The effect of different concentrations of microalga *Chlorella vulgaris* supplementation on ruminal fermentation and blood indices in cows

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Abstract

This study examined the effects of dietary *Chlorella vulgaris* supplementation on ruminal fermentation, blood biochemistry, and protozoal composition in dairy cows. Four ruminally cannulated Simmental cows (6.00 ± 0.83 years; 521.00 ± 9.51 kg body weight) were assigned to a 4 × 4 Latin square over 72 days. Cows received a basal diet (ALG0) or the same diet supplemented with 2.76 (ALG1), 8.22 (ALG2), or 16.3 (ALG3) g/kg odf dry matter (DM) of lyophilized *C. vulgaris*. Alga supplementation affected ruminal pH ($P = 0.013$), which was highest in ALG2 (6.69) and lowest in ALG3 (6.23). Nitrogenous compounds increased with algae inclusion, with NH_3-N peaking in ALG2 (8.82 mmol/l; $P = 0.001$) and total nitrogen highest in ALG3 (5.8 g/kg; $P < 0.001$). Protozoal counts rose significantly with supplementation ($P = 0.001$), especially in ALG3. Volatile fatty acid concentrations and molar proportions were unaffected ($P > 0.10$), and the acetate-to-propionate ratio remained stable. Blood biochemical indices were largely unchanged ($P > 0.05$). Total protein tended to increase, while glucose showed a non-significant decline at the highest alga dose. Other markers, including 3-hydroxybutyric acid (BHB), nonesterified fatty acids (NEFA) and liver enzymes, remained within physiological limits. These results suggest that *C. vulgaris* can enhance nitrogen metabolism and protozoal activity without adversely affecting metabolic health, supporting its potential as a functional feed additive in dairy nutrition.

Algae, animal nutrition, blood metabolites, ruminants

To improve the sustainability of both the dairy and beef industries, manipulation of the ruminal microbiome to improve feed utilization, reduce methane emission, and enhance animal growth performance is a major research challenge (Patra et al. 2012). Using algae materials (e.g., algae extract, dried biomass) may provide a new, alternative option in this matter. Various species of algae with favourable biological activity have been reported as acceptable for inclusion in the diets of monogastric and ruminant animals (Choi et al. 2021).

Microalgae represent a diverse group of photosynthetic, unicellular, or simple multicellular organisms occurring in seawater and freshwater. These microorganisms can be cultivated on marginal or nonarable land, providing the opportunity to repurpose idle land for food and feed production (Wild et al. 2019). Some microalgae species have promising nutritional properties due to high crude protein concentrations, up to 70% of dry matter (Becker 2007), or the occurrence of omega-3 fatty acids (Ryckebosch et al. 2012), for which they are regarded as alternative feed resources for cattle. The nutritional value of a feedstuff is determined by its nutrient composition and the animal's utilization of the nutrients. Detailed information on the nutrient composition and utilization of a feedstuff is required for a nutrient supply meeting the animal's requirements (Wild et al. 2019).

The nutrient utilization in ruminants is primarily determined by microbial fermentation in the rumen. Numerous studies have reported the beneficial effect of micro- and -macro-

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algae on animal physiology and welfare (Kholif et al. 2021; Altomonte et. al. 2018; Bature et al. 2022). Thanks to significant breakthroughs in algal biotechnology, they have a high potential to become an efficient 'cell factory' for food production, accelerating the expansion of the algal bioeconomy in the food and feed sectors. Furthermore, renewable biofuels made from microalgae have a great chance of eventually dispensing with fossil fuels whose supplies are running low. Microalgae-based biofuel production is currently more expensive than fossil fuels. Still, with developing technologies, it could soon become profitable, simultaneously allowing for the mass production of defatted microalgae residues for livestock.

Algae supplementation can provide plenty of beneficial effects in cattle, e.g., improved growth and body weight, enhanced immune response and durability towards illness, antibacterial and antiviral action, as well as enrichment of livestock products with bioactive compounds (Madeira et al. 2017; Kusmayadi et al. 2021).

However, scientific data regarding the nutritional value of microalgae and their dietary impact on animal health, digestion, and performance are scarce. Most of the previous studies conducted on ruminants concern the application of docosahexaenoic acid-rich microalgae for the alteration of fatty acid profiles of milk (Boeckaert et al. 2008; Glover et al. 2012) or the inhibition of methanogenesis and mitigating CH_4 enteric emissions (Boeckaert et al. 2006; Elghandour et al. 2017).

Therefore, this study was conducted to evaluate the effects of dietary inclusion of microalga *C. vulgaris* on key ruminal and systemic physiological indices in ruminants. Specifically, the research focused on assessing the impact of graded levels of microalga supplementation on: (1) ruminal fermentation characteristics (pH, ammonia nitrogen, total and individual volatile fatty acids), (2) the composition and abundance of ruminal ciliated protozoa, and (3) blood biochemical markers including energy metabolites (glucose [GLU], 3-hydroxybutyric acid [BHB], nonesterified fatty acids [NEFA]), protein metabolites (urea, total protein, albumin), and liver function indicators (aspartate aminotransferase [AST], gamma-glutamyl transferase [GGT], bilirubin). These variables were selected to elucidate the role of microalgae in modulating rumen microbial activity, nutrient utilization, and systemic metabolic responses. The findings are expected to contribute novel insights into the potential of microalgae as a sustainable and functional dietary additive in ruminant nutrition.

Materials and Methods

Experimental design and animal's diet

Four Simmental cows (6.00 ± 0.83 years of age; 521.00 ± 9.51 kg body weight), each fitted with a permanent ruminal cannula, were used in this study. The cows were housed in individual pens (6.7 m²) at the experimental facility of Agrovýzkum Rapotín (Czech Republic) and had access to water *ad libitum*. All animal procedures were approved by the Ministry of Agriculture of the Czech Republic (approval no. 14608/2021-MZE-18134). Cows were assigned to four dietary treatments in a 4 × 4 Latin square design. The treatments consisted of a control diet (ALG0; basal diet only), and the basal diet supplemented with lyophilized *Chlorella vulgaris* at 2.76 g/kg dry matter (DM) (ALG1), 8.22 g/kg DM (ALG2), or 16.3 g/kg DM (ALG3). Each experimental period lasted 24 days, including a 10-day adaptation phase and a 14-day measurement phase. Cows were fed individually twice daily at 06:00 h and 16:00 h, receiving 60% and 40% of their daily feed allowance, respectively. All diets were formulated to meet the nutrient and energy requirements according to NRC (2016) guidelines.

The proportions of feed ingredients and their chemical composition are shown in Tables 1 and 2.

Sampling, data collection, and chemical analyses

Chopped meadow hay, maize silage, and granulated feed mixture were sampled weekly to determine DM and nutrient composition. The chemical compositions of all diets (Table 2) were analysed for DM, crude protein, crude fat, crude fibre, and ash according to the EC Commission Regulation (2009). The neutral detergent fibre (NDF) concentration was determined using an ANKOM A200 Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) according to the methodology supplied by the company, which is based on the methods described by Van Soest et al. (1991).

Table 1. Composition of the experimental diets.

	Diet			
	ALG0	ALG1	ALG2	ALG3
Ingredients, % of DM				
Meadow hay	67.32	72.63	72.23	71.64
Maize silage	16.84	17.59	17.49	17.35
Granulated feed mixture[1]	15.84	9.51	9.46	9.38
Lyophilized microalga *C. vulgaris*[2]	0.00	0.28	0.82	1.63
Total	100.00	100.00	100.00	100.00

ALG0 – control diet without *C. vulgaris* supplementation; ALG1 – diet containing 2.76 g of lyophilized *C. vulgaris*/kg of dry matter (DM); ALG2 – diet containing 8.22 g of lyophilized *C. vulgaris*/kg DM; ALG3 – diet containing 16.3 g of lyophilized *C. vulgaris*/kg DM.

[1]Granulated feed mixture BIOSTAN (Biokron, s.r.o., Czech Republic), (quantity per kg of product): barley (15%), oat mill feed (65%), wheat (15%), malt flower (10%), sunflower expellers (5%), extracted soybean meal (3%).

[2]The nutritional composition of *C. vulgaris* lyophilized biomass: Ca (78 mg/100 g), Mg (326 mg/100 g), Fe (56.5 mg/100 g), K (924 mg/100 g), P (1190 mg/100 g), Zn (6.9 mg/100 g), beta-carotene (14 mg/100 g), vitamin B12 (0.9 μg/100 g).

Table 2. Chemical characteristics of major feed ingredients.

	Ingredients			
Nutrient composition	Meadow hay	Maize silage	GFM	*C. vulgaris*
DM, %	92.78	92.87	91.58	93.95
Crude protein, %	8.60	8.10	13.37	46.90
Ash, g/kg DM	66.10	3.80	7.60	46.10
Crude fibre, g/kg DM	284.80	183.8	5.62	15.37
Fat, %	1.75	3.42	2.68	0.83
NDF, %	35.10	37.18	19.60	0.22

GFM – granulated feed mixture BIOSTAN; DM – dry matter; NDF – neutral detergent fibre.

Rumen fluid samples were collected from each cow once per week throughout the experimental period, resulting in a total of 32 samples (4 cows × 4 treatments × 8 sampling weeks). Sampling was performed 3 h after the morning feeding to standardize postprandial fermentation conditions. Samples were collected via a rumen cannula with a probe connected to a vacuum pump and sent to the laboratory for further analysis. Tests of rumen fluid included the measurement of pH, physical characteristics, concentration of total nitrogen (N_{tot}), nitrogenous compounds (NC) and ammonia (NH_3-N), determination of the total number of protozoal ciliates (TPC), the generic composition of the ciliates protozoa, the molar proportions of volatile fatty acids (VFAs), and the total amount of VFAs.

The pH was measured immediately after sample collection using a portable pH meter (EUTECH CyberScan PC510 pH/Conductivity Bench Meter, USA). Twenty ml of ruminal fluid aliquots were stored at −20 °C for subsequent analysis of VFAs, N_{tot}, NC, and NH_3-N. Samples for rumen protozoa analysis were preserved in 1 ml of 10% formaldehyde solution, stained with Brilliant Green Dye, and allowed to stand overnight. The density of the rumen protozoa per ml was obtained using a Bürker counting chamber in a fluorescent optical microscope (INTRACO FL200, Czech Republic) at a × 40 magnification according to the procedure described by Dehority (2004). Identification of protozoa genera present in each sample was performed according to the phenotypical criteria described by Ogimoto and Imai (1981), Baraka (2012), and Dehority (2018).

Volatile fatty acids were determined by gas chromatography (Agilent 6820 Gas Chromatograph System; Agilent Technologies, Santa Clara, USA) using the method described by Filipek and Dvorak (2009). The Kjeldahl method was used to determine the amount of nitrogenous compounds and ammonia, as described by Chen et al. (1987).

Blood samples were collected from each cow once per week throughout the experimental period, excluding the adaptation phase. This resulted in a total of 32 samples (4 cows × 4 dietary treatments × 8 sampling weeks). Samples were drawn via the jugular vein using serum collection tubes (HEMOS H-02; GAMA Group, České Budějovice, Czech Republic) without anticoagulant, and were used for the determination of serum biochemical indices.

After the sampling, serum was allowed to clot at room temperature, separated after centrifugation at 3,000 *g* for 10 min, and stored at −70 °C until the analysis. The following indicators were determined in the cows' serum: albumin (ALB), total proteins (TPROT), GLU, NEFA, BHB, AST, and GGT.

Serum NEFA and BHB were measured using standardized kits supplied by Randox Laboratories Ltd. (United Kindom). The other biochemical indices in serum were determined using commercial kits (Biovendor, Czech Republic). An automatic Konelab 20XT biochemical analyser (Thermo Fisher Scientific, Finland) was used for determination.

Statistical analysis

The software Statistica 14.0.0. (TIBCO, USA) was used to perform the statistical analysis. Data were analysed with a one-way analysis of variance (ANOVA). If statistical significance was observed, *post hoc* comparison analysis via *post hoc* Tukey's test was performed. $P < 0.05$ was considered significant, and the trend was $0.05 \leq P < 0.01$. The normality of the distribution of variables was tested using Shapiro-Wilk test, and all variables were normally distributed.

Results

Rumen fermentation variables

The impact of microalga dietary inclusion on the ruminal fermentation indices is represented in Table 3. Alga supplementation affected ruminal pH values, which were higher in cows fed diet ALG2 compared to diets ALG1 and ALG3 ($P = 0.013$).

At the dosage of 8.22 g/kg DM, the alga-supplemented diet increased pH in the rumen and, at the same time, significantly affected the amount of NC, total protozoa, and ammonia concentrations ($P < 0.005$). Interestingly, no impact was observed on the total and individual VFAs concentrations.

Table 3. Rumen fermentation indices in rumen of cows fed a basal diet with different microalga supplementation schemes.

Indicator	ALG0	ALG1	ALG2	ALG3	SEM	*P* value
pH	6.53	6.28^{a}	6.69^{b}	6.23^{ac}	0.22	0.013
Nitrogenous compounds (g/kg)	2.81^{a}	2.70^{c}	4.23^{ab}	5.80^{d}	1.50	< 0.001
Total rumen protozoa (10^4 cells/ml)	32.10^{a}	28.90^{ab}	36.70^{ac}	39.90^{b}	2.26	0.001
NH_3-N (mmol/l)	5.07^{a}	5.77^{ac}	8.82^{b}	6.78^{abc}	1.86	0.001
VFA_{tot} (mmol/l)	99.39	95.60	108.20	103.00	2.03	1.12
Acetic acid (mmol/l)	79.70	76.60	82.70	86.70	1.74	0.20
Propionic acid (mmol/l)	11.70	12.80	12.94	11.27	0.51	0.60
Butyrate (mmol/l)	8.40	8.63	6.43	7.31	1.40	1.10
Acetate/propionate	6.67	5.96	6.85	5.93	0.37	0.20

[a,b]Means with different superscripts in the same row differ from each other significantly ($P < 0.05$).

SEM – standard error of the mean; VFA_{tot} – the total volatile fatty acids; NH_3-N – ammonia-nitrogen. ALG0 – control diet without *C. vulgaris* supplementation; ALG1 – diet containing 2.76 g of lyophilized *C. vulgaris*/kg of dry matter (DM); ALG2 – diet containing 8.22 g of lyophilized *C. vulgaris*/kg DM; ALG3 – diet containing 16.30 g of lyophilized *C. vulgaris*/kg DM.

The effect of alga supplementation on blood metabolites

The impact of microalga dietary inclusion on the blood biochemical traits is presented in Table 4. Microalga dietary inclusion did not affect most of the measured blood indices, except for significantly higher glucose concentrations ($P = 0.02$) in the ALG2 diet compared to ALG1. It decreased in diet ALG3 compared to the control diet (ALG0).

Rumen protozoal community at different levels of microalga dietary inclusion

The most abundant rumen protozoa genera observed in the present study are presented in Fig. 1 (Plate IV), and their relative abundance in the cows' rumen is shown in Fig. 2 (Plate IV).

Table 4. The effect of microalga supplementation on blood biochemical indices.

Indicator	ALG0	ALG1	ALG2	ALG3	SEM	*P* value
ALB (g/l)	38.80	36.70	34.50	33.60	0.80	0.07
AST (μkat/l)	1.46	0.96	1.06	0.95	0.29	0.09
BHB (mmol/l)	0.36	0.40	0.44	0.32	0.02	1.18
GGT (μkat/l)	0.22	0.20	0.24	0.19	0.03	0.34
GLUC (mmol/l)	3.17[a]	2.97	3.08[ac]	2.58[b]	0.28	0.02
NEFA (mmol/l)	0.20	0.16	0.12	0.24	0.08	0.45
TPROT (g/l)	63.84	64.20	71.30	69.9	5.51	0.21
Urea (mmol/l)	3.66	3.05	3.46	2.54	0.27	0.53

[a,b]Different superscripts within a row indicate significant differences ($P < 0.05$); ALG0 – control diet without *C. vulgaris* supplementation; ALG1 – diet containing 2.76 g of lyophilized *C. vulgaris*/kg of dry matter; ALG2 – diet containing 8.22 g of lyophilized *C. vulgaris*/kg of dry matter; ALG3 – diet containing 16.30 g of lyophilized *C. vulgaris*/kg of dry matter; SEM – standard error of the mean; ALB – albumen; AST – aspartate aminotransferase; BHB – beta-hydroxybutyrate; GGT – gamma-glutamyl transferase; GLUC – glucose; NEFA – non-esterified fatty acids; TPROT – total proteins.

Discussion

During rumen fermentation, pH is considered to be a major factor affecting microbial growth, enzyme activity, VFAs concentration, and even methane production (Bhatta et al. 2006). In general, cows maintain rumen pH values between 5.5 and 7.5 through a complex acid-base regulation system depending on the type of diet provided (Zheng et al. 2020). According to Kopecny and Wallace (1982), the optimal pH of rumen proteolytic enzymes ranges from 5.5 to 7.0; however, protein degradation is reduced at the lower end of the ruminal pH environment. Two dual flow continuous culture fermentation studies compared high forage vs. high concentrate rations at pH ranging from 4.9 to 7.0, demonstrated that protein degradation was reduced as pH decreased with both types of rations (Calsamiglia et al. 2002). In the present study, ruminal pH values were within the physiological range. Nevertheless, the dietary alga inclusion at the dosage of 8.22 g/kg DM increased ruminal pH. The concentration of ammonia nitrogen (NH_3-N) was higher for cows fed with alga diets (ALG1, ALG2, and ALG3).

Location, method, time of sampling, type of diet, and rumen fluid volume are the main factors that affect the concentration of NH_3-N in rumen fluid. Thus, these factors should be considered when interpreting the concentrations of NH_3-N in the rumen. Some proteins tend to be more soluble in the rumen fluid, and thus, they are more easily degraded by the rumen microbes (Wohlt et al. 1976). Protozoa play an important role in rumen protein degradation mainly due to their ability to engulf large molecules, proteins, or ruminal bacteria. In addition, they contribute to N-turnover in the rumen and supple soluble protein for microbial growth (Van Soest 1994). Protozoa are not able to use NH_3-N and a fraction of engulfed proteins are later returned to rumen fluid in the form of soluble protein (Bach et al. 2005). This might be the result of decreasing NH_3-N with the absence of protozoa in defaunation experiments (Eugene et al. 2004).

Differences in NH_3-N concentrations may have been due to differences in the protein solubility of the dietary proteins and greater protozoal activity in the rumen. Additionally, the susceptibility of protein to microbial protein degradation in the rumen can also be affected by interactions with other nutrients and depends on the predominant microbial population (Bach et al. 2005). The presence of rigid cell walls might particularly restrict the susceptibility of microalgae protein to ruminal protein degradation (Wild et al. 2019). However, there is no detailed study conducted regarding the investigation of the microalgal

protein solubility in the rumen or the protein structure of microalgae; therefore, the cause of the low ruminal degradation of microalgae protein cannot be clarified yet.

In the present study, the significant increase in the NH_3-N concentration was concurrent with an increase in pH and total protozoa in the rumen fluid (Table 3).

Treatment of male laboratory mice with the ethanol extract of *C. vulgaris* caused a significant decrease in the glucose serum concentration in the high- and low-dose groups compared to the control (Ghwenm et al. 2020). The authors suggest that this decrease can be caused by the fact that the alga extract influenced the beta-pancreatic cells, as it worked to stimulate them to produce insulin and increase the entry of glucose into the adipocytes. Phenolic compounds may have stimulated the non-affected beta cells to produce insulin as beta cells are not equal in their sensitivity to alloxan, which led to a decrease in the level of sugar in the blood. Algae extract may increase both protein glucose transporters (GLUT4) in cell membranes and insulin receptors, which may have led to the escalation of glucose entry to the body tissues.

In the present study, glucose concentrations in animal serum supplemented with a higher dosage of alga (ALG3) caused a significant decrease in blood sugar concentrations compared to the control. However, serum glucose was higher in animals fed with ALG2 diet compared to ALG3. Glucose concentrations in all animals were within the physiological range and did not show an adverse effect on blood biochemical traits.

Ciliate protozoa populations differ with changes in diet and can represent up to 50% of the total rumen biomass (Williams and Coleman 1997). They perform essential functions such as fibre degradation, oxygen scavenging, regulation of the bacterial protein turnover through bacteria predation as well as influencing methane emissions (Williams and Coleman 1997; Newbold et al. 2015). According to broad research conducted by Henderson et al. (2015), *Entodinium* and *Epidinium* dominate the rumen ecosystem, representing 54.7% of protozoal sequence data.

In the current study, the most abundant species in all animals were *Entodinium*, with a relative abundance of 35 to 60%, and *Dasytricha,* whose presence ranged from 20 to 30% (Fig. 2, Plate IV). Cosmopolite protozoal genera, such as *Entodinium* spp., *Isotricha* spp., and *Dasytricha* spp., stayed at the same concentrations in all diets as other genera. The alga diet had no significant impact ($P < 0.05$) on the distribution of protozoal species in the rumen. In contrast, the total number of protozoa was significantly affected by alga supplementation (Table 3).

These results indicated the rumen protozoal community's stability and adaptability to the new diet after the alga inclusion. The lack of shifts in the protozoal community may also demonstrate a more stable ruminal fermentation. Higher concentrations of ammonia nitrogen and higher total protozoa caused by alga supplementation can be explained by the fact that protozoa significantly contribute to protein degradation and deamination processes. This is in line with a study that showed that ruminal NH3-N tends to be lower in faunated animals than defaunated animals (Males and Purser 1970). In another study, lambs with a faunated rumen produced significantly higher total VFA and ruminal ammonia concentrations compared to defaunated lambs (Luther et al. 1966), demonstrating the stimulatory role of protozoa in rumen fermentation. Eugène et al. (2004) also reported a ruminal NH3-N decrease after defaunation. These results suggest that there might be a linear relationship between the protozoal activity and ammonia concentrations in the rumen.

In conclusion, the present study demonstrates that dietary supplementation with microalga *C. vulgaris* can beneficially modulate ruminal fermentation dynamics in cows without negatively affecting systemic metabolic homeostasis. Specifically, supplementation at moderate levels (8.22 g/kg DM) elevated ruminal pH, ammonia-nitrogen, nitrogenous

compounds, and total protozoal counts, suggesting enhanced microbial activity and nitrogen metabolism. Despite these improvements in ruminal indicators, no significant alterations were observed in volatile fatty acid concentrations or acetate-to-propionate ratios, indicating maintained fermentation stability. Furthermore, blood biochemical indices, including glucose, protein metabolites, and liver enzymes, remained within physiological ranges across all dietary treatments, signifying no adverse systemic metabolic responses to microalga inclusion. Therefore, dietary *C. vulgaris* at moderate supplementation levels represents a feasible nutritional strategy for optimizing ruminal microbial activity and nitrogen utilization, with potential implications for improving feed efficiency and animal performance. Nonetheless, additional research involving larger animal cohorts, diverse production conditions, and varying microalgae formulations is recommended to fully elucidate the long-term implications and practical applicability of *C. vulgaris* in ruminant nutrition.

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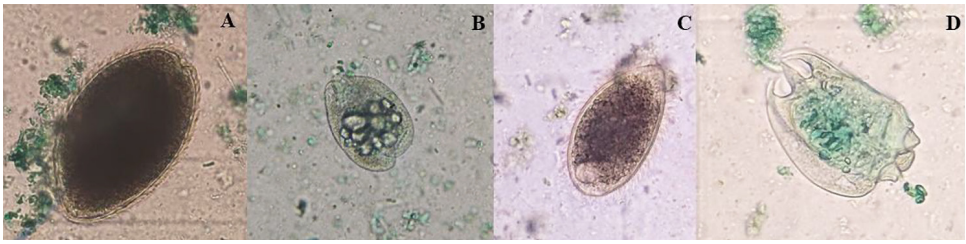

Fig. 1. Photomicrographs of the most abundant protozoa genera observed (at × 40 magnification) in the ruminal fluid of animals supplemented with different concentrations of *Chlorella vulgaris* supplementation. (A) *Isotricha* spp. (B) *Entodinium* spp. (C) *Dasytricha* spp. (D) *Diplodinium* spp.

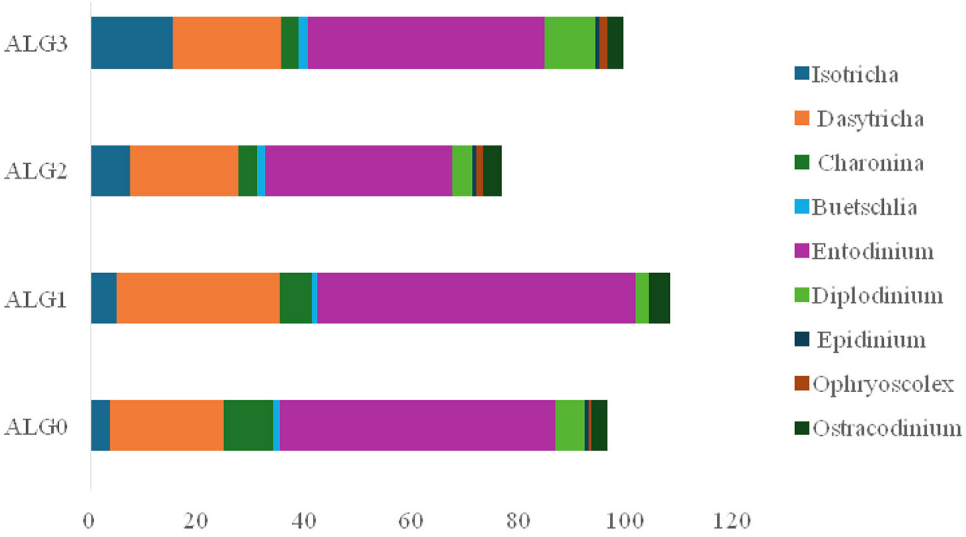

Fig. 2. The relative abundance of observed protozoal genera in the cows' rumen fed with diets (ALG0, ALG1, ALG2, ALG3). ALG0 – control diet without *Chlorella vulgaris* supplementation; ALG1 – diet containing 2.76 g of lyophilized *C. vulgaris*/kg of dry matter; ALG2 – diet containing 8.22 g of lyophilized *C. vulgaris*/kg of dry matter; ALG3 – diet containing 16.30 g of lyophilized *C. vulgaris*/kg of dry matter.