The T-RFLP research method in the study of rumen microbiota in dairy cows with subclinical ketosis

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Abstract. The article presents the results of researches related to the study of the possibility of using the modern molecular genetics method -T-RFLP-analysis (terminal restriction fragment length polymorphism) to identify the community of microorganisms in the contents of the rumen in clinically healthy highly productive cows and cows with subclinical ketosis. The research methodology is based on the analysis of the conservative regions in the microorganisms' genome variability. The results showed the high efficiency of the method used for the identification of microorganisms in the rumen contents in the studied animals. A large community of bacteria, archaea, protozoa and anaerobic fungi is determined in the rumen contents from highly productive cows with subclinical ketosis. The data obtained allow us to significantly amplify the information on the pathogenesis of subclinical ketosis in cows with high milk productivity. The presence of conditionally pathogenic and pathogenic microflora in sick cows in the rumen contents indicates a violation of rumen digestion, which leads to the development of concomitant non-contagious diseases in animals.

1 Introduction

Active longevity and health of cows with the highest possible milk productivity is one of the most urgent tasks of practical veterinary science. In the Russian Federation today there is a tendency to breed highly productive animals in industrial dairy complexes, since this technology is the most promising, due to the fact that much less feed, labor, material resources, etc. spent on milk production. The introduction of intensive technologies for feeding and keeping highly productive cows into livestock breeding practice leads to the occurrence and development of metabolic diseases, such as ketosis. The most common form of the disease is subclinical. As a rule, in highly productive animals, especially in the post-partum period, as a result of a lack of metabolic energy, a violation of protein, carbohydrate and fat metabolism occurs. Subclinical ketosis of cows is accompanied by increased formation, accumulation and excretion from the body with urine and milk of

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ketone bodies: acetone, hydroxybutyric and acetoacetic acids, a decrease in alkaline reserve, and impaired rumen digestion [1, 2, 3].

It is well known that the nutrition of the ruminants' organism occurs mainly with the active participation of rumen symbiotic microorganisms, as well as other parts of the digestive tract. The rumen is a complex symbiotic ecosystem containing up to 1010-1011 bacteria, 107-109 archaea, 106 protozoa, and 104-105 anaerobic fungi per milliliter of rumen fluid. The symbiosis between rumen microorganisms and the animal organism, formed during the evolutionary development of the ruminant digestive system, is obligate – the existence of one partner is impossible without the other [1, 4, 5]

In turn, as noted above, a change in the composition of the feed ration leads to a change in the ratio of certain types of microorganisms in the ecosystem of the gastrointestinal tract of cattle [6, 7, 8]. This causes a violation of the traditional biochemical orientation of the microflora and, as a result, the development of various metabolic disorders, in particular subclinical ketosis [9, 10]. In this regard, the study of the species composition of the ruminant microbiocenosis, as well as the specific role of each species in increasing productivity of cattle, is certainly of great interest to researchers [11, 12, 13].

2 Materials and methods

The studies were performed on the basis of the St. Petersburg State University of Veterinary Medicine, at the Department of Internal Animal Diseases named after A.V. Sinev and JSC "Krasnoselskoe" in the Leningrad region. Laboratory work on the study of rumen contents microbiocenosis was carried out in the molecular genetics laboratory of the research and production company PLC "BIOTROF", St. Petersburg. To assess the state of rumen microbiota, most researchers use classical methods of microbiological research, such as cultivation on various nutrient media. The classical bacteriological method makes it possible to detect no more than 1.0–5.0% of the total microflora of the ruminants' gastrointestinal tract. In our work, to identify microorganisms, we used a modern molecular genetics method - T-RFLP analysis (terminal restriction fragment length polymorphism). It is based on the analysis of the microbial genome conserved regions variability. The main essence of the technique is to isolate the all microbial DNA present in the rumen contents, multiply its amount using the polymerase chain reaction (PCR) technique, followed by enzymatic cleavage of DNA into fragments and their separation on an automatic sequencer. When interpreting the results, the taxonomic affiliation of all isolated bacteria is determined in accordance with the terminal gene fragments lengths using the Fragment Sorter program. T-RFLP analysis was performed according to the method.

The experiments were carried out on black-motley cows with high milk productivity. The cows involved in the experiment were kept in similar conditions, tethered. Animals were under constant observation and subjected to a comprehensive clinical examination, which was carried out according to generally accepted methods. Laboratory analysis of biological fluids was carried out using unified methods. Sampling of rumen contents was carried out using a probe developed by us (patent RU 208268U1 "Probe for obtaining samples of rumen contents in cattle") 3 hours after the first (morning) feeding. All groups of animals were formed taking into account the physiological state according to the principle of pair-analogues. The conditions of keeping, feeding and care in which the animals of the experimental and control groups were kept were the same. Based on express tests of blood, urine and milk, cows with signs of subclinical ketosis were identified, their content of ketone bodies was above 1.0 mmol/l. Rapid blood tests were performed using a Free Style Optium keto-glucometer and Free Style Optium β-Ketone test strips for the determination of β-hydroxybutyric acid. Based on clinical examination, two groups were formed: 1st (control) healthy animals and 2nd (experimental) cows with subclinical ketosis.

The obtained results were ran through the statistical processing in Excel Windows Office XP and IBM SPSS Statistics 26 (USA) with the calculation of the arithmetic mean and its standard error (M ± m), as well as using Student's t-test for independent and paired samples with a normal distribution, the Mann-Whitley U-test for independent samples, and the Wilcoxon test for paired samples that differ from normal distribution and ordinal variables. Qualitative nominal values were analyzed by Pearson's chi-squared test. The normality of the determination was determined using the Shapiro-Wilk test. The critical level of significance when testing statistical hypotheses is $p \leq 0.05$ [14, 15].

3 Results

The results of the T-RFLP study of the rumen contents of clinically healthy cows and cows with subclinical ketosis on the fifth day of lactation are presented in Tables 1, 2 and 3.

Table 1. The content of normal flora in samples of the rumen contents of cows (T-RFLP-analysis), % M±m

Parameter	Reference values	Control group (n=10)	Experimental group (n=10)
Bacteroids	2-17	5.65±0.58	4.39±0.40
Succinivibrio	0-2	0.19±0.03	0.01±0.00***
Lachnospira	≥4	18.83±1.47	11.62±0.22***
Ruminococci	≥2	6.09±0.73	6.07±1.18
Eubacteria	≥1	3.50±0.34	3.22±0.35
Clostridia	≥2	5.03±0.97	1.83±0.13*
Thermoanaerobacter	≥0,5	1.32±0.10	0.45±0.12***
Selenomonads	≥3	8.65±0.81	5.50±0.49***
Bacilli	≥7	16.69±1.19	12.86±0.45**
Bifidobacteria	≥0,5	0.49±0.05	0.23±0.01***

Note: statistically significant in comparison with the control group when:
* - $p \leq 0.05$
** - $p \leq 0.01$
*** - $p \leq 0.001$

Analyzing the data obtained in Table 1, we can conclude that, on average, the representatives proportion of the normal microflora in the rumen contents, both in the control and in the experimental group of animals (cellulolytic bacteria, bacilli, selenomonads, succinivibrio) in the studied samples corresponded to the reference values.

Despite the fact that the proportion of some cellulolytic bacteria, that break down plant fiber and other feed carbohydrates, in the experimental group was normal and slightly fluctuated compared to the control group of animals, the ratio of other types of cellulolytic bacteria: lachnospir, thermoanaerobacteria and clostridia significantly differed from same levels in the control group or even went out of reference values range. The lahnospir group in the rumen contents in cows with subclinical ketosis was 62.0% lower than in the control animals. The number of clostridia in the experimental group did not rise on average above the lower limit of the norm and fell in relation to the control group to 36.0%. Selenomonas responsible for breaking down organic acids in the rumen tended to decrease, but their number corresponded to the reference values. The level of bifidobacteria, as well as bacilli with antimicrobial activity against pathogenic microorganisms of the rumen and other useful properties, in all the studied groups corresponded to the reference values, while at the same time in the control group it came close to the minimal limit.

Table 2. The content of opportunistic microflora in samples of the rumen contents of cows (T-RFLP-analysis), % M±m

Parameter	Reference values	Control group (n=10)	Experimental group (n=10)
Lactobacilli	≥2	0.32±0.05	0.68±0.04***
Enterobacteria	≥10	2.15±0.22	4.80±0.42***
Actinobacteria	≥10	3.11±0.22	6.46±0.84**

Note: statistically significant in comparison with the control group when:
* - p≤0.05
** - p≤0.01
*** - p≤0.001

Based on the results presented in Table 2, it can be concluded that the proportion of opportunistic lactobacilli, enterobacteria and actinobacteria in the rumen contents of experimental animals was within the reference values, but compared with the control had a pronounced tendency to increase. This indicates a noticable dysbacteriosis in rumen digestion in cows with subclinical ketosis. According to many researchers, lactobacilli actively participate in the fermentation of monosaccharides to lactic acid, enterobacteria can provoke gastroenteritis in animals, and actinobacteria lead to the development of actinomycosis.

Table 3. The content of pathogenic and transit microflora in samples of the rumen contents of cows (T-RFLP-analysis), % M±m

Parameter	Reference values	Control group (n=10)	Experimental group (n=10)
Fusobacteria	≤3	1.51±0.15	6.00±0.85***
Staphylococci	≤2,5	0.63±0.09	1.06±0.28
Peptococci	≤1	0.39±0.08	0.79±0.15*
Campylobacter	≤3	0.63±0.07	0.99±0.26
Clostridium botulinum	≤1	0.16±0.02	0.32±0.09
Pseudomonas	≤10	0.90±0.18	1.64±0.32
Uncultivated bacteria	5-55	23.76±0.66	31.09±1.55***

Note: statistically significant in comparison with the control group when:
* - p≤0.05
** - p≤0.01
*** - p≤0.001

Analyzing the content of pathogenic and transit microflora in the rumen of the studied animals (table 3), it can be stated that in the control group there is a statistically significant increase in the content of fusobacteria, which, can lead to the development of necrobacteriosis in cows. In the experimental group, their proportion was 100.00% higher than the upper limit, compared with the control group. Staphylococci, peptococci and campylobacter are the causative agents of purulent-inflammatory processes in the body, and despite the fact that their level was within the reference values, in the experimental group there was an increase in relation to the control. The causative agent of dangerous food intoxication *Clostridium botulinum* did not go beyond the reference values, however, in the experimental group of animals, that is, cows with subclinical ketosis, its level was 0.32 ± 0.09%, and in clinically healthy cows it was up to 0.16 ± 0.02%. Pseudomonas differed

slightly from the control group and did not have a significant effect on rumen digestion. The role of nonculturable bacteria has not been fully established.

4 Conclusion

Thus, based on the study results of the species composition and quantitative content of microorganisms in the rumen content of healthy and subclinical ketosis cows using the T-RFLP molecular genetics method, significant imbalances in the ratio of microorganisms (bacteria, fungi and archaea) were established. The data obtained allow us to significantly amplify the information on the pathogenesis of subclinical ketosis in cows with high milk productivity. The presence of conditionally pathogenic and pathogenic microflora in sick cows in the rumen contents indicates a violation of rumen digestion, which leads to the development of concomitant non-contagious diseases in animals.

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