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The Influence Of A Bacillus Subtilis Probiotic On The Cecal Microbial Communities, Exocrine Pancreatic Function, And Productivity Parameters In Broiler Chicks.

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ABSTRACT

The changes in cecal microbial communities in broiler chicks (Gallus gallus) fed diets supplemented with a Bacillus subtilis probiotic strain were determined using NGS and RT-PCR techniques. Cecal microbiota in control treatment included species obligate for the typical avian microbiotas, a number of unidentified taxons, and certain Lachnospiraceae and Ruminococcaceae species considered specific for ruminal microbiotas. Streptococci, enterococci, and bifidobactera (considered obligate for avian species) were presented only as minor communities; typical avian pathogens (Camphylobacter spp., Staphylococcus spp.) were not found. Supplementation of the diet with a B. subtilis probiotic led to the six-fold increase in cecal Bacillus spp. community, the decrease in total cecal microbial population, and the shifts in the proportions of different communities. Significant increases (1.1-1.47 times) were found for the cecal communities of beneficial species. These changes in cecal microbiota positively affected the efficiency of the digestion and productivity parameters in broilers. The activity of pancreatic amylase and lipase in blood serum increased in broilers fed probiotic, probably due to the secretion of the respective digestive enzymes by the probiotic strain. The decrease in proteolytic activity in serum can be related to the competition between proteases of host and proteases produced by B. subtilis.

Keywords: broiler chicks, probiotic, microbiota, digestive enzymes, productivity, digestibility and availability of dietary nutrients.

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INTRODUCTION

The importance of microbial communities of the gastrointestinal tract (GIT) for health and productivity in chicken is presently universally recognized; microbial species supply to the host a range of enzymes lacking in avian digestive system (cellulases, etc.), antibiotic substances, proteins, hormones, vitamins, etc. [1, 2]. At the same time GIT microbiota is a system highly sensitive to certain external factors (host age, diet composition, inclusion of antibiotic growth promoters) and the reactions of this system to these factors are not inevitably beneficial for the metabolism and health in host [3].

Our earlier knowledge on chicken intestinal microbiotas have been based exclusively on the classic microbiologic methods (culturing); according to this earlier research main obligate species in the avian intestine are Bifidobacteria, Streptococci, Lactobacteria and other lactic acid bacteria, Eubacteria, Bacteroides, and Enterobacteria [1, 2]. Modern molecular genetic methods of research opened the unique possibilities to expand our knowledge on the intestinal microbiotas. Species from more than 140 bacterial genera were found in the avian intestine; only 10% of this biodiversity were identified using microbial 16S rRNA genes while other bacterial species still remain unidentified and can even belong to presently unknown genera [4]. The new techniques of real-time polymerase chain reaction (RT-PCR) and next-generation sequencing (NGS) allow the simultaneous analysis of few thousands of microbial sequences; this approach can provide the authoritative information on the responses of intestinal microbiomes to certain exogenous factors.

The aim of the study presented was the evaluation of the effects of the supplementation of vegetable diets for broiler chicks with a bacterial probiotic on the cecal microbial population, exocrine pancreatic function, and certain productivity parameters. Cecal communities were chosen since the cecum is the main site of microbial proteolysis and microbial digestion of dietary cellulose and starch [1].

MATERIALS AND METHODS

The study was performed on Cobb-500 broiler chicks in conditions of Center for Genetic Selection "Zagorskoye EPH" (Sergiev Posad). The broilers (35 birds per treatment) were kept in cage batteries from 1 to 41 days of age. The regimes of lighting, temperature and humidity, feeding and drinking as well as the nutritive values of the diets at all ages accorded with the breeder's recommendations. Control treatment was fed vegetable (corn-wheat) basic diet (BD); experimental treatment was fed BD supplemented with Bacillus subtilis strain 1-85 (1000 ppm). The composition and nutritive parameters of the BD are presented in **Table 1**.

Table 1: The composition and nutritive value of the basic diet (BD) for broilers

Ingredient, %	Age periods (d)		
	1-14	**15-21**	**22-41**
Corn	22.000	20.566	17.000
Wheat	27.806	36.292	40.355
Soybean meal	20.107	8.526	2.950
Sunflower cake	15.000	20.000	25.000
Corn gluten	7.171	5.709	4.064
Soybean oil	4.000	5.000	6.977
Salt	0.216	0.219	0.217
Monocalcium phosphate	0.864	0.780	0.617
Limestone	1.536	1.508	1.470
Lysin	0.300	0.400	0.350
Premix	1.000	1.000	1.000
Nutritive parameters, %			
Metabolizable energy, Kcal/100 g	305.000	311.000	320.000
Crude protein	24.840	21.378	19.786
Crude fiber	5.029	4.996	5.211
Calcium	0.938	0.889	0.809

Total P	0.773	0.728	0.683
Available P	0.499	0.469	0.429
Sodium	0.160	0.160	0.160
Chlorine	0.282	0.305	0.297
Potassium	0.778	0.636	0.587
Total amino acids:			
Lysine	1.425	1.231	1.078
Methionine	0.793	0.749	0.732
Methionine+cystine	1.199	1.104	1.064
Threonine	0.950	0.798	0.735
Tryptophan	0.289	0.245	0.232
Arginine	1.459	1.238	1.188
Available amino acids:			
Lysine	1.257	1.088	0.939
Methionine	0.600	0.558	0.538
Methionine+cystine	0.931	0.847	0.809

Cecal content was sampled from 6 birds (3 birds per treatment) slaughtered at 37 days of age by cervical dislocation; the procedure and sterility conditions accorded with the respective guidelines for poultry producing branch [5]. At the same age a balance trial was performed (3 birds per treatment) to determine the digestibility of dietary nutrients.

Total DNA from the samples was isolated using "Genomic DNA Purification Kit" (Fermentas, Inc., Lithuania) according to the manufacturer's guidelines. The NGS analysis of bacterial communities was performed on sequencer MiSeq (Illumina, Inc., USA) with MiSeq Reagent Kit v.3 and primers 343F/806R (5'-CTCCTACGGRRSGCAGCAG-3', 5'-GGACTACNVGGGTWTCTAAT–3'). The reads were processed using bio-informative platform "CLC Bio GW 7.0" (Qiagen, the Netherlands).

Total bacterial counts were determined using RT-PCR technique, "Reagent kit for RT-PCR in the presence of intercalating dye EVA Green" (Sintol Co, Ltd., Russia), primers Eub338/Eub518 (5'-ACTCCTACGGGAGGCAGCAG-3', 5'-ATTACCGCGGCTGCTGG-3'). The amplifier DT Lite-4 (NPO DNA-Technologies, Russia) was used with the parameters as follows: 95^0C for 3 min (1 cycle), 95^0C for 13 sec, 57.6^0C for 13 sec, 72^0C for 30 sec (40 cycles).

The efficiency of the intestinal digestion was evaluated on broilers with chronic duodenal T-shape fistulae. At 15-20 days of age 10 broilers (5 birds per treatment) were operated to implant the fistulae into the duodenum near Meckel's diverticulum; after 5 days of recovery the birds were used in the experiment. During 10 days of the experiment the birds after night starvation (14 hrs) were fed 30 g of feed per bird; samples of the duodenal digesta (5.0 ml) were taken before feeding and 1 hr later and centrifuged at 5,000 rpm for 3 min. The supernatant was diluted 10-fold with cold Ringer's solution for subsequent analysis of enzymatic activities. The samples of blood were taken in parallel to determine enzymatic activities in serum. Blood samples (2-3 ml) were taken from the axillary vein of broilers before feeding and 1 hr after the feeding. After the addition of aqueous sodium citrate (3.2%, 0.2 ml) the samples were centrifuged at 5000 rpm for 5 min to obtain the serum.

Amylase activity in duodenal digesta (mg of starch hydrolyzed by 1 ml of sample during 1 min) was determined using Roy-Smith method [6] on colorimeter KFK-3 (ZOMZ, Russia) with wavelength 670 nm. Activity of pancreatic proteases in duodenal digesta (mg of casein hydrolyzed by 1 ml of sample during 1 min) was determined on colorimeter KFK-3 with wavelength 450 nm [7]. Activity of lipase in duodenal digesta was determined on semi-automatic biochemical analyzer Sinnowa BS 3000P (China) using lipase reagent kit DIAKON-VET (Russia). Enzymatic activities in serum were determined on automatic analyzer ChemWell 2900(T) (USA) using reagent kits for amylase and proteases (Human, Germany) and on Sinnowa BS 3000P (China) using lipase reagent kit DIAKON-VET (Russia) [8].

Live bodyweight in broilers was determined individually at 7, 14, 21 and 41 days of age; feed consumption was assessed by weighing fresh feed and residues form the feeders [9].

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The results were processed statistically using Microsoft Excel 2010 software; the data are presented as M±m.

RESULTS AND DISCUSSION

The substantial part of the genomes obtained from the cecal digesta remained unidentified after the taxonomic analysis of the sequences (6.31% of all genomes in control treatment, 4.59% in experimental treatment; **Table 2**). Similar results reported earlier [3] evidence the lack of knowledge concerning these taxons and the absence of the attempts of their investigation.

Table 2: The occurrence of certain bacterial taxons in cecal microbial community in broilers fed diets supplemented or not supplemented with a B. subtilis probiotic (n = 3)

Taxons	Treatment	
	control	experimental
Genomes per 1 g of cecal digesta		
Total bacterial count	$1.46x10^{11} \pm 7.01x10^{9}$	$4.81x10^{10} \pm 2.16x10^{9}$**
Cecal occurrence, %		
Phylum Bacteroidetes	18.94 ± 0.94	24.89 ± 1.19**
Phylum Firmicutes	71.59 ± 0.75	66.45 ± 3.18**
Family Clostridiaceae	7.50 ± 0.36	8.98 ± 0.46**
Family Eubacteriaceae	0.27 ± 0.01	0.30 ± 0.01**
Family Ruminococcaceae	19.96 ± 0.89	29.33 ± 1.52**
Family Lachnospiraceae	0.63 ± 0.02	0.72 ± 0.03**
Genus Lactobacillus spp.	11.51 ± 0.55	10.93 ± 0.49
Genus Enterococcus spp.	0.87 ± 0.05	0.40 ± 0.02**
Genus Streptococcus spp.	0.01 ± 0.0004	–
Genus Bacillus sp.	0.02 ± 0.001	0.12 ± 0.006**
Phylum Actinobacteria	0.85 ± 0.04	0.65 ± 0.03**
Family Bifidobacteriaceae	0.19 ± 0.009	0.11 ± 0.005**
Family Coriobacteriaceae	0.66 ± 0.01	0.53 ± 0.02**
Phylum Proteobacteria	2.30 ± 0.06	3.17 ± 0.16**
Genus Sutterella spp.	1.12 ± 0.05	0.77 ± 0.03**
Genus Escherichia spp.	0.16 ± 0.006	0.86 ± 0.04**
Phylum Tenericutes	0.01 ± 0.0004	*
Phylum TM7	*	0.25 ± 0.01
Unidentified species	6.31 ± 0.07	4.59 ± 0.22**
Number of genera	32.0 ± 1.60	33.0 ± 1.50

* – below NGS sensibility.

** – the difference with control is significant, P<0.05.

The total bacterial count in cecal digesta in control treatment was $1.46x10^{11}$ genomes per 1 g of the digesta; cecal bacterial population was dominated by families Ruminococcaceae (19.96%) and Clostridiaceae (7.50%) producing certain amylo- and cellulolytic enzymes, and phylum Bacteroidetes (18.94%) producing amylo- and proteolytic activities. Minor fractions of other amylo- and cellulolytic bacteria were identified: families Lachnospiraceae (0.63%) and Eubacteriaceae (0.27%). It should be noted that Lachnospiraceae and Ruminococcaceae species earlier have been considered specific for ruminal microbiotas [2].

The significant community of obligate genus Lactobacillus was also found (11,51%) that can competitively replace pathogenic species due to the secretion of organic acids and bacteriocins [1]. Despite the traditional opinion, the cecal communities of other species with similar activity (Bifidobacteriaceae, Streptococcus spp., Enterococcus spp., Escherichia spp.) were minor (<1%). The communities of phyla Actinobacteria and Tenericutes and family Erysipelotrichaceae were also minor. It is interesting to note the total absence in cecal microbiota of certain lactic acid bacterial genera which are traditionally considered

obligate for poultry (Selenomonas spp., Megasphaera spp.), as well as certain typical avian GIT pathogens (Campylobacter spp., Staphylococcus spp.).

Supplementation of the diets with a B. subtilis probiotic led to the 6-fold increase of the cecal population of Bacillus spp. (presumably due to the reproduction of the bacilli from the probiotic), 3.04-fold reduction of total bacterial count in cecal digesta, and changes in the proportions of certain cecal microbial communities. First of all, the significant increases were found in the communities related to the processes of the hydrolysis of fiber, starch, and protein from vegetable diet: phylum Bacteroidetes (1.31 times compared to control), families Clostridiaceae (1.2 times), Ruminococcaceae (1.47 times), Eubacteriaceae (1.1 times), Lachnospiraceae (1.14 times). The increase in non-pathogenic Escherichia spp. community (capable of the synthesis and secretion of vitamin K and certain antimicrobials) was 5.38 times compared to control. The proportions of family Coriobacteriaceae and genus Sutterella (which can induce dysbioses in poultry) in experimental treatment were significantly lower compared to control.

The investigation of the activities of the digestive enzymes in the duodenal digesta and blood serum can elucidate the mechanism of the beneficial effect of the probiotic (**Table 3**).

Table 3: The influence of a B. subtilis probiotic on the activities of pancreatic enzymes in the duodenal digesta and blood serum in broilers (n = 5)

Enzyme	Treatments		Δ, % to control
	control	experimental	
Duodenal digesta			
Amylase, mg/mL/min	341±27.3	354±33.3	+3.8
Lipase, U/L	1734±215.4	1455±161.8	-16.1
Proteases, mg/mL/min	33±1.0	31±0.9	-6.1
Blood serum			
Amylase, U/L	244±37.2	455±56.1*	+86.5
Lipase, U/L	20±4.1	31±5.0	+55.0
Proteases, U/L	29±2.1	21±2.9*	-27.6

** – the difference with control is significant, P<0.05.

The data from **Table 3** evidence that there were no significant changes in the activities of the pancreatic enzymes in the duodenal digesta in broilers fed probiotic-supplemented diets. A trend was found to decreased lipase activity (by 16.1% compared to control). These changes in enzymatic activities cannot substantially affect the efficiency of the intestinal digestion.

The activity of pancreatic amylase in blood serum significantly increased in broilers fed probiotic-supplemented diets (by 86.5%, P<0.05) while the activity of lipase increased by 55.0%; these increases evidence the intensification of metabolism of fat and carbohydrates, probably due to the secretion of the respective digestive enzymes by the probiotic strain. The decrease in proteolytic activity in serum can be related to the competition between endogenous proteases of host and proteases produced by B. subtilis, especially at the finisher age period.

Productivity parameters in broilers are presented in **Table 4**. Mortality levels in both treatments were 0%. Live bodyweight in experimental treatment was insignificantly higher compared to control at all studied ages, in males and in females. Feed conversion ratio in broilers fed probiotic was better by 2.44%.

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Table 4: Productivity parameters in broilers fed diets supplemented or not supplemented with a B. subtilis probiotic

Parameter	Treatment		Δ, % to control
	control	experimental	
Mortality, %	0	0	
Avg. live bodyweight (g) at: 1 day of age	42.0	42.0	
7 days of age	137.0±2.3	138.6±2.2	+1.0
14 days of age	344.8±9.2	353.3±7.2	+2.5
21 days of age	637.2±12.6	658.5±13.1	+3.3
41 days of age	2282.50	2354.51	+3.2
including males	2383.0±45.7	2459.3±27.1	+3.2
females	2182.0±26.6	2249.6±34.8	+3.1
Cumulative feed consumption, kg/bird	3.855	3.883	
Feed conversion ratio	1.721	1.679	-2.44
Eviscerated carcass yield, %	73.5	73.9	

Better weight gains in the treatment fed probiotic were presumably achieved due to the normalization of intestinal microbiota and decrease in the activity of pathogenic species. This competitive mechanism is especially important at the earlier stage of postnatal development of chicks (from 1 to 14 days of age) when the active functional evolvement of the digestive system (including the pancreas) occurs [10].

The results of the balance trial at 37 days of age evidenced that improvements in weight gains in broiler fed diets supplemented with the probiotic were related to the corresponding improvements in the digestibility of basic dietary nutrients. Digestibility of dry matter was higher by 2.31% compared to control; assimilation of nitrogen higher by 2.87%; digestibility of crude fiber by 3.2%; crude fat by 1.8%. There were no differences between treatments in digestibility of calcium (47.92 and 48.01%) and phosphorus (44.17 and 44.41%), as well as in the yields of eviscerated carcasses.

CONCLUSIONS

Cecal microbiome in chicken was found to be taxonomically diverse, contrary to the traditional opinion. It included species obligate for the avian GIT – families Clostridiaceae, Eubacteriaceae, Lactobacillaceae, phylum Bacteroidetes; a number of unidentified taxons; and bacteria from families Lachnospiraceae and Ruminococcaceae which were considered specific for the ruminal microbiotas. Streptococci, enterococci, and bifidobactera (considered obligate for avian species) were presented only as minor populations; typical avian pathogens (Camphylobacter spp., Staphylococcus spp.) were not found.

Deep analysis of the cecal microbiota in broilers using NGS and RT-PCR techniques evidenced that supplementation of diets with a Bacillus subtilis probiotic beneficially affected the composition of intestinal microbial communities and the productive performance in broilers.

Supplementation of the diet with a B. subtilis probiotic led to the six-fold increase in cecal Bacillus spp. community, the decrease in total cecal microbial population, and the shifts in the proportions of different communities. Significant increases were found for the cecal communities of phylum Bacteroidetes (1.31 times compared to un supplemented control), families Clostridiaceae (1.2 times), Ruminococcaceae (1.47 times), Eubacteriaceae (1.1 times), Lachnospiraceae (1.14 times); some of these species produce certain enzymes digesting the fibrous substances from vegetable diet components. Significant increase was also found in the cecal community of non-pathogenic Escherichia spp. (normally presented in the chicken GIT) while the communities of Coriobacteriaceae spp. and Sutterella spp. (that can induce dysbioses in poultry) were found to be significantly lesser in compare to control. These changes in cecal microbiota positively affected the efficiency of the digestion and productivity parameters in broilers.

The activity of pancreatic amylase and lipase in blood serum increased in broilers fed probiotic-supplemented diets; these increases evidence the intensification of metabolism of fat and carbohydrates,

probably due to the secretion of the respective digestive enzymes by the probiotic strain. The decrease in proteolytic activity in serum can be related to the competition between endogenous proteases of host and proteases produced by B. subtilis, especially at the finisher age period.

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