of more pronounced excretion of C. difficile with animal feces, as observed in a study by other researchers who used vancomycin to induce dysbiosis in mice. The administration of vancomycin, as well as Am and Cl in our study, made mice likely to spread C. difficile while no clinical signs of specific infection were observed in animals. At the same time, it was possible to ascertain that, despite the use of the antibiotic in mice, in which C. difficile colonized the intestines more efficiently, we observed similarity with normobiotic animals in their ability to get rid of bacteria over time.

The study of the mice microbiota we carried out using the 16S rRNA sequencing method revealed a significant decrease in the absolute biodiversity of bacteria in fecal samples and Shannon index at the end of 10-day antibiotic administration. The diversity of microbiota has been significantly reduced to almost two families Clostridiales unknown families and Anaeroplasma. The observed changes in the microbiota of mice are generally consistent with the data of other researchers, who associate such changes with an increased risk of C. difficile colonization.31 Similar effects on changes in the composition and diversity of the microbiota of mice were also observed with vancomycin. However, when cefoperazone was used in mice, a significant decrease in biodiversity was observed, but at the same time, they developed clinical signs of CDI. Moreover, we noted that C. difficile itself can significantly affect the diversity of the microbiota of mice. Accordingly, we found a notable decrease in the Shannon index for fecal samples from the group of infected animals, compared with intact ones after 21 days from the start of the study. A decrease in the biodiversity of the microbiota of mice feces after colonization with *C.* difficile was also noted by other researchers. The administration of lactitol in our study did not recover microbiota diversity, which is consistent with the data of other researchers who did not observe a significant recovery of the diversity of the fecal microbiota when using probiotics and prebiotic in mice infected with *C.* difficile after pretreatment with cefoperazone.33 Nonetheless, we observed an indicative increase in the abundance of the families Erysipelotrichaceae and Lactobacilliaceae in AmCl+Lactitol/CD group as compared to AmCl/CD group, and a significant increase in the abundance of Lachnospiraceae in the group of animals receiving lactitol as compared to the Intact group. In studies by other investigators, increased abundance of the families Erysipelotrichaceae, Lactobacilliaceae, and Lachnospiraceae correlated with the elimination of *C.* difficile. Interestingly, in our study, in the AmCl/CD group of animals, a significant increase in the abundance of the family Lachnospiraceae was also observed after 21 days, at the same time, a.i., it was noted that *C.* difficile bacteria eliminated from animal feces by themselves.

In the AmCl/CD and AmCl+Lactitol/CD groups, a significant increase in the abundance of the genus Bifidobacterium was observed on the 21st day of the study compared to the intact group and the Intact/CD group. Apparently, the observed effect can be associated with the spontaneous recovery of microbiota due to the diet used. However, it is known that Bifidobacterium by themselves inhibit the colonization of C. difficile. Probably, an increase in the proportion of this species in the intestinal microbiota may also contribute to the observed spontaneous elimination of *C.* difficile. In the AmCl+Lactitol/CD group, a significant decrease in the abundance of Clostridia from the Clostridiaceae family was also observed as compared to AmCl/CD group, which is consistent with the literature data, where it was shown in clinical studies that lactitol inhibits populations of bacteria of the genus Clostridium in feces, as well as the abundance of Clostridium cluster XIVab in a synbiotic combination with Lactobacillus acidophilus. Furthermore, in the lactitol group, we observed a probable indicative antagonistic effect to the antibiotic action due to a decrease in the number of the family Paenibacillaceae, the genera Paenibacillus, Clostridium, and Clostridium unknown species, however, in the available literature, we did not find any mention of the special role of these bacterial taxa in the recovery of the microbiota.

Taking into consideration the observations described above, the development of CDI and its severity are probably associated not only with the compositional disorder of the microbiota but also with other factors. Several studies indicate an important role of the immune system, which can be damaged by antibiotics that can lead to the development of disease.39 Thus, in Myd88–/– mice with impaired innate immune response in mucous membranes, the disease caused by C. difficile proceeded with a vivid clinical picture and lesions in the intestine in comparison with wild mice.30 It is known that prebiotics such as lactitol can change the composition of the host microbiota, stimulating the growth of bifidobacteria and lactobacilli, inhibiting the growth of bacteria from the families *Clostridiaceae and Enterobacteriaceae,* as well as influencing the production of butyrate, maintaining the viability of enterocytes and stimulating the body's

immune response through the induction of immunoglobulin A and transforming growth factor beta (TGF-β). In the study in mice, we tested the lactitol administered before the development of clinical signs of the disease, i.e. in a preventive regimen. When using such a regimen of the co-use of lactitol with a mixture of Am and Cl, a significant decrease by 10 times in the concentration of *C.* difficile in samples of intestinal contents was observed compared to the group receiving only the antibiotic before infection. This may be associated with the main effect of lactitol being metabolized by beneficial bacteria, and activating their growth, despite the antimicrobial effect. The influence of the recovery of normal intestinal microbiota on *C.* difficile colonization has also been shown in studies on the effectiveness of fecal transplantation, where the administration of intestinal contents of healthy animals to infected ones significantly reduced their fecal excretion of Clostridia [5].

To assess the impact of *C.* difficile on the composition of immune cells in mice with induced dysbiosis against the background of prophylactic use of lactitol, we conducted a study, which determined the number of leukocytes, lymphocytes, and granulocytes in the blood 10 days a.i. As a result, we observed a significantly lower concentration of the level of lymphocytes in the group receiving lactitol for prophylaxis compared to the group of infected animals without lactitol. The infection with *C.* difficile in mice, even without additional antibiotic pretreatment, showed a tendency to an increase in the content of leukocytes and lymphocytes in comparison with intact animals. Apparently, the observed effects are likely realized through the reaction of blood cells to the release of inflammatory cytokines (interferons α and γ), TNF-α, and colony-stimulating factors, such as granulocyte-macrophage colony-stimulating factor (GM CSF), macrophage colony-stimulating factor (M CSF), and granulocyte colony-stimulating factor (G CSF) in the focus of Clostridial infection, that lead to hematopoiesis and the observed increase in the number of leukocytes in the blood. In our study, a blood test was performed 10 days a.i. *C.* difficile. This time interval allowed us to observe an increase in leukocytes and lymphocytes in the blood in response to the expression of inflammatory factors, which is consistent with the data of other researchers. However, in the group of mice that received lactitol in a prophylactic regimen, an increase in the number of immune cells was not observed, and most likely this is associated with the ability of lactitol to stimulate the expression of TGF-β, which is a pleiotropic cytokine produced by different types of cells and regulates various cellular functions as a suppressor of immune response and cell growth. In the intestinal immune response, TGF-β suppresses the inflammatory response to bacterial antigens in the intestinal lumen, which leads to a less pronounced proliferative response from leukocytes and lymphocytes that we observed in our study.

To assess the changes in the humoral immune response against the background of C. difficile infection, we studied the relative level of nonspecific IgG in the blood of mice, which made it possible to observe an increase in its content in the group of mice receiving lactitol as prophylactic agent compared with other groups, and it can also be explained by the probable role of stimulation of TGF-β with lactitol. In one of the studies, it was shown that TGF-β is capable of enhancing IgA production; however, in the work of other authors, it was found that TGF-β is also capable of enhancing IgG production in mice. There was no significant difference in the IgG content between the groups of C. difficile infected animals with the antibiotic pretreatment that received or did not receive lactitol. Nevertheless, we observed a trend towards more pronounced IgG accumulation in the group of mice with lactitol.

In this study, we have not assessed the levels of IgA, which preferentially accumulates in the intestinal mucosa, however, the data of histological examination of mucosal samples from the cecum of mice from the different groups, obtained 8 days after *C.* difficile infection, allowed to discover signs of a less pronounced inflammatory reaction (infiltration) of the mucous membrane in mice from the group of lactitol. This difference in the group of lactitol versus the non-lactitol group apparently responds to more efficient neutralization of bacterial antigens by IgA molecules, the level of which was probably increased by lactitol. As shown in one of the studies, secretory IgA accumulates on the outer surface of the intestinal mucosa, actively interacting with bacteria and their toxins.

The results of studying effects of lactitol, obtained using the model of C. difficile colonization in mice, helped us to conduct similar experiments on a model with pronounced clinical signs. Therefore, we carried out a study in golden hamsters, pretreated with the antibiotic i/g and then infected with C. difficile. This study showed the ability of lactitol in a therapeutic and prophylactic regimen (i.e. lactitol was administered before the development of clinical signs of the disease and after their appearance) to significantly prolong the average time of life (the number of days) in these animals, in comparison with animals that did not receive the prebiotic. As a result, the use of lactitol did not increase the survival rate of animals, which died in 100% of cases in the period from the first to the fourth day a.i. in all experimental groups. Judging by the rate of death in hamsters, especially in the group that received only the antibiotic before infection, these animals are highly susceptible to the strain ATCC-43255 used. Overall, our results are consistent with the available literature data and confirm the high sensitivity of hamsters to C. difficile infection.50,51 Thus, the development of clinical signs of the disease caused by C. difficile were observed at different time points depending on the type of antibiotics used after their withdrawal, this time point probably depended on MIC and the rate of elimination of the antibiotic from the body of animals. In our study, the administration of a low dose of the antibiotic, which did not cause significant suppression of lacto- and bifidobacteria in hamsters, led to a faster death of animals in the group, as compared to the group administered a high dose of the antibiotic. Probably, the delay of death of animals when using a higher dose of the antibiotic is associated with its longer residual antibacterial effect, inhibiting the growth of C. difficile, which persisted for more than 2 days after the completion of the antibiotic administration in our study. It is known that this antibiotic is capable of suppressing Clostridia at relatively low concentrations of MIC = 0.03-2 mg/L.53

Considering the observed effects on the rate of death from the administration of different doses of the antibiotic to hamsters prior to their infection with C. difficile, it could be assumed that the severity of the disease and the rate of death of animals is most likely related to the ability of these pathogenic bacteria to reproduce in the intestines of animals. However, the determined counts of pathogens in the feces of hamsters were consistent with those previously found in the asymptomatic mice. Such a difference in the clinical manifestations of the disease caused by C. difficile is probably due to differences in the natural sensitivity of mice and hamsters to C. difficile toxins,

among which the main are exotoxins A and B. Furthermore, these molecules are able to enhance the toxic effect of each other for the cells of the intestinal mucosa.54 It was found that hamsters are 100-1000 times more susceptible to toxins A and B than mice.55

According to our data, lactitol was not able to protect animals from the action of *C.* difficile toxins; however, it significantly slowed down the rate of death in these animals by 33% and significantly decreased the concentration of *C.* difficile in fecal samples (by 2.5 times). Apparently, as in the case of studies in mice, this effect could be realized due to the ability of lactitol to increase the levels of short-chain fatty acids in the intestine, which are known to suppress toxin production and reproduction of *C.* difficile. To determine the probable effects of lactitol on the parameters of the cellular immune system in hamsters, as well as in mice, we conducted a study to see the changes in the concentrations of certain immune cells in the blood of hamsters, assessed one day a.i. *C.* difficile. These studies revealed a significant shift in levels of granulocytes and lymphocytes in the group of infected animals treated with the antibiotic without lactitol. We observed a significant increase in the concentration of granulocytes and a decrease in the concentration of blood lymphocytes in comparison with the group of the intact animals. In this case, the use of lactitol did not significantly change the proportion of lymphocytes to granulocytes in the blood of hamsters in comparison with the group of the intact animals. The observed effect of granulocytes and lymphocytes in the group of animals that received only the antibiotic and then infected with *C.* difficile indicates the probable role of cytokines (interferons α and γ) and G CSF.58 At the same time, a significant decrease in lymphocytes in the group of infected animals, pretreated with the antibiotic, is probably associated with the apoptosis of these cells, which is also observed in sepsis.59 It is known that in response to the action of C. difficile toxins, a diversity of inflammatory products are released, such as interleukins (IL-6, IL-8, IL-1β), tumor necrosis factor α (TNF-α), and colony-stimulating factors.60-62 The dependence of the ratio of granulocytes (neutrophils) to lymphocytes and the severity of the infectious process was also observed.63,64 Noteworthy is the fact that lactitol was able to prevent the development of a severe inflammatory reaction in hamsters, which resulted in the normalization of the ratio of granulocytes to lymphocytes. Perhaps, this effect of lactitol could be realized through the induction of TRP-β, as in mice. What is more, the role of IL-10 cannot be ruled out, the enhancement of induction was observed upon administration of butyrate to animals, and lactitol is capable of increasing the concentration of this substance in the intestine.65 Expression of IL-10 triggers the proliferation of B lymphocytes and suppresses inflammation, T lymphocytic and macrophage activity by inhibiting the production of interferon-γ, interleukins (IL-2, IL-12, IL-18), TNF-α and other factors.66,67 It should be noted that differences in the pattern of changes in the levels of immune blood cells in mice and hamsters are associated with different time intervals that were used to study the cellular composition. At the same time, in hamsters, a picture of an acute reaction of white blood cells (leukocytes) to inflammation that arose soon a.i. (1 day after) was observed, and in mice, a picture of a distant reaction of white blood cells in response to C. difficile was observed (10 days a.i.).

Signs of suppression of the inflammatory response were also noted after a histological examination of sections from cecum samples obtained from hamsters treated with lactitol i/g in a scheme of therapeutic and prophylactic usage. It was noted that in these animals there are no signs of pathological changes in the mucous membrane of the wall of the cecum, and the infiltration in the mucous membrane consisted mainly of plasma cells and lymphocytes. At the same time, in animals that had received only the antibiotic before infection, more noticeable signs of inflammation were observed in the wall of the intestinal mucosa: distinctly dilated, plethoric; swelling of the stroma of the mucous membrane; desquamation of some cells of the epithelial layer in the apex of the crypts. In the cellular infiltrate, along with plasma cells and lymphocytes, polymorphonuclear leukocytes were found, a similar pathomorphological picture of the mucous membrane of hamsters was also observed in studies of other authors.

In conclusion, in this study, we developed a model of C. difficile colonization of intestines in C57BL/6 mice, as well as a model of lethal infection in golden hamsters caused by a 10-day administration of the mixture of Am and Cl at different doses. According to our study, microbiota disorders were caused to a greater extent by the C. difficile colonization of the intestines of animals rather than by the severity of the disease caused by the pathogen, which also depended on the species of animals. Use of lactitol together with antibiotic showed suppression of intestinal C. difficile colonization in all the studied animals, including through stimulation of the bacterial families such as Erysipelotrichaceae, Lactobacilliaceae, Lachnospiraceae, genus Bifidobacterium and inhibition of the bacterial families Clostridiaceae and Paenibacillaceae In addition, it also showed immune-mediated beneficial effects, expressed in the potentiation of the immune response due to the increase of IgG production and normalization of cellular immune responses. All the positive effects of lactitol observed in our study may indicate the possibility of its further co-administration with antibiotics for the purpose of complex prevention of antibiotic-associated diarrhea caused by C. difficile. In addition, we suggest lactitol to be used in combination regimens with antibacterial therapy against CDI in patients to achieve a more pronounced therapeutic effect and support the immune system for fighting against the pathogen. Taking into account that hamsters are very susceptible to CD much more than humans the addition of lactitol would be more beneficial for humans.

Materials and Methods

Bacterial strains

The strain of Clostridium difficile (ATCC® 43255™)68 was used for the model development of the intestinal clostridial infection in mice and hamsters. For the growth of C. difficile, we used a nutrient agar consisting of the Clostridium difficile Agar Base (HiMedia, India) with 7% of fresh defibrinated sheep blood and a selective additive of Cycloserine-cefoxitin (Oxoid, USA); Brain Heard Infusion Broth (Hi-Media, India) with a selective additive of Cycloserine-cefoxitin; thioglycolic medium (FBIS SRCAMB, Russia) with a selective additive of Cycloserine-cefoxitin. Sporulation of C. difficile was induced by cultivation on selective enrichment media under anaerobic conditions. The initial suspension of spores was prepared by suspending individual colonies in saline, which grew on the fifth day of incubation on a solid culture medium under anaerobic conditions at 37°C. The suspension for infection was prepared using a densitometer, adjusting the optical density of the suspension of the

initial culture of C. difficile to 2 McFarland units, which corresponded to 1.0×109 CFU/ml. Then the prepared suspension was diluted to the desired concentration needed to infect animals i/g.

Laboratory animals

In the experiments, specific-pathogen-free (SPF) mice of the C57BL/6 line (females/males, at the age of 8 - 9 weeks with a bodyweight of 18 - 28 g) were used, obtained from the nursery of laboratory animals Pushchino, the branch of the Shemyakin and Ovchinnikov Institute of bioorganic chemistry (FBIS IBCh RAS, Russia). Experiments with C57BL/6 mice were carried out in accordance with the VP-2017/6 protocol, approved by the Bioethics Commission of the FBIS SRCAMB. Golden Syrian hamsters (females/males, at the age of 6 - 8 weeks with a bodyweight of 60 -80 g) were also used, obtained from the nursery of laboratory animals Stolbovaya, the branch of the Scientific Center for Biomedical Technologies (FBIS SCBMT FMBA, Russia). All experiments with these laboratory animals were carried out in accordance with the VP-2019/2 protocol approved by the Bioethics Commission of the FBIS SRCAMB.

Experimental animals were kept in standard conditions in accordance with SR 2.2.1.3218-14 Sanitary and epidemiological requirements to arrangement, equipment and maintenance of biological clinics (vivariums),69 and Directive 2010/63/EU of the European Parliament and the Council of the European Union of September 22, 2010, on the protection of animals used for scientific purposes, as well as Guide for the Care and Use of Laboratory Animals.70 All animals underwent quarantine before participating in the experiments. The manipulations with the animals were carried out in class 2 microbiological safety boxes. The animals were euthanized by CO2 inhalation.

Drugs to be examined and their dosing regimens

In studies with mice, anhydrous lactitol was used – batch No. 1942839316 (Beneo-Orafti, Belgium), which was administered i/g twice a day. A single dose of lactitol was1740 mg/kg. Mice received lactitol in a prophylactic regimen; it was given starting from the first day of antibiotic administration and then for 21 days. We used lactitol to study the effects of the prebiotic in hamsters – batch No. 1943297164, manufactured by Danisco (USA), in a single dose of 2116 mg/kg/day. Hamsters received lactitol in a therapeutic and prophylactic regimen, starting 12 days before and continuing until infection, on the day of infection, and after C. difficile infection until the end of the experiment or death of the animal.

Modeling of antibiotic-induced dysbiosis in animals

A mixture of antibiotic substances was used as antibacterial drugs for the induction of dysbiosis in mice: Am (batch No. 1011702241402, North Chinese pharmaceutical group Semisintek, China) and Cl with siloid (1:1) (batch No. ACS0150, CKD Bio Corporation, Korea). In studies in hamsters, a mixture of antibiotic substances: Am (batch No. V352210, North Chinese pharmaceutical group Semisintek, China) and Cl (batch No. BP1M180850, DSM Sinochem Pharmaceuticals, Netherlands) were used. A solution of the substances of Am and Cl was administered into mice i/g using a metal gastric tube (Stanford Equipment Corp, USA). The volume of the substance solution received by the animals was 200 µl. A single dose of Am and Cl was 585 mg/kg and 83.62 mg/kg, respectively. The drugs were administered twice a day for 10 days with a dosing interval of at least 8 hours. Hamsters were administered with a solution of Am and Cl i/g using a metal gastric tube. The volume of the substance solution administered to the hamsters was 0.5 ml. Depending on the experiment, a single dose of Am and Cl was either 13.34 and 1.89 mg/kg, respectively, or 2.67 and 0.38 mg/kg, respectively. The drugs were administered twice a day for 10 days with a dosing interval of at least 8 hours.

Infection of animals with *Clostridium difficile*

Mice were infected i/g by administering 0.2 ml of a bacterial suspension of C. difficile with a concentration of 5.0-10.0×104 CFU/ml. Hamsters were infected i/g by administering 0.5 ml of a bacterial suspension of C. difficile with a concentration of 4.0×104 CFU/ml. In the groups of animals that received the antibiotic, mice were infected in 1 day, and hamsters in 2 days after the completion of the antibiotic administration. Throughout the experiment, each animal was examined daily, and clinical signs of disease or death were recorded.

Blood cells analysis

Blood of the animals for a blood cells count was taken from the retroorbital venous sinus into tubes with K2-EDTA (SC Sanguis Counting, Germany) in a volume of 200 µl using a Pasteur pipette. The complete blood cells count was performed within 30-90 minutes after taking a blood sample. The samples were analyzed on an automatic hematological analyzer PCE-90Vet (High Technology, USA) in accordance with the instructions for use.

Quantitative determination of nonspecific Immunoglobulin G

The blood of the mice was taken into separate dry microcentrifuge tubes to obtain serum. After separating the blood clot from the walls of the tube, the samples were placed in a refrigerator overnight and then centrifuged at 3000 g for 10 minutes. The resulting serum was collected in labeled tubes and frozen at minus 20°C until analysis. The concentration of total protein in blood serum was determined using the Total protein-olvex kit (Olvex Diagnosticum, 006.001). The concentration of nonspecific IgG was evaluated using the Easy-Titer™ Mouse IgG Assay Kit (ThermoFisher, 23300). The studies were carried out using a Tecan Infinite M1000 multifunctional reader. Blood sera were stored at minus 800C prior to their use in the study. The IgG content in animal serum samples is presented as a portion to the total protein content.

Fecal sampling and microbiological analysis

Fecal samples of the animals were taken at 250 mg into labeled Eppendorf microcentrifuge tubes. The animal feces were sampled for routine bacteriological analysis.71Samples of intestinal contents were taken after euthanasia of mice to assess the content of C. difficile. The number of cells of Lactobacterium spp., Escherichia coli, Enterococcus spp., Candida spp., Clostridium difficile in the feces of

mice and hamsters were determined by inoculating suspensions of the samples to be examined on solid nutrient media. The cell concentration of Bifidobacterium spp. in the samples was assessed by inoculation on a semi-liquid culture medium. The species composition of the intestinal microbiota in mice and hamsters was determined using differential diagnostic media: medium No. 1 GRM (FBIS SRCAMB, Russia), growth medium for cultivation and isolation of bifidobacteria (FBIS SRCAMB, Russia), growth medium for cultivation and isolation of lactobacilli (FBIS SRCAMB, Russia), Endo agar for isolation of enterobacteria (FBIS SRCAMB, Russia), sorbitol agar (FBIS SRCAMB, Russia), Enterococcus agar (FBIS SRCAMB, Russia), culture medium No. 10 (FBIS SRCAMB, Russia), Sabouraud maltose agar (FBIS SRCAMB, Russia).

To identify bifidobacteria and lactobacilli, the inoculations were cultivated in BBL GasPak System anaerostats with the Anaero Gen Termo Scientific gas generating sachet under strictly anaerobic conditions. The isolated cultures of bacteria were identified by MALDI - TOF MALDI Biotyper mass spectrometer based on Microflex BRUKER Daltonik Gmb mass spectrometer (BRUKER, Germany) using the BRUKER TAXONOMY automatic program.

Sequencing of mouse fecal samples

Fecal samples of the animals were taken in 250 mg into labeled Eppendorf microcentrifuge tubes and frozen at -20°C immediately after sampling and stored until DNA extraction. Total DNA from the fecal samples was isolated using a commercial QIAamp® DNA Stool Mini Kit (50) (QIAGEN, Germany) according to the manufacturer's instructions. The DNA concentration in the isolated samples was assessed using NanoDrop ND-1000 spectrophotometer according to the manufacturer's instructions. The assessment of the composition of the microbiota was carried out specifically for the hypervariable regions V3-V4 of the 16S ribosomal ribonucleic acid (rRNA) gene using the pair-end method. For 16S library preparation, microbial DNA from fecal samples was diluted to a final concentration of 5 ng/µl. The regions to be amplified were combined with the Nextera® XT Index Kit v2 Set A, B, and C barcodes (Illumina, USA). Sequencing rounds were performed using the MiSeq 600 cycle Reagent Kit v3 and the MiSeq Illumina system instrument (Illumina, USA).

The quality control of the sequences was carried out using the FastQC application, primer trimming of reads and the quality of nucleotides-using the Trimmomatic tool with the parameters HEADCROP:20 SLIDINGWINDOW:4:12 MINLEN:200. Assembling read pairs was carried out using the VSEARCH tool, no more than 5 mismatches were allowed in the overlap region. The assembled read pairs were mapped to the Greengenes v13.8 database with a specified minimum identity threshold of 97%.72 Taxonomic names indicated in square brackets are suggested by the Greengenes database v13.8.

Pathomorphological studies

The necropsy of animals from the experimental groups was performed for three euthanized animals from each group. Fragments of different parts of the intestine of the animals, including the cecum, were exsected and placed in a vial with 50 ml of a 4% paraformaldehyde solution. After a 24-hour exposure of the samples, the paraformaldehyde solution was replaced with a fresh portion. The mucous membrane of the small and large intestines of the animals was studied. Histological sections were stained with hematoxylin and eosin according to the standard procedure. In order to study pathomorphological changes, microscopy of prepared and stained sections of intestinal tissue samples was carried out using a Nikon Eclipse 80i microscope equipped with a photographic device and a NIS Elements F 4.60.00 image analyzer. The sections were studied at 10x and 20x magnifications.

Statistics

The data of bacterial concentration in fecal samples are presented as the mean and its standard deviation, or 95% confidence interval. Statistical analysis of bioinformatics data was carried out by assessing the index of absolute variation used as a measure of “richness”. The index was calculated as the average number of nonzero observations in the hypergeometric distribution in a sample with a depth of 1700 reads and compositional parameters n, equivalent to the number of reads in the sample. Shannon entropy, a measure of alpha diversity (“evenness”), was also evaluated. Furthermore, for the calculations, the data were filtered – bacteria that are present on a level above 0.1% on at least one of the days in less than 30% of the samples were excluded. Comparisons of mean values of indices between groups were carried out using the Student's t-test with Benjamini-Hochberg's adjustment to control the proportion of false positives (False Discovery Rate, FDR) at the level of 0.05.74 A comparison of the bacterial abundances between groups by species and genera was carried out using the ALDEx2 method, 75,76 implemented in the R package of the same name.77 ALDEx2 estimates distributions of bacterial abundances using a Monte Carlo sampling from the Dirichlet distribution. The number of samples in the Monte Carlo sampling was set equal to 1000. Abundances are normalized to the geometric mean for all nonzero features. The size of the effects was assessed using the relative abundance of bacteria in the compared groups. Within ALDEx2, Monte Carlo sample distributions were compared using Student's t-test for related or independent samples. The Benjamini-Hochberg method was used to correct for multiple comparisons.

Conclusion

Data of hematological parameters are presented as median values and confidence intervals for the median at the 95% significance level, according to tables for small samples. IgG content data are presented in the figure as median values in the form of a segment within a rectangle, 3-quantile intervals are limited by the perimeter of the rectangle, error bars determined the intervals for 1.5 interquantile ranges. Intergroup comparisons of hematological parameters and IgG content were performed using the Mann-Whitney U-test with correction for multiple comparisons, at $p<0.05$ for multiple comparisons with the Kruskal-Wallis test for the selected parameter.

Acknowledgements

The authors express their gratitude to the company Ksivalue for the assistance in mapping the initial raw data obtained from the results of 16S rRNA sequencing of fecal samples of animals to the taxonomic database.

References

1. Leffler, Daniel A, and Lamont J Thomas. "Clostridium Difficile Infection." New Eng J Med 372 (2015): 1539-1548.
2. Bouza, E, Munoz P, and Alonso R. "Clinical Manifestations, Treatment and Control of Infections Caused by Clostridium Difficile." Clin Microbiol Infect 11 (2005): 57-64.
3. Chilton, Caroline H, Freeman Jane, Crowther Grace S, and Todhunter Sharie L, et al. "Co-Amoxiclav Induces Proliferation and Cytotoxin Production of Clostridium Difficile Ribotype 027 in a Human Gut Model." J Antimicr Chemoth 67 (2012): 951-954.
4. Hutton, Melanie L, Mackin Kate E, Chakravorty Anjana, and Lyras Dena. "Small Animal Models for the Study of Clostridium Difficile Disease Pathogenesis." FEMS Microbiol Lett 352 (2014): 140-149.
5. Buckley, Anthony M, Spencer Janice, Candlish Denise, Irvine June J, et al. "Infection of Hamsters with the UK Clostridium Difficile Ribotype 027 Outbreak Strain R20291." J Med Microbiol 60 (2011): 1174.

How to cite this article: A. A. Sergeev, N. A. Mnafki, E. A. Ryabenko, A. A. Nikitina, C. Shammas, E. Nikolaou, A.V. Dikovskiy, T.I. Kombarova, O. V. Korobova, E. A. Ganina, I. P. Mitsevits, A. I. Borzilov, M. V. Khramov, and L. C. Zacharia. "Immune-mediated Effects of Lactitol in Animals with Antibiotic-induced Dysbiosis after Infection with Clostridium Difficile". *J Antimicro Agent* (7): (7)(2021) :253