Application of new technologies to assess the effectiveness of feed materials for ruminants

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Abstract. The study presents the results of the influence of coconut and flaxseed oils by the in-vitro method on the microbiome of scar fluid in young cattle. During the experiment, a decrease in the number of bacteria for Firmicutes phylum by 37.2 and 35.2 % using vegetable oils with the control sample and growth for the large phylum Bacteroidia, the bacterial abundance was higher than in control by 28.1 and 25.1 % for coconut and flaxseed oils, respectively. Also, the use of coconut and flaxseed oils has led to an increase in the number of bacteria for filum Candidatus Saccharibacteria and phylum Verrucomicrobia.

1. Introduction
In the last decade, the problem of the microbiome in connection with changing environmental conditions has received particular development for various animal species [1, 2], including agricultural in general and cattle in particular [3].

Many approaches, such as improving the quality of feed, increasing the proportion of concentrates in the diet and additional feeding of other feeds, have been studied to control the rumen microbiome [4, 5]. Although these solutions gave different results, it was repeatedly shown that feeding strongly affects the rumen microbiome [6].

One of the most important tools for managing the ruminant rumen microbiome, including to reduce methane formation, is the practice of feeding vegetable oils [7].

However, supersaturation of diets with lipids leads to the disruption of the flow of biosynthetic processes in the scar [8]. This process is caused by significant changes in the rumen microbiome, the inhibitory effect of oils on enzymatic processes [9].

Meanwhile, due to many reasons, including the need to increase the energy concentration in diets against the background of increasing the genetic potential of modern breeds and crosses of cattle; reducing the formation of methane in the rumen and increasing the proportion of lipids in animal diets in the coming years, there will be no alternative. In this connection, studies on the study of the microbiome of cattle when introduced into the diet of vegetable oils seem relevant.

2. Materials and methods
2.1 Object of study
Cicatricial fluid, microbiome.
2.2 Scheme of the experiment

The study of the rumen microbiome was carried out on a model of red steppe goby with a rumen fistula (n = 3), the animals were 12 months old, and their live weight was 250–300 kg. Studies were performed using the Latin square method. The contents of the animals and the procedures during the experiments corresponded to the requirements of the instructions and recommendations of the Russian regulations (Order of the Ministry of Health of the USSR No. 755 of 08/12/1977) and "The Guide for Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996)." Every effort has been made to minimize animal suffering and reduce the number of samples used.

The studies used coconut oil samples with a content of 9.9 % – caprylic acid, 4.2 % – capric acid, 47.5 % – lauric acid, 17.3 % – myristic acid, 5.8 % – palmitic acid, 3.6 % – stearic acid, 9.6 % – oleic acid, 1.1 % – linoleic acid, 0.5 % – arachinic acid and 0.5 % – behenic acid.

The fatty acid composition of linseed oil was represented by the following carboxylic acids: palmitic acid – 6.2 %, stearic acid – 4.4 %, oleic acid – 16.9 %, linoleic acid – 16.6 %, linolenic acid – 55.5 %, arachinic acid – 0.4 %.

Studies of the scar microbiome were performed using the in-vitro method using the "artificial scar" model.

For studying the effect of vegetable oils on cicatricial microorganisms in fistulous animals, cicatrical samples were taken 3 hours after feeding. Samples were filtered through 4 layers of gauze. The studied oil samples were added to the scar fluid at a dosage of 1 ml per 1 litre of scar fluid or 6.6 %, in separate baths for each sample and incubated in an artificial scar at a constant T = 39 0 C for 24 hours in a thermostat (TS-1/80 SPU). After incubation, scar fluid was sampled with a syringe dispenser (Ecohim OPA-2-20) into microtubes (1.5 ml Eppendorf).

2.3. Metagenomic analysis of scar microorganisms

Microflora was analyzed by metagenomic sequencing (Illumina MiSeq, Illumina, USA) using the MiSeq Reagent Kit v3 reagent kit (600 cycles). For bio information processing of the results, a pair program (Pair-and Assembler, PEAR v0.9.8) was used.

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

Statistical processing was performed using the SPSS Statistics 20 program (IBM, USA); mean (M), standard deviations (± σ), standard deviation errors (± S.E.) were calculated. For compare, the options used non-parametric analysis method. Differences were considered statistically significant at ≤ 0.05.

3. Results

The study of the cicatrical microbiome of the control group showed that the dominant phyla were Firmicutes (54.6 %), which was represented by the classes Clostridia (28.6 %), Bacilli (15.1 %), Negativicutes (10.5 %) and the phylum Bacteroidetes (39.2 %) the largest the class is marked by Bacteroidia (38 %) (Table 1). A small number of bacteria was noted for phylum Candidatus Saccharibacteria, Spirochaetes, Proteobacteria no more than 2 % of the total number of selected taxa.

The predominant number of bacteria isolated belonged to the family Ruminococaceae (7.6 %), represented by the genus Ruminococcaceae (4.7 %). The remaining specific genera belonging to the Ruminococaceae family did not exceed 2 % for each taxon: Clostridium IV (0.3 %), Ruminococcus (1.2 %), Saccharofermentans (1.2 %). In the Lachnospiraceae family (16.5 %), Lachnospiraceae (9.2 %), Butyrivibrio (2.3 %) were most pronounced. The genus Streptococcus (13.7 %) belonged to the class Streptococaceae (13.7 %).

The number of taxa identified in a cicatrical fluid using coconut oil identified three main phyla Firmicutes (17.4 %), Bacteroidetes (67.7 %), Verrucomicrobia (6.3 %) (Table 2).

The genus unclassified Ruminococaceae and the genus Oscillibacter, which belong to the family Ruminococaceae, showed a slight increase relative to the control by 0.5 % - 1.0 %, respectively. The remaining classes, families and genera, in the composition of Firmicutes phylum, were distinguished
by a significant decrease in the number of bacteria. The *Lachnospiraceae* family decreased by 13.4 % relative to the control. The number in the class *Negativicutes* decreased by 7 %, the family *Acidaminococcaceae* by 6.8 %, the genus *Succiniclasticum* 6.8 %.

Table 1. Taxonomic composition of the microbiome of cicatricial fluid in vitro, control sample, %

| Phylum              | Class               | Family                | Genus                        |
|---------------------|---------------------|-----------------------|------------------------------|
| Firmicutes          | Clostridia          | Ruminococcaceae       | Unclassified_Ruminococcaceae |
| (54.6 %)            | (28.6 %)            | (7.6 %)               | (4.7 %)                      |
|                     |                     | Lachnospiraceae       | Unclassified_Lachnospiraceae |
|                     |                     | (16.5 %)              | (9.2 %)                      |
|                     |                     |                       | Butyribivirio                |
|                     |                     |                       | (2.3 %)                      |
|                     |                    | Unclassified_Clostridales | (3.4 %)                      |
| Bacilli             | Streptococcaceae    | Streptococcus         |                              |
| (15.1 %)            |                     | (13.7 %)              |                              |
| Negativicutes       | Acidaminococcaceae  | Succiniclasticum      |                              |
| (10.5 %)            |                     | (9.7 %)               |                              |
| Bacteroidetes       | Bacteroidia         | Prevotellaceae        | Prevotella                   |
| (39.2 %)            | (38 %)              | (25 %)                | (22.4 %)                     |
|                     |                     | Unclassified_Bacteroidales | (10.6 %)                 |
|                     | Other *             | Other *               | Other *                      |
| (6.2 %)             | (7.7 %)             | (13.4 %)              | (23.9 %)                     |

* This group includes taxa, the number of each of which does not exceed 2 % of the total

Table 2. Taxonomic composition of the microbiome of scar fluid in vitro using coconut oil, %

| Phylum              | Class               | Family                | Genus                        |
|---------------------|---------------------|-----------------------|------------------------------|
| Firmicutes          | Clostridia          | Ruminococcaceae       | Unclassified_Ruminococcaceae |
| (17.4 %)            | (13.6 %)            | (8.6 %)               | (5.2 %)                      |
|                     |                     | Lachnospiraceae       | Unclassified_Lachnospiraceae |
|                     |                     | (3.09 %)              | (1.5 %)                      |
| Negativicutes       | Acidaminococcaceae  | Succiniclasticum      |                              |
| (3.5 %)             |                     | (2.9 %)               |                              |
| Bacteroidetes       | Bacteroidia         | Prevotellaceae        | Prevotella                   |
| (67.7 %)            | (56.9 %)            | (33.2 %)              | (29.4 %)                     |
|                     |                     | unclassified_Bacteroidales | (18.08 %)                 |
|                     | Porphyromonadaceae  | Genus unknown         | (18.08 %)                    |
|                     | (4.25 %)            | Unclassified_Porphyromonadaceae | (4.0 %)               |
| Verrucomicrobia     | Subdivision 5       | Family unknown        | Genus unknown                |
| (6.3 %)             | (6.2 %)             | (6.2 %)               | (6.2 %)                      |
|                     | Other *             | Other *               | Other *                      |
| (8.6 %)             | (8.6 %)             | (12.2 %)              | (14.03 %)                    |

* This group includes taxa, the number of each of which does not exceed 2 % of the total

In the phylum *Bacteroidetes*, the number of taxa included in its composition increased by 28.5 % compared to the control. The highest growth was found in the families *Prevotellaceae* (33.2 %), unclassified *Bacteroidales* (18.0 %) and *Porphyromonadaceae* (4.2 %).

It is worth noting that there is a definite increase for the phylum *Verrucomicrobia* (6.3 %) and its member class *Subdivision 5* (6.2 %), compared to the control it is more by 5.4 %.
A study using linseed oil on the effect on cicatricial microorganisms revealed phylums *Firmicutes* (19.4 %), *Bacteroidetes* (64.3 %), *Candidatus Saccharibacteria* (2.9 %), *Verrucomicrobia* (5.3 %). The *Ruminococcaceae* family (10.5 %) slightly exceeded the control sample by 2.9 % and more than in the experiment with coconut oil by 1.9 % (Table 3).

### Table 3. The taxonomic composition of the in vitro scar fluid microbiome using linseed oil

| Phylum       | Class               | Family               | Genus                        |
|--------------|---------------------|----------------------|------------------------------|
| *Firmicutes* | Clostridia          | Ruminococcaceae      | Unclassified_Ruminococcaceae |
| (19.4 %)     | (15.3 %)            | (10.5 %)             | (6.25 %)                     |
|              |                     | Lachnospiraceae      | Unclassified_Lachnospiraceae |
|              |                     | (3.2 %)              | (1.34 %)                     |
|              |                     | Acidaminococcaceae   | Succiniclasticum             |
|              |                     | (2.6 %)              | (2.6 %)                      |
|              |                     | Family unknown       | Genus unknown                |
|              |                     | (14.6 %)             | (14.6 %)                     |
| *Bacteroidetes* | Bacteroidia       | Prevotellaceae        | Unclassified_Porphyromonadace |
| (64.3 %)     | (56.7 %)            | (36.9 %)             | (4.1 %)                      |
|              |                     |                      | Genus unknown                |
|              |                     | (4.3 %)              | (5.3 %)                      |
|              |                     |                      | Other*                       |
|              |                     |                      | Other*                       |
| *Candidatus* | Saccharibacteria    | Family unknown       | Genus unknown                |
| *Saccharibacteria* | genera_incertae    | (2.9 %)              | (2.9 %)                      |
| (2.9 %)      | sedis               |                      |                              |
| *Verrucomicrobia* | Subdivision 5    | Family unknown       | Genus unknown                |
| (5.3 %)      | (5.3 %)             | (5.3 %)              | (5.3 %)                      |
| Other*       | Other*              | Other*               | Other*                       |
| (8.1 %)      | (8.3 %)             | (11.8 %)             | (17.03 %)                    |

* This group includes taxa, the number of which does not exceed 2 % of the total

The family of *Lachnospiraceae* (3.2 %) and *Acidaminococcaceae* (2.6 %) under the influence of linseed oil decreased their presence by 13.3 % and 7.1 %, in contrast to the control sample. The presence of the genus *Succiniclasticum* was (2.6 %), less than in control by 7.1 %.

![Figure 1. The distribution of bacteria in groups for phyla Candidatus Saccharibacteria and Verrucomicrobia](image-url)
As in the experiment with coconut oil, phylum *Verrucomicrobia* (5.3 %) and the class Subdivision 5 isolated in it, using the natural form of linseed oil, grew by 4.5 % in comparison with the control experiment.

The taxa that make up one large phylum of *Bacteroidetes* also had higher values than in control. Metagenomic sequencing revealed an increase in the number, for the families *Prevotellaceae* (36.9 %), unclassified *Bacteroidales* (14.6 %), *Porphyromonadaceae* (4.3 %).

Comparing the results using coconut and linseed oil with a control sample of scar fluid, an increase in the content for Phylum *Candidatus Saccharibacteria* and Phylum *Verrucomicrobia* should be noted. In the control sample, the presence of *Candidatus Saccharibacteria* phylum was 1.2 %, and 0.8 % for *Verrucomicrobia* phylum, which is 0.4 % less than in the experimental groups, 5.5 % in the experiment with coconut oil and 1.7 % less, 4.5 % for the group with linseed oil (Fig 1).

4. Conclusion

Earlier, the addition of oil to the diet of ruminants was proposed as a potential means of reducing methane emissions [10, 11].

It should be noted the pronounced effect of oils on the rumen microbiome, which primarily affected the ratio of *Firmicutes / Bacteroidetes* by changing the value of this indicator from 1.39: 1 in control to 1: 3.9 and 1: 3.3 when using coconut and linseed oil. These dynamics demonstrate a change like digestion and a decrease in the activity of microorganisms that decompose structural carbohydrates [12]. A general decrease in the *Firmicutes* share was accompanied by the degradation of individual taxa of this phylum so that it would be noted for the classes *Clostridia, Negativicutes*, the families *Lachnospiraceae, Acidaminococcaceae*. These classes of microorganisms are capable of digesting complex polysaccharides – fibre, pectin, using nitrogen-containing substrates as well. In particular, *Acidaminococcaceae* can use amino acids as their sole source of energy for their growth and development [13].

Against the background of a general decrease in *Firmicutes* from 54.6 % in control to 17.4–19.4 % in the experimental groups, one of the families of this phylum, *Ruminococcaceae*, showed positive growth.

One of the primary and significant phyla identified in the experimental groups was the phylum *Bacteroidetes*. All taxa isolated in this type were significantly higher relative to the control group. From literature data, it is known that this type of bacteria is capable of fermenting various hydrocarbons with the formation of succinic, acetic, and formic acids [14].

When entering the study, the class was accompanied by an increase in the number of bacteria of the *Prevotella* genus, which is the most represented by the bacterial genus in the scrub [15]. The high resistance of the *Prevotella* bacterium allowed [17; 18] us using strains of *Prevotella bryantii* to prevent subacute acidosis.

The growth of phylum *Verrucomicrobia* in experiments with vegetable oils, in our case with coconut and linseed oil, maybe due to several reasons while acidification of cicatricial fluid could also occur due to the increased number of *Bacteroidetes* bacteria.

Also, phylum *Verrucomicrobia* possibly influenced the increase in the phylum population of *Candidatus Saccharibacteria*, since *Verrucomicrobia* can oxidize methane, thereby contributing to the growth of *Candidatus Saccharibacteria*, which themselves can destroy various organic compounds [19].

Also, cattle treated with vegetable oils were characterized by a decrease in the species richness of mushrooms, which is consistent with previously obtained results [20-22].

The ratio of saturated and unsaturated carboxylic acids contained in vegetable oils, in particular in coconut and linseed oil, can affect the abundance of bacteria capable of hydrogenating short-chain and long-chain fatty acids, changing the ratio of polyunsaturated fatty acids.

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