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CHARACTERIZATION OF MICROALGAE-BASED FEED SUPPLEMENT AND THEIR POSSIBLE INFLUENCE ON CATTLE RUMEN MICROBIAL ECOSYSTEM

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Rumen is a complex ecosystem where feed consumed by animals is digested by the help of rumen protozoa. They plays an important role in contributing nutrients to the host animal. This study aimed to investigate the effects of dietary inclusion of unicellular microalgae *Chlorella vulgaris spp.* on total count and generic composition of protozoa in cows rumen. In this study, dietary treatments were tested during three 21-d experimental periods. Each of period content different amount of microalgae supplement. In the first experimental period in cows feed was added 30g (3,14g/kg of DM) of *Chlorella*, in second period-90g (9,6g/kg of DM) of *Chlorella* and in third period-170g (18,7 g/kg of DM). As a result of this study, ciliates of 10 genera were detected and identified. The number of this genera and total amount of ciliates were counted. The analysis of rumen protozoa population in tested cows shows visible effect of the presence of microalgae in cows dietary. In particular, the density of ciliates protozoa of the cows treated with 90 and 170 g of algal supplement was visibly higher compare to results from the control diet. Microalgae-based supplement diet had stimulative effect on ruminal protozoa population and caused increasing of many protozoa genera such as *Isotricha, Dasytricha, Charonina, Buetschlia, Ostracodinium, Ophryoscolex*.

Keywords: Identification, ruminal protozoa, ruminant, microalgae feed supplement, Chlorella vulgaris.

INTRODUCTION

Microalgae based feeds in ruminant diets are suggested in order to supplement the ration, as a primary source of a broad range of metabolites that are suitable for animal feed, such as proteins, amino acids, vitamins, polysaccharides, polyunsaturated fatty acids (PUFA) and other organic compounds. The definition of microalgae commonly includes all unicellular and simple multicellular photosynthetic microorganisms, both prokaryotic microalgae (cyanobacteria) and eukaryotic microalgae (green algae, red algae and diatoms) (Brennan and Owende, 2010). Bioactive compounds synthetized by microalgae are an important contemporary research area to create substitutes for feed additives due to their potential to modify rumen fermentation in purpose to improve feed utilization, feed conversion efficiency, and animal performance. It is well known that green algae are most biotechnologically relevant microalgae that are traded commercially as nutritional feed additives for humans and animals (Andrade et al., 2018). Chlorella is a genus of unicellular freshwater microalgae that are used as additives with high nutritional value in feed for agriculturally important animals (Abdelnour et al., 2019). This microalga contains the highest amount of chlorophyll of any common plant, with protein content of about 600 g/kg dry matter (DM) and 18 amino acids as well as vitamins and minerals, carotenoids, glycoproteins (Knolif et al., 2016). Moreover, *C. vulgaris* carries a phyto-nutrient called Chlorella Growth Factor (CGF), which comprised of nucleic acids and other essential substances, with detoxification and antioxidant properties (Han. et al., 2002). This unique complex was found in the cell nucleus of Chlorella. CGF is a remarkable nutritional supplement that promotes growth, stimulate immune system, detoxifies. The rumen is a dynamic fermentation container with a highly complex and competitive microbial ecosystem within. Rumen ciliates plays an enormous role in digestive functions of ruminants as fermentation microorganisms and range in size from 10 to 250 μm in length. Besides of representing approx. 50% of rumen viable biomass, ciliates degrade approx. 20 % of the proteins of all proteins received by host animal. In addition, its 7.5 to 15 % of total lipids are produced by ciliates (Purevtsogt et al., 2016). The feedstuff consumed by the animal is fermented to volatile fatty acids and microbial cell proteins to supply energy and proteins. In particular, the plant materials such as celluloses, hemicelluloses, pectines, starches and other polysaccharides are hydrolysed by the rumen microorganisms to monomers or dimers of sugars and are finally fermented to provide a variety of products such as acetic acid, propionic acid, butyric acid etc. (Puniya et al., 2015). Existence of ciliate protozoa in the rumen is important for cellulose digestion and a stable internal environment. They protect sugars and starches from bacterial utilization (for example by engulfing starch granules) so that organic acids are not produced immediately after the feeding of animals. So, they protect the rumen from acidosis by controlling the carbohydrate

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fermentation. Rumen environment might have satisfactory conditions for microalgae to provide important nutrients for protozoa activity in the rumen. Therefore, investigation of counts and genera compositions of ciliates in the rumen is seen to be important for evaluation of their functions in the rumen. Density of ruminal protozoa and their generic composition can be affected with the nature of their diet, frequency of feeding and other factors. The present work aimed to study the use of microalgae *Chlorella vulgaris* as a feed supplement and to investigate its influence on ruminal protozoa community.

RESEACH METHODS

Rumen contents were obtained from four cattle of beef breeds, all with rumen cannulas. In our study, cows were tested during 3 experimental periods each of every endured 21 days. Tested cannulated cows were divided – first cow was the control and had diet without microalgae supplement, second three animals were the experimental group. All animals were fed once daily in the morning. Water and trace mineral salt were available free-choice. The total mixed ration (TMR) consisted of grass silage (11 kg) and cereal daily concentrate (1.2kg).

Experimental cows had 3 treatment diets with lyofilized unicellular microalgae *Chlorella vulgaris* supplemented in different amount: first diet included TMR and 30g (3.14 g kg^{-1} of DM) of chlorella, second diet had TMR and 90g (9.6 g kg^{-1} of DM) of chlorella and third- TMR and 170g (18.7 g kg^{-1} of DM) of chlorella. Duration of every treatment was 21 day. Rumen liquor samples were collected three hours after the morning feeding with the help of ruminal probe through cannula. The pH of ruminal fluid was immediately measured (EUTECH CyberScan PC510 pH/Conductivity Bench Meter, USA) and ranged from 6.20 to 7.06 throughout the study. Samples were filtered through the synthetic cloth (119µm, Uhelon 59 S, Silk & Progress, Ltd.) and evaluated for physical, chemical and microscopic examination for protozoa in all the cattle. Sample filtration helps to remove larger particles from the sample and makes the visualization of the ciliates protozoa easier than without filtration. The rumen content samples were fixed in 18.5 % formaldehyde solution and stained with Brilliant Green Dye. The samples were mixed and allowed to stand overnight. The density of the rumen protozoa per mL was obtained using Bürker counting chamber in an optical microscope at magnification 40x. Microscopic examination of protozoa is widely accepted as golden standard for analyzing ciliate community structure and can provide useful information about physiological processes in rumen associated with animal nutrition. Ciliates present in each sample were identified based on the criteria described by Imai & Ogimoto (1981), Baraka T.A. (2012) and Burk A. Dehority (2018).

RESULTS AND DISCUSSION

The total protozoal counts in rumen fluid in cows fed with a diet with varying levels of Chlorella treatments are presented in Table 1 and in Figure 4. The density of ciliates in cows treated with 30 g of Chlorella supplement was 24.05×10^{4}/ml (SE±6.77) in contrast with control cow which had 26.84 x 10^4/ml (SE ± 5,01). On the Fig.4 is visible that dietary treatment of chlorella 30g caused the decrease of total protozoal density, but higher algal treatments (90, 170g of Chlorella) had the opposite effect. This can be explained by higher intensity of fermentative processes of digestion, what might be provoked by microalgae diet treatment.

Table 1. Percentage (%) composition of protozoal genera with different Chlorella diet treatments (data are reported as mean ± standard deviation)

Percentage (%) composition of protozoal genera	**Chlorella 30g (3,14 g kg^{-1} DM) diet treatment**		**Chlorella 90g (9.6 g kgv DM) diet treatment**		**Chlorella 170g (18.7 g kgb DM) diet treatment**	
	Control diet	Experimental group	Control diet	Experimental group	Control diet	Experimental group
Entodinium	51.52 ± 12.63	59.42 ± 6.76	48.51 ± 16.29	34.90 ± 8.10	42.24 ± 8.85	44.33 ± 1.64
Isotricha	4.97 ± 2.11	3.72 ± 1.92	7.50 ± 4.88	11.97 ± 6.21	8.81 ± 2.98	12.27 ± 1.18
Dasytricha	30.32 ± 13.13	21.08 ± 4.27	20.31 ± 8.07	32.81 ± 5.51	13.15 ± 0.90	25.71 ± 1.41
Charonina	6.06 ± 2.55	9.35 ± 1.73	3.33 ± 1.48	7.73 ± 4.57	2.44 ± 1.39	4.98 ± 1.03
Buetschlia	1.07 ± 0.27	1.14 ± 0.60	1.65 ± 0.54	3.20 ± 1.58	1.64 ± 0.43	2.15 ± 0.33
Ostracodinium	2.99 ± 0.11	3.75 ± 0.59	2.84 ± 0.65	4.06 ± 1.89	3.57 ± 1.81	3.39 ± 0.38
Diplodinium	4.58 ± 2.90	1.82 ± 0.33	15.27 ± 2.81	3.71 ± 1.33	7.10 ± 3.52	8.23 ± 3.07
Epidinium	0.45 ± 0.10	0.09 ± 0.11	1.10 ± 0.67	0.67 ± 0.12	1.25 ± 0.38	0.88 ± 0.14
Ophryoscolex	0.17 ± 0.12	0.21 ± 0,20	0.55 ± 0.15	1.46 ± 0.58	1.79 ± 0.78	1.65 ± 0.16

During this experiment the following genres of rumenal ciliates were observed: genus *Entodinium* (Fig. 2), genus *Isotricha* (Fig. 1), genus *Dasytricha*, g. *Charonina,* g. *Ophryoscolex* (Fig. 3), g. *Epidinium* (Fig. 3), g. *Diplodinium* (Fig.

2), g. *Buestchlia*, g. *Ostracodinium* (Fig. 1, 2), g. *Metadinium* (Fig. 1). Genus of *Metadinium* was observed only several times in the samples. On the figure 1 is *Metadinium medium* pictured and there are visible two skeletal plates which run parallel to each other. The genus *Entodinium* is mainly characterized by having a single ciliary zone which enriches the mouth. The ciliary band is thought to have a function in both locomotion and food digestion (Dehority Bur, 2003). From the genera of ciliates identified, *Entodinium* was the most abundant. Larger percent of protozoa from the family Isotrichidae (*Isotricha* and *Dasytricha)* were observed in animals maintained by Chlorella 90 and 170 g dietary treatments.

Several studies (Karnati et al., 2009; Newbold et al., 2015) reported the lower digestibility and VFA (volatile fatty acids) concentration in defaunated animals (the removal of protozoa from the rumen using a wide variety of chemicals and physical techniques) what seem to indicate the role of protozoa in the synthesis of VFA and feed degradation. During the microscopic study of impact of microalgae supplement on protozoal community we observed that ciliates of almost all represented genera (Fig. 1,2,3) had chloroplasts and other nutrients from Chlorella engulfed in their body. Thus, protozoa appear to protect chloroplast unsaturated fatty acids from the rumen biohydrogenation increasing the intestinal flows of mono and polyunsaturated fatty acids. Huws et al. (2012) used steers fed diet with different chlorophyll levels and it was demonstrated that the high levels of polyunsaturated fatty acids in protozoal cells appears to be associates with ingestion of chloroplasts.

Figure 1. Genus of *Isotricha* (on the left side)*, Ostracodinium clipeolum* (in the middle), *Metadinium medium* (on the right side); (40x, photo by Svetlana Malyugina)

Figure 2. *Ostracodinium mammosum* (on the left side), *Diplodinium dentatum* (in the middle), *Entodinium bimastus* (on the right side). (40x, photo by Svetlana Malyugina)

Figure 3. Genus of *Epidinium* (on the left side), Genus of *Ophryoscolex* – in the middle of the figure ciliate is stained with Brilliant Green Dye, ciliate on the right side pictured without staining (40x, photo by Svetlana Malyugina)

Figures 1, 2, 3 show intracellular chloroplasts observed during microscopic study in genera *Isotricha*, *Entodinium*, *Ophryoscolex*, *Ostracodinium*, *Epidinium*.

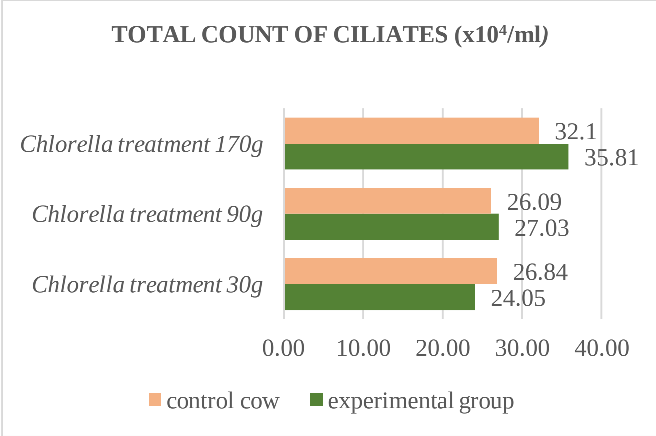

Figure 4. Total count of ciliates (x10^4/ml)

The density of ciliates in cows treated with 90g of Chlorella supplement was 27.03 x 10^4/ml (SE ± 6.47) in contrast with control cow which had 26.09 x104/ml (SE ± 5.45) and the total amount of ciliates in cows treated with 170 g of Chlorella supplement was 35.81 x104/ml (SE ± 16.58) in contrast with control cow which had 32.1 x104/ml (SE ± 7.61). The microalgae diet shows evident impact on the count of protozoa in the cow's rumen.

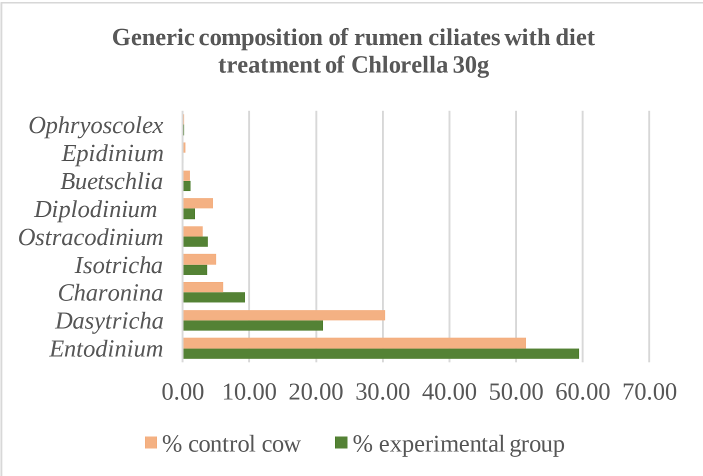

Figure 5. Generic composition of rumen ciliates with diet treatment of Chlorella 30 g (3. 14g kg^{-1} DM)

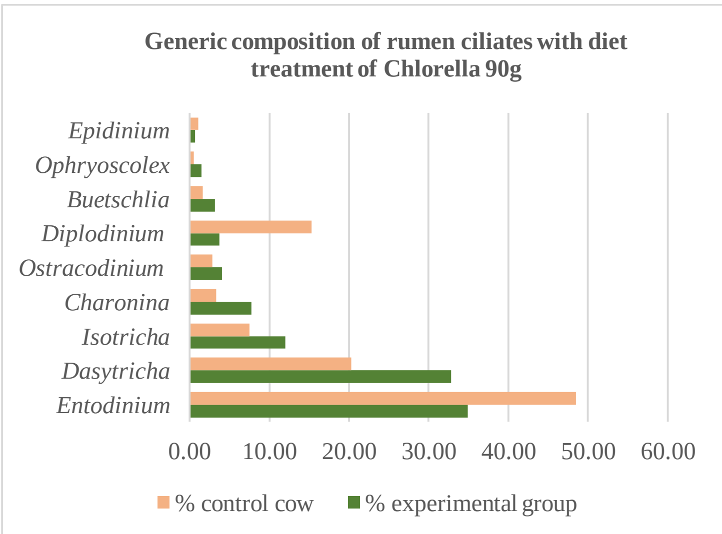

Figure 6. Generic composition of rumen ciliates with diet treatment of Chlorella 90g (9.6 g kg^{-1} DM)

On Fig. 5, 6, 7 is seen that the rumen protozoa population structure was dominated mainly by the genus *Entodinium* and *Dasytricha.* In the ruminal fluid samples collected during the second experimental period, during which tested animals were fed with 90 g of algal treatment, were observed apparent growth of genus *Isotricha, Dasytricha,* *Buetschlia* and *Charonina.* In the third experimental period the difference in the amount of ciliates between control diet and tested animals is still noticeable.

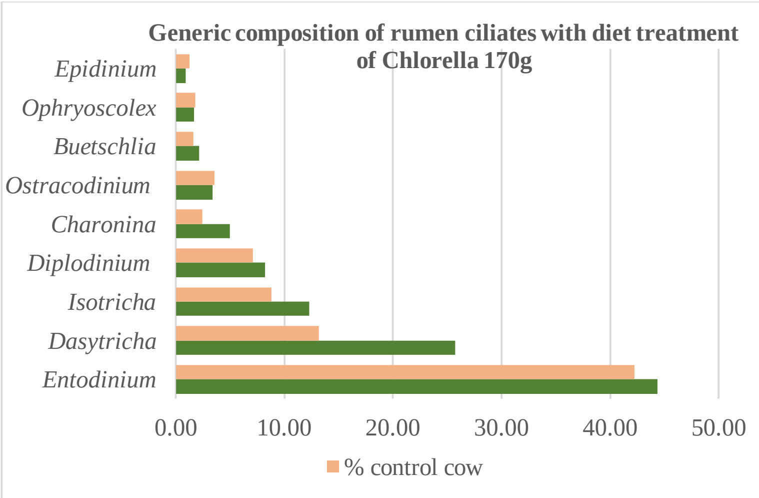

Figure 7. Generic composition of rumen ciliates with diet treatment of Chlorella 170g (18,7 g/kg of DM)

CONCLUSIONS

The aim of this study was to evaluate the possible influence of microalgae on ruminal protozoa community. During an *in vivo* trial with four rumen-cannulated cows, the effect of dietary supplementation of freshwater unicelular miroalgae *Chlorella vulgaris* on the ruminal protozoa population was investigated. Algal supplementation resulted in higher concentration of total protozoa in tested cows during second (27.03 x10^4/ml) and third (35.81 x10^4/ml) experimental periods (with Chlorella dietary treatments 90 g and 170 g) in contrast with control animal. Its concluded that dietary microalgae supplement have stimulative effect on rumenal protozoa population and caused increasing of many protozoa genera such as *Isotricha*, *Dasytricha*, *Charonina*, *Buetschlia*, *Ostracodinium*, *Ophryoscolex*. Despite the fact that ciliate protozoa contribute large part of the rumen biomass, their role in ruminal fermentation is still unclear. Therefore, it's obviously that additional studies are needed to understand the real interactions between microalgae and ruminal microorganisms and functions of certain protozoa genera and species in ruminal ecosystem. This results suggests that microlgae Chlorella vulgaris had impact on protozoal community in the rumen.

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