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STUDY OF FAUNA AND ECOLOGY OF SPECIES BELONGING TO ENTODYNIMORPHA IN THE REGIONS OF NAKHCHIVAN AUTONOMOUS REPUBLIC

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ИЗУЧЕНИЕ ФАУНЫ И ЭКОЛОГИИ ВИДОВ, ПРИНАДЛЕЖАЩИХ К ENTODYNIMORPHA В РАЙОНАХ НАХЧИВАНСКОЙ АВТОНОМНОЙ РЕСПУБЛИКИ

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Abstract. Rumen Protozoa are strictly anaerobic and highly specialized ciliates and can live only in the rumen and similar habitats. These Ciliata play an important role in feed utilization and indicate the environmental impact (methane emissions and nitrogen excretion) of ruminant production. Digestion of feed in ruminants depends on the life activity of microorganisms. Without their help, ruminants could not assimilate cellulose with simple non-protein compounds. These microorganisms located in rumen decompose the organic substances in the feed and form volatile fatty acids, ammonia, amino acids, which animals use for their development and reproduction. In the rumen of domesticated cattle and sheep, rumen cilia constitute 20-50% of the total microbial biomass. Rumen Ciliata develop symbiotic relationships with their animal hosts and both symbiotic and predator-prey relationships with other members of the rumen microbiota. The lack of axenic cultures of rumen Ciliata has forced researchers to use other methods to determine the metabolism and functions of rumen Protozoa, such as comparing rumen fermentation and microbial profiles of faunal and non-ciliated cattle or sheep or using *in vitro* cultures.

Аннотация. Простейшие рубца являются строго анаэробными и высокоспециализированными инфузориями и могут жить только в рубце и аналогичных средах обитания. Эти ресничные инфузории играют важную роль в использовании корма и оказывают воздействие в симбиозе со жвачными животными на окружающую среду (выбросы метана и выделение азота). Переваривание кормов у жвачных животных зависит от жизнедеятельности микроорганизмов. Без их помощи жвачные животные не смогли бы усвоить целлюлозу с простыми небелковыми соединениями. Эти микроорганизмы, находящиеся в рубце, разлагают органические вещества корма и образуют летучие жирные кислоты, аммиак, аминокислоты, которые животные используют для своего развития и размножения. В рубце домашнего крупного рогатого скота и овец рубцовые ресничные инфузории составляют 20–50% общей микробной биомассы. Инфузории рубца развивают симбиотические отношения со своими животными-хозяевами, а также симбиотические отношения и отношения хищник-жертва с другими членами микробиоты рубца. Отсутствие аксенических культур инфузорий рубца вынудило исследователей использовать другие методы для определения метаболизма и функций простейших рубца, такие как сравнение ферментации рубца и микробных профилей крупного рогатого скота или овец или использование культур *in vitro*.

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Keywords: Entodiniomorpha, rumen fluid, protozoa, species.

Ключевые слова: энтодиниморфы, рубцовая жидкость, простейшие, виды.

Material and Methodology of the Research

Research was conducted in the regions of Nakhchivan Autonomous Republic. The research object was sheep. The Ciliophora effect have been studied by regularly conducting research on sheep. Oligotrichous Protozoa from sheep rumen, mainly *Entodinium caudatum*, were maintained in culture in vitro and split every 2 days at a density of 15,000-30,000 protozoa/ml, rice starch, dried grass, 10% (v/v) rumen fluid (fresh or autoclaved). and 50 μg is included in the medium for several months. The effect of changing each of these factors in turn and using different growth conditions on these organisms is described. Protozoa were also cultured from an inoculum containing 100,900/ml. It had a density of more than 50,000/ml for 10 days. In specifying the species Freeliving ciliates of Azerbaijan [1], Basics of Animal Husbandry [4], other numerous identifiers, works of I. Kh. Alekperov [2] were used.

Discussion and Conclusions of the Study

Species of protozoa belonging to a group living in the intestinal tract of ungulates were studied in the regions of the Nakhchivan Autonomous Republic. These ciliated primitives are present in all wild and domesticated ruminants. Rumen ciliated protozoa are acquired by young ruminants directly from mature animals or by eating food contaminated with the saliva of such animals. Rumen protozoa are one of the important groups of strictly anaerobic microbes living in the rumen. Strict anaerobic conditions are required to maintain and grow the viability of rumen protozoa, and only a few laboratory conditions equipped with protozoological expertise and anaerobic facilities can grow rumen protozoa. For the same reason, only a few species have been grown and maintained as laboratory cultures for research. Anaerobic rumen protozoa can also be grown aerobically if antioxidants are included in the media based on the research conducted. Indeed, our experiments have shown that both *Entodinium caudatum* and *Epidinium caudatum*, two major rumen protozoal species, can be successfully cultivated in an aerobic medium supplemented with ascorbic acid and glutathione. Thanks to the fermentation properties, anaerobic fermentation was maintained, and the microbial populations changed to some extent under aerobic conditions. Antioxidants also enhanced the recovery of frozen stock cultures of both rumen protozoal species. The results of this study may facilitate and encourage future studies in which rumen protozoa need to be cultured in the laboratory. Evidence has been obtained that it is the particles in the rumen fluid that are important for growth. The aim of this work is to describe experiments in which *Entodinia spp.*, mainly *Entodinium caudatum* from sheep rumen, were kept in frequent splits for more than several months. The standard medium consisted of 30 ml amount of added autoclaved mineral salts solution $Na_2S \times 9H_2O$ to a final concentration of 0.01% (w/v), c. 15 mg dried herb (55° for 3 days) finely chopped; 15 mg of rice starch is added as a suspension of known concentration in water; 3.0 ml prepared fresh rumen liquid; The final concentration of chloramphenicol is 50 μg/ml. The final volume of the complete medium c. It was 35 ml. Tubes of complete medium were vigorously gassed for 3 min with a mixture of 95% (w/v) N_2 + 5% (w/v) CO_2 and stopped immediately. All media were warmed to 38° before use. Fresh rumen fluid was usually prepared and used on the same day, unless obtaining fresh rumen contents was inconvenient. At this time, the prepared material was stored at −15° under 95% (w/v) N_2 + 5% (w/v) CO_2 for no more than 4 days and warmed to 38° before use. Autoclaved rumen fluid was prepared by autoclaving in sealed McCartney bottles under 95% (w/v) N_2 + 5% (w/v) CO_2.

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The procedures involved in initial inoculation from fresh rumen contents and subsequent manipulations resulting in oligotrichous protozoan cultures split every 2-4 days have already been described. The conditions used in the experiments described below differed initially in that the cultures were split every 48 h. Subsequently, maintenance was further simplified by omitting the initial centrifugation and changing the supernatant liquid on days when the cultures were diluted with an equal volume of fresh medium.

Fresh grass with or without rice starch and other medium components had to be added daily for successful maintenance of protozoa. Cultures are diluted with an equal volume of fresh medium every 1-4 days, depending on the conditions, and then the diluted cultures are split each to generate two identical cultures. All described experiments were carried out on one member of such a pair of tubes, the other serving as a control. Three different growth conditions were used. Manipulations were carried out on the days when the culture was diluted and, on the days, when the volume was not changed, that is, on the non-diluted days. Condition A undiluted day: the culture is centrifuged, the supernatant liquid is removed and replaced with an equal volume of fresh medium. Dilution day: the culture was centrifuged, the supernatant fluid was removed and replaced with twice its volume of fresh medium.

Condition B non-rinse day: rice starch (up to dilution day concentration) and 15 mg of dried grass only added. On the day of dilution: the culture was centrifuged, the supernatant fluid was removed and replaced with twice the volume of fresh medium containing rice starch and grass. Condition C non-dilution day: rice starch and 15 mg only dried grass is added. Dilution day: culture diluted with equal volume of fresh medium containing rice starch and grass. All centrifugations were 2 min at 500 g. The frequency of dilution of the culture under any growth condition is given in days by the number following the letter. Unless otherwise stated, the numbers shown always refer to the number of protozoa present immediately before dilution of the culture. When the effect of a change in growth conditions is reported, allow at least 3 weeks to count the number of protozoa present.

Stable population density of Entodinia spp. The medium under different growth conditions consisted of 30 ml basal mineral salts containing 0.01% $Na_2S \times 9H_2O$ + indicated materials: R = 0.5 mg rice starch/ml; R/2 = 0.25 mg rice starch/ml; G = c. 15 mg dried grass /ml; FRF = 10% fresh rumen fluid; ARF = 10% autoclaved rumen fluid; CAP = 50 μg chloramphenicol/ml.

Table 1

Medium	*Growth conditions*	*That growth under conditions of dilution number*	*Immediately without dilution population density before protozoa/ml)*
1. R, G, FRF, CAP	A_2	40	28,000
2. R/2, G, FRF, CAP	A_2	16	13,000
3. R, G, FRF, CAP	B_2	155	32,000
4. R, G, FRF, CAP	D_2	53	14,000
5. R/2, G, FRF, CAP	D (dil. (3 days from 4)	70	4000 before the third serial dilution
6. R (120°, 24 hours), G (autoclaved), FRF, CAP	B_2	117	26.000
7. R/2, G, ARF, CAP	A_2	17	16.000-26.000
8. R/2, G, ARF, CAP	C_2	171	24.000
9. R/2, G, 1% ARF, CAP	C_2	32	10.000
10. R/2 (120°, 24 hours), G (autoclaved), ARF, CAP	C_2	95	18.000
11. R, G	D_3	53	14.000

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As can be seen from the table, the optimal concentration of rice starch was 0-5 mg/ml and 28,000 organisms/ml with growth occurring in the A_2 condition. participated (Table 1). When the starch content was halved, the total was 13,000 protozoa/ml; doubling the starch concentration caused an initial increase in numbers, followed by a steady decline, probably due to increased bacterial numbers. Omission of rice starch resulted in the death of the protozoa within 3 days. Rice starch could not be replaced by soluble starch or autoclaved rice starch, although in the presence of the latter there were persistently reduced organisms for 46 days (100 protozoa/ml after 15 dilutions).

The effect of heating rice starch was investigated because it was hoped to grow the protozoa in axenic culture using sterile starch. When dry starch was heated in an oven to 160° for 1 hour, the protozoa soon died before use. Starch heating at 140° for 1 hour did not affect the protozoa when the number was greater than 15,000-20,000/ml, but resulted in their death when the number was less than 5000-10,000/ml. Finally, the least effective method for protozoa was to heat dry starch in an oven at 120° for 24 hours.

The release of the dried herb was followed by the death of the organisms within ten days of normal dilution of the culture; low numbers of protozoa (i.e., 5000 protozoa/ml) sometimes persisted for more than 50 days when the culture was not diluted. Within 4 days, it completely replaced the dried grass, but after that the number of sprouts decreased due to the absence of grass. The dried herb could not be replaced by 0.5 ml, the supernatant liquid obtained after autoclaving can be sterilized by autoclaving in 1 g dried herb, 20 ml water, herb 30 ml. In any case, the use of sterilized grass caused an initial depression in the growth rate of the protozoa, but this gradually reverted to the original culture. After a 6-month acclimation period, almost grass-grown cultures were obtained. The use of rice starch and dried grass dry-heated at 140° for 1 hour before use gave inconsistent results or only slow growth, sometimes resulting in the death of the protozoa. The optimal concentration of chloramphenicol is 50 μ g/ml; An increase to 150 μ g./ml killed the protozoa. When the antibiotic was omitted, the organisms grew normally for 3μ 4 days, but then, even when the culture was not diluted, the numbers declined, and the protozoa died or were present in low numbers. Bacterial growth under these conditions was much more severe than in the presence of chloramphenicol, which may account for the lack of growth of the protozoa.

When fresh rumen fluid was released, the protozoa grew normally for 3-4 days and then steadily decreased in number because the culture was not diluted. When fresh rumen fluid was replaced by an equal volume of autoclaved rumen fluid, the culture could be maintained under A_2 condition with 16,000-26,000 protozoa/ml, in the presence of only 0.25 mg rice starch/ml. was the optimal concentration under these conditions. Increasing rice starch to 0.5 mg./ml increased bacterial growth without improving protozoan growth, and this heavier bacterial growth was sometimes associated with sudden protozoan death. Under condition A_2, reproducible growth in the presence of 10% (w/v) autoclaved rumen fluid was difficult to obtain, and cultures often contained many dead protozoa; Consistent growth was achieved with 24,000 protozoa/ml under C_2 condition. Condition C_2 was adopted for all experiments with autoclaved rumen fluid thereafter. A reduction in autoclaved rumen fluid concentration from 10% (w/v) to 1% reduced the protozoan count to 10,000/ml. Fresh rumen fluid was fractionated as follows to determine the presence of supernatant fluid or bacterial bodies, a major part of autoclaved rumen fluid. Normally prepared fresh rumen fluid was centrifuged at 30,000 g for 50 min and the supernatant fluid was removed. The sediment (bacterial body fraction) was washed twice with basal salts solution in a centrifuge and reconstituted with the same volume of basal salts solution as the original rumen fluid. Two fractions were autoclaved in sealed McCartney bottles under a gas mixture of 95% (w/v) N_2+ 5% (w/v) CO_2 and

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tested at a concentration of 10% (w/v) under C_2 condition. Autoclaved rumen fluid could be replaced by the supernatant fluid fraction for more than 80 days, although the number of organisms was 10-40% lower. The fraction of bacterial bodies completely replaced the autoclaved rumen fluid within 15 days, but after that the protozoan count steadily decreased to only 300 protozoa/ml. After 30 days, the use of sterile grass and rice starch instead of non-sterile material in the presence of 10% (w/v) autoclaved rumen fluid reduced protozoan counts to 18,000/ml. When rumen fluid and chloramphenicol 15 mg, rice starch +15 mg. dried grass was added to a standard culture containing, for example, 16,000 protozoa/ml after daily dilution, the protozoan count increased to 36,000/ml without replacing the supernatant liquid, and then could be maintained by treatment under D_3 condition. Bacterial growth was more severe under these conditions than in the presence of fresh rumen fluid and chloramphenicol. Attempts to increase the dilution rate of the culture every 2 days were unsuccessful because the culture eventually perished.

Preliminary experiments showed that it was necessary to change the supernatant fluid every 24 hours to maintain the protozoa. Later, it was found that daily addition of grass and rice starch maintained the organisms for at least a week, and that the culture in standard medium under condition A_2 could be diluted without initial centrifugation and replacement of the supernatant liquid, i.e., with a slight improvement in growth rate from condition B_2. Further simplification of the procedure by changing to D_2 resulted in a reduction of the protozoan count from 32,000/ml to 14,000/ml. On undiluted days under D_2 condition, when no medium was added, the protozoa grew normally for 4 days but died after 8 days. Cultures were maintained under three conditions by growing regularly every week with 1.5% (w/v) inoculum of fresh medium under the conditions to be used for the inoculated tube; The average number of protozoa present on the seventh day is shown in Table 2. After 25 weeks of keeping the supernatant without daily replacement under both conditions, 50-80% of the protozoa were *Entodinium longinucleatum*, with most of the remainder being *E. caudatum* (Figure).

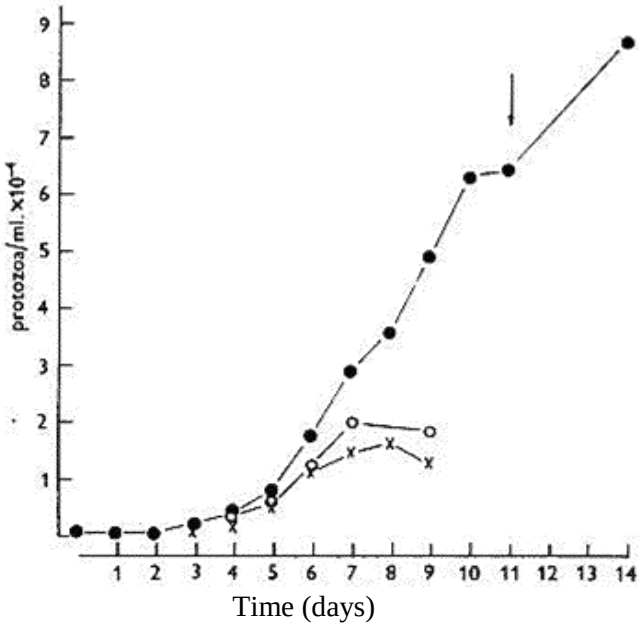

Figure. *Entodinia spp.* from a small inoculum. The inoculum was 900 protozoa/ml. 1.0 ml was added. A culture grown in standard medium under condition A_2. All media contained only 0.25 mg rice starch/ml except as specified. ●—● = inoculated onto standard medium and maintained in condition A (using standard medium) without dilution; the concentration of rice starch increased to 0.5 mg./ml. on the axis. ○—○ = inoculated into standard medium and maintained under condition C without dilution, × —× = inoculated into standard medium without chloramphenicol or rumen fluid and maintained under condition C without dilution.

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Entodinia spp. A small inoculum maintained by weekly transfer using a 1.5% (w/v) inoculum is grown from *Entodinia* under the same conditions as would be used for the inoculated tube. Culture 1 was maintained under condition A with standard medium containing 0.25 mg undiluted. rice starch/ml. Culture 2 was inoculated onto standard medium containing only 0.25 mg rice starch/ml. and then kept in C without dilution but with the addition of rice starch to a concentration of 0.25 mg/ml. Culture 3 was identical to culture 2 except that chloramphenicol and fresh rumen fluid were omitted from the primary tube of medium.

Table 2

	Weekly issue	*Mean number of protozoa/ml after growth*
Culture	Transfers	1 week
1	40	38.000
2	41	18.000
3	27	10.000

Results

1. As a result of the conducted research, it was determined that the rumen protozoa system is diverse and complex, consisting of a system that acts symbiotically to break down the feed consumed by ruminants. They also affect the quality of the final product (milk and meat). High-throughput sequencing-based methods allow us to determine which microbes are there and what they are doing, depending on the approach taken. Understanding the rumen protozoan system and its relationship with the ruminant itself is essential to producing quality products, increasing profitability and reducing environmental impact. Individual ciliates (*Entodinium caudatum*, *Entodinium simplex*) have different effects on ruminal fermentation, and when these species are combined, they sometimes have different effects, so that the proteins in the body of digested microorganisms have high biological nutritional value.

2. Rumen protozoa are one of the important groups of strictly anaerobic microbes living in the rumen. Strict anaerobic conditions are required to maintain and grow the viability of rumen protozoa, and only a few laboratory conditions equipped with protozoological expertise and anaerobic facilities can grow rumen protozoa. For the same reason, only a few species have been grown and maintained as laboratory cultures for research. Anaerobic rumen protozoa can also be grown aerobically if antioxidants are included in the media based on the research conducted.

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