ANIMAL HUSBANDRY

DOI: https:/doi.org/10.23649/jae.2019.4.12.18

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Received: 16.11.2019; Accepted: 02.12.2019; Published: 16.12.2019

LACTOGENIC EFFECTS OF PROBIOTIC PREPARATION ON THE BASE OF RECOMBINANTLACTOBACILLI WITH GROWTH HORMONE RELEASING FACTOR GENE IN RUMINANTS

Research article

Abstract

For the first time, lactogenic action of parenteral administration of the extract from the pituitary gland was detected in Russia (Azimov, Krouze, 1937). However, stimulation of milk production with the help of growth hormone injections is associated with a number of shortcomings. The aim of this work was to study the effects of oral administration of genetically engineered recombinant strain of *Lactobacillus* sp. 8 RAZ (pLF-SL2), Reg. No. B-7495, expressing synthesis of growth hormone releasing factor (GH-RF) in digestive tract, to enhance the milk production in ruminants. In the experiment I, conducted on goats, the recombinant preparation was given as feed additive to 5 goats on the 3rd month of lactation daily for 6 weeks at a dose of 25×10^9 CFU per day, 5 goats served as control. In experiment II, a recombinant preparation was given to 8 cows at 4-5 months of lactation at a dose of 26×109 CFU per day for 2 months (group 1), the original non-recombinant strain was given to 8 cows at the same dose (group 2), 8 cows fed basic diet served as control. Experiment III was carried out on three groups of cows at the 3-4th month of lactation, 10 cows each. The cows of group 1 were fed a recombinant probiotic daily at a dose of 200×10^9 CFU per day, and group 2 at a dose of 1000×10^9 CFU daily. The control group of cows received only the basic diet. In all experiments, the recombinant preparation stimulated milk production and increased the conversion of feed into milk components after three-week lag period. In goats of experimental group, the daily milk yield (DMY) during the last 2-3 weeks was higher by 23.5% ($P<0.05$) than in control group. In experiment II, during the last 5 weeks, DMY in group 1 was more by 10% ($P<0.05$) compared to group 2 and by 8.1% ($P<0.05$) compared to group 3. In experiment III, DMY in groups 1 and 2 was higher by 6.3 and 13.2% ($P<0.05$), respectively, in comparison with control. The data obtained show that the use of recombinant strains of lactic acid bacteria expressing GH-RF may be an alternative to the use of injections of growth hormone in order to increase the milk productivity and quality of milk in ruminants.

Keywords: goats, cows, recombinant Lactobacillus strain, growth hormone releasing factor, milk production.

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Получена: 16.11.2019; Доработана: 02.12.2019; Опубликована: 16.12.2019

ЛАКТОГЕННОЕ ВЛИЯНИЕ ПРОБИОТИЧЕСКОГО ПРЕПАРАТА РЕКОМБИНАНТНЫХ ЛАКТОБАЦИЛЛ С ГЕНОМ СОМАТОЛИБЕРИНА У ЖВАЧНЫХ

Научная статья

Аннотация

Впервые лактогенное действие парентерального введения экстракта из гипофиза было обнаружено в России (Азимов, Круз, 1937). Однако стимуляция молочной продуктивности с применением перорального введения гормона роста связана с рядом недостатков. Цель данной работы - изучение эффектов применения рекомбинантного штамма лактобацилл с геном соматолиберина в качестве кормовой добавки для повышения молочной продуктивности жвачных животных. В первом опыте, проведенном на козах, препарат рекомбинантного штамма *Lactobacillus* sp. 8 РАЗ (pLF-SL2), рег. № В-7495 с геном соматолиберина скармливали в составе комбикорма 5 козам на 3-м месяце лактации ежедневно в течение 6 недель в дозе 25×10^9 КОЕ на голову, 5 коз служили контролем. Во втором опыте

рекомбинантный пробиотик давали 8 коровам на 4-5 месяце лактации в дозе 26×10^9 КОЕ на голову в течение 2 месяцев (1-я группа), исходный не рекомбинантный штамм давали 8 коровам в той же дозе (2-я группа), 8 коров служили контролем. Третий опыт проведен на трех группах коров на 3-4-м месяце лактации по 10 голов в каждой. Первой группе животных ежедневно скармливали рекомбинантный пробиотик в дозе 200×10^9 КОЕ на голову, 2-й группе – в дозе 1000×10^9 КОЕ на голову. Контрольная группа коров получала только основной рацион. Во всех опытах препарат стимулировал молокообразование и повышал конверсию корма в компоненты молока. У коз, получавших препарат, суточный удой в течение последних 2-3 недель был выше на 23,5% ($P<0.05$), чем в контроле. Во втором опыте на протяжении последних 5 недель удой в 1-й группе был больше на 10% ($P<0,05$) по сравнению со 2-й группой и на 8,1% ($P< 0,05$) по сравнению с 3-й группой. В третьем опыте удой в 1-й и 2-й группах был выше на 6,3 и 13.2% соответственно в сравнении с контролем. Заключили, что применение рекомбинантных штаммов молочнокислых бактерий с геном соматолиберина может быть реальной альтернативой применению инъекций и имплантаций соматотропного гормона с целью повышения молочной продуктивности жвачных животных.

Ключевые слова: козы, коровы, пробиотики, лактобациллы, рекомбинантные штаммы, соматолиберин, молочная продуктивность.

1. Introduction

For the first time, lactogenic action of the extract from the pituitary gland was detected in Russia [1]. With periodic parenteral administration of the preparation from the pituitary to cows, an increase in milk yield was observed. In subsequent years, numerous studies have established that somatotropic hormone (STH, GH) has a pronounced stimulating effect on the milk production in ruminants [2,], [3], [4], [5], [6], [7], [8], [9]. Growth hormone releasing factor (GH-RF) is a 44 amino acid peptide hormone, produced in the arcuate nucleus of the hypothalamus. The arcuate nucleus of the hypothalamus plays an important role in the dopaminergic control of the secretion of growth hormone, vasopressin, prolactin and regulation of the estrous cycle. GH-RF is released from the neurosecretory nerve endings of arquate neurons and is transferred by the hypothalamic-pituitary portal system to the anterior pituitary gland, where it stimulates the secretion of growth hormone.

The stimulation of milk production with GH injections is associated with a number of shortcomings, but in a number of studies, including long chronic experiments, it has been shown that injections of GH-RF increase the level of endogenous GH in the blood and milk production in cows [10], [11]. It was previously shown that the absorption of low molecular weight peptides into blood is an important physiological process in animals, including ruminants [12], [13]. In this connection, it has been promising to use live recombinant strains of bacteria producing GH-RF by oral administration.

In previous studies, it was found that when feeding probiotic recombinant strains of lactobacilli carrying a genetic engineering construct with GH-RF gene, biological effects are observed in experimental animals, suggesting an increase in the secretion of GH − an increase in body weight gain, an increase in the proportion of muscle tissue, and a decrease fat in the carcass, an increase in the ploidy of the hepatocyte nuclei and another effect [14].

The aim of this work was to study the possibility of using a recombinant strain of *Lactobacillus* sp. 8 RAZ (pLF-SL2), expressing formation of GH-RF in digestive tract, to enhance the milk production in ruminants.

2. Material and methods

Characteristic of the recombinant lactobacilli preparation.

Preparations of lactobacilli were prepared in the laboratory of biotechnology in Institute of Animal Physiology, Biochemistry and Nutrition, Borovsk, Russian Federation. Method for the production of genetic engineering recombinant bacterial strains have been described previously [15], [16]. The principle is to use the rep-operon transcribed in any recipient of plasmids of the pLF family. In the spacer region between the repA and repB genes, the HindIII and HindII restriction enzyme sites are located, and any sequence cloned into these sites, including the synthetic sequence encoding GH-RF, will be transcribed if the cloning does not interfere with plasmid replication. Selection of recombinants was carried out on selective plates with chloramphenicol. The analysis of the obtained transforms allowed selecting the target construct - plasmid pLF-SL2. It contains a double dose of GH-RF gene and before each of the duplicated coding regions there is a bacterial signal of translation.

Experiments on lactating ruminants. Three experiments were performed using recombinant strain of *Lactobacillus* sp. 8 RAZ (pLF-SL2), one on the goats and two on the cows.

Experiments on goats. Experiment I was conducted on two groups of goats, 5 goats each, on 3rd month of lactation. The experiment consisted of two periods, the initial (2 weeks) and main (6 weeks). At the end of the initial period, groups of animals were formed, analogues for live weight, productivity, lactation phase and age. Animals were contained in individual cells. Goats of the control and experimental groups received the basic diet. In the main period, each goat of the experimental group fed 35 ml of a liquid preparation of recombinant *Lactobacillus* sp. 8 times (pLF-SL2), B-7495 with a GH-RF gene at a dose of 25×10^9 CFU per day. In the course of the experiment, milk productivity and feed intake were determined daily. The content of protein and fat in milk was determined twice a week. The dry matter content of the animal feeds was evaluated and its consumption for milk produced (corrected for 4% fat content) were calculated.

Experiments on cows. Experiment II was carried out by the method of groups and periods on 3 groups of cows, 8 cows each, on the 4^{th}-5^{th} month of lactation. The groups were balanced for milk productivity, lactation phase and age. All cows received a diet containing hay, straw, molasses, sunflower meal, gluten, concentrate and contained dry matter − 15,2 kg, metabolizable energy −162 MJ, crude protein − 1952 g, fiber − 2792 g.

In the main period of experiment, group 1 of animals fed within 2 months a recombinant Lactobacillus sp. 8 time (pLF-SL2) at a dose of 26 billion CFU per head (test group), and group 2 fed preparation of the original strain Lactobacillus sp. in

the same dose (control 1). The cows of group 3 fed only basic diet). Feed intake, daily milk yield (DMY) weekly, fat and protein content in milk were determined once every two weeks. In the main period of the experiment, blood probes were taken from the jugular vein to determine the concentration of NEFA.

Experiment III was carried out by the method of groups and periods on three groups of cows, 10 cows each, on the 3-4th month of lactation. During 6 weeks in the preliminary period, the mean DMY and milk composition were determined to adjust the balancing groups. All cows received a basic diet containing hay grass, herbage silage, corn flakes (oats, vetch), cornmeal, sunflower cake, concentrates (oats, barley) and contained dry matter − 16,68 kg, metabolizable energy −149.9 MJ, crude protein − 1809 g. In the main period, in group 1 daily within 2 months, the preparation of the recombinant *Lactobacillus* sp. strain with GH-RF gene was given as feed additive at a dose of 200 billion CFU per cow, and in group 2 at a dose of 1000 billion CFU per cow. Cows of control group were fed only the basic diet. In the course of the experiment, DMY, feed consumption weekly, and content of protein and fat in milk biweekly were measured.

3. Results

Experiment on goats. The addition of recombinant lactobacilli to the feed of goats did not affect significantly the live weight of animals and feed intake during the experiment. Milk productivity in all animals decreased during the course of the experiment, however feeding of the recombinant preparation inhibited this decline (table 1). The animals of both groups did not differ in the mean DMY in the initial period and in the first two weeks of the main period of the experiment. In the next two-week period (5-6 weeks) of the experiment, there was a clear trend towards an increase in DMY by 20% in the goats of experimental group, compared to the control group. Over the next two weeks of the main period of the experiment, this difference became even more contrasting (by 26.5%, P<0.05).

Table 1 – Daily milk yield and milk composition in goats (experiment I, M±SE, n=5)

Periods of experiment	Weeks	Control group			Experimental group		
		DMY, G	**PROTEIN, %**	**FAT, %**	**DMY, G**	**PROTEIN, %**	**FAT, %**
Initial	1-20	1388±186	3,56±0,05	**4,21±0,17**	1375±113	**3,36±0,16**	**4,34±0,21**
Main	3-4	1287±190	3,22±0,08	**3,65±0,16**	**1292±109**	**3,22±0,11**	**3,81±0,14**
	5-6	987±83	3,10±0,09	**3,56±0,50**	**1186±110**	**3,29±0,18**	**3,82±0,16**
	7-8	942±70	3,09±0,07	**3,92±0,14**	1192±93*	**3,43±0,11***	**4,03±0,09**

*Note: * P<0.05 by t-test vs control.*

In experimental group, the milk protein content in the first two weeks of the main period decreased in the same way as in the control animals. However, after 5 weeks of using recombinant preparation, the decrease in milk protein concentration ceased and it began to increase. During the last two weeks of the main period, the level of milk protein in the goat of experimental group increased by 11% vs control (table 2, P<0.05). The use of the recombinant probiotic preparation reduced the consumption of feed dry matter for 1 kg of milk, corrected for 4% fat content.

In the initial period of the experiment, the goats of the control and experimental groups did not differ significantly in either the production of milk, corrected for 4% fat content, or in the efficiency of using feed dry matter for milk production. The daily production of fat corrected milk in both groups of goats decreased during the course of the experiment, while the consumptions of feed dry matter per 1 kg of milk increased.

So, there were no significant differences between groups of animals on these traits in the first two weeks of the main period of experiment. However, in the following weeks, the experimental group significantly exceeded the control group for the production of fat corrected milk (P<0.05), while the dry feed consumption for its synthesis significantly decreased compared to the control group (table 2, P<0.05). During the last two weeks of the main period, 1.9 g of feed dry matter was required to synthesize 1 g of milk in the control group, and only 1.5 g (P<0.05) in the experimental group.

Table 2 – Effect of feeding recombinant probiotic on the milk productivity and costs of feed dry matter for 1 kg of fat corrected milk in goats (M±SE, n = 5)

Periods of experiment	Weeкs	Control group		Experimental group	
		MILK PRODUCTION, G	**FEED/ MILK+**	**MILK PRODUCTION, G**	**FEED/ MILK^{+}**
	1–20	1439±178	**1,40±0,11**	1463±94	**1,34±0,07**
Initial	3–4	1137±116	**1,67±0,10**	**1223±100**	**1,57±0,09**
	5–6	879±41	**2,02±0,04**	**1118±81***	**1,64±0,06***
	7–8	937±69	**1,89±0,07**	1202±101*	**1,49±0,07***

*Notes:+ g feed dry matter/g milk; * P <0.05 vs control.*

Thus, the results of the experiment on goats indicate that the feeding of recombinant *Lactobacillus* preparation inhibits the decrease in milk production during lactation and raises the efficiency of using the feed for the synthesis of milk components.

Experiment on cows. In the initial period of the experiment II, the mean DMY in the experimental, control 1 and control 2 groups was the same (20.0-20.4 kg). During the first three weeks after the initiation of feeding recombinant preparation, there was no significant intergroup difference in mean DMY (table 3), but from the 4th week of the main period, the DMY began to increase in the experimental group. During the last five weeks of the main experiment period, the yield in the experimental group increased by an average by 10% (P<0.05) compared with the control 1 group, in which the original lactobacillus preparation was fed and by 8.1% (P<0.05) as compared to control 2 group (table 4).

Table 3 – Effect of feeding lactobacilli preparations on milk yield in cows (experiment II) (M±SE, n = 8)

Groups	Initial period	Main period		
	Mean DMY, kg	Mean DMY, kg		Mean difference between experimental and control cows-analogues over subsequent 5 weeks ± SE†
		For first 3 weeks	In the subsequent 5 weeks	
Experimental	20,0±0,70	21,6±0,90	22,8±0,91	
Control 1 [b]	20,4±0,79	21,7±1,02	20,7±0,96	2,01±0,84* [a-b]
Control 2 [c]	20,4±0,78	21,5±1,08	21,1±0,75	1,65±0,68* [a-c]

*Notes: [a] preparation of recombinant lactobacilli strain; [b] preparation of initial lactobacilli strain; [c] basic diet; † for cows-analogues for productivity in the experimental and control groups; *P <0.05 by t-test when comparing variants [a-b] and [a-c].*

The experimental animals of all three groups did not differ significantly in the protein content of the milk. Its concentration was the lowest in the initial period of the experiment, and the highest at the end of the main period (table 4). Feeding recombinant strain promoted a tendency to increase in the fat content in milk. In the main period of the experiment, the concentration of milk fat decreased in the control 1 and control 2 groups, and it increased in the experimental group.

At the beginning of the main period, there were no significant differences between the groups on the feed consumption for milk production, and in the next five weeks they increased in 1 and 2 control groups by 14.5 (P<0.05) and 10.4%, respectively (P<0.05) as compared to the experimental group (table 5).

It is known that when an exogenous GH is administered to animals in large doses, the content of nonesterified fatty acids (NEFA) in the blood increases substantially as a result of enhanced lipolysis (Etherton, Bauman, 1998, Tucker, 2000). However, when small amounts of GH are administered, the growth of milk production may not be accompanied by an increase in the concentration of NEFA (Baldi et al., 2002; Chiado et al., 2000). In our experience, in the group of animals that received the recombinant *Lactobacillus* strain, there was a tendency for an increase in the NEFA content in the blood plasma relative to both control groups.

Table 4 – Effect of feeding lactobacilli preparations on milk composition in cows (experiment II) (M±SE, n = 8)

Periods	Control 1 [b]		Control 2 [c]		Experimental group [a]	
	Protein, %	Fat, %	Protein, %	Fat, %	Protein, %	Fat, %
Initial	3,31±0,13	4,16±0,17	3,33±0,08	4,27±0,38	3,49±0,12	4,54±0,28
Main, first 3 weeks	3,48±0,10	4,07±0,23	3,47±0,07	3,91±0,22	3,70±0,08	4,67±0,28
next 5 weeks	3,74±0,09	4,19±0,32	3,73±0,06	4,11±0,20	3,91±0,09	5,04±0,07

Notes: [a] recombinant strain of lactobacilli; [b] initial strain of lactobacilli; [c] basic diet.

Table 5 – Effect of feeding lactobacilli preparations on feed dry matter consumption for milk production in cows (experiment II) (M±SE, n = 8)

Groups	Main period		
	Feed consumption, kg for 1 kg of milk		Mean difference between experiment and control over the past 5 weeks ± SE†
	For first 3 weeks	In past 5 weeks	
Experimental [a]	0,830±0,034	0,801±0,036	
Control 1 [b]	0,823±0,043	0,917±0,047	0,115±0,035* [a-b]
Control 2 [c]	0,846±0,045	0,884±0,041	0,082±0,032* [a-c]

*Notes: [a] preparation of the recombinant strain of lactobacilli; [b] preparation of an initial strain of lactobacilli; [c] basic diet; † for analogous animals in the experimental and control groups; *P <0.05 by t-test when comparing variants [a-b] and [a-c].*

In the experiment III, conducted on cows, the average daily yield in the control 1, control 2 and experimental groups in the initial period were the same (13.9, 14.0 and 13.7 kg, respectively). Feeding the recombinant preparation did not significantly affect milk composition, but had a positive effect on mean DMY and production of milk protein and fat (table 6, 7).

Table 6 – Effect of feeding recombinant lactobacilli preparations on composition of milk in cows (experiment III, M±SE, n=10)

Groups	Initial period		Main period	
	Fat, %	Protein, %	Fat, %	Protein, %
Experimental [a]	4,02±0,14	2,76±0,07	4,42±0,14	3,10±0,06
Experimental [b]	4,37±0,18	2,77±0,08	4,56±0,22	3,07±0,07
Control [c]	3,71±0,22	2,72±0,08	4,31±0,23	3,12±0,05*

*Notes: [a] dose of 200 billion CFU per day; [b] dose of 1000 billion CFU per day; [c] basic diet; *P<0.05 by t-test vs control.*

The recombinant preparation at a dose of 200 billion CFU per day increased the average daily yield by 6.3%, and in a dose of 1000 billion CFU − by 13.2% (P<0.05) compared to the control (table 6). The production of milk fat increased by 12.3 and 11.5% (P<0.05), respectively, and milk protein by 4.5 and 13.6% (P<0.05), respectively, compared with control (table 7, 8).

Thus, the results of our experiments showed that feeding preparation of recombinant lactobacilli increases the milk productivity and efficiency of using feed for the synthesis of milk components. Since this effect was manifested when compared with both control groups, it can be concluded that the stimulation of milk production and efficiency of feed conversion into milk components was due to an increase in the level of growth hormone as an effect of releasing GH synthesized in recombinant lactobacilli.

Table 7 – Effect of feeding recombinant lactobacilli preparations on milk yield in cows (experiment III, M±SE, n=10)

Groups	Initial period	Main period	
	Mean DMY, kg	Mean DMY, kg	Mean difference experiment- control ± SE[†]
Experimental [a]	14,0±0,63	15,3±0,88	0,9±0,99
Experimental [b]	13,7±0,61	16,3±0,66	1,9±0,77*[b-c]
Control [c]	13,9±0,91	14,4±1,19	

*Notes: here and in table 8: [a] dose of 200 billion CFU per day; [b] dose of 1000 billion CFU per day; [c] basic diet; [†] for analogous animals in the control and experimental groups; * P <0.05 by t-test vs control.*

Table 8 – Effect of feeding recombinant lactobacilli preparations on milk fat and protein production in cows (experiment III, M±SE, n=10)

Groups	Initial period		Main period			
	Fat, g	Protein, g	Fat, g	Mean difference experiment-control ± SE	Protein, g	Mean difference experiment-control ± SE
Experimental [a]	582±40	384±15	683±48	75,0±70,2	468±25	20,4±20,5
Experimental [b]	508±30	375±25	678±33	69,9±31,6	509±25	61,0±17,0*
Control [c]	545±49	383±26	608±80		448±32	

4. Discussion and conclusion

The disadvantage of known methods of stimulating productivity of animals through the introduction of STH is the need for periodic injections, the high cost of preparations and the laboriousness of these methods. Therefore, the development of inexpensive ways to increase the secretion of endogenous STH in productive animals is of practical interest in terms of increasing productivity and improving product quality. At the same time, further research is needed to realize the potential merits of this approach. If the use of protein and peptide hormones by parenteral administration is widely approved, the use of live recombinant strains of bacteria expressing the formation of these hormones by oral administration, by some researchers is considered to date as problematic. Supporters of this point of view motivate their position by the fact that protein molecules and peptides are not absorbed in intact form from the intestines of animals. However, this view is obsolete, since the absorption of proteins from the intestine was established by numerous experiments on animals and isolated tissues, and some researchers have demonstrated the passage of high-molecular peptides through the isolated jejunum of animals [12], [[13], 20].

The physiological effect of recombinant bacteria nesting the digestive tract may depend on a variety of factors, including the level of transcription of the cloned gene and the efficiency of translation of foreign mRNA, the efficiency of transport of the synthesized peptide from the intestine, and other factors.

The absence of a lactogenic effect of recombinant lactobacilli during the first three weeks after the initiation of their feeding in our experiments seems to be explained by the fact that during this period, the content of the rumen becomes infested with bacteria, and stable associative links in its ecosystem are created that contribute to the growth of their population.

Colonization activity of bacteria is characterized by their ability to adhere to epithelial cells of the intestinal mucosa. Bacteria that grow slowly but attach to the intestinal wall, can colonize the intestine, while non-adherent species are compensated for by increasing the growth rate. Attachment provides a microorganism resistance to leaching from the intestine during peristaltic flow of contents. It follows from this that if a bacterial strain can occupy places of adhesion on the intestinal wall, as is characteristic of many species of lactobacilli, then it takes root in the digestive tract [21]. Important from the point of

view of biological safety is that the obtained recombinant strains can not transfer the vector plasmid further, which minimizes the danger of its dissemination in nature with large-scale use of strains [15].

On the whole, the data obtained in this investigation, suggest that oral administration of genetically engineered recombinant preparation of lactic acid bacteria expressing GH-RF in digestive tract may be an alternative to the use of hormone injections with the aim to increase the milk productivity and improving the quality of products in ruminant animals.

Conflict of Interest

None declared.

Конфликт интересов

Не указан.

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