

UDC 636.4:636.084:636.087.8:579.64

doi: 10.15389/agrobiol.2017.2.418rus

doi: 10.15389/agrobiol.2017.2.418eng

DIETARY PROBIOTIC *Lactobacillus plantarum* L-211 FOR FARM ANIMALS. II. THE ADDITIVE FOR PIGLETS

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The authors declare no conflict of interests

Acknowledgements:

Supported financially under the subsidy agreement with Ministry of Education and Science of the Russian Federation № 14.579.21.0021 dated 05.06.2014

Received October 3, 2016

Abstract

The lack of lysine in the diet of pigs negatively affects appetite, weight gain, metabolism and immunity of animals. Most feeds for pigs are deficient in lysine. Synthetic amino acids, as feed additives, make feeds significantly more expensive. In this regards, the biologics based on microbial producers able to synthesize lysine in the gastrointestinal tract of animals are promising. However, common producers of lysine, *Brevibacterium lactofermentum*, *Escherichia coli* and the genus *Corynebacterium*, are conditionally pathogenic as a causal agents of opportunistic infections. In the present study, we first examined the changes in intestinal bacterial community and the productive performance in Large White pigs («Novgorod bacon», Novgorod Province) under the influence a lysine producing strain *Lactobacillus plantarum* L-211 (JSC «Bioreactor», Moscow). Taxonomic composition of microorganisms was determined by T-RFLP (terminal restriction fragment length polymorphism) analysis. For surveillance we used two groups of pigs from 28- to 84-day age, fed with the basic diet ($n = 715$, group 1, control) and the same diet supplemented with *L. plantarum* L-211 at the dose not less than 10^9 CFU per animal added to water ($n = 657$, group 2). Microbial community in the pigs' large intestine was taxonomically divers and included a number of unidentified phylotypes. Here, the predominating bacteria were representatives of the phylum *Firmicutes*, including mainly *Clostridia* possessing cellulolytic and amylolytic properties, as well as the members of order Negativicutes able to ferment acids. The phyla *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* were less abundant. The counts of genus *Lactobacillus* was lower than previously assumed, moreover, there was a complete absence of enterococci and bifidobacteria, which are usually attributed to the autochthonous microbiota of the large intestine of animals and birds. Lysine synthesizing strain *L. plantarum* L-211 had a high probiotic effect resulting in a significant increase in the counts of genera *Lactobacillus* (2.94-fold, $P < 0.01$) and *Bacillus* (3.29-fold, $P < 0.01$), of phylum *Bacteroidetes* (5.29-fold, $P < 0.01$), and class *Clostridia* (2.05-fold, $P < 0.01$), whereas the proportions of pathogens from *Staphylococcus* genus and *Campylobacteriaceae* family were below the T-RFLP sensitivity, and the family *Pasteurellaceae* decreased in number 1.41-fold ($P < 0.05$). Both the survival and the average daily weight gain ($P < 0.05$) in pigs, as influenced by the probiotic strain *L. plantarum* L-211, were higher. *L. plantarum* L-211 also improved feed conversion efficiency as compared to the control pigs.

Keywords: lysine, intestinal microflora, pigs, bacterial community, T-RFLP, probiotic, *Lactobacillus plantarum*, productivity, pigs' survival, feed conversion

In baby pigs, digestive system is formed and live weight is intensively increased which is important for obtaining highly productive livestock [1, 2]. In this, full amino acid composition of the diet, including essential amino acids, plays a special role. The lack of lysine is associated with a decrease in appetite and productivity of animals, weight loss, impaired calcification of bones, general

exhaustion and anemia [3, 4]. In addition, immunity suppression occurs and susceptibility to infectious diseases increases [5]. Due to the lack of lysine in vegetable fodder mixtures, their use in feeding pigs is ineffective. The main raw materials in production of domestic mixed fodders for pigs are the components deficient in the lysine (grain, by-products of their processing, sunflower meal), therefore, as a rule, it is not possible to provide enough lysine level without the use of synthetic amino acids, which considerably increases the fodder cost [6, 7].

The development of probiotics synthesizing lysine in the gastrointestinal tract of animals [8, 9] performed in Russia and abroad, are considered as the most promising approach in the formation of the optimal intestinal microflora. In this, *Brevibacterium lactofermentum*, *Escherichia coli* and representatives of the genus *Corynebacterium* [10-12] were mainly studied as lysine producers, but the listed microorganisms are conditionally pathogenic and capable of causing opportunistic infections, so their use as probiotics is undesirable. Some strains of the genus *Lactobacillus* are also able to synthesize lysine [8, 9, 13] and positively affect the growth, carcass quality, animal immunity [14, 15]. The positive effect of dietary lactobacilli in pigs is also associated with the synthesis of organic acids and bacteriocins that inhibit the growth and development of various pathogens, i.e. *Salmonella*, *Proteus*, *Staphylococcus*, *E. coli*, *Pseudomonads*, *Streptococci* [15, 16].

In recent years, successful use of molecular genetic approaches to study the microbial community of the digestive tract of pigs has been reported, including differences in the composition of microbiocenosis of healthy pigs and animals with intestinal disorders [17, 18]. The T-RFLP (terminal restriction fragment length polymorphism) and NGS (next generation sequencing) methods allow a detailed description of the microbial community, revealing not only taxonomic dominants, but also minor components, including non-cultivated microorganisms, whose proportion in different ecosystems can reach 90 % [19, 20]. However, studies of the intestinal microbiom of pigs carried out to date are extremely few [21, 22], and data on the complex analysis of the bacterial community, as influenced by probiotic lactobacilli, are absent. The strain *Lactobacillus plantarum* L-211 is described as a lysine producer with a fairly high yield in culture [8, 9].

For the first time, using the T-RFLP method, we determined the composition of the bacterial community in the thick intestine of the baby pigs when the preparation of the lysine-producing strain of lactobacilli was supplemented with water and revealed its high probiotic activity which was accompanied by positive changes in the zootechnical indices, i.e. an increased survivability and daily live weight gain.

The purpose of this work was to study the effect of dietary lysine-producing strain *Lactobacillus plantarum* L-211 on the bacterial community of the intestine and the productive indices in baby pigs during early growth.

Technique. Farm experiment was carried out on two groups of baby Large White pigs from day 28 to day 84 of life (OOO Novgorodsky Bacon, Novgorod region). The feeding and housing pigs from groups I (control, $n = 715$) and II ($n = 657$) were in compliance with all technological parameters and equivalent feed compositions (OOO Novgorodsky Bacon, Novgorod region) for lysine corresponding to the norms for Large White breed. Baby pigs of group II were additionally given dietary *Lactobacillus plantarum* L-211 (OOO Bioreactor, Moscow) in a dose of at least 10^9 CFU per animal. Survivability of livestock, the live weight of baby pigs at the age of 28 and 84 days in individual weighing, its daily gain, feed consumption and feed costs per 1 kg of live weight were recorded.

Contents of the large intestine from three slaughtered pigs of each group were collected on day 84 with strict sterility for molecular genetic studies. T-RFLP analysis of bacterial community was performed according to description

[23]. Total DNA was isolated from the samples using Genomic DNA Purification Kit (Fermentas, Inc., Lithuania) according to manufacturer's recommendations. PCR was performed on a Verity DNA amplifier (Life Technologies, Inc., USA) with eubacterial primers 63F CAGGCCTAACACATGCAAGTC labeled at the 5'-end (WellFed D4 fluorophore, Beckman Coulter, Inc., USA) and 1492R TACGGHTACCTTGTTACGACTT. The fluorescently labeled amplicons of 16S rRNA gene was purified as described [24], the restriction (30-50 ng DNA) with endonucleases HaeIII, HhaI and MspI was performed following the manufacturer's recommendation (Fermentas, Inc., Lithuania). Restricts were analyzed using CEQ™ 8000 (Beckman Coulter Inc., USA) according to the manufacturer's protocol. The taxonomic attribution was performed in the Fragment Sorter program (<http://www.oardc.ohiostate.edu/trflpfragsort/index.php>).

The data were processed by an analysis of variance. Differences with the control were considered significant at $P < 0.05$; $P < 0.01$ and $P < 0.001$.

Results. T-RFLP analysis of the microbial community of thick intestine where the most important digestion of carbohydrates, including fiber, occurs with participation of intestinal microbiota and formation of volatile fatty acids (VFA) and other metabolites [25], revealed a number of taxonomic groups (Table 1).

1. The ratio of bacterial taxa (%) in the thick intestine in 84-day old the Large White pigs receiving probiotic *Lactobacillus plantarum* L-211 ($\bar{X} \pm x$, OOO Novgorodsky bacon, Novgorod region)

Taxon	Group I (control, n = 3)	Group II (n = 3)
Phylum <i>Bacteroidetes</i>	0.17±0.01	0.90±0.04**
Phylum <i>Firmicutes</i>	35.46±1.69	65.38±2.95**
class <i>Clostridia</i>	5.35±0.21	10.96±0.43**
family <i>Lachnospiraceae</i>	1.48±0.06	0.69±0.03**
family <i>Eubacteriaceae</i>	1.07±0.04	8.18±0.03***
family <i>Ruminococcaceae</i>	0.84±0.03	0.47±0.02**
family <i>Clostridiaceae</i>	1.89±0.09	1.62±0.07
genus <i>Peptostreptococcus</i>	0.07±0.01	Brd
genus <i>Lactobacillus</i>	7.83±0.33	23.07±1.13**
genus <i>Bacillus</i>	2.93±0.13	9.65±0.61**
genus <i>Staphylococcus</i>	0.25±0.01	Brd
order <i>Negativicutes</i>	19.10±0.97	21.70±1.03
Phylum <i>Actinobacteria</i>	0.16±0.01	Brd
Phylum <i>Proteobacteria</i>	2.04±0.03	2.64±0.06**
family <i>Enterobacteriaceae</i>	0.87±0.23	1.95±0.15*
family <i>Campylobacteriaceae</i>	0.21±0.01	Brd
family <i>Pseudomonadaceae</i>	0.96±0.04	0.21±0.01***
genus <i>Acinetobacter</i>	Brd	0.48±0.02
family <i>Pasteurellaceae</i>	2.60±0.14	1.84±0.08*
Phylum <i>Fusobacteria</i>	0.07±0.01	0.14±0.01*
Unclassified sequences	59.49±2.98	29.10±1.39**

N o t e. Description of the groups is given in the *Technique* section.
 Brd — below the limit of reliable determination by the method of T-RFLP (terminal restriction fragment length polymorphism).
 *, **, *** Differences with control are statistically significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Identified bacteria belonged to five phylums, of which the members of the phylum *Firmicutes*, including *Clostridia* and the order of *Negativicutes*, predominated. Traditionally, the bacteria of the families *Lachnospiraceae*, *Clostridiaceae*, *Ruminococcaceae*, *Eubacteriaceae* belonging to class *Clostridia*, are considered as the main producers of enzymes (cellulase, hemicellulase, amylase, etc.) necessary to metabolize carbohydrates from plant fodder. As a rule, the VFAs formed during this process are used by representatives of the order of *Negativicutes*, including bacteria *Megasphaera*, *Selenomonas*, and others. Note that these processes were previously described primarily for ruminants [25]. The phyla of *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* were less abundant.

Some gut microorganisms found were opportunistic and pathogenic. Bacteria of the genus *Fusobacterium*, revealed in both groups, have traditionally been regarded as causative agents of cattle necrobacteriosis [26], and their presence in the intestine, various organs, on the skin in animals and humans has now been confirmed using molecular genetic methods [27]. Representatives of the families *Enterobacteriaceae* and *Campylobacteriaceae* detected in the intestine in small quantities are typical pathogens of the dysbiosis in animals. Of interest is the fact that bacteria of the family *Pasteurellaceae*, the pathogens of respiratory tract in animals and poultry, have been detected in the intestines of baby pigs

[16]. At that, bacteria of the genus *Staphylococcus* common in intestinal contents of animals were practically absent in baby pigs.

A part of the bacterial community could not be taxonomically identified which agrees with foreign and domestic studies of the microbiom of the digestive tract in various species of animals and poultry [23, 28]. As to the identified bacteria, the results obtained using the T-RFLP method also did not contradict the known concepts [16, 21, 25] with some exceptions. Thus, the bacteria of the genus *Lactobacillus*, which are commonly considered to be the dominant inhabitants of pig intestines, we found in small amounts. In addition, enterococci and bifidobacteria, previously described as representatives of the autochthonous microbiota of the large intestine in animals and birds, were completely absent in the intestines of these baby pigs [16, 21].

Dietary probiotic *L. plantarum* L-211 which produces an average of 148.4 ± 4.45 mg/l lysine in minimal media culture [8, 9], when given with water to pigs once a week at a dose of at least 10^9 CFU per animal, changed microbiota of the large intestine. The percentage of genus *Lactobacillus* representatives increased 3 times ($P < 0.01$), which was probably due to the good persistence and growth of the introduced lactobacilli in intestinal contents. The ability of a number of lactobacilli to adhere to the intestinal walls is known which allows them to colonize the digestive tract [29] and occupy free ecological niches in its microbiome, exerting a probiotic effect. In addition, *L. plantum* strain L-211 contributed to a 3-fold increase in the number of bacteria of genus *Bacillus* ($P < 0.01$) which also, due to the synthesis of organic acids and bacteriocins, are capable of competitive exclusion of pathogens [16]. *L. plantarum* L-211 also affected the multiplication of bacteria from the phyla *Bacteroidetes* and *Clostridia*, producing cellulolytic and amylolytic enzymes, and caused 5-fold ($P < 0.01$) and 2-fold ($P < 0, 01$) increase, respectively, in their number in the large intestine compared to control. The proportion of bacteria from the order *Negativicutes* under the influence of probiotic lactobacilli did not change significantly.

2. Zootechnical indices in 84-day-old Large White pigs receiving probiotic *Lactobacillus plantarum* L-211 ($\bar{X} \pm x$, OOO Novgorodsky bacon, Novgorod region)

Parameter	Group I (control, n = 3)	Group II (n = 3)
Total weight, kg	8567	6753
Death loss:		
by number, heads	13	8
by weight, kg	203	116
Sanitary butchering:		
by number, heads	9	11
by weight, kg	181	299
Transmitted for fattening:		
by number, heads	693	638
by weight, kg	21550	19875
Weight, kg per head ($\bar{X} \pm x$)	31.10 ± 1.48	31.15 ± 1.39
Preservation, %	98.18	98.78
Gross increment, kg	13367	13537
Age at transfer, day	83	84
Feed-days, total	28675	27288
Average daily weight gain, g ($\bar{X} \pm x$),	466.20 ± 11.29	$496.10 \pm 10.14^*$
Fodder consumption, kg:		
total	26700	26400
mixed fodder CK-4	17890	18300
mixed fodder CK-5	8810	8100
Conversion of feed ($\bar{X} \pm x$), kg	1.997 ± 0.040	1.950 ± 0.050
Consumption of mixed fodder for the feed-day, kg	0.931	0.967

N o t e. Description of the groups is given in *Technique* section.
* Differences with control are statistically significant at $P < 0.05$.

Importantly, *L. plantarum* L-211 positively affected opportunistic and pathogenic bacteria which percentage decreased. The counts of *Staphylococcus* and *Campylobacteriaceae* bacteria decreased to the levels not detectable by T-RFLP, and the *Pasteurellaceae* was 1.41-fold less abundant ($P < 0.05$). Also, the counts of *Pseudomonadaceae* bacteria, transit microorganisms entering intestine with food, decreased 4.57 times ($P < 0.001$). Unidentified bacteria decreased 2.05 times ($P < 0.01$) compared to control.

Zootechnical indicators (Table 2) confirmed an increased viability, the daily weight gain ($P < 0.05$), and the efficiency of feed conversion due to *L. plantarum* L-211.

Thus, *Lactobacillus plan-*

tarum L-211 synthesizing lysine has a high probiotic activity with a positive effect on the composition of the bacterial community of the large intestine of baby pigs. When feeding the probiotic, the normoflora (genera *Lactobacillus* and *Bacillus*) significantly increased, and the number of microorganisms traditionally associated with intestinal dysbiosis in humans and animals (the family *Campylobacteriaceae*, *Pasteurellaceae*, genus *Staphylococcus*) decreased. The change in the microbial community positively affected zootechnical indicators of pigs which survivability and the daily weight gain ($P < 0.05$) increased at better feed conversion. Therefore, this probiotic used during early growing can provide for highly productive livestock and reduces the costs of compound feed per unit of production.

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