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EFFECT OF A COMPLEX FEED ADDITIVE ON THE COMPOSITION AND FUNCTION OF THE *Oryctolagus dominis* CAECUM MICROBIOME

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Abstract

There is a growing interest in the study of natural multicomponent feed additives to regulate gut microbiome composition and improve immune and physiological status of rabbits. In the present work, for the first time, it was bioinformatically found that a complex probiotic biological product affects the change in the predicted metabolic pathways of the rabbit intestinal microbiome. The aim of the work was to study the joint action of a complex containing minerals and a probiotic on physiological status, composition and functional potential of gut microbiome in rabbits. For the study (the vivarium of the FGBU VO SPKhFU of the Ministry of Health of Russia, St. Petersburg, 2021), ten Soviet chinchilla rabbits of 2.5 months of age (5.37-5.53 kg bw) were allocated to two groups of five rabbits each. Control group I received the recommended basal diet (BD, RAAS norms 2003), test group II was fed with the BD supplemented with a complex feed additive (30 mg per animal day^{-1}) consisting of the microelement preparation Silaccess at 5 mg/kg of bodyweigh (LLC TECHNOLOG 2D, Russia) and the probiotic strain *Bacillus subtilis* 1-85. On days 30 and 60, the animals were weighed before morning feeding, and blood was sampled to evaluate natural resistance parameters (bactericidal function, including lysozyme activity, and phagocytic activity of neutrophils). Chyme samples of the caecum for microbiome studies aseptically collected at the end of the experiment were immediately placed in sterile plastic tubes. Total DNA was isolated using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., USA). The bacterial community was assessed by NGS sequencing on a MiSeq automated sequencer (Illumina, Inc., USA) using primers to the V3-V4 region of the 16S rRNA gene which allows us to identify microorganisms to the species level: the forward primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ and the reverse primer, 5′-GTCTCCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. The reconstruction and prediction of the functional content of the metagenome was performed using the PICRUSt2 (v.2.3.0) software package (https://github.com/picrust/picrust2). Mathematical and statistical processing was carried out by the multivariate analysis of variance procedure using Microsoft Excel XP/2003, R-Studio (Version 1.1.453) (https://rstudio.com). In group II compared to control, the phagocytic index was higher ($p \le 0.05$) by 1.8 %, the phagocytic number by 32.3 % ($p \le 0.05$). NGS sequencing revealed the values of the Chao1, Shannon and Simpson biodiversity indices to be higher ($p \le 0.05$) in group II compared to group I. Taxonomic analysis of caecum microbial community disclosed 12 phyla of the kingdom *Bacteria* among which representatives of the phylum *Firmicutes* dominated (80.2±6.2 % in group I, 78.2±7.4 % in group II). In group II, there was

a 1.3-2.6-fold increase in the abundance of phyla *Verrucomicrobiota*, *Actinobacteriota*, *Patescibacteria*, *Proteobacteria*, *Desulfobacterota* and a 4.8-fold decrease in the abundance of the phylum *Campylobacterota* ($p \leq 0.05$). In the caecum of test rabbits, the genus *Bacillus* spp. increased 2.82 times compared to control ($p \leq 0.05$). *Staphylococcus sciuri* was found in group I (0.075±0.006 %) but not in group II. Data processing using the PICRUSt2 software tool (v.2.3.0) revealed 370 predictable metabolic pathways in the rabbit gut microbial community, 36 of which differed ($p \leq 0.05$) between the groups. In group II, the intestinal microbiome pathways related to the degradation of aromatic compounds and xenobiotics, protein, carbohydrate, and energy metabolism, alcohol biosynthesis, photorespiration, assimilation of formaldehyde, degradation of myo-, chiro- and scillo-inositol, cell wall synthesis and spore formation activated compared to group I ($p \leq 0.05$). The dominant proportion (15 pathways) of enhanced potential metabolic pathways was associated with the degradation of aromatic compounds and xenobiotics. Thus, a complex dietary additive based on the probiotic strain of *Bacillus subtilis* 1-85 and microelements has a multiple positive effect both on gut microorganisms (fewer pathogens, metabolic regulation) and on the macroorganism (higher values of immunity parameters, a better growth performance of Soviet chinchilla rabbits).

Keywords: probiotic, trace elements, resistance, domestic rabbits, microbiome, NGS, metabolic pathways

Rabbits (*Oryctolagus dominis*) are farm animals with a short pregnancy period, high fecundity, and good feed conversion [1, 2]. Rabbit meat is high in protein, low in fat, cholesterol, and sodium, and is easily digestible, which makes it a dietary choice [3]. In addition, rabbits are often used for experimental purposes as model animals.

In many countries, rabbit farming is becoming an important growing subsector [4]. The main factors hindering the development of rabbit breeding are viral and bacterial diseases, leading to mass mortality and significant economic losses [5].

The microbial populations inhabiting the gastrointestinal tract of animals form the microbiome, a complex ecosystem capable of autoregulating its homeostasis under favorable conditions. The mammalian gut microbiome plays an important role in digestive, metabolic, physiological and immunological processes, affects host susceptibility to many immune-mediated diseases and disorders (6), and affects productivity [7, 8]. This was also confirmed by the example of rabbits, whose economically valuable traits are also affected by the intestinal microbiota [8].

Due to the rapid development of sequencing technologies, several interesting studies have been carried out to analyze the microbial communities of the rabbit gut. In 2008 and 2012, the bacterial community of the caecum of rabbits was studied using high-throughput sequencing of the V3-V4 amplicon of the 16S rRNA gene [9, 10]. In 2018, changes in the microbiota of the rabbit by intestinal tract were described, with a focus on the microbiota of the caecum and faeces [11]. In 2019, the composition of the microbiota of the gallbladder of rabbits of different ages was studied [12], in 2020, the structure of the intestinal microbiota in commercial rabbits was studied in dynamics from weaning to the end of rearing [13]. More recently, the composition of the bacterial microbiota throughout the gastrointestinal tract in New Zealand rabbits has been characterized [5]. Despite the existence of microbial populations in the proximal and distal sections of the rabbit gastrointestinal tract [14], the caecum is the main organ where the most active enzymatic processes occur.

In modern conditions of rabbit breeding, factors such as overcrowding of a significant number of animals in a limited area, violation of the principles of the feeding system and keeping technology, changing diets, poor quality feed, uncontrolled use of antibiotics and other stresses, significantly increase the risk of infectious diseases. The weaning and rearing periods, when there is a transition from mother's milk to solid foods, stress from mother absence and regrouping, are critical [15]. This leads to enteritis and gastrointestinal infections, in particular those caused by the bacteria *Clostridia* spp. and *Escherichia coli*, *Lawsonia intracellulari*s, *Salmonella* spp. [5, 16]. One of the most dangerous disease of domestic rabbits is

epizootic enteropathy. It is a multifactorial gastrointestinal syndrome with a 30-95% mortality rate regardless of breed, with an increasing incidence in the post-weaning period [17).

Intestinal diseases often manifest as inflammation of the digestive system [18], which leads to a violation of the wall integrity [19]. Inflammation and damage to the intestines cause a redistribution of nutrients, which leads to a decrease in animal productivity and a significant increase in economic losses [20]. Studies using chromatographic methods have shown that a number of differential metabolites are involved in five metabolic pathways associated with intestinal inflammation (tryptophan metabolism, pyrimidine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, lysine degradation, and bile secretion) [21]. In turn, an increase in the number of pathogenic and opportunistic bacteria (*Escherichia coli*, *Clostridium* spp., *Bacteroides* spp.) and a decrease in the presence of beneficial bacteria (*Lactobacillus casei*, *Bifidobacterium* spp. and *Lactobacillus* spp.) is associated with the release of pro-inflammatory signaling factors (cytokines, IL-6 and TNF-α), as well as increased secretion of immunoglobulin A (IgA). This process has been associated with decreased short-chain fatty acids, inhibition of intestinal ion and water absorption, and inflammation of the intestinal mucosa [22-24].

Strategies for regulating the microbiome and preventing digestive disorders include introducing feed additives such as probiotics into diets. During the last decade, several studies have been published on the effect of probiotics on the performance of rabbits [25-27]. Some authors have considered specific and non-specific immune responses to probiotic dietary supplements. Various hematological parameters were analyzed: total protein, immunoglobulins, leukocytes and lymphocytes [28, 29]. It has been established that probiotics have a positive effect on the composition of the microbiome, reducing the number of pathogens [30, 31].

Previously, in a study [32] based on bioinformatics processing of data from NGS sequencing of the 16S rRNA gene in the microbiome of the rumen of dairy cows we revealed that changes in the taxonomic structure of rumen microorganisms under the influence of the Cellobacterin+ probiotic were associated with metabolic shifts in the functional potential of microorganisms. In addition, our findings confirmed the role of this probiotic in maintaining metabolic homeostasis. In 2021, a genotype-dependent alteration of potential metabolic pathways of the gut microbiome based on 16S rRNA gene sequencing was demonstrated in rabbits, revealing potential biomarkers important for improving meat rabbit breeds [33].

However, experiments related to the evaluation of the effect of probiotics on the composition and potential metabolic pathways of the intestinal microbiome of rabbits have not been previously performed. That is, the mechanism by which microorganisms and probiotics interact at a metabolic level in the gut of these animals is unclear.

Dietary trace elements have a positive effect on various functions in rabbits, e.g., acid-base balance, nutrient metabolism and immunity. Iron deficiency in rabbits reduces animal activity, leads to loss of appetite, and deterioration of the skin and coat [34). Iodine is necessary for the proper functioning of the thyroid gland, while cobalt is directly related to the absorption of vitamins in fur-bearing animals [35, 36].

It is of interest to create and study the effectiveness of new complex feed additives that will make it possible to obtain environmentally friendly rabbit products of higher quality and reduce the risk of infectious diseases.

Here, we revelaled, based on bioinformatic methods, that a complex probiotic biological product affects the change in the predicted metabolic pathways of the rabbit intestinal microbiome.

This work aimed to investigate how a dietary complex containing minerals

and a probiotic affects physiological parameters of rabbits, gut microbiome composition and its functional potential.

Materials and methods. Experimetns were carried out in 2021 in the vivarium of the St. Petersburg Chemical and Pharmaceutical University (veterinary state registration certificate for the vivarium No. 78-0713/2). Feeding and keeping conditions corresponded to the guidelines for rabbits [37].

Ten Soviet chinchilla rabbits (2.5 months of age, 5.37-5.53 kg body weight) were divided into two groups (5 rabbits each). Animals of control group I received the basal diet (BD) as per recommended detailed norms of the Russian Academy of Agricultural Sciences (2003), experimental group II received BD with the addition of a complex feed additive (30 mg/animal daily). The complex feed additive contained the microelement preparation Silaccess (OOO TECHNOLOG 2D, Russia) [38], consisting of a mixture of mineral components of silicon (35-42.7%), iron (3.5-4.5%), copper (0 08-0.12%) and zinc (0.04-0.055%) in a stabilizing agent (GOST 12.1.007.-76. Moscow, 2007). The dosage of the Silaccess component was 5 mg/kg body weight. In addition, the supplement included the water-soluble probiotic Likvipro (OOO BIOTROF, Russia) at a ratio of 0.5 g/10 l of water. The introduction of the probiotic component was carried out around the clock into the drinking system using a Dosatron D25RE5 dispenser (Dosatron, France). Liquipro is based on the *Bacillus subtili*s 1-85 strain, the drug is produced in dry form in the form of a powder. The duration of the experiment was 75 days.

The growth rate of the animals was controlled by individual weighing on an empty stomach before morning feeding in 30 and 60 days (at 3.5 and 4.5 months of age) after the start of the experiment. The live weight of experimental rabbits was determined using an electronic balance for weighing animals Momert 6551 (MOMERT Co Ltd., Hungary) with an error of up to 10 g, the average daily gains for the noted periods were calculated using the generally accepted formula:

$$A = \frac{W1-W0}{t},$$

where A is the average daily gain in live weight, g; W_0 - live weight at the beginning of the experiment, kg; W_1 - live weight at the end of the experiment, kg; t is the time period, days.

The clinical and physiological state of the rabbits was assessed during daily examination. Attention was paid to the behavior, palatability of food, the condition of the coat.

Thirty days after the start of the experiment and at the end of the experiment (60 days), blood was taken from the rabbits on an empty stomach from the tail vein into two types of vacuum tubes (with the anticoagulant heparin and with a coagulation activator). Determined indicators of natural resistance (bactericidal activity, including lysozyme, phagocytic activity of neutrophils). The phagocytic activity of pseudoeosinoyls was assessed by counting phagocytic cells from 100 neutrophils. When determining the bactericidal activity of blood serum, the method of I.M. Karput [39]. Lysozyme activity in blood serum was determined by the nephelometric method according to V.G. Dorofeichuk [40]. Clinical (hematological) blood analysis was performed using an ABXMICRO 60-OT18 apparatus (Roche, France).

Chyme samples for microbiome studies were manually collected from the caeca at the end of the experiment under aseptic conditions as possible and immediately placed in sterile plastic tubes. All samples were frozen at –20 °C and sent in dry ice to the molecular genetic laboratory of the OOO BIOTROF company for DNA extraction.

Total DNA was isolated from the samples using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc., USA) according to the attached

instructions. The method is based on selective detergent-mediated precipitation of DNA from a substrate using solutions for cell wall lysis and DNA precipitation, 1.2 M sodium chloride, and chloroform.

The bacterial community was assessed by NGS sequencing, which makes it possible to identify microorganisms to the species level, on a MiSeq automatic sequencer (Illumina, Inc., USA) using primers for the V3-V4 region of the 16S rRNA gene: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' (forward primer), 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3' (reverse primer). PCR conditions were as follows: 3 min at 95 °C; 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C (elongation) (25 cycles); 5 min at 72 °C (final elongation). Sequencing was performed using Nextera® XT IndexKit library preparation reagents (Illumina, Inc., USA), Agencourt AMPure XP purification PCR products (Beckman Coulter, Inc., USA), and MiSeq® ReagentKit v2 sequencing reagents. (500 cycle) (Illumina, Inc., USA). The maximum length of the resulting sequences was 2×250 bp.

Automatic bioinformatic analysis was performed using QIIME2 ver. 2020.8 (https://docs.qiime2.org/2020.8/). After importing sequences in the .fastq format from the sequencing instrument and creating the necessary mapping files containing the metadata of the studied files, paired read lines were aligned. Next, the sequences were filtered for quality using the default settings. Noise sequences were filtered using the DADA2 package built into QIIME2, which includes information about the quality of sequences in the error model (filtering of chimeric sequences, artifacts, adapters), which makes the algorithm resistant to a sequence of lower quality. In this case, the maximum length of the pruning sequence was used, equal to 250 bp (https://benjjneb.git-hub.io/dada2/tutorial.html). To construct de novo phylogeny, multiple sequence alignments were performed using the MAFFT software package, followed by a masked alignment to remove positions that differed significantly. For taxonomy assignment, the QIIME2 software was used, which assigns sequences a taxonomic identification based on ASV data (using BLAST, RDP, RTAX, mothur and uclust methods) using the Silva 138.1 16s rRNA database (https://www.arb-silva.de /documentation/release-138.1/).

Based on the obtained table of operational taxonomic units (OTU; operational taxonomic unit, OTU), using plugins of the QIIME2 package, biodiversity indices were calculated, and a graph of the dependence of the number of OTUs on the number of reads was plotted. In the statistical analysis of diversity indices, their additional transformation was not carried out.

The reconstruction and prediction of the functional content of the metagenome, gene families, and enzymes was carried out using the PICRUSt2 (v.2.3.0) software package (https://github.com/picrust/picrust2) [41]. We worked with the program according to the recommended scenario of actions, all settings were used by default. The OTUs of each sample were arranged according to their content, from largest to smallest, and the values were converted using the logarithmic transformation of Log2. The MetaCyc database (https://metacyc.org/) was used to analyze metabolic pathways and enzymes. The predicted profiles of MetaCyc metabolic pathways were assessed by the abundance of ASV (Amplicon Sequence Variants) [42]. Data visualization and calculation of statistical indicators were performed using the Phantasus v1.11.0 web application (https://artyomovlab.wustl.edu/phantasus/), which, in addition to the main visualization and filtering methods, supports R-based methods such as like k-means clustering, principal component analysis, or differential expression analysis with the limma package.

Mathematical and statistical processing of the obtained results was carried out by the method of multifactor analysis of variance (multifactor ANalysis Of VAriance, ANOVA) in Microsoft Excel XP/2003, R-Studio (Version 1.1.453)

(https://rstudio.com). Data are presented as means (*M*) and standard errors of means (±SEM). Significance of differences was assessed by Student's *t*-test, differences were considered statistically significant at $p \leq 0.05$. Means were compared using Tukey's Significantly Significant Difference (HSD) test and the TukeyHSD function in the R Stats Package.

Results. Clinical blood test parameters are extremely sensitive to physiological influences, and therefore the blood profile is a fairly accurate reflection of such influences [43-45]. In animals of the control and experimental groups, the content of hemoglobin and erythrocytes corresponded to age norms, only some minor deviations were noted: the number of erythrocytes and hemoglobin in the II experimental group after 60 days of the experiment (Table 1). In addition, the average content of hemoglobin in erythrocytes in both groups exceeded the norm. In both groups, age-related changes in blood parameters were observed at different stages of development of the rabbit organism. Thus, in 60 days, the amount of hemoglobin in test group II increased by 8.2% ($p \leq 0.05$) compared to the beginning of the experiment, in control group I by 4.45% ($p \leq 0.05$).There were no statistically significant differences in these indicators between the control and experimental groups ($p > 0.05$) (see Table 1).

1. Blood clinical analysis in Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements ($n = 5$, M±SEM; the vivarium expeeriment, St. Petersburg, 2021)

Parameter	Days of experiment	Group I (control)	Group II	Norm
Erythrocytes, $\times 10^{12}$/l	1	7.01±0.43	7.24±0.31	5.2-7.8
	30	7.24±0.59	7.42±0.77	
	60	7.28±0.33	8.20±0.93	
Hemoglobin, mmol/l	1	143.80±8.80	152.20±4.09	100.5-160.0
	30	143.80±4.30	152.00±7.31	
	60	150.20±6.02	164.60±9.24	
The average content of hemoglobin in erythrocytes, pg/l	1	19.72±1.80	21.06±0.71	9.3-15.3
	30	20.70±0.79	21.32±0.73	
	60	20.44±0.58	22.14±2.34	

N o t e. For a description of the groups, see the Materials and methods section.

Regarding the fact that at an early age of rabbits their gastrointestinal tract is not sufficiently prepared for the digestion of solid feed, and the body during the growing period is exposed to stress factors that affect the immune system, we studied the indicators of natural resistance in experimental animals.

Based on the indicators that indirectly reflect the levels of innate immune mediators in the blood (lysozyme content, bactericidal characteristics of blood, phagocytic activity, phagocytic index, phagocytic number), natural resistance in rabbits treated with a complex feed additive was higher than control ($p \leq 0, 05$) (Table 2). Thus, the phagocytic index (the average number of phagocytosed microorganisms per one active leukocyte), reflecting the intensity of phagocytosis, was higher in group II compared to group I by 1.8 ($p \leq 0.05$), the phagocytic number (the ratio of the number of phagocytized leukocyte bacteria to the total number of counted leukocytes) was higher by 32.3% ($p \leq 0.05$).

An increase in natural resistance in rabbits treated with a complex feed additive is natural. It has long been known that blood contains antimicrobial components that provide rapid responses to infection [46]. Innate responses to microbial infections in mammals are mediated by signaling molecules, including Toll-like receptors, cytosolic kinases, nuclear factor (NF)-kB, and transcription factors [47]. Pathogen entry induces rapid expression of several genes encoding antimicrobial proteins and peptides [48, 49]. In turn, there are molecular mechanisms that provide a cross-relationship between the microbiome and the expression of host genes, primarily immunity genes [50]. The introduction of a probiotic strain

of bacteria in combination with trace elements into the intestines of rabbits could affect the expression of host genes associated with immunity. An increase in the content of total protein and globulins in the blood serum of rabbits under the influence of a probiotic has been previously demonstrated [28]. Data obtained by A.F. Mohamed et al. [51] indicate that the introduction of a probiotic strain of Lactobacillus acidophilus into the diet did not have a significant effect on the globulin content, but led to an increase in the number of leukocytes and the amount of total protein in the blood serum. It has been suggested that micronutrients play an important role in various physiological processes and are critical for the proper functioning of the immune system [52].

2. Natural resistance parameters in Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements (day 60, $n = 5$, $M \pm$SEM; the vivarium expeeriment, St. Petersburg, 2021)

Parameter	Group I (control)	Group II
Lysozyme, %	36.18±1.04	46.29±1.36*
Bactericidal activity of blood serum, %	38.45±1.09	46.68±1.27*
Phagocytic activity, %	43.45±1.19	63.27±1.33*
Phagocytic index	5.33±0.10	7.13±0.15**
Phagocytic number	2.88±0.10	3.81±0.11*
Phagocytic capacity, thousand microbial bodies	73.12±1.53	75.89±1.21

N o t e. For a description of the groups, see the Materials and methods section.
* and ** Differences between the test and control groups based on Student's *t*-test are statistically significant at $p \le 0.05$ and $p \le 0.01$, respectively.

3. Daily weigh gain in Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements ($n = 5$, $M \pm$SEM; the vivarium expeeriment, St. Petersburg, 2021)

Group	Days of experiment	Daily weigh gain, g
I (control)	1-30	5.53±0.32
	31-60	5.21±0.15
II	1-30	6.08±0.49
	31-60	5.90±0.21

N o t e. For a description of the groups, see the Materials and methods section.

Weighing data allow us to assume that the studied complex feed additive based on microelements and probiotics does not have a negative effect on bodyweight gain in rabbits (Table 3). However, it should be noted that the number of animals in the experimental groups did not provide a sufficient level of evidence, since the groups were formed according to the main goal of our study - to analyze the composition and functions of the microbiome.

Previously, the positive effect of probiotics and trace elements on the performance of rabbits was noted. A.A.A. Abdel-Wareth et al. [25] studied a complex preparation from a mixture of fenugreek seeds and a probiotic (AmPhi-Bact, American Pharmaceutical Innovations Company, LLC, USA) containing cultures of *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Bifidobacterium bifidum*, metabolites of *Bacillus subtilis* and *Aspergillus niger*, on 45-day-old New Zealand white rabbits for 6 weeks. The authors noted an improvement in feed conversion, a higher digestibility of crude protein and an increase in meat productivity in the groups treated with the drug. M. Lopez-Alonso et al. [53] opined that trace elements coordinate many biological processes and are therefore essential for the maintenance of animal productivity.

At the next stage, we studied the composition of the chyme microbiome from rabbit cecum by NGS sequencing. A total of 57.56 sequenced 16S rRNA gene sequences were generated (with median reads of 9.59 at min = 7.69 and max = 12.86). When comparing the control and experimental groups for the Chao1, Shannon and Simpson indices, it turned out that the values had statistically

significant differences ($p \leq 0.05$) (Fig. 1). In the trest group, the α-biodiversity indices were higher. That is, a complex feed additive, which included a probiotic strain and a mixture of trace elements, had a positive effect on increasing the species diversity of the microbiome in the intestines of rabbits. These results are consistent with other reports [54, 55].

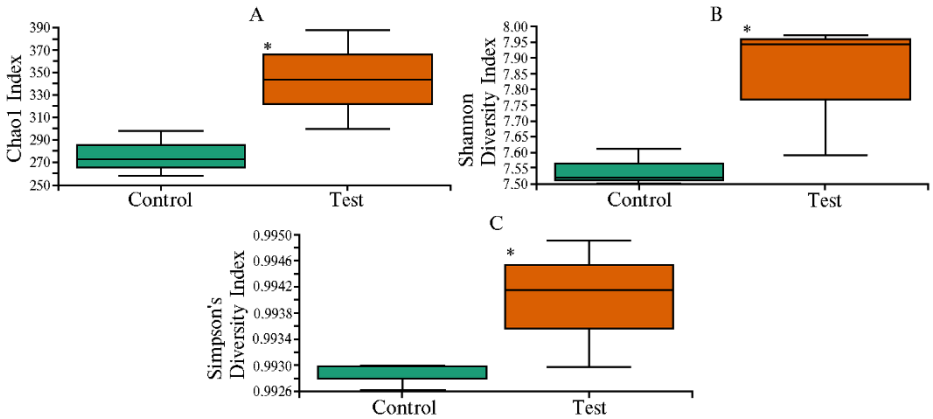

Fig. 1. Absolute values of α-biodiversity indices Chao1 (A)**, Shannon** (B)**, and Simpson** (C) **divericity indexes for the caecal microbiome of Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements** (n = 5, M±SEM; the vivarium expeeriment, St. Petersburg, 2021). Calculated using Qiime2 ver. 2020.11. For a description of the groups, see the Materials and methods section.
* Differences between the test and control groups based on Student's t-test are statistically significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

When studying the composition of microorganisms of the blind processes of the intestines of rabbits, 12 phyla of the kingdom Bacteria were identified (Fig. 2), among which representatives of the phylum *Firmicutes* dominated in number (80.2±6.2% in group, I.78.2±7.4% in group II), which indicates their ecological and functional importance of the phylum in the digestive tract. The main function of *Firmicutes* is the ability to degrade complex polysaccharides with subsequent formation of short-chain fatty acids [56]. Based on previous studies, the dominance of representatives of this phylum in the contents of the blind processes is quite typical for rabbits [11].

In our experiment, the phylum *Bacteroidetes* was the second most common in the intestines of rabbits (13.3±1.2% in group I, 12.3±1.8% in group II). Bacteroidetes have previously been shown [9, 57] to stimulate the development of gut-associated immune tissue in the digestive system. Results similar to ours were also obtained in wild rabbits, as well as in domestic Rex rabbits [58, 59]. On the whole, the quantitative representation of phyla in the intestines of rabbits corresponds to that of other monogastric herbivores [60, 61].

In the experimental group, compared with the control group, there was an increase in abundance of the phyla *Verrucomicrobiota*, *Actinobacteriota*, *Patescibacteria*, *Proteobacteria*, *Desulfobacterota* by 1.3-2.6 times and a decrease in the representation of the phylum *Campylobacterota* by 4.8 times ($p \leq 0.05$). In all likelihood, these data indicate a positive effect of the complex feed additive on the composition of the microbiome. For example, members of the phylum *Verrucomicrobiota*, such as *Akkermansia muciniphila*, have probiotic activity by modulating gut mucus thickness and enhancing intestinal barrier integrity [62]. Bacteria of the phylum *Actinobacteriota* produce antimicrobial substances active against pathogens [63]. Among the bacteria of the phylum *Campylobacterota*, which was represented by the only genus *Campylobacter* (Fig. 3), there are pathogenic forms associated

with rabbit proliferative enteropathy [64].

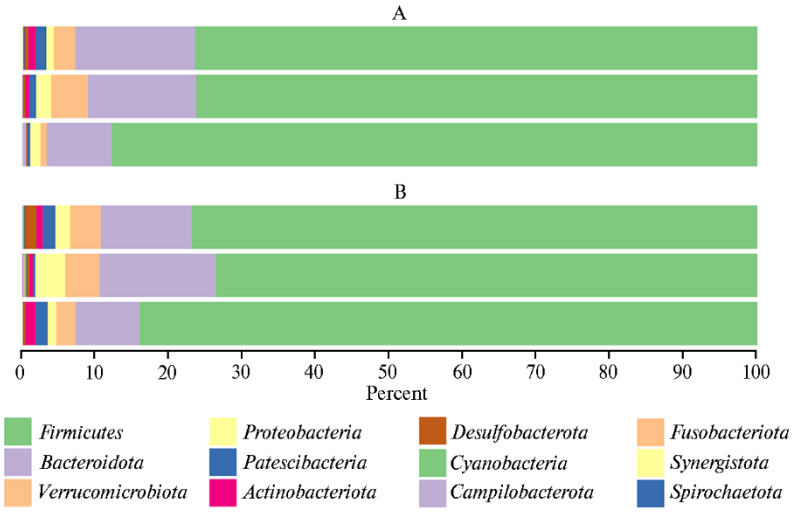

Fig. 2. The composition of caecal microbiome (at the bacterial phylum level) of Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements (day 60, Next-Generation Sequencing of 16S rRNA gene amplicons; $n = 5$, $M \pm$SEM; the vivarium expeeriment, St. Petersburg, 2021). For a description of the groups, see the Materials and methods section.

In the caecum of rabbits from group II, an increase in the number of bacteria of the genus *Bacillus* spp. by 2.82 times compared to the control ($p \leq 0.05$) (see Fig. 3), which may indicate the survival and increase in the number of probiotic microorganism introduced as part of a complex feed additive. This is an important finding, since introduced strains of microorganisms may have different ability to survive in the aggressive environment of the host intestine [65]. Previously, RAPD-PCR proved the survival of probiotic bacteria *Bacillus clausii* in the gastrointestinal tract for 12 days [66].

It is important to note that in the caecum of the intestines of rabbits, we have identified a significant number of genera, including uncultivated ones, belonging to the cellulolytic bacteria of the families *Thermoanaerobacteraceae*, *Ruminococcaceae*, *Clostridiaceae*, *Eubacteriaceae*, *Lachnospiraceae*, which is consistent with the data obtained by C. Вдuerl et al. [67]. Cellulosolytic bacteria are the most relevant microorganisms of the phylum *Firmicutes*, contributing to the breakdown of fiber in plant foods. S. Combes et al. [68] found that bacteria of the genus *Ruminococcus* dominate in healthy rabbits, and when a disease occurs, the number of these microorganisms decreases. E. Cotozzolo et al. [5] also showed that the abundance of members of the families *Ruminococcaceae* and *Lachnospiraceae* serve as important indicators of intestinal health in rabbits. According to the authors, a higher abundance of Lachnospiraceae is characteristic of healthy animals, which is associated with the stimulation of caecotrophic behavior. Cecotrophy is an important feature of the order *Lagomorpha*, which makes it possible to increase the digestibility of low-nutrient plant feeds [69].

The fact is that in the process of digestion of rabbits, two types of feces are formed: solid, poor in nutrients, and soft, consisting of protein, vitamins, inorganic salts and containing a significant number of microorganisms. The latter are called cecotrophs and are eaten by *Lagomorpha* in the process of cecotrophy. Therefore, there is an assumption that cecotrophy is a phenomenon that ensures the formation of the correct composition of the intestinal microbiome. However, in our study, no significant difference was found in the content of genera of

cellulolytic microorganisms between the control and experimental groups ($p > 0.05$).

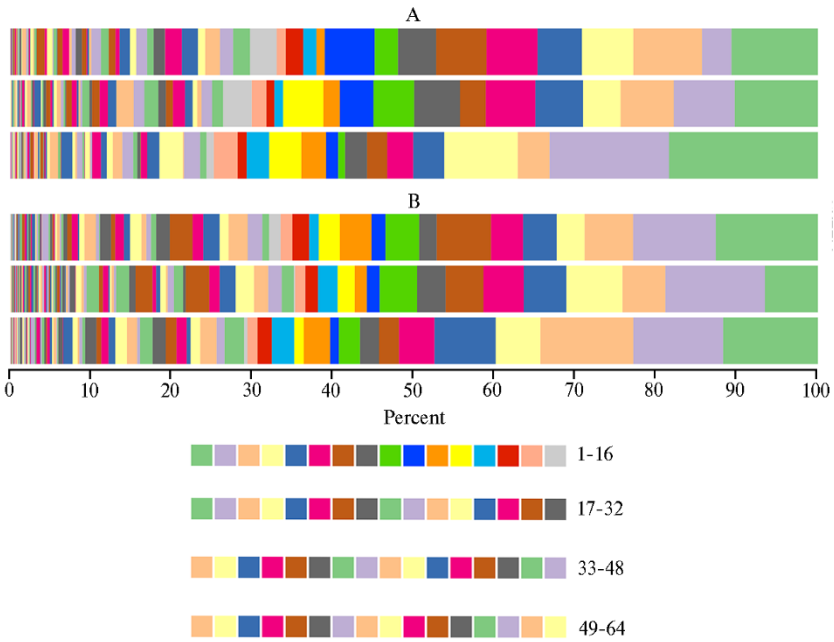

Fig. 3. The composition of caecal microbiome (at the bacterial genera levels) of Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements: 1 — f. *Lachnospiraceae* g. unclassified, 2 — g. *Clostridia* UCG-014, 3 — g. *Oscillospiraceae* NK4A214, 4 — g. *Ruminococcus*, 5 — g. *Monoglobus*, 6 — g. *Oscillospirales* UCG-010, 7 — g. *Clostridia* vadinBB60, 8 — g. *Bacteroides*, 9 — g. *Akkermansia*, 10 — g. *Rikenellaceae* dgA-11 gut, 11 — f. *Bacilli* RF39 g. unclassified, 12 — g. *Lachnospiraceae* NK4A136, 13 — g. *Christensenellaceae* R-7, 14 — g. *Eubacterium coprostanoligenes*, 15 — g. *Oscillospiraceae* V9D2013, 16 — f. *Lachnospiraceae* g. unclassified, 17 — g. *Ruminococcaceae* Incertae Sedis, 18 — f. *Oscillospiraceae* g. unclassified, 19 — f. *Eubacteriaceae* g. unclassified, 20 — g. *Eubacterium siraeum*, 21 — f. *Barnesiellaceae* g. unclassified, 22 — g. *Alistipes*, 23 — g. *Rikenellaceae* RC9 gut, 24 — g. Candidatus *Saccharimonas*, 25 — g. *Subdoligranulum*, 26 — g. *Oscillospiraceae* UCG-005, 27 — g. *Tyzzerella*, 28 — g. *Campylobacter*, 29 — f. *Ruminococcaceae* g. unclassified, 30 — g. *Ruminiclostridium*, 31 — g. *Vibrionimonas*, 32 — g. *Muribaculaceae*, 33 — g. *Desulfovibrio*, 34 — g. *Colidextribacter*, 35 — f. *Anaerovoracaceae* g. unclassified, 36 — g. *Pelomonas*, 37 — g. *Papillibacter*, 38 — f. *Atopobiaceae* g. unclassified, 39 — g. *Staphylococcus*, 40 — f. *Acidaminococcaceae* g. unclassified, 41 — f. *Peptococcaceae* g. unclassified, 42 — g. *Pseudomonas*, 43 — g. *Eubacterium nodatum*, 44 — g. *Anaerovoracaceae* XIII AD3011, 45 — f. *Sutterellaceae* g. unclassified, 46 — g. *Stenotrophomonas*, 47 — g. *Oscillospiraceae* UCG-007, 48 — g. *Ruminococcaceae* CAG-352, 49 — g. *Prevotella*, 50 — g. *Lachnospiraceae* NK4B4, 51 — g. *Izemoplasmatales*, 52 — f. *Eggerthellaceae* g. unclassified, 53 — g. *Ruminococcaceae*, 54 — g. *Defluviitaleaceae* UCG-011, 55 — g. *Ruminococcaceae* UCG-001, 56 — g. *Holdemania*, 57 — g. Oscillibacter, 58 — f. *Erysipelatoclostridiaceae* g. unclassified, 59 — g. *Escherichia-Shigella*, 60 — g. *Odoribacter*, 61 — f. *Xanthobacteraceae* g. unclassified, 62 — g. *Parabacteroides*, 63 — g. *Lachnospiraceae* FCS020, 64 — g. *Parasutterella* (day 60, Next-Generation Sequencing of 16S rRNA gene amplicons; $n = 3$, $M \pm$SEM; the vivarium experiment, St. Petersburg, 2021). For a description of the groups, see the Materials and methods section.

Nevertheless, the positive effect of the complex feed additive on the composition of the microbiome was manifested in a decrease in the number of a number of opportunistic and pathogenic microorganisms in the digestive system of rabbits. Thus, the species *Staphylococcus sciuri* [70] ($0.075 \pm 0.006\%$) was present in the intestines of animals from the control group, while it was not present in the experimental group. Rabbit staphylococcosis is a dangerous disease that leads to pododermatitis, subcutaneous abscesses, mastitis, abscesses of internal organs, mainly lungs, liver, uterus. Arthritis, periodontitis, sinusitis and otitis media have also been described [71]. Despite the great importance of the species *Staphylococ-*

cus aureus in causing staphylococcosis in rabbits, the clinical significance of *Staphylococcus sciuri* seems to be increasing as the bacterium has been associated with various infections such as endocarditis, peritonitis, septic shock, urinary tract infection, endophthalmitis, inflammatory diseases of the reproductive systems [72]. The absence of *S. sciuri* in the experimental group indicates the positive effect of the feed additive, which included a probiotic strain and a mixture of trace elements, which probably act in synergy.

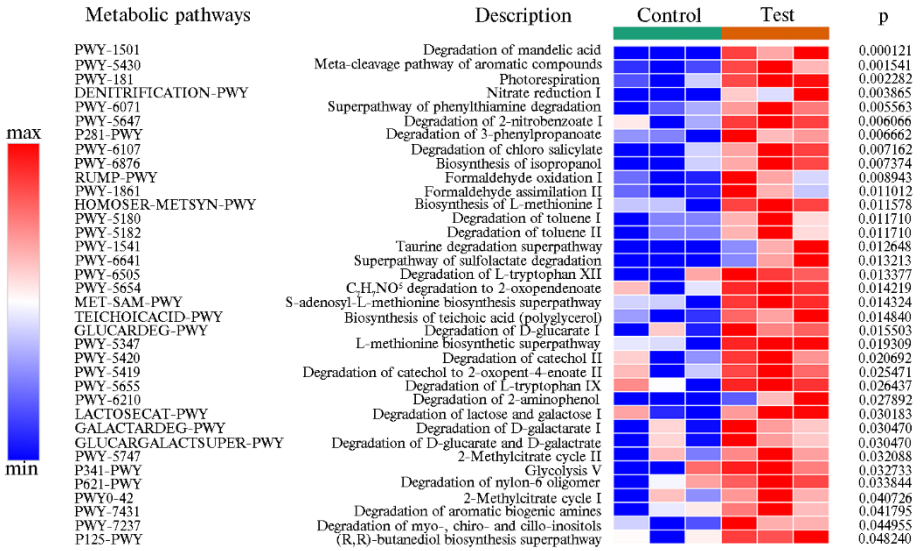

Fig. 4. Functional annotation of potential methabolic pathways in caecal microbiome of Soviet chinchilla rabbits (*Oryctolagus dominis*) fed a complex feed additive based on the probiotic *Bacillus subtilis* 1-85 strain and trace elements (day 60, Next-Generation Sequencing of 16S rRNA gene amplicons; $n = 3$, $M \pm$SEM; the vivarium expeerim, St. Petersburg, 2021). The data were obtained using the PICRUSt2 software package (v.2.3.0) (https://github.com/picrust/picrust2). The scale reflects the intensity of potential metabolic pathways of the microbiome: blue is the lowest (minimum) intensity, red is the highest (maximum).For a description of the groups, see the Materials and methods section.

As a result of the analysis carried out using the PICRUSt2 (v.2.3.0) software package, we found 370 predicted metabolic pathways in the intestinal microbial community of rabbits of the studied groups. Statistically significant differences between the experimental groups ($p \leq 0.05$) were revealed in 36 pathways (Fig. 4). In the intestinal microbiome of rabbits from group II, compared to group I, there was up to 4-fold activation ($p \leq 0.05$) of 8 pathways related to protein metabolism (biosynthesis and conversion of amino acids, nitrogenous compounds), 4 pathways related to carbohydrate metabolism (breakdown of various sugars), 3 to energy metabolism (methylcitrate cycle and glycolysis), 2 to the biosynthesis of alcohols, 1 to photorespiration, 1 to the assimilation of formaldehyde, 1 to the degradation of myo-, chiro- and scillo-inositol, 1 to the synthesis of the cell wall and spore formation (teichic acid biosynthesis pathway). Interestingly, the dominant number of potential metabolic pathways (15 pathways) was associated with the degradation of aromatic compounds and xenobiotics, including the degradation of toxicants such as catechin, formaldehyde, 3-phenylpropanoate, and nylon-6 oligomer [73, 74]. An increase in the potential for degradation of xenobiotics in the intestines of rabbits from the experimental group could be associated with an increase in the number of bacteria *Bacillus* spp. *Bacillus* spp. have long been considered as potential biodestructors and bioremediators capable of decomposing various toxic substances due to the synthesis of various enzymes [75, 76].

An increase in the degradation pathway of myo-, chiro-, and scyllo-inositol

(PWY-7237) in the second experimental group compared to the control ($p \leq 0.05$) could also be associated with an increase in the abundance of *Bacillus* spp. Previous studies have identified in *Bacillus subtilis* 1-85 a number of genes required for myo-inositol catabolism, including the *iolABCDEFGHIJ* and *iolRS* operons [77], as well as the *iolT* gene [78]. As for the enhancement of potential pathways involved in protein metabolism in the experimental group, it is of interest to activate three pathways at once (HOMOSER-METSYN-PWY, MET-SAM-PWY, and PWY-5347) associated with the synthesis of L-methionine. This echoes the conclusions of foreign researchers [79], who suggested that methionine and threonine are produced by microorganisms of the caecum and enter the body of rabbits in the process of caecotrophy of soft feces.

It is worth noting that in growing rabbits, an excess of dietary protein can lead to a higher incidence of mucoid enteropathy [80, 81]. Current recommendations for feeding rabbits tend to reduce the amount of protein in the diet and increase the fiber content in order to prevent digestive disorders [82]. Nevertheless, industrial rabbit breeding is interested in growing highly productive animals. According to G.G. Partridge et al. [83], there is a positive relationship between rabbit weight and protein requirements. Therefore, the possibility of reducing the protein content in the diet while maximizing the efficiency of synthesis and assimilation of amino acids in the digestive system as a result of the use of feed additives seems to be extremely relevant.

Previously, a study [33] was conducted to identify differences in gut microbiota functionality in two commercial rabbit breeds, Elco and Ira, based on 16S rRNA gene sequencing. An increase in the functional potential of the gut microbiome associated with bacterial chemotaxis, the conversion of pentose phosphate, fructose, mannose, and branched chain amino acids was revealed in Elco rabbits compared to Ira rabbits. The effect of feed additives on the metabolic potential of rabbits has not been studied before. However, similar work was carried out on cattle [32], pigs [84], and poultry [85]. On the example of Hyline Brown laying hens, it was shown [85] that a dietary probiotic based on *Bacillus subtilis* DSM 32324, *Bacillus subtilis* DSM 32325 and *Bacillus amyloliquifaciens* DSM 25840 provided an increase in the activity of pathways associated with the metabolism of vitamin B_6, retinol, phosphonates and phosphinates, tyrosine, biosynthesis of phenylpropanoids, monobactams, pantothenates and CoA, RNA degradation.

Thus, a complex feed additive which includes the strain *Bacillus subtilis* 1-85 and a mixture of mineral components of Si, Fe, Cu contributed to a change in the blood levels of innate immune mediators of the Soviet chinchilla rabbits. The content of lysozyme and other blood bactericidal parameters, phagocytic activity, phagocytic index, phagocytic number in rabbits of test group II fed a complex feed additive were higher than in control group I ($p \leq 0.05$). The NGS sequencing revealed higher values of the α-biodiversity indices Chao1, Shannon and Simpson ($p \leq 0.05$) in the test group vs. control. In the composition of the microbiome of the caecum of the intestines, 12 phyla of the kingdom *Bacteria* were found, among which *Firmicutes* dominated ($80.2 \pm 6.2\%$ in the control group, $78.2 \pm 7.4\%$ in the test group). In the tes group, the abundance of *Verrucomicrobiota*, *Actinobacteriota*, *Patescibacteria*, *Proteobacteria*, and *Desulfobacterota* increased and the representation of *Campylobacterota* decreased. In test rabbits, the number of the genus *Bacillus* increased by 2.82 times compared to the control group ($p \leq 0.05$), which probably indicates colonization of the intestinal chyme by a strain of a probiotic microorganism introduced into the diet as part of a complex feed additive. *Staphylococcus sciuri* was found in the intestines of control animals ($0.075 \pm 0.006\%$). In the rabbit gut microbial community, we revealed 370 predicted metabolic pathways, 36 of which showed differences between the experimental groups ($p \leq 0.05$).

In the intestinal microbiome of rabbits from the test group, compared to control, there was activation ($p \leq 0.05$) of pathways related to the degradation of aromatic compounds and xenobiotics, to the protein, carbohydrate, energy metabolism, alcohol biosynthesis, photorespiration, formaldehyde assimilation, and degradation of myo-, chiro- and scillo-inositol, cell wall synthesis and spore formation. The dominant number of enhanced potential metabolic pathways is associated with the degradation of aromatic compounds and xenobiotics. It seems interesting to further study other aspects of the beneficial effects of the introduced bacterial strain and the microelement complex on the host, in particular, the assessment of productive indicators and the development of technologies for introducing the presented feed additive into the digestive tract.

REFERENCES

1. Lebas F., Coudert P., de Rochambeau H., Thébault R.G. *The rabbit: husbandry, health, and production*. Food and Agriculture Organization of the United Nations, Rome, 1997.
2. Cullere M., Dalle Zotte A. Rabbit meat production and consumption: State of knowledge and future perspectives. *Meat Science*, 2018, 143: 137-146 (doi: 10.1016/j.meatsci.2018.04.029).
3. Usachev I.I. *Vestnik Buryatskoy gosudarstvennoy sel'skokhozyaystvennoy akademii im. V.R. Filippova*, 2008, 1: 26-29 (in Russ.).
4. McNitt J.I., Lukefahr S.D., Cheeke P.R., Patton N.M. *Rabbit production*. CABI, Wallingford, UK, 2013.
5. Cotozzolo E., Cremonesi P., Curone G., Menchetti L., Riva F., Biscarini F., Marongiu M.L., Castrica M., Castiglioni B., Miraglia D., Luridiana S., Brecchia G. Characterization of bacterial microbiota composition along the gastrointestinal tract in rabbits. *Animals*, 2020, 11(1): 31 (doi: 10.3390/ani11010031).
6. Flint H.J., Scott K.P., Louis P., Duncan S.H. The role of the gut microbiota in nutrition and health. *Nature Reviews Gastroenterology & Hepatology*, 2012, 9(10): 577-589 (doi: 10.1038/nrgastro.2012.156).
7. Heinrichs J., Lesmeister K.E. Rumen development in the dairy calf. *Advances in Dairy Technology*, 2005, 17: 179-187.
8. Drouilhet L., Achard C.S., Zemb O., Molette C., Gidenne T., Larzul C., Ruesche J., Tircazes A., Segura M., Bouchez T., Theau-Clément M., Joly T., Balmisse E., Garreau H., Gilbert H. Direct and correlated responses to selection in two lines of rabbits selected for feed efficiency under ad libitum and restricted feeding: I. Production traits and gut microbiota characteristics. *Journal of Animal Science,* 2016, 94(1): 38-48 (doi: 10.2527/jas.2015-9402).
9. Monteils V., Cauquil L., Combes S., Godon J.J., Gidenne T. Potential core species and satellite species in the bacterial community within the rabbit caecum. *FEMS Microbiology Ecology*, 2008, 66(3): 620-629 (doi: 10.1111/j.1574-6941.2008.00611.x).
10. Massip K., Combes S., Cauquil L., Zemb O., Gidenne T. High-throughput 16SDNA sequencing for phylogenetic affiliation of the caecal bacterial community in the rabbit: Impact of the hygiene of housing and of the intake level. *Proc. VIIIth INRA-RRI Symposium on Gut Microbiology. Gut microbiota: friend or foe?* Clermont-Ferrand, France, 2012: 57.
11. Velasco-Galilea M., Piles M., Vinas M., Rafel O., González-Rodríguez O., Guivernau M., Sanchez J.P. Rabbit microbiota changes throughout the intestinal tract. *Front. Microbiol.*, 2018, 9: 2144 (doi: 10.3389/fmicb.2018.02144).
12. Xing Y., Liu J., Lu F., Wang L., Li Y., Ouyang C. Dynamic distribution of gallbladder microbiota in rabbit at different ages and health states. *PLoS ONE*, 2019, 14(2): e0211828 (doi: 10.1371/journal.pone.0211828).
13. Fang S., Chen X., Pan J., Chen Q., Zhou L., Wang C., Xiao T., Gan Q. F. Dynamic distribution of gut microbiota in meat rabbits at different growth stages and relationship with average daily gain (ADG). *BMC Microbiol.*, 2020, 20(1): 116 (doi: 10.1186/s12866-020-01797-5).
14. Gouet P.H., Fonty G. Changes in the digestive microflora of holoxenic rabbits from birth until adulthood. *Annales de Biologie Animale Biochimie Biophysique*, 1979, 19(3A): 553-566.
15. Carabaco R., Badiola I., Licois D., Gidenne T. The digestive ecosystem and its control through nutritional or feeding strategies. In: *Recent advances in rabbit sciences*. L. Maertens, P. Coudert (eds.). ILVO, Melle, Belgium, 2006: 211-228.
16. Marlier D., Dewrée R., Lassence C., Licois D., Mainil J., Coudert P., Meulemans L., Ducatelle R., Vindevogel H. Infectious agents associated with epizootic rabbit enteropathy: isolation and attempts to reproduce the syndrome. *Journal of Veterinary Diagnostic Investigation,* 2006, 172(3): 493-500 (doi: 10.1016/j.tvjl.2005.07.011).
17. Lebas F. Entérocolite: situation en juin 2001. *Cuniculture,* 2001, 28: 183-188.
18. Lord B. Gastrointestinal disease in rabbits 2. Intestinal diseases. *In Practice*, 2012, 34(3): 156-162 (doi: 10.1136/inp.e973).
19. Olkowski A.A., Wojnarowicz C., Chirino-Trejo M., Drew M.D. Responses of broiler chickens

orally challenged with Clostridium perfringens isolated from field cases of necrotic enteritis. *Research in Veterinary Science*, 2006, 81(1): 99-108 (doi: 10.1016/j.rvsc.2005.10.006).
20. Lu H., Adedokun S.A., Adeola L., Ajuwon K. Anti-inflammatory effects of non-antibiotic alternatives in *Coccidia* challenged broiler chickens. *Journal of Poultry Science*, 2014, 51(1): 14-21 (doi: 10.2141/jpsa.0120176).
21. Tang T., Li Y., Wang J., Elzo M. A., Shao J., Li Y., Xia S., Fan H., Jia X., Lai S. Untargeted metabolomics reveals intestinal pathogenesis and self-repair in rabbits fed an antibiotic-free diet. *Animals (Basel)*, 2021, 11(6): 1560 (doi: 10.3390/ani11061560).
22. Deng Z., Han D., Wang Y., Wang Q., Yan X., Wang S., Liu X., Song W., Ma Y. Lactobacillus casei protects intestinal mucosa from damage in chicks caused by *Salmonella pullorum* via regulating immunity and the Wnt signaling pathway and maintaining the abundance of gut microbiota. *Poultry Science*, 2021, 100(8): 101283 (doi: 10.1016/j.psj.2021.101283).
23. Liu B., Liu Q.-M., Li G.-L., Sun L.C., Gao Y.-Y., Zhang Y.-F., Liu H., Cao M.-J., Liu G.-M. The anti-diarrhea activity of red algae-originated sulphated polysaccharides on ETEC-K88 infected mice. *RSC Advances*, 2019, 9: 2360-2370 (doi: 10.1039/C8RA09247H).
24. Karamzadeh-Dehaghani A., Towhidi A., Zhandi M., Mojgani N., Fouladi-Nashta A. Combined effect of probiotics and specific immunoglobulin Y directed against Escherichia coli on growth performance, diarrhea incidence, and immune system in calves. *Animal*, 2021, 15(2): 100124 (doi: 10.1016/j.animal.2020.100124).
25. Abdel-Wareth A.A.A., Elkhateeb F.S.O., Ismail Z.S.H., Ghazalah A.A., Lohakare J. Combined effects of fenugreek seeds and probiotics on growth performance, nutrient digestibility, carcass criteria, and serum hormones in growing rabbits. *Livestock Science*, 2021, 251: 104616 (doi: 10.1016/j.livsci.2021.104616).
26. Lam Phuoc T., Jamikorn U. Effects of probiotic supplement (*Bacillus subtilis* and *Lactobacillus acidophilus*) on feed efficiency, growth performance, and microbial population of weaning rabbits. *Asian-Australas. J. Anim. Sci.*, 2016, 30(2): 198-205 (doi: 10.5713/ajas.15.0823).
27. Dimova N., Laleva S., Slavova P., Popova Y., Mihaylova M., Pacinovski N. Effect of probiotic "Zoovit" on productivity of rabbits. *Macedonian Journal of Animal Scienc*, 2017, 7: 123-127.
28. Fathi M., Abdelsalam M., Al-Homidan I., Ebeid T., El-Zarei M., Abou-Emera O. Effect of probiotic supplementation and genotype on growth performance, carcass traits, hematological parameters and immunity of growing rabbits under hot environmental conditions. *Anim. Sci. J.*, 2017, 88(10): 1644-1650 (doi: 10.1111/asj.12811).
29. El-Shafei A.A., Younis T.M., Al-Gamal M.A., Hesham A.M. Impact of probiotic (*Lactobacillus planterium* L) supplementation on productive and physiological performance of growing rabbits under Egyptian conditions. *Egyptian Journal of Rabbit Science*, 2019, 29(1): 125-148 (doi: 10.21608/ejrs.2019.48188).
30. Wlazło Ł., Kowalska D., Bielański P., Chmielowiec-Korzeniowska A., Ossowski M., Łukaszewicz M., Czech A., Nowakowicz-Dębek B. Effect of fermented rapeseed meal on the gastrointestinal microbiota and immune status of rabbit (*Oryctolagus cuniculus*). *Animals (Basel)*, 2021, 11(3): 716 (doi: 10.3390/ani11030716).
31. Shen X.M., Cui H.X., Xu X.R. Orally administered *Lactobacillus casei* exhibited several probiotic properties in artificially suckling rabbits. *Asian-Australas. J. Anim. Sci.*, 2020, 33: 1352-1359 (doi: 10.5713/ajas.18.0973).
32. Yyldyrym E.A., Laptev G.Yu., Il'ina L.A., Dunyashev T.P., Tyurina D.G., Filippova V.A., Brazhnik E.A., Tarlavin N.V., Dubrovin A.V., Novikova N.I., Soldatova V.V., Zaytsev S.Yu. The influence of a dietary *Enterococcus faecium* strain-based additive on the taxonomic and functional characteristics of the rumen microbiota of lactating cows. *Sel'skokhozyaistvennaya biologiya* [*Agricultural Biology*], 2020, 55(6): 1204-1219 (doi: 10.15389/agrobiology.2020.6.1204eng).
33. Ye X., Zhou L., Zhang Y., Xue S., Gan Q. F., Fang S. Effect of host breeds on gut microbiome and serum metabolome in meat rabbits. *BMC Vet. Res.*, 2021, 17(1): 24 (doi: 10.1186/s12917-020-02732-6).
34. Alekseeva L.V., Arsanukaev D.L. *Materialy Mezhdunarodnoy nauchno-prakticheskoy konferentsii «Konkurentosposobnost' i innovatsionnaya aktivnost' APK regionov»* [Proc. Int. Conf. «Competitiveness and innovative activity of the agro-industrial complex of the Russian Federation regions»]. Tver', 2018: 89-92 (in Russ.).
35. Alekseeva L.V., Makarevskiy A.A., Subbotenko T.V. *Materialy Mezhdunarodnoy nauchno-prakticheskoy konferentsii «Nauchnye prioritety v APK»* [Proc. Int. Conf. «Scientific priorities in the agro-industrial complex»]. Tver', 2019: 146-148 (in Russ.).
36. Alekseeva L.V., Luk'yanov A.A., Belyakova S.V. *Materialy Mezhdunarodnoy nauchno-prakticheskoy konferentsii «Konkurentosposobnost' i innovatsionnaya aktivnost' APK regionov»* [Proc. Int. Conf. «Competitiveness and innovative activity of the agro-industrial complex of the Russian Federation regions»]. Tver', 2017: 132-134 (in Russ.).
37. *Guide for the care and use of laboratory animals.* The National Academy press, Washington, D.C, 2011.
38. Alekseeva L.V., Demenik F.G., Savina A.S. *Vestnik tverskogo gosudarstvennogo universiteta. Seriya: biologiya i ekologiya*, 2018, 2: 274-285 (in Russ.).
39. Karput' I.M. *Immunologiya i immunopatologiya bolezney molodnyaka* [Immunology and immunopathology of diseased young animals]. Minsk, 1993 (in Russ.).

40. Dorofeychuk V.G. *Laboratornoe delo*, 1968, 1: 67 (in Russ.).
41. Douglas G.M., Maffei V.J., Zaneveld J.R. Yurgel S.N., Brown J.R., Taylor C.M., Huttenhower C., Langille M.G.I. PICRUSt2 for prediction of metagenome functions. *Nature Biotechnology*, 2020, 38: 685-688 (doi: 10.1038/s41587-020-0548-6).
42. Tracey K.J., Wei H., Manogue K.R., Fong Y., Hesse D.G., Nguyen H.T., Kuo G.C., Beutler B., Cotran R.S., Cerami A. Cachectin/tumor necrosis factor induces cachexia, anemia, and inflammation. *The Journal of Experimental Medicine*, 1988, 167(3): 1211-1227 (doi: 10.1084/jem.167.3.1211).
43. Ovsyannikov A.G. *Anemiya krolikov (etiopatogenez, diagnostika, lechenie). Kandidatskaya dissertatsiya* [Anemia in rabbits (etiopathogenesis, diagnosis, and treatment. PhD Thesis]. St. Petersburg, 2019 (in Russ.).
44. Glagoleva O.N. *Vesti MANEB v Omskoy oblasti*, 2013, 2(2): 13-15 (in Russ.).
45. Kraemer K., Zimmermann B. *Nutritional anemia*. Sightand Life Press, Basel, Switzerland, 2007.
46. Levy O. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. *Blood*, 2000, 96(8): 2664-2672.
47. Modlin R.L., Brightbill H.D., Godowski P.J. The toll of innate immunity on microbial pathogens. *N. Engl. J. Med.,* 1999, 340(23): 1834-1835 (doi: 10.1056/NEJM199906103402312).
48. Hoffmann J.A. Innate immunity of insects. *Current Opinion in Immunology*, 1995, 7(1): 4-10 (doi: 10.1016/0952-7915(95)80022-0).
49. Hoffmann J.A., Reichhart J.M., Hetru C. Innate immunity in higher insects. *Current Opinion in Immunology*, 1996, 8(1): 8-13 (doi: 10.1016/s0952-7915(96)80098-7).
50. Nichols R.G., Davenport E.R. The relationship between the gut microbiome and host gene expression: a review. *Human Genetics*, 2021, 140(5): 747-760 (doi: 10.1007/s00439-020-02237-0).
51. Mohamed A.F., El-Sayiad G.A., Reda F.M., Ashour E.A. Effects of breed, probiotic and their in-teraction on growth performance, carcass traits and blood profile of growing rabbits. *Zagazig Journal of Agricultural Research*, 2017, 44(1): 215-227 (doi: 10.21608/zjar.2017.53947).
52. Lukác N., Massányi P. Vplyv stopoýtch prvkov na imunitný systém [Effects of trace elements on the immune system]. *Epidemiol. Mikrobiol. Imunol.*, 2007, 56(1): 3-9.
53. López-Alonso M. Trace minerals and livestock: not too much not too little. *International Scholarly Research Notices*, 2012, 2012: 704825 (doi: 10.5402/2012/704825).
54. Kim H.B., Borewicz K., White B.A., Singer R.S., Sreevatsan S., Tu Z.J., Isaacsona R.E. Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. *Proceedings of the National Academy of Sciences*, 2012, 109(38): 15485-15490 (doi: 10.1073/pnas.1205147109).
55. Pajarillo E.A., Chae J.P., Balolong M.P., Kim H.B., Seo K.-S., Kang D.-K. Characterization of the fecal microbial communities of duroc pigs using 16S rRNA gene pyrosequencing. *Asian-Australas. J. Anim. Sci.,* 2015, 28(4): 584-591 (doi: 10.5713/ajas.14.0651).
56. den Besten G., van Eunen K., Groen A.K., Venema K., Reijngoud D.-J., Bakker B.M. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research*, 2013, 54(9): 2325-2340 (doi: 10.1194/jlr.R036012).
57. Abecia L., Rodríguez-Romero N., Yañez-Ruiz D.R., Fondevila M. Biodiversity and fermentative activity of caecal microbial communities in wild and farm rabbits from Spain. *Anaerobe,* 2012, 18(3): 344-349 (doi: 10.1016/j.anaerobe.2012.04.004).
58. Fu X., Zeng B., Wang P., Wang L., Wen B., Li Y., Liu H., Bai S., Jia G. Microbiome of total versus live bacteria in the gut of Rex rabbits. *Front. Microbiol.*, 2018, 9: 733 (doi: 10.3389/fmicb.2018.00733).
59. Crowley E.J., King J.M., Wilkinson T., Worgan H.J., Huson K.M., Rose M.T., McEwan N.R. Comparison microbial population in rabbits and guinea pigs by next generation sequencing. *PLoS ONE,* 2017, 12(2): e0165779 (doi: 10.1371/journal.pone.0165779).
60. Bangsgaard Bendtsen K.M., Krych L., Sørensen D.B., Pang W., Nielsen D.S., Josefsen K., Hansen L.H., Sørensen S.J., Hansen A.K. Gut microbiota composition is correlated to grid floor induced stress and behavior in the BALB/c mouse. *PLoS ONE*, 2012, 7(10): e46231 (doi: 10.1371/journal.pone.0046231).
61. An C., Kuda T., Yazaki T., Takahashi H., Kimura B. FLX pyrosequencing analysis of the effects of the brown-algal fermentable polysaccharides alginate and laminaran on rat cecal microbiotas. *Applied and Environmental Microbiology*, 2013, 79(3): 860-866 (doi: 10.1128/AEM.02354-12).
62. Zhou K. Strategies to promote abundance of *Akkermansia muciniphila*, an emerging probiotics in the gut, evidence from dietary intervention studies. *Journal of Functional Foods*, 2017, 33: 194-201 (doi: 10.1016/j.jff.2017.03.045).
63. Glukhova A.A., Karabanova A.A., Yakushev A.V., Semenyuk I.I., Boykova Y.V., Malkina N.D., Efimenko T.A., Ivankova T.D., Terekhova L.P., Efremenkova O.V. Antibiotic activity of actinobacteria from the digestive tract of millipede *Nedyopus dawydoffiae* (*Diplopoda*). *Antibiotics*, 2018, 7(4): 94 (doi: 10.3390/antibiotics7040094).
64. Hotchkiss C.E., Shames B., Perkins S.E., Fox J.G. Proliferative enteropathy of rabbits: the intracellular Campylobacter-like organism is closely related to *Lawsonia intracellularis*. *Lab. Anim. Sci.*, 1996, 46(6): 623-627.
65. Corcoran B.M., Stanton C., Fitzgerald G.F., Ross R.P. Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Applied and Environmental Microbiology*, 2005, 71(6): 3060-3067 (doi: 10.1128/AEM.71.6.3060-3067.2005).

66. Ghelardi E., Celandroni F., Salvetti S., Gueye S.A., Lupetti A., Senesi S. Survival and persistence of *Bacillus clausii* in the human gastrointestinal tract following oral administration as spore-based probiotic formulation. *J. Appl. Microbiol.*, 2015, 119(2): 552-559 (doi: 10.1111/jam.12848).
67. Bäuerl C., Collado M.C., Zúñiga M., Blas E., Pérez Martínez G. Changes in cecal microbiota and mucosal gene expression revealed new aspects of epizootic rabbit enteropathy. *PLoS ONE,* 2014, 9(8): e105707 (doi: 10.1371/journal.pone.0105707).
68. Combes S., Gidenne T., Cauquil L., Bouchez O., Fortun-Lamothe L. Coprophagous behavior of rabbit pups affects implantation of cecal microbiota and health status. *Journal of Animal Science*, 2014, 92(2): 652-665 (doi: 10.2527/jas.2013-6394).
69. Fekete S. Recent findings and future perspectives of digestive physiology in rabbits: a review. *Acta Vet. Hung.*, 1989, 37(3): 265-279.
70. Hu X., Zheng B., Jiang H., Kang Y., Cao Q., Ning H., Shang J. Draft genome sequence of *Staphylococcus sciuri* subsp. *sciuri* strain Z8, isolated from human skin. *Genome Announcements*, 2015, 3(4): e00714-15 (doi: 10.1128/genomeA.00714-15).
71. Hermans K., Devriese L.A., Haesebrouck F. Rabbit staphylococcosis: difficult solutions for serious problems. *Veterinary Microbiology*, 2003, 91(1): 57-64 (doi: 10.1016/s0378-1135(02)00260-2).
72. Dakić I., Morrison D., Vuković D., Savić B., Shittu A., Ježek P., Hauschild T., Stepanović S. Isolation and molecular characterization of Staphylococcus sciuri in the hospital environment. *Journal of Clinical Microbiology*, 2005, 43(6): 2782-2785 (doi: 10.1128/JCM.43.6.2782-2785.2005).
73. Mayer J., Huhn T., Habeck M., Denger K., Hollemeyer K., Cook A.M. 2,3-Dihydroxypropane-1-sulfonate degraded by *Cupriavidus pinatubonensis* JMP134: purification of dihydroxypropanesulfonate 3-dehydrogenase. *Microbiology*, 2010, 156(5): 1556-1564 (doi: 10.1099/mic.0.037580-0).
74. Yurimoto H., Hirai R., Yasueda H., Mitsui R., Sakai Y., Kato N. The ribulose monophosphate pathway operon encoding formaldehyde fixation in a thermotolerant methylotroph, *Bacillus brevis* S1. *FEMS Microbiol. Lett.*, 2002, 214(2): 189-193 (doi: 10.1111/j.1574-6968.2002.tb11345.x).
75. Arora P.K., Srivastava A., Singh V.P. Diversity of 4-chloro-2-nitrophenol-degrading bacteria in a waste water sample. *Journal of Chemistry*, 2016, 2016: 7589068 (doi: 10.1155/2016/7589068).
76. Singh B., Singh K. *Bacillus*: as bioremediator agent of major environmental pollutants. In: *Bacilli and agrobiotechnology*. M. Islam, M. Rahman, P. Pandey, C. Jha, A. Aeron (eds.). Springer, Cham, 2016: 35-55 (doi: 10.1007/978-3-319-44409-3_2).
77. Yoshida K.I., Aoyama D., Ishio I., Shibayama T., Fujita Y. Organization and transcription of the myo-inositol operon, iol, of *Bacillus subtilis*. *Journal of Bacteriology*, 1997, 179(14): 4591-4598 (doi: 10.1128/jb.179.14.4591-4598.1997).
78. Yoshida K.-I., Yamamoto Y., Omae K., Yamamoto M., Fujita Y. Identification of two myo-inositol transporter genes of *Bacillus subtilis*. *Journal of Bacteriology*, 2002, 184(4): 983-991 (doi: 10.1128/jb.184.4.983-991.2002).
79. Abe Y., Sakoda T., Goto H., Ikeda S., Sukemori S. Cecotrophy contribute methionine and threonine requirements of rabbits. *Journal of Pet Animal Nutrition*, 2014, 17: 6-12 (doi: 10.11266/jpan.17.6).
80. Gidenne T., Kerdiles V., Jehl N., Arveux P., Eckenfelder B., Briens C., Stephan S., Fortune H., Montessuy S., Muraz G. Protein replacement by digestible fibre in the diet of growing rabbits. 2: Impact on performances, digestive health and nitrogen output. *Animal Feed Science and Technology*, 2013, 183(3-4): 142-150 (doi: 10.1016/j.anifeedsci.2013.03.013).
81. Martínez-Vallespín B., Martínez-Paredes E., Ródenas L., Moya V.J., Cervera C., Pascual J.J., Blas E. Partial replacement of starch with acid detergent fibre and/or neutral detergent soluble fibre at two protein levels: Effects on ileal apparent digestibility and caecal environment of growing rabbits. *Livestock Science*, 2013, 154(1-3): 123-130 (doi: 10.1016/j.livsci.2013.02.012).
82. Trocino A., García J., Carabaco R., Xiccato G. A meta-analysis on the role of soluble fibre in diets for growing rabbits. *World Rabbit Sci.*, 2013, 21(1): 1-15 (doi: 10.4995/wrs.2013.1285).
83. Partridge G.G., Garthwaite P.H., Findlay M. Protein and energy retention by growing rabbits offered diets with increasing proportions of fibre. *Journal of Agricultural Science*, 1989, 112(2): 171-178 (doi: 10.1017/S0021859600085063).
84. Shin D., Chang S.Y., Bogere P., Won K., Choi J.Y., Choi Y.J., Lee H.K., Hur J., Park B.Y., Kim Y., Heo J. Beneficial roles of probiotics on the modulation of gut microbiota and immune response in pigs. *PLoS ONE*, 2019, 14(8): e0220843 (doi: 10.1371/journal.pone.0220843).
85. Khan S., Chousalkar K.K. Functional enrichment of gut microbiome by early supplementation of *Bacillus* based probiotic in cage free hens: a field study. *Animal Microbiome*, 2021, 3(1): 50 (doi: 10.1186/s42523-021-00112-5).