Cattle’ microbiocoenosis of rumen while various feed ultrafine particles release

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Abstract. Changes in the cattle’ microbiocoenosis of the rumen directly impact the animals’ productivity. And the main role here is played by the feeding type, the animal’s diet, with a predominance of certain components in different periods of cows housing, which create the necessary conditions for the existence and activity of symbiotic microflora, as well as the development of associated pathologies. Earlier detection of these errors will help to avoid many abnormalities of metabolic processes in the body and prevent the decline in the animals’ productive qualities. That is why it is relevant to study the effect of ultrafine particles on the composition of cattle’ microbiota of rumen. In this article, we analyzed the cattle’ bacterial microbiocoenosis in response to the introduction of ultrafine particles (FeCo and silicon oxide SiO₂ alloy) into their diet. It is revealed that these ultrafine particles do not have a significant qualitative change in cattle’ microflora of the rumen, but shift the bacterial communities balance towards those microorganisms that improve the food digestibility, which allows them to be recommended as food additives to improve the farm animals productivity.

1. Introduction

Today, more and more research is associated with increased productivity, improved product quality and animal welfare conditions. It is known that the inclusion into the farm animals’ diet of mineral nutrients leads to an increase in the intensity of metabolic processes, the degree of hydrolysis of nutrients in the digestive tract. The main role in these studies plays the use of nanomaterials as biological additives in order to improve the biochemical processes and, as a result, to obtain higher quality products [1-3]. It is the use of nanomaterials that solves the problem of optimizing the farm animals’ mineral nutrition [4, 5]. The development and promotion of such drugs, especially ultrafine particles of metals, today is an integral part of modern science in the agriculture field [6-8]. It is already known that ultrafine particles of metals stimulate growth and development [9, 10], have an immune-enhancing effect, increase the safety of livestock and increase the productivity of farm animals [11]. Of particular interest are researches for the study of ultrafine particles mechanisms of action on the qualitative and quantitative composition of microflora [12], which is especially important when used in feeding ruminants. The first studies on the use of ultrafine materials in cattle nutrition have already demonstrated the prospects for such a solution [13].

In this regard, the purpose of this work was to analyze the ruminants’ microbiocoenosis of the rumen with the inclusion of ultra-dispersed particles into their diets.
2. Materials and methods

The study of microbiocenosis of rumen was carried out in red-steppe bulls. The bulls were kept at the production site of the Pokrovsky Agricultural College Branch of the Orenburg State Agrarian University. All animals were standardized for body weight, an average of 300-310 kg, age, 13 months. The animals were in the preparatory period for 10 days. During the preparatory period of the experiment, the bulls were transferred to tethered contents, individual feeding, to rations based on detailed feeding norms developed by A. P. Kalashnikov and others. (2003). For the experiment, groups were formed (n = 9), randomly divided into control I (K) and two experimental ones – II (FeCo), III (SiO₂). All groups of animals were kept under the same conditions. The duration of the main accounting period was 15 days, falling on the preparatory stage. Animals of the control group were treated with food without adding ultradispersed particles of metals to their diet, while animals of the experimental groups were introduced into the feed ultrafine particles in the morning. Animals of the second group received ultrafine particles at a dosage of 13 mg / kg, and the third - 5 mg / kg body weight during the entire accounting period.

In experimental studies, ultrafine particles of SiO₂ and FeCo alloy were used as sources of microelements, which are spherical preparations synthesized by the gas-phase method of OOO “Perevdovye poroshkovye teknologii”, Tomsk.

These ultrafine particles underwent material science certification by electronic scanning JSM 7401F and transmission JEM-2000FX microscopy (JEOL, Japan), as well as by X-ray phase analysis on a multifunctional diffractometer DRON-7 (NPP Burevestnik, Russia) and corresponded to physical and chemical characteristics described in table 1.

**Table 1. Physical and chemical description of the used ultrafine particles (UUP)**

| UUP            | Size, nm | Chemical and phase composition                      | Method of obtaining | Specific surface (Sₚ, m²/g) | Z-potential | Particle size in the aqueous phase, nm |
|----------------|----------|------------------------------------------------------|---------------------|-----------------------------|-------------|--------------------------------------|
| SiO₂           | 40,9     | SiO₂: 99.8 %; Cl₂: <0.2%                              | vapor phase         | 55,4                        | -31±0,5     | 89,6±16,6                            |
| FeCo (alloy)   | 62,5     | 70% iron and 30% cobalt                              | vapor phase         | 15,5                        | 15±0,2      | 123,3±12,9                           |

The preliminary preparation of the UUP drugs consisted in suspending them in isotonic solution and additional ultrasonic treatment for 30 minutes with a frequency of 35 kHz (power 300 (450) W, oscillation amplitude 10 µm) using an ultrasonic disperser UZDN-2T (NPP “Akademprigor”, Russia). The time of exposure to ultrasound was chosen experimentally in order to obtain particles of aqueous lysols homogeneous in size [23].

To assess the microbial communities of the cattle rumen, ruminal fluid was collected. For this, samples of ruminal contents were taken from fistula animals in the amount of 300 ml before feeding, 3 after feeding was started. Samples were placed in sterile microtest tubes with snap-on lid of “Eppendorf” type (Nuova Aptaca S.R.L., Italy), frozen at -70 °C (ULUF65 “ARCTICO” freezer, Denmark) and stored without re-freezing.

DNA isolation from the obtained samples was performed by chemical extraction. To do this, each sample was incubated in 300 µl of sterile lysis buffer, which was made by mixing 20 mmol / l EDTA, 1400 mmol / l NaCl, 100 mmol / l Tris-HCl, pH = 7,5, with the addition of 50 µl lysozyme in concentration 100 mg / ml, at 37 °C for 30 minutes. Then 10 µl of protease K was added at a concentration of 10 mg / ml and sodium dodecyl sulfate to its final concentration of 1.0% and incubated for 30 min at 60 °C.

DNA purification was performed with phenol-chloroform mixture, sedimented by adding sodium acetate and three volumes of absolute ethanol at -20 °C for 4 hours. After extraction with phenol-
chloroform-isoamyl alcohol (25: 24: 1) and chloroform-isoamyl alcohol (24: 1) DNA in the aqueous phase was sedimented with ammonium acetate (1 mol / L to 10% by volume) and three times volume of anhydrous ethanol during nights at -20 °C. After centrifugation and double washing with 80% ethanol, the DNA was dried and dissolved in TE buffer.

In order to evaluate the purity of the foreign DNA extraction, negative parallel control was established by treating 100 μl of autoclaved deionized water using the same method described above. DNA purity was checked by electrophoresis in a 1.5% agarose gel. DNA concentration was quantified using a Qubit 2.0 fluorometer with a high sensitivity dsDNA assay (Life Technologies).

The preparation of genomic library, as well as sequencing, was carried out at the Core facilities Centre “Persistence of Microorganisms” of the Institute of Cellular and Intracellular Symbiosis, Ural Branch of the Russian Academy of Sciences (Orenburg, Russia). The 16S DNA libraries were prepared in accordance with the Illumina workflow (http://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) with primers targeted on the V3- and V4- areas of the SSU rRNA gene, such as direct SD-Bact-0341-bS-17 and reverse SD-Bact-0785-aA-21. The libraries were sequenced in MiSeq (Illumina) using the MiSeq V3 panel with 2 × 300 base pairs.

Data analysis was performed using USEARCH v8.0.1623_win32 and included the paired reads fusion, quality filtering and the amplicon size choice (minimum size of 415 base pairs). During filtering, reading with N or total Q-score <15 was discarded. Evaluation of filtration quality was carried out using FastQC v 0.11.3. As a result of decomposition and clustering with USEARCH, OTU was formed when single and two-ton were removed. OTU was determined using similarity levels between sequences of at least 97% to classify the microorganism at the species level. Chimera detection was performed through UCHIME (Edgar 2010) using USEARCH and chimeric sequences were removed. Contaminated OTUs were found and removed using the USEARCH command-articular sequence corresponding to sequences from negative control samples. A taxonomic classification of sequences was carried out using VAMPS and the SILVA reference database (Huse 2014).

**Purpose of the study:** the examination of the bacterial microflora of cattle’ rumen while feed ultrafine particles release

3. Results and discussion

In the microflora of the cattle’ rumen using 16s rRNA as a marker, it was revealed that *Bacteria* is the dominant taxon, and its occurrence is about 99.89% of the entire analyzed sample in group I (k) (Figure 1). At that, 7 phylums were classified, of which *Bacteroidetes* and *Firmicutes* were allocated, representing the majority (42.87% and 41.98% of the total, respectively), and also included *Proteobacteria*, which content was 5.05%, and *Cyanobacteria*, *Actinobacteria*, *Thermotogae* and *Verrucomicrobia*, but their content was 1.28, 1.22, 0.81, 0.80% of the total, respectively, which did not exceed 3.5%.

The phylum *Bacteroidetes* taxonomic diversity was represented by 3 classes: *Bacteroidia* (35.34% of the total) and *Sphingobacteria* (5.09% of the total). The amount of third-class *Flavobacteria* was less than 3.5%. Other classes, which amount exceeded 3.5%, were represented by the taxon *Firmicutes*, – *Clostridia*, whose amount was 27.05% and *Bacilli*, which amount was 13.71%. The other classes’ amount was less than 3.5%, and the total content of unclassified classes was 7.61%.

The exact dominant cattle’ rumen family was not found, however, among all certain families, *Prevotellaceae* (19.07% of the total), *Streptococcaceae* (10.82% of the total), *Lachnospiraceae* (10.80% of the total) and *Bacteroidaceae* (10.26% of the total), can be distinguished, while all of them belonged to either the phacum *Bacteroidetes* or the *Firmicutes*.

The number of *Prevotella* genus bacteria in the metagenome of the cattle rumen community was 19.07%, *Streptococcus* – 10.79%, *Bacteroides* – 10.26%, *Succiniliclasticum* – 5.18%, *Blautia* – 4.58%, *Pedobacter* – 3.89 %. Minor taxa (less than 3.5%) were the *Butyrvibrio* genus representatives.
The species diversity of the cattle’ rumen microflora was represented by 714 morphologically distinct bacterial species, only one of which was significant – Streptococcus bovis, whose number was 8.52% of the total, at the same time, unclassified species were in a large proportion (58.02% of the total).

Figure 1. Metagenomic analysis of samples taken from the rumen of cattle in the control group

The study of cattle’ rumen microbiocenosis in groups II (FeCo) and III (SiO2) made it possible to identify certain changes in the amount of previously defined species. So, with the introduction of FeCo nanoparticles into the diet of animal species, the taxon Bacteria also remained dominant (99.96% of the total), but 25 phylums were classified, where Bacteroidetes (39.02%) and Firmicutes (47.64 %) dominated, notably, that while in the control the reliable did not differ from each other, then the addition of nanoparticles leads to the prevalence of bacteria belonging to the phylum Firmicutes. While the Proteobacteria phyla number did not tend to change and amounted to only 4.52%, which is almost identical to its content in the control. Moreover, all classified phyla (Cyanobacteria, Actinobacteria, Thermotogae and Verrucomicrobia) in the control were also identified in this experimental group and their number did not exceed 3.5%.
In group II (FeCo), the *Bacteroidetes* taxon was mostly represented by the *Bacteroidia* class, occupying 32.86% of the bacterial communities’ total number and more than half of this taxon total number, while the *Sphingobacteria* class was much less numerous and amounted to 4.24% of the total identified bacteria. The *Firmicutes* taxon was mostly represented by two classes – *Clostridia* and *Bacilli*, whose numbers were 27.04 and 13.71%, respectively. At the same time, 41 classes were identified, four of them were significant: the classes presented above included bacteria, whose numbers were above 3.5%.

**Figure 2.** Metagenomic analysis of samples taken from the cattle’ rumen while feed ultrafine FeCo particles release

Among the identified families (181) in group II (FeCo), 7 families were significant, among which the family *Prevotellaceae* accounted for 18.89% of the total, *Streptococcaceae* – 15.99%, *Lachnospiraceae* – 10.28%. The other families, such as *Bacteroidaceae*, *Veillonellaceae*, *Sphingobacteriaceae*, *Clostridiaceae*, were small in number and accounted for 25.2% of the total.

420 genera were identified in group II (FeCo), only five of them were significant - *Prevotella* (18.89%) and *Bacteroides* (8.92%) belonging to the *Bacteroidia* class, *Streptococcus* (15.98%) belonging to the *Bacilli* class, *Blautia* (4.23%) and *Succinichystalicum* (6.78%), belonging to the *Clostridia* class.

The species diversity of the cattle rumen microflora in response to ultrafine FeCo particles release into their diets was represented by 613 morphologically different bacterial species. But only one type of
Streptococcus bovis was significant, at the same time, unclassified species accounted for more than half – 55.4%, and in general, were undefined or insignificant, including less than 3.5%, - 87.78%.

In group III (SiO$_2$), represented by samples from the cattle rumen, in which diet ultrafine SiO$_2$ particles were introduced twice, 99.97% of the total number of taxonomic groups allocated occupied Bacteria taxon, where Firmicutes was the most significant phylum, occupying more than 50% of the selected taxa total number. The Bacteroidetes phylum (32.86%) was also considerable; Proteobacteria accounted for only 4.28%, the number of other 22 classified phyla did not exceed 3.5%.

The Firmicutes taxon was characterized by two classes – Clostridia and Bacilli, however, while the number of Clostridia class did not have significant differences from the control values, the number of Bacilli class underwent significant changes in response to the addition to the diet of ultrafine SiO$_2$ particles, and which is 1.83 times higher compared to the control. The Bacteroidetes taxon was mainly represented by a single Bacteroidia class, which number decreased by 8.64% compared to the control and 6.16% compared to the experimental group II (FeCo) and amounted to 26.70% of the total. In another class, represented by the Sphingobacteriia taxon, the abundance varied within 1% of their amount in the control group. Other classes identified were characterized by numbers not exceeding 3.5%.

![Figure 3. Metagenomic analysis of samples taken from the cattle’ rumen while feed ultrafine SiO$_2$ particles release](image-url)
In the further taxonomic analysis of the cattle rumen contents, 8 out of 188 classified families should be mentioned, which number exceeded 3.5%. These included the Clostridiaceae, Lachnospiraceae, Veillonellaceae, Streptococcaceae, Prevotellaceae, Bacteroidaceae and Sphingobacteriaceae. At the same time, the introduction of ultrafine SiO$_2$ particles into the diet led to a change in the dominant family, which resulted in a significant increase in the occurrence of the Streptococcaceae family members by 2.07 times and a decrease in the Prevotellaceae proportion by 1.29 times. The occurrence of the other significant families did not undergo significant changes.

Taxonomic diversity at the genus level in group II (SiO$_2$) was characterized by 440 identified genera, five of them were significant and exceeded 3.5% in occurrence. These included the Streptococcus genus, whose number was 22.41%, the genus Prevotella was the second most significant and its number was 14.83%, and the Bacteroides, Sphingobacteriaceae and Blautia genus were few in number – 7.38-4.15%. The total content of unclassified genus was 15.15%.

The species diversity of the cattle’ rumen, when ultrafine SiO$_2$ particles were introduced into their diets, included 685 taxonomic units, while the type of Streptococcus bovis turned out to be significant, as it was in the previous cases. The remaining species were small and their content was less than 3.5%. It should be noted that while the number of the dominant Streptococcus bovis species increased by 7.46%, when introducing ultradispersed FeCo particles into the cattle ration, the introduction of ultrafine SiO$_2$ particles led to an even more pronounced increase in the number of this species – from 8.52% in the control up to 16.53% in the experimental group III (SiO$_2$).

The rumen’s ecosystem consists of a wide microorganisms’ variety that are in symbiotic relationships under strict anaerobic conditions [14, 15]. The scar microbiocenosis consists of rumen bacteria, protozoa and fungi. Bacterial populations are most vulnerable to the rumen’s physicochemical properties [16], to those nutrients and food additives that farm animals get in their diets [17, 18]. Therefore, it is especially important to consider the change in bacterial composition when changing the farm animals’ diet. The important point is that food additives used in the diet of animals were non-toxic to the microflora inhabiting the rumen and the environment. That is why these studies emphases the examination of the bacterial cattle’ rumen microbiocenosis while feed ultrafine particles release.

In the course of the research it was found that more than 99% of the rumen content is represented by the Bacteria phylum. In this case, the dominant phyla were the Bacteroidetes and Firmicutes. The main bacteria classes that inhabit the cattle’ rumen were Bacteroidia and Sphingobacteria. Among the families that were able to be classified, Prevotellaeceae, Streptococcaceae, Lachnospiraceae and Bacteroidaceae were distinguished. In assessing the generic diversity, 6 genera were identified, which number exceeded 3.5%. However, when identifying rumen contents at the species level, Streptococcus bovis prevails.

The introduction into the animal ration of mineral additives in the form of ultrafine particles did not lead to a qualitative change in the microbiocenosis, but shifted the established balance towards those microorganisms that are responsible for the best feed digestibility [13, 19, 20, 21, 22], in particular, an increase in Streptococcus bovis in the series control-FeCo-SiO$_2$.

4. Conclusion
Thus, the mineral additives in the form of ultrafine particles release is accompanied by an increase in the number of microorganisms involved in the feed digestibility, which allows recommending them as a feed additive to improve the farm animals’ performance.

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