Changes in the taxonomic composition of the rumen microbiome during the dietary supplements administration

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Abstract. The degree of the dry matter (DM) digestibility of the diet and the effect of additional administration of linseed oil on the composition of the rumen microbiome of cattle were studied. The in vitro method was used using the incubator "ANKOM Daisy II "(modifications D200 and D200I) according to a specialized technique. Microflora analysis was performed using MiSeq ("Illumina", USA) by the new generation sequencing method (NGS) with a set of MiSeq reagents. With the introduction of linseed oil, an increase in the digestibility of DM feed by 2.3% relative to the control (wheat bran) was noted. In the experimental group the level of species diversity was 45% higher than in the control group. The Simson dominance index in the control group was 0.44, in the experimental group it is 0.54. The Shannon index was equal to E=0.3, which shows a greater alignment of the microbial community in the ruminal fluid when flaxseed oil was included. There was an increase in the number of bacteria of the phylum Bacteroidetes (64.2 %) and a decrease in Firmicutes (19.3 %)., There was an increase in the number of microorganisms belonging to the classes Saccharibacteria, Spirochaetia, Mollicutes, Lentisphaeria, Elusimicrobia in the experimental group, relative to the control. Also it is an increase in representatives of all classes of the phylum Proteobacteria in comparison with the control by 44 % (p < 0.05).

1. Introduction

Mammals can synthesize all the fatty acids (FA) necessary for the normal course of metabolic processes, with the exception of polyunsaturated fatty acids (PUFA), which must be supplied with the diet. PUFAs, which are part of flaxseed oil, are involved in many chemical reactions in the body, so their addition to the diet is of particular interest in the cattle feeding [1]. The inclusion of flaxseed oil (60 g / kg of dry matter) in the diet based on barley silage contributed to a greater (P < 0.01) accumulation of useful biohydrogenation intermediates (C18: 2 trans-11, cis-15; C18: 1 cis-15), which reflects the ability of flaxseed oil to produce more desirable FA [2, 3]. Flaxseed oil is considered one of the most useful for livestock and poultry. It tastes softer, more pleasant and easier to digest than others, and has a high content of mucous substances and dietary value. Due to the significant content of protein and fat, the nutritional value of the feed can be regulated [4]. Zubtsov V. A. (2015) found that a apozem of linseed cake and seed is easily absorbed by the animal's body. It envelops the intestinal walls, prevents the absorption of harmful substances released by intestinal microbes, and helps to remove them from the body. Flaxseed proteins are characterized by high biological value [5]. Flaxseed is the richest source of the lignan compound secoisolaricyresinol diglucoside, which is found in the outer fibrous layers of flax.

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The rumen appears to be the main site of conversion of secoisolaricryresinol diglucoside to enterolignans, enterodiol, and enterolactone. However, there is limited information about the types of rumen microbiota involved in the metabolism of the diglucoside secoisolaricryresinol [6]. Microcapsules of oil can not only improve the structure of the intestine, but also increased the number of bacteria Paenibacillus and decreased the number of harmful bacteria Pseudoalteromonas, as well as Roseovarius in the intestine. Therefore, the attention of specialists is increasingly attracted to full-fat flaxseed and rapeseed seeds as sources of polyunsaturated fatty acids (PUFA Q-3 and Q-6) [7].

The purpose of our work was to study the degree of digestibility of the dry matter (DM) of the diet and the effect on the composition of the rumen microbiome of additional administration of linseed oil.

2. Materials and methods

2.1. Object of research
Ruminal fluid, microbiom.

2.2. Experiment scheme
The study of the rumen microbiome was performed on a model of gobies with a rumen fistula (n=3). The studies were carried out using the Latin square method.

The studies used unrefined linseed oil - STO 40490379-001-2015, TP TS 024/2011 Technical Regulations for fat and oil products. Made by order of OOO "Planet of Taste", Russia, OOO "Businessoil", 142138, Moscow, Shchapovskoye settlement, Kurilovo settlement, Rabochaya str., 5. The studied oil was added in an amount of 3% of the dry matter by mixing into the feed (wheat bran was used as a substrate - control). The content of saturated fatty acids in this oil was 11%, oleic acid is 16.9 %, linoleic acid is 16.6 %, linolenic acid is 55.5 %.

Studies of the rumen microbiome were carried out by the in vitro method using the "artificial rumen" model using the "ANKOM Daisy II" incubator unit (modifications D200 and D200f) according to a specialized technique. Distilled water was chosen as the dispersion medium.

In order to study the effect of linseed oil on the rumen microbiome in fistula animals samples of the rumen contents were taken in 3 hours after feeding, which were filtered through 4 layers of gauze and incubated in an artificial rumen at a constant T=39°C for 24 hours. At the end of incubation, the samples were washed and dried at a temperature of +60 °C to a constant weight.

The in vitro dry matter digestibility coefficient was calculated as the weight difference between the feed sample and the pouch before and after two-stage incubation. After incubation, the ruminal fluid was collected with a syringe dispenser ecohim OPA-2-20 of the company "Ecroschem", St. Petersburg, in micro-tubes of the Eppendorf type with a volume of 1.5 ml. For the analysis, 1.5 ml of the substrate of the ruminal fluid was taken, one sample for each exponent. The maintenance of animals and the procedures for performing experiments complied with the requirements of the instructions and recommendations of the Russian regulations (Order of the Ministry of Health of the USSR № 755 of 12.08.1977) and "The Guide for Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996)". Every effort was made to minimize the suffering of the animals and to reduce the number of exponent used.

2.3. Metagenomic analysis of rumen microorganisms
Microflora analysis was performed using MiSeq (Illumina, USA) by the new generation sequencing method (NGS) with the MiSeq Reagent Kit v3 (600 cycle). For The Bioinformatic Processing Of The results, The PEAR (Pair-End AssemblY, PEAR v0.9.8) program is used (Zhang J., Kobert K., Flouri T., Stamatakis A. PEAR: A fast and accurate Illumina Paired-End reAd merger. Bioinformatics, 2014, 30(5): 614-620 (doi: 10.1093/bioinformatics/btt593)). Filtering, dereplication, deleting of null sequences, clustering, sorting (singletons clipping), deleting of contamination is performed in the USEARCH program (usearch v8. 0. 1623_i86linux32, http://drive5.com/usearch). The fastq filter
algorithm is used for filtering, the derep prefix algorithm is used for replication, and the cluster otus algorithm is used for clustering and deleting chimeric sequences.

The sequencing results were processed using the Microsoft Excel 10 data analysis package, Microsoft Office software.

2.4. Statistical processing

The numerical data were processed using the SPSS program "Statistics 20" (IBM, USA), the mean (M), standard deviations (±σ), standard deviation errors (±SE) were calculated. A nonparametric analysis method was used to compare the variants. The differences were considered statistically significant at P<0.05. The Shannon (H'), Simpson (D), and Pielu (E) biodiversity indices were also calculated [8].

3. Study results

With the introduction of linseed oil, an increase in the digestibility of DM feed by 2.3% relative to the control (wheat bran) was noted.

According to the results of the metagenomic sequencing of the contents of the calves rumen, the values of the parameters of α-biodiversity are presented (figure 1). When comparing the groups according to the Shannon index (H') and Simson dimination index (D), it turned out that their values changed with the additional inclusion of linseed oil in the diet. It was found that the level of species diversity in the experimental group was 45% higher than in the control group. The Simson dominance index in the control group was 0.44, in the experimental group it is 0.54. Based on the Shannon index, we calculated the Pielu equalization index (E), this index in the control was 0.2, and in the experimental group it is E=0.3. It shows a greater equalization of the microbial community in the ruminal fluid when flaxseed oil was included. The most pronounced change in the level of diversity in the experimental group compared to the control due to an increase in energy in the feed when the oil is turned on. In our study, the Shannon index (p < 0.05) increased compared to the control group and amounted to 0.76.

![Figure 1](image-url)

**Figure 1.** Parameters of α -biodiversity: A-Shannon index, B-Simson index, C-Pielu equalization index: I-control group, II-experimental group.

The increase in the α-biodiversity of the rumen microbiome in the experimental group did not affect the β-diversity, so the Jacquard and Sorensen indices were calculated. They were equal to 1, which indicates a complete coincidence of the communities in the control and experimental groups.
In order to assess whether variations in α-biodiversity were associated with changes in the composition and function of the microbiota, changes in the taxonomy of the rumen microbiota were evaluated based on NGS sequencing data. Metagenomic sequencing of the cattle rumen contents of the control group showed the presence of 12 phylum, where 2 phylum dominant, in particular, phylum Firmicutes 54.6%, Bacteroidetes 39.2%. These two taxonomic groups can be considered as the "core of the bacterial microbiome", since they are present in significant amounts in all the exponent studied. The dominant bacteria found in the control exponent are presumably responsible for the transformation of the main components of the diet - cellulose, hemicellulose, starch, organic acids, and proteins (figure 2). When a high-energy component (flaxseed oil) is introduced into the diet against the background of an increase in the production of VFA in the rumen, an increase in the number of acid-resistant bacteria of the phylum Bacteroidetes (64.2%) and a decrease in the number of Firmicutes (19.3%) is observed (figure 3) [10].

In the experimental group, relative to the control, an increase in the number of microorganisms belonging to the classes Saccharibacteria_genera_incertae_sedis by 2.8 times, Spirochaetia by 5 times, Actinobacteria by 1.3 times, Mollicutes by 2.2 times, Lentisphaeria by 2.1 times, and Elusimicrobia by 17 times was noted. There was also an increase in representatives of all classes of the phylum Proteobacteria in comparison with the control by 44% (p<0.05).

When introducing linseed oil, the predominance of representatives of the Prevotellaceae family was noted, which accounted for 63.1% of the total number of representatives of all families, while the number of these microorganisms was 40% higher than the control values. In the experimental group, relative to the control, an increase in the number of microbial composition of the rumen of the families Bifidobacteriales, Porphyromonadaceae, Rikenellaceae, uncultured Bacteroidales and Subdivision5_genera_incertae_sedis was also noted.
When flaxseed oil was included, the number of Streptococcus bovis amylolytic microorganisms decreased by 99% (p < 0.05) relative to the lactate-producing control, which leads to an increase in VFA-producing bacteria and the intensity of VFA synthesis. Also in the study, when flaxseed oil was added, the presence of archaea domain in the ruminal fluid, in particular Methanomassiliicoccales, whose concentration in the experimental group was 97% higher compared to the control.

4. Results discussing

Linseed oil, rich in linoleic acid, is one of the most effective sources of lipids used in strategies to reduce methane emissions [9]. Flaxseed lipids (and fats in general), added to the diet, replace some of the dietary carbohydrates and, since the rumen microbes do not ferment them, less hydrogen is produced. It was found that the number of protozoa decreases with the addition of linseed oil [10], although this effect was not always observed [11].

In the inclusion of linseed oil, we found an increase in the number of microorganisms by 11.6% relative to the control diet. Oleic acid (an average of 20% of linseed oil fatty acids) stimulated the growth of Selenomonas ruminantium in pure cultures [12]. However, in vivo studies, the results are contrasting: Selenomonas was among the genera explaining the differences in bacterial community structure between lambs fed a flaxseed diet and those fed a control diet, but there was no change in the abundance of Selenomonas when cows were fed sunflower oil (30% oleic acid) [13]. In this study, a 62% decrease in the number of the order Selenomonadales in the ruminal fluid of calves was found with the additional administration of linseed oil. In contrast, the addition of linseed oil increased the abundance of Bacteroidetes by 35.8% (p < 0.05) relative to the control group. The addition of flaxseed oil also affected the abundance, but not the diversity of the rumen methanogenic community, so the
antimetanogenic potential of flaxseed oil fatty acids was related to the number of archaea in the rumen, not the species composition.

Ruminococcaceae are an important group of bacteria that live in the rumen, and are able to decompose polysaccharides of the plant cell wall for metabolized energy. This means that increasing this rumen community increases the breakdown of dietary fiber. In the present studies, the addition of flaxseed oil tended to increase the digestibility of the DM diet and made a difference of 2.3%. The current study found no relationship between the number of important taxa and feed efficiency. This is not surprising, given the high ability of microbial populations to adapt to dietary changes, which could offset differences in feed efficiency under the influence of changes in microbial communities [14].

The ratio of Firmicutes to Bacteroidetes (F: B) is closely related to the feed efficiency and optimize strategies for reducing methane emissions into the environment.

5. Conclusion
The additional inclusion of linseed oil in the rumen diet contributed to an increase in the species diversity of the rumen microbiome, an increase in the number of bacteria of the phylum Bacteroidetes, as well as a significant increase in representatives of the archaea domain involved in rumen methanogenesis. The data obtained can be used in the development of diets for young cattle on fattening to improve feed efficiency and optimize strategies for reducing methane emissions into the environment.

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Conflict of Interest
Authors declares that they have no conflict of interest.

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