Journal of Agriculture and Food Research 19 (2025) 101663

Contents lists available at ScienceDirect

Journal of Agriculture and Food Research

journal homepage: www.sciencedirect.com/journal/journal-of-agriculture-and-food-research

The effect of supplementing *Nannochloropsis oculata* microalgae on ruminal fermentation, methane production and microbial population

Zahra Salehian [a], Hamed Khalilvandi-Behroozyar [a,**], Rasoul Pirmohammadi [a], Nasrollah Ahmadifard [b], Hadi Almasi [c], Germán Buendía Rodríguez [d], Maximilian Lackner [e,***], Abdelfattah Z.M. Salem [f,*]

[a] Department of Animal Science, Faculty of Agriculture, Urmia University, P. O. Box 165, Urmia, Iran
[b] Department of Fisheries, Faculty of Natural Resources, Urmia University, Urmia, Iran
[c] Department of Food Science and Technology Engineering, Faculty of Agriculture, Urmia University, Urmia, Iran
[d] Campo Experimental Valle de México, CIRCE, Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP), Mexico
[e] Faculty of Industrial Engineering, University of Applied Sciences Technikum Wien, Hoechstaedtplatz 6, 1200, Vienna, Austria
[f] Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado México, Toluca, Estado de México, Mexico

ARTICLE INFO

Keywords:
Eicosapentaenoic acid
Fermentation
Methanogenic archaea
Microalgae
Rumen Microbial community

ABSTRACT

This study aimed to evaluate the dietary supplementation of *Nannochloropsis oculata (N. oculata)* microalgae in different levels (1-, 2-, 3-, 4-, and 5- g/100 g dry matter (DM)) on the kinetics of gas production (GP), methane (CH_4) production, dry matter apparent digestibility, volatile fatty acids (VFA), and microbial population by *in vitro* method. The findings of this study indicated that adding *N. oculata* at levels of 1–5 g/100 g DM did not have an impact on GP at different incubation hours. However, a diet with 5 g/100 g DM *N. oculata* resulted in a consistent increase in GP rate (part c) compared to the control treatment ($P < 0.0001$). Supplementing of *N. oculata* to the diet decreased the CH_4 production ($P < 0.001$), methane to gas production ratio in 24 h ($P < 0.0001$), protozoa population ($P < 0.002$), total VFA ($P < 0.005$), *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Prevotella* spp., *Anaerovibrio lipolytica*, and *Butyrivibrio proteoclasticus*, and increased the partitioning factor ($P < 0.0001$), pH ($P < 0.001$), *Ruminococcus albus*, *Butyrivibrio fibrisolvens*, *B. fibrisolvens*, *S. ruminantium,* and fungi population ($P < 0.0001$). The addition of *N. oculata* did not impact on the levels of dry matter digestibility, ammonia nitrogen, acetate, propionate, and butyrate. However, this study demonstrated that *N. oculata* effectively decreased methane production and the presence of methanogenic archaea by more than 3 g/100 g DM in the diet. Therefore, *N. oculata* can be considered as a beneficial biological supplement for reducing methane emissions in animal feed. Nevertheless, further experiments are required to determine the optimal supplementation level for practical application.

1. Introduction

Microalgae can be classified as photoautotrophic unicellular or multicellular microorganisms that have a size of less than 400 μm. Recent studies focusing on using microalgae biomass as a supplement for feeding livestock that produces food for humans have presented a promising approach towards enhancing human health [1,2]. With the growing global population and the rising need for energy, exploring renewable energy sources has become imperative [3]. Microalgae have emerged as a potential solution, offering benefits such as animal feed, medicinal properties, and an alternative fuel source [4,5]. Recently, researchers have shown a growing interest in employing microalgae as a feed additive [7,8]. As a result, in most studies conducted so far, biomass or algae extract has not been considered as a primary food source, but rather as an addition to feed rations [9]. According to recent research, the feed industry accounts for 30 % of global algae production. The

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: h.khalilvandi@urmia.ac.ir (H. Khalilvandi-Behroozyar), maximilian.lackner@technikum-wien.at (M. Lackner), salem@uaemex.mx, asalem70@yahoo.com (A.Z.M. Salem).

https://doi.org/10.1016/j.jafr.2025.101663
Received 29 November 2024; Received in revised form 9 January 2025; Accepted 20 January 2025
Available online 21 January 2025

predominant species include *Schizochytrium* sp., *Chlorella* sp., *Arthrospira* sp., *Isochrysis galbana (I. galbana), and Porphyridium* sp [8,10–14].The consumption of microalgae worldwide is steadily increasing. The incorporation of small quantities of microalgae biomass into animal feed can enhance the animals' physiology, boosting immune response, disease resistance, antiviral and antibacterial activity, intestinal function, and promoting probiotic colonization [10]. Microalgae are a food source that has been less commonly used to feed ruminants. Based on the structural and compositional characteristics of algal biomass, ruminants should be expected to be suitable consumers for them, as they should be able to break even the wall of unprocessed algal cells due to their unique digestive system [15]. In addition, ruminants can use complex polysaccharides and non-protein eukaryotic nitrogen. However, adverse effects on ruminal fermentation have been reported using microalgae in feed [8,14].

However, it is noteworthy that some researchers' results show that using some microalgae species as animal feed reduces the production of greenhouse gases [16] and methane [12–14]. Salehian et al. [8] reported that varying levels of the *I. galbana* microalgae led to a significant reduction in methane emission *in vitro* method, without adversely impacting feed digestibility. In the recent century, controlling and reducing the amount of methane emission and greenhouse gases produced and its importance has increased. The results of various reports show that ruminants have an influential role in CH_4 emission. Therefore, the use of healthy food products and their study on their impact on animal health, nutritional value, and reduction of CH_4 emission by livestock is critical.

The *N. oculata* microalgae contain protein, providing essential amino acids [17] and polyunsaturated fatty acids (PUFA) with omega-3, especially eicosapentaenoic acid (EPA) [18,19]. Additionally, the *N. oculata* microalgae are known for their high nutritional value; however, there is insufficient information regarding their effect on rumen function [11]. Therefore, the main purpose of this experiment was to investigate the effect of supplementing *N. oculata* at different levels to the diet on the kinetics of GP, CH_4 emission, VFA, bacterial, fungal, and protozoa populations *in vitro* method.

2. Materials and methods

The present research was conducted in the Animal Research Station of Agriculture College of Urmia University, Urmia, Iran. The research protocol was approved by the Animal Care and Use Committee of Urmia University.

2.1. Microalgae preparation and chemical composition analysis

The *N. oculata* microalgae was prepared by the Artemia and Aquaculture Research Institute of Urmia University [20]. The methods of AOAC (2000) [6] were used to determine the dry matter (DM), ash, and crude protein (CP). Total fat was determined according to Bligh and Dyer (1959) [21], using methanol and chloroform in an ultrasonic water bath.

2.2. In vitro fermentation experiment

Three rumen-fistulated Holstein bulls weighing 640 ± 10 kg were used for rumen fluid collection. All of the animals used in this experiment were maintained according to the Guide for the Care and Use of Farm Animals in Animal Science Research [22]. Animals were fed individually, twice a day, with a fully mixed ration to provide metabolizable energy to the extent of 120 % of maintenance requirements of the NRC [23]. Throughout the experiment, the animals had free access to drinking water and with mineral-vitamin supplements. Total mixed ration samples were prepared with incremental levels of *N. oculata* replaced with soybean meal (control, no algae supplement; 1–5 g/100 g DM for treatments 1 to 5, respectively). The chemical composition of *N. oculata* is shown in Table 1.

In vitro, the gas production technique was conducted in two runs and three replicates in each run [24] using a digital pressure gauge [25]. The ingredients and chemical composition of the experimental diet used in the *in vitro* experiment were reported in Table 1. Cumulative gas production kinetics and corresponding parameters were recorded during two separate runs. In each of the runs, three laboratory replications were used for each of the experimental diets (n = 3). The rumen contents were collected from both the liquid and the solid phase and handled from three fistulated cows fed on a diet containing alfalfa hay and concentrate after morning feeding [24]. Collected rumen fluid was filtrated through four layers of cheesecloth and transported to the laboratory by (39 °C) insulated flasks. The experimental diet was milled (Wiley mill) to pass through a 1 mm screen and 500 mg of diet was weighed into incubation flasks. Fifty mL of the incubation medium was dispensed anaerobically into 120 mL screw caps vials. The ratio of rumen fluid to buffer medium was 1:2 (v/v). The cumulative volume of produced gas was estimated using gas pressure with pressure transducers at 2, 4, 8, 10, 12, 24, 48, 72, 96, 120, and 144 h of incubation. The volume of gas produced in the bottles containing feed samples at any time was calculated by the regression relationship between gas volume and pressure. The Mitscherlich model [26] was used for fitting the data.

$$GP = A\,(1 - e - c\,(T - L)) \quad (1)$$

where GP is the cumulative gas production at a given time (mL); A is the potential cumulative gas production (mL); c is the gas production rate (mL/h); T is the time of fermentation (h); and L is the lag time (h).

Separate incubation flasks were considered for methane and fermentation parameter determination after 24 h of incubation. The CH_4 production was determined by analyzing the headspace collected gas after 24 h of incubation from three bottles in each run. Headspace gas was sampled with gas-tight syringes (Hamilton, Reno, NV, USA), and 30 μL of each gas sample was injected into a gas chromatograph (Agilent 6820 series; Agilent Technologies Inc., Santa Clara CA) equipped with a thermal conductivity detector and an HP-PLOT Q capillary column (Agilent Technologies Inc.) as described by Patra et al. [27]. The ratio of CH_4 production to total GP (mL of CH_4 production/mL total gas 24 h) and CH_4 production per g of true digested organic matter (TDOM; mL/g TDOM) was also calculated.

After headspace gas sampling, incubation flasks were opened, and pH was measured (Schott Titrator Titroline easy) To measure the counting of protozoa population under a light microscope, three subsamples from each of the incubation bottles were filtrated through four layers of cheesecloth and were fixed with 50 % formalin solution and stained with methylene blue. Cheese-cloth filtered incubation media [28]. Samples for VFA profiling were collected after centrifugation of the incubation media at 4000 g for 15 min. Supernatants were acidified by sulphuric acid and kept at −20 °C until further analysis. A gas chromatograph (6820, Agilent Technologies, Santa Clara, CA) equipped with an HP-FFAP column (J&W HP-FFAP GC Column, 30 m, 0.25 mm, 0.25 μm, 7-in cage, Agilent) was used to determine the effects of microalgae supplementation on rumen VFA concentration. The injection site and detector temperature were set at 250 °C and 300 °C, respectively. The column temperature was programmed to rise 20 °C/min from 60 to 200 °C and hold at the final temperature for 10 min. Nitrogen was used as a carrier gas with a flow of 1 mL/min. Samples were automatically injected at a 50:1 split ratio.

Fatty acid profiles of the dried microalgae samples were also determined as described in Folch et al. [29]. For fatty acid analysis, an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, California, United States) equipped with an autoinjector (Agilent 7683 series, Santa Clara, California, United States) and FID detector was used.

To measure the apparent digestibility coefficients, bottle contents were filtered through an ash-less filter paper and placed in a forced air oven at 80 °C for 48 h. Filter paper remaining materials were corrected

Table 1
Ingredients and chemical composition of diets.

Ingredients, g/100 g DM	Levels of *N. oculate*, g/100 g DM					
	Control	1	2	3	4	5
Alfalfa hay	50.5	50.5	50.5	50	50	50
Corn silage	16.6	16.6	16.6	16.6	16.6	16.6
Barley	25	25	25	25	25	25
Soybean meal	4	3.4	3	2.4	2.4	2
N. oculata microalgae	0	1	2	3	4	5
Calcium carbonate	1	1	1	1	1	1
Calcium phosphate	0.5	0.5	0.5	0.5	0.5	0.5
Multivitamin	1	1	1	1	1	1
Salt	1	1	1	1	1	1
Chemical composition						
Metabolizable energy, Mcal	4.7	4.8	4.9	4.96	5.03	5.1
NDF	34.5	34.3	34.1	33.9	33.8	33.6
CP	16.54	16.54	16.54	16.54	16.54	16.54
Ash	11	11	11	11	11	11
Fat	2.4	2.8	3.3	3.5	3.9	4.3
N. oculata composition						

†CP	†Fat	†NDF	†Ash	††SFA	††UFA	††MUFA	††PUFA
37	39.05	4.3	12	28.2	70.9	28.9	41.1

CP: Crude protein.
NDF: Neutral detergent fiber.
†: g/100g DM.
††: g/100 g FA.
SFA: Saturated fatty acids.
UFA: Unsaturated fatty acids.
MUFA: Monounsaturated fatty acids.
PUFA: Polyunsaturated fatty acids.

for corresponding blanks. The partitioning factor (PF) was calculated from the ratio of true digested organic matter (TDOM) to GP at 24 h of incubation [30]. The NH_3-N concentration was also measured by the method of Broderick and Kang [31].

2.3. Microbial population

2.3.1. DNA extraction, real time-PCR analyses

After 24 h of incubation, a uniform aliquot (2 mL) of the syringe's contents was transferred to an Eppendorf tube and kept at −80 until DNA extraction; Total DNA extraction was done using phenol and chloroform followed by saline-alcohol precipitation [32,33]. Extracted DNA was solubilized in sterile Tris-EDTA, processed using DNase-free RNase, and purified Using DNA Clean and Concentrator™-25, ZYMO (CORP. Irvine, USA. RESEARCH) due to manufacturer's manual. The yield purity of extracted DNA and RNA was assessed using a Nanodrop Spectrophotometer (Thermo-Fisher Scientific, Wilmington, USA); The different microbial groups were determined in the samples using an SYBR green rtPCR assay; Characterization of the Primers used for PCR amplification of total bacteria, total fungi, methanogenic archaea, major cellulolytic, amylolytic, and proteolytic bacteria was shown in Table 2; Quantification of the target gene was performed on microbial DNA using a Step One apparatus (Biosystems® 7500 Real-Time PCR); Reactions were run in triplicate in 48-well plates, using PCR master mixtures (final volume of 20 μL) containing 2 μL of primer pairs (4 P mol/μL from each of forward and reverse primer), 10 μL of SYBR mix (Fermentas, Cat. No: K0221), 4 μL of PCR water and 4 μL of DNA solution (20 ng of DNA). Negative controls without templates were run in each assay to assess overall specificity; PCR conditions were as follows: one cycle of 10 min at 95 °C, 40 PCR cycles of 15 s at 95 °C, 10 s at the annealing temperature of the primers, and 60 s at 72 °C. Plates read every 0.1 °C from 55 °C to 95 °C for drawing melting curves, and then the reactions were ended with an extension of 5 min at 72 °C. The populations of target bacteria/microbial groups were expressed relative to the total bacterial populations; The comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) was calculated by subtracting Ct of the target gene from the Ct value of the reference gene (16S rDNA of total bacteria at 24 h) [34].The shifts in microbial communities due to the supplementation of different levels of GTEE were determined by taking the microbial population in the Control group as 100 [35].

Table 2
Primer design characteristics used for PCR amplification of rumen microorganisms.

	FO- Primer	RE - Primer	Annealing temperature
Total bacteria	GTG STG CAY GGY TGT CGT CA	GAG GAA GGT GKG GAY AC GT	60
Total protozoa	CAYGTCTAAGTATAAATAACTAC	CTCTAGGTGATWWGRTTTAC	61
Total fungi	GAGGAAGTAAAAGTCGTAACAAGGTTTC	CAAATTCACAAAGGGTAGGATGATT	60
Methanogens	CCGGAGATGGAACCTGAGAC	CGGTCTTGCCCAGCTCTTATTC	60
F. succinogenes	GGTATGGGATGAGCTTGC	GCCTGCCCCTGAACTATC	62
R. albus	GTTTTAGGATTGTAAACCTCTGTCTT	CCTAATATCTACGCATTTCACCGC	60
R. flavefaciens	TCTGGAAACGGATGGTA	CCTTTAAGACAGGAGTTTACAA	62
A. lipolytica	TGGGTGTTAGAAATGGATTCC	CTCTCCTGCACTCAAGAATT	59
B. fibrisolvens	TAACATGAGTTTGATCCTGGCTC	CGTTACTCACCCGTCCGC	62
B. proteoclasticus	TCCTAGTGTAGCGGTGAAATG	TTAGCGACGGCACTGAATGCCTAT	62
M. elsdenii	GACCGAAACTGCGATGCTAGA	CGCCTCAGCGTCAGTTGTC	60
Prevotella spp.	CACGGTAAACGTGGAT	GGT CGG GTTGCA GAC C	57
P. ruminicola	GGTTATCTTGAGTGAGTT	CTGATGGCAACTAAAGAA	55
S. ruminantium	TGCTAATACCGAATGTTG	TCCTGCACTCAAGAAAGA	53
S. bovis	TGTTAGATGCTTGAAAGGAGCAA	CGCCTTGGTGAGCCGTTA	60

2.4. Calculations and statistical model

In this study, 3 experimental replications were used for each treatment (each replication was made with one of the microalgae production replications). To compare the effect of different levels of *N. oculata* on the VFA profile and microbial population, a completely randomized design (Equation No. 2) was used to analyze in a GLM procedure (SAS, 9.4) [36]. The averages of intra-run and inter-run replications in each experimental replication were used for statistical comparison. Cumulative gas production kinetics were analyzed as repeated measures and the effect of incubation time (h) and the interaction of incubation time and treatment was considered in the statistical model (Equation No. 3). Data were analyzed in a mixed model procedure and first order variance-covariance structure was chosen based on the smallest Schwarz's Bayesian information criterion [36]. Tuckey corrected the least square means were compared for possible statistically significant differences by the PDIFF option. Data were reported in the tables as least square means and corresponding SEM. A regression analysis was also done to determine linear or quadratic effects of incremental levels of microalgae in diets.

$$Y_i = \mu + T_i + e_i \quad (2)$$

$$Y_{ij} = \mu + T_i + It_j + (T \times It)_{ij} + e_{ij} \quad (3)$$

where, Yi: observation i, μ: average of all observations, T_i: effect of treatment, It_j: effect of incubation time; TIt_{ij}: interaction effect of incubation time and type of processing, and e_{ij}: experimental error effect.

3. Results

3.1. Gas production

The results obtained from this research showed that different levels of *N. oculata* in diet did not affect the volume of gas production (Fig. 1). As shown in Table 3, the gas production potential of the insoluble fraction (coefficient b) was not affected by supplementing of *N. oculata* and the gas production rate constant (coefficient c) in treatment 5 was the highest amount (Table 3; $P < 0.0001$). The coefficient b and coefficient c have a linear and quadratic response to incremental levels of *N. oculata* supplementation ($P < 0.0001$).

3.2. Methane production and rumen fermentation parameters

N. oculata caused a significant decrease in CH_4 production and the CH_4 production to the produced gas in 24 h of incubation ($P < 0.001$). Increasing the level of *N. oculata* in the diet caused a linear decrease in CH_4 production to the produced gas in 24 h of incubation and protozoa population ($P < 0.002$). Dry matter apparent digestibility and NH_3-N were not affected by the addition of different levels of *N. oculata.* The supplementation of *N. oculata* to the diet increased the partition factor and pH (Table 4; $P < 0.001$). The ratio of CH_4 production produced to feed organic matter affected *N. oculata* supplemented at different levels (Fig. 2; $P < 0.0001$). The VFA, acetate to propionate ratio, and acetate + butyrate to propionate ratio were not affected by different levels of *N. oculata* in diets. However, the total VFA decreased linearly with increasing levels of *N. oculata* supplementing (Table 5; $P < 0.005$).

3.3. Rumen microbial population

The population of protozoa and methanogenic archaea decreased, and anaerobic fungi increased respectively, with increasing levels of *N. oculata* in diet (Panel A, Fig. 3; $P < 0.0001$). In our study, supplementation *N. oculata* decreased simultaneously *F. succinogenes* and *R. flavefaciens* and in contrast, the population of *R. albus* in the treatment containing 5 g/100 g DM *N. oculata* showed the highest increase (Panel B, Fig. 3; $P < 0.0001$). *A. lipolytica* and *B. proteoclasticus* were also affected by the supplementing of *N. oculata* and the population of these bacteria decreased. In addition, the population of *B. fibrosolvens* showed a significant increase (Panel C, Fig. 3; $P < 0.0001$). Population of proteolytic bacteria, *Prevotella* spp. and *P. ruminicola,* unlike *S. ruminantium*, showed a significant decrease with increasing *N. oculata* in the diet. However, the population of *S. ruminantium* increased significantly (Panel D, Fig. 3; $P < 0.0001$).

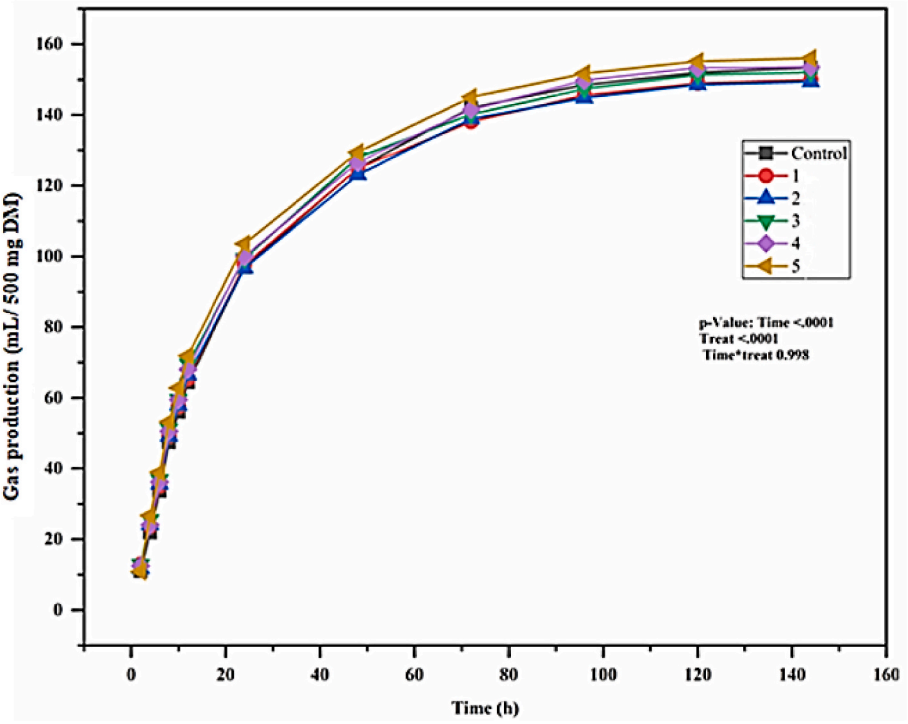

Fig. 1. Effect of adding different levels of *N. oculata* on the volume of cumulative gas production (mL/500 mg DM).

Table 3
The effect of adding different levels of *N. oculata* on gas production coefficients of insoluble section, and constant gas production rate in complete diets (mL/500 mg of DM).

Coefficients	Levels of *N. oculate*, g/100 g DM						*P*-value	SEM	Polynomial regression analysis	
	Control	1	2	3	4	5			Linear	Quadratic
b	150	147	154	148	150	152	0.06	2.21	0.0013	<0.0001
c	0.042^{bc}	0.044^{ab}	0.044^{ab}	0.046^{ab}	0.044^{ab}	0.048^{a}	<0.0001	0.0031	0.0001	<0.0001

b: The amount of insoluble gas production.
c: Constant gas production rate.
Different superscripts within a row indicate a significant difference ($P < 0.05$).

Table 4
Fermentation parameters diets supplemented with different levels of *N. oculata*.

Parameters	Levels of *N. oculate*, g/100 g DM						*P*-value	SEM	Polynomial regression analysis	
	Control	1	2	3	4	5			Linear	Quadratic
Methane[†]	42.2^{a}	36.7^{b}	35.3^{bc}	33.7^{cd}	32.8^{de}	31.7^{e}	0.001	0.295	0.0001	0.003
Gas 24 h[†]	96.9	97.6	96.7	99.4	100	103.6	0.121	2.824	0.07	0.18
methane: 24 h gas	0.436^{a}	0.376^{b}	0.366^{b}	0.339^{b}	0.328^{c}	0.306^{c}	<0.0001	0.0042	0.0001	0.009
Apparent digestibility[†]	60.05	59.4	59.3	59.5	59.7	59.8	0.178	0.168	0.001	0.641
PF[ǂ]	3.52^{d}	3.55^{d}	3.58^{d}	3.76^{c}	3.88^{b}	4.21^{a}	<0.0001	0.008	0.0001	0.0007
Protozoa $\times 10^5$	14.7×10^{5a}	13.5×10^{5b}	13.05×10^{5bc}	12.6×10^{5cd}	12.3×10^{5bc}	12.3×10^{5d}	0.002	0.196×10^5	0.0007	0.022
pH	6.4^{c}	6.6^{ab}	6.5^{ab}	6.6^{ab}	6.6^{ab}	6.6^{a}	0.001	0.016	0.0002	0.069
Ammonia nitrogen[ɛ]	18.6	18.5	18.5	18.3	18.2	18.421	0.07	0.114	0.306	0.021

†: ml/500 mg DM.
ŧ: g/100 g DM.
PF = partitioning factor.
ǂ: mg/ml.
ɛ: mmol/L.
Different superscripts within a row indicate a significant difference ($P < 0.05$).

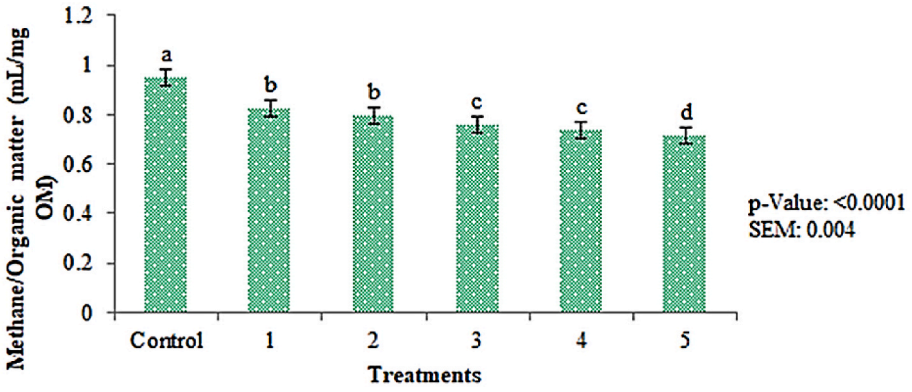

Fig. 2. The ratio of methane production to digested organic matter (mL/mg organic matter) in diets containing different levels of *N. oculata* at 24 h of *in vitro* incubation.

Table 5
The effect of different levels of *N. oculata* on the volatile fatty acids' concentration (VFA, mmol/dL) and profile after 24 h of incubation.

Parameters	Levels of *N. oculate*, g/100 g DM						*P*-value	SEM	Polynomial regression analysis	
	Control	1	2	3	4	5			Linear	Quadratic
Acetate	44. 1	43.9	40.7	42.5	43.9	45.8	0.57	1.91	0.147	0.343
Propionate	37.3	38.9	41.7	39.7	37.3	34.9	0.44	2.22	0.132	0.226
Butyrate	12.2	10.8	11.1	11.2	11.2	11.9	0.44	0.496	0.158	0.145
Valrate	5.38	5.15	5.38	5.4	6.53	6.21	0.32	0.447	0.763	0.954
Isobutyrate	0.363	0.463	0.464	0.472	0.391	0.401	0.07	0.0235	0.866	0.747
Isovalerate	0.592	0.651	0.552	0.683	0.714	0.671	0.16	0.0381	0.142	0.039
Total VFA	84.8^{a}	81.8^{b}	80.6^{bc}	$79. 9^{bcd}$	79.4^{cd}	77.8^{d}	0.005	0.376	0.002	0.001
Acetate/Propionate	1.18	1.13	0.97	1.07	1.18	1.31	0.49	0.111	0.27	0.12

Different superscripts within a row indicate a significant difference ($P < 0.05$).

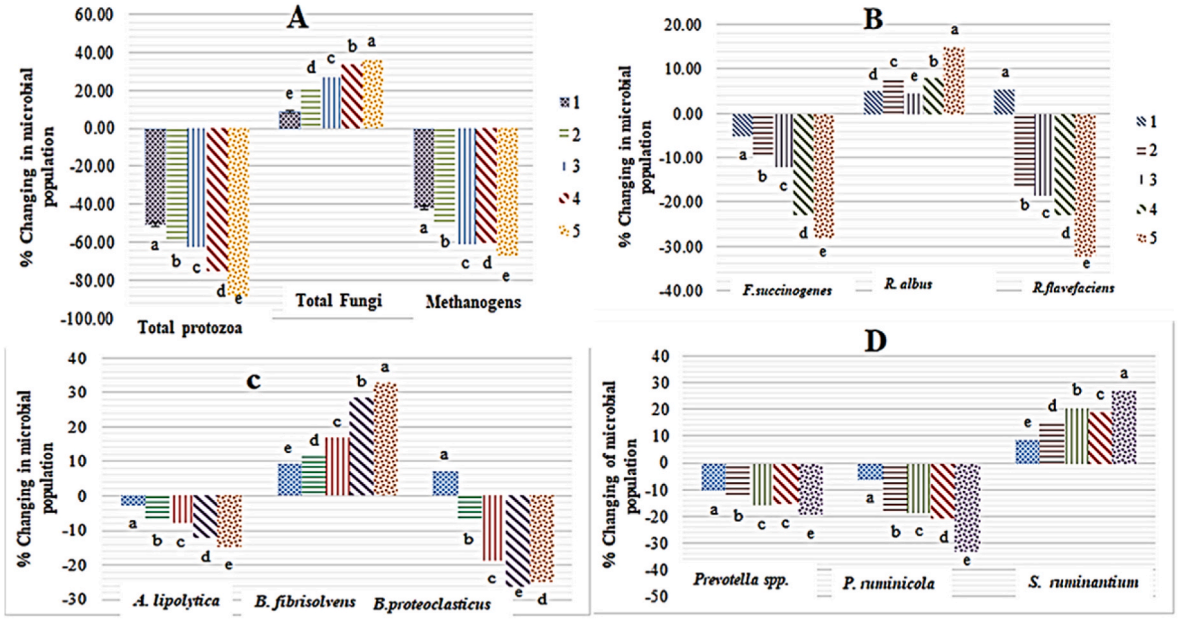

Fig. 3. Microbial population in diets supplemented with *N. oculata* (Panel A, B, C, D).
Panel A: SEM for total protozoa, total fungi and methanogens are respectively: 1.054, 0.249, 0.367; $P < 0.0001$.
Panel B: SEM for *F. succinogenes, R. albus,* and *R. flavefaciens* are respectively: 1.029, 0.906, 1.211; $P < 0.0001$.
Panel C: SEM for *A. lipolytica, B. fibrisolvens,* and *B. proteoclasticus* are respectively: 0.431, 0.152, 0.634; $P < 0.0001$.
Panel D: SEM for *Prevotella* spp., *P. ruminicola,* and *S. ruminantium* are respectively: 1.04, 1.001, 0.843; $P < 0.0001$.

4. Discussion

4.1. Gas production

The results obtained from the effect of supplementation *N. oculata* at different levels of diet on gas production (GP) were consistent with the results of Marrez et al. [37]. The amount of GP in the incubation and fermentation process is a reflection of the composition of the ratio components and their nature. However, the supplementation of 5 g/100 g DM levels of *N. oculata* increased the rate of GP in this treatment compared to the control. Probably, the increase in the level of *N. oculata* in the diet over time has caused its cell wall to be broken by microorganisms and release its nutrient contents and the consumption of nutrients by microorganisms, resulting in more gas production and an increase in gas rate.

4.2. Methane production and rumen fermentation parameters

Recent research shows that microalgae in diet can inhibit CH_4 production [13,14,38], and species with high levels of unsaturated fatty acids (UFA) reduce CH_4 production levels produced in the rumen [14]. Rumen microorganisms use free H_2 to convert UFA to saturated fatty acids (SFA), reducing the amount of H_2 available to methanogenic archaea decreases CH_4 production. The results of this experiment were similar to those of Meehan et al. [14]. In this study, the CH_4 production (mL/g organic matter (OM)) in the diet based on corn silage and containing 10 g/100 g DM of *N. oceanica* was reduced. The UFA in fish oil reduced protozoa viability [39]. Microalgae species, the composition of diet components used in the incubation process, algae culture medium, harvesting, and processing are also factors affecting the fermentation process and ruminal parameters [40–42]. Ciliate protozoa are among the H_2 suppliers needed by methanogens to produce methane. In the structure of *N. oculata* Omega-3 UFA and especially EPA is high [20,43]. In our study, the level of PUFA and UFA was high in *N. oculata* (Table 1). Increasing the level of microalgae in the diet may have increased the UFA released from the microalgae and the coverage of the fibers by the fat. As a result, LCUFA harms the survival of protozoa. In the present study, linear increasing the level of *N. oculata* in the diet and UFA decreased the population of these protozoa and CH_4 emissions.

Increasing the partition factor in a food item indicates an improvement in its efficiency [44], and a decrease in it indicates a decrease in the efficiency of microbial protein synthesis in the culture medium. This means that most digested food is used for GP instead of microbial protein synthesis [45]. In addition, the results related to the partition factor, and the ratio of methane to OM indicate that with the increase of *N. oculata* supplemented the consumption of OM by rumen microorganisms has progressed towards the production of long-chain fatty acids (LCFA) or microbial protein. Therefore, the consumption of carbon and H_2 produced in the environment by methanogens is reduced and these substances are out of their reach and CH_4 production was reduced.

In a study conducted on increasing levels of *Schizotrium* in goats' diets, their ruminal pH increased [46]. Merrez et al. [37] reported that which used levels of 0, 2, 4, and 6 g/100 g DM of *N. limnetica* in the diet, the pH increased with increasing levels of microalgae. The *N. oculata* contains long-chain unsaturated fatty acids (LCUFA), including EPA. Probably due to the decrease in total volatile fatty acids (VFA; Table 5). The nature of microalgae species in terms of the type of fatty acids has affected the fermentation process and the activity of microorganisms.

The results showed that with increasing *schizotrium* levels in the goat diet, total VFA decreased [46]. The VFA produced in the rumen reflects the fermentation process of dietary nutrients in the rumen. Decreasing ruminal pH indicates an increase in rapidly fermenting carbohydrates and VFA production in the rumen. In this experiment, by increasing the level of *N. oculata* in the diet, the pH also increased, which means a decrease in the amount of VFA in the fermentation process. Research also shows that VFA levels and methane production are correlated, and a decrease in VFA is associated with a decrease in methane production [47]. In the present study, VFA and methane emission in the treatment containing different levels of *N. oculata* were significantly reduced compared to the control treatment. The research indicates that due to the combination of microalgae fatty acids, the fat production process in these conditions may have tended towards the producing of LCFA and the biohydrogenation process [37] where the VFA was reduced. Thus, maybe the *N. oculata* cell wall structure, the availability of intracellular

compounds of microalgae for microorganisms, and its effect on the fermentation process during incubation have affected fat metabolism and VFA production. Increased the partition factor may be due to decreased TDOM and this inclined to produce VFA, and this is more likely to increase the microbial protein mass.

Examining the results of the linear regression analysis indicates a relationship between the linear increase of *N. oculata* in the diet and the corresponding linear increase and decrease of certain rumen parameters. Conversely, the findings from this project's researchers, based on previous studies regarding the composition of unsaturated fatty acids and PUFA in this type of microalgae, have been documented in other articles [20]. It appears that increasing the level of *N. oculata* in the diet led to changes in rumen parameters.

4.3. Rumen microbial population

In a study by Gomma et al. [12] on the levels of 1–5 g/100 g DM *N. oculata*, the results showed that the level of 5 g/100 g DM in a total mixed diet reduced the protozoa population. However, the results obtained in the present study from the microscopic examination of the protozoa population were similar to the results of the protozoa rDNA examination. The UFA is toxic for rumen microorganisms and their increase in the rumen environment and incubation causes a decrease in the number of protozoa [48] and methanogenic archaea [12,49,50]. The decrease in the population of methanogenic archaea in this experiment was one of the main reasons for the decrease in methane following the increase in the level of *N. oculata* in the diet. The noteworthy point is that different rumen microorganisms show different reactions to UFA in the rumen environment. Increasing the level of fat in the diet increases the coverage of fibers by fat, and as a result, the conditions for the growth of these cellulolytic bacteria and the digestion of fibers become unfavorable. The decrease in the population of cellulolytic bacteria such as *F. succinogenes* and *R. flavefaciens* as a result of the increase in fat in the diet may be related to the increase in the level of *N. oculata*. The increase in the population of *R. albus* bacteria was probably due to the different responses of bacteria to environmental conditions. Probably, the increase in the population of *R. albus*, along with the increase in anaerobic fungi, has prevented the decrease in feed digestibility.

The *Butyrivibrio* species are very effective in the process of biohydrogenation of bacteria and increase when biohydrogenation is completed and formed at 18:00 [9]. The *Butyrivibrio* species has the ability of phospholipase and lysophospholipase. *B. proteoclasticus* are most sensitive to UFA in the environment. It is may that the decrease in the population of these bacteria was in response to the increase in the level of microalgae *N. oculata* in the diet and the increase in UFA in the environment, which harmed their survival and activity.

P. ruminicola is one of the major bacteria in the breakdown of ruminal protein. Increased UFA do not affect the survival rate of *S. ruminantium* and *P. ruminicola* [51] In this study, *S. ruminantium* species increased as the level of microalgae *N. oculata* increased in the environment, but *P. ruminicola* decreased. This is probably due to some antibacterial compounds in the structure of microalgae that have affected the growth and survival of these bacteria. Hence increases the population of *S. ruminantium* and decrease *Prevotella* spp. and *P. ruminicola* were probably in response to the ecosystem changes of the incubation environment due to the presence of *N. oculata* and the increase in the level of UFA at the same time as the increase in the level of microalgae in the diet. In addition to biohydrogenation activity, various bacteria of the genus *Butyrivibrio* are also involved in the breakdown of protein, starch, and cell walls [52,53]. The effect of *N. limnetica* microalgae was performed at different levels on ruminal parameters [37], in this study results showed that the use of 6 g/100 g DM microalgae in the diet reduces the number of bacteria. Gomma et al. [12] reported that 2 g/100 g DM of *N. oculata* supplementation to total mixed diet increased the bacterial population, which contradicts the results of the present study. This discrepancy seems to be due to the amount of fat in the microalgae species *N. oculata* tested in the present study and the research of Gomma et al. [12]. Because the amount of fat of *N. oculata* in that study was 10.2 g/100 g DM but in our study, it was 39.1 (g/100 g DM). It seems that increasing the level of microalgae in the diet of the present experiment has increased the amount of UFA in the environment and has affected the growth, activity, and survival of bacteria. The reason that the population of some bacterial species such as *R. albus*, *B. fibrisolvens,* and *S. ruminantium* increase was due to the differences in the adaptation of bacterial species to changes in the environment [28].

5. Conclusion

This study found that dietary supplementation of *N. oculata* at a highest level (3–5 g/100 g DM) has positive effects on nutrient availability while reducing CH_4 production *in vitro*. In addition, incremental levels of *N. oculata* decreased the population of protozoa, methanogenic archaea, *A. lipolytica*, *Prevotella* spp., *R. flavefaciens*, *F. succinogenesis* and increased the population of *R. albus*, *B. fibrisolvens*, *S. ruminantium*, and fungus, while not affecting dry matter digestibility. The unsaturated fatty acids in *N. oculata* and their antibacterial compounds appear to influence the population of methanogenic archaea, thereby reducing methane emissions. The regression analysis in this research indicated that the linear increase in the level of *N. oculata* microalgae in the diet leads to a significant linear decrease in methane emission, microbial population, and certain rumen parameters. Therefore, considering the costs associated with the project and evaluating the results of the treatment involving 3 g/100 *N. oculata*, it is recommended for future studies. Considering the growing use of microalgae in animal feed as a valuable nutritional supplement to reduce methane emissions, it is recommended that researchers conduct further studies at various levels and explore additional parameters. Furthermore, *in vivo* experiments are essential to obtain results related to nutritional value, animal health, and methane emission reduction in ruminants.

Author contributions

Z. S. Project administration, data analysis, writing the original draft. H. Kh.B. Project administration and Supervision, Conceptualization of the idea, Data Validation, writing the original draft, editing, and review. R. P. Supervision, Conceptualization of the idea. N. A. Data analysis. H. A.; M.L.; G.B.R. and A.Z.M.S. Writing the original draft.

Declaration

All the animals used in this experiment were maintained according to the Guide for the Care and Use of Farm Animals in Animal Science Research and approved by the animal care and ethics committee of Urmia University. All of the co-authors have read and confirmed the final version of the manuscript before submission and have complete consent for publication in the Journal of Agriculture and Food Research. The authors declare that there is no conflict of interest regarding the publication of this paper. Financial support is done by the deputy of research and technology at Urmia University (Grant No. 4163). Also, partial financial support was done by Kimiya Danesh Alvand (industry relation grant the number of 10–1219).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

References

[1] J. He, Y. Yang, H. Xu, X. Zhang, X.M. Li, Olanzapine attenuates the okadaic acidinduced spatial memory impairment and hippocampal cell death in rats, Neuro. Psychopharmacol. 30 (8) (2005) 1511–1520, https://doi.org/10.1038/sj.npp.1300757.

[2] M. Koller, M. Alexander, B. Gerhart, Microalgae as versatile cellular factories for valued products, Algal Res. 6 (2014) 52–63, https://doi.org/10.1016/j.algal.2014.09.002.

[3] S.M.M. Souza, T.S. Bastos, M.C. dos Santos, Microalgae use in animal nutrition: a review, Res., Soc. Dev. 10 (16) (2021) e53101622986, https://doi.org/10.33448/rsd-v10i16.22986.

[4] K. Skjånes, C. Rebours, P. Lindblad, Potential for green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process: a Review, Crit. Rev. Biotechnol. 33 (2) (2013) 172–215, https://doi.org/10.3109/07388551.2012.681625.

[5] A. Banerjee, N. Kumar, S.J. Varjani, C. Guria, R. Bandopadhyay, P. Shukla, C. Banerjee, Computational Modelling and Prediction of Microalgae Growth Focused towards Improved Lipid Production, Springer, Singapore, 2018, pp. 223–232, https://doi.org/10.1007/978-981-10-7434-9_13.

[6] Association of Official Analytical Chemists, Official Methods of Analysis, seventeenth ed., Association of Official Analytical Chemists, Washington D.C., 2000.

[7] R.E. Austic, A. Mustafa, B. Jung, S. Gatrell, X.G. Lei, Potential and limitation of a new defatted diatom microalgal biomass in replacing soybean meal and corn in diets for broiler chickens, J. Agric. Food Chem. 61 (30) (2013) 7341–7348, https://doi.org/10.1021/jf401957z.

[8] Z. Salehian, H. Khalilvandi Behroozyar, R. Pirmohammadi, N. Ahmadifard, H. Almasi, M. Ramin, Investigating the effect of supplementing different levels of Isochrysis galbana on in vitro rumen fermentation parameters, Anim. Sci. J. 95 (1) (2024) e13929, https://doi.org/10.1111/asj.13929.

[9] A. Skrede, L.T. Mydland, Ø. Ahlstrøm, K.I. Reitan, H.R. Gislerød, M. Øverland, Evaluation of microalgae as sources of digestible nutrients for monogastric animals, J. Anim. Feed Sci. 20 (1) (2011) 131–142, https://doi.org/10.22358/jafs/66164/2011.

[10] W. Becker, Microalgae in human and animal nutrition, in: A. Richmond (Ed.), Microalgal Culture, Blackwell Publishing Ltd., Oxford, UK, 2004, pp. 312–351, https://doi.org/10.1002/9780470995280.ch18.

[11] G.G. DaSilva, E. Ferreira de Jesus, C.S. Takiya, T.A. Del Valle, T.H. da Silva, T.H. A. Vendramini, E.J. Yu, F.P. Rennó, Partial replacement of ground corn with algae meal in a dairy cow diet: milk yield and composition, nutrient digestibility, and metabolic profile, J. Dairy Sci. 99 (11) (2016) 8880–8884, https://doi.org/10.3168/jds.2016-11542.

[12] A.S. Gomaa, A.E. Kholif, A.M. Kholif, R. Salama, H.A. El-Alamy, O. Olafadehan, Sunflower oil and *Nannochloropsis Oculata* microalgaeas sources of unsaturated fatty acids for mitigation of methane production and enhancing diets' nutritive value, J. Agric. Food Chem. 66 (8) (2018) 1751–1759, https://doi.org/10.1021/acs.jafc.7b04704.

[13] A. Kiani, C. Wolf, K. Giller, L. Eggerschwiler, M. Kreuzer, A. Schwarm, *In vitro* ruminal fermentation and methane inhibitory effect of three species of microalgae, Can. J. Anim. Sci. 100 (3) (2020) 1–9, https://doi.org/10.1139/cjas-2019-0187.

[14] D.J. Meehan, A.R.J. Cabrita, L.J. Silva, J.M. Antonio Fonseca, R. Margarida, G. Maia, Effects of *Chlorella vulgaris, Nannochloropsis oceanica* and *Tetraselmis* sp. supplementation levels on *in vitro* rumen fermentation, Algal Res. 56 (2021) 102284, https://doi.org/10.1016/j.algal.2021.102284.

[15] R.J. Shields, I. Lupatsch, Microalgal biotechnology, in: C. Posten, C. Walter (Eds.), Integration and Economy: Algae for Aquaculture and Animal Feeds, Publisher: De Gruyter. Swansea University, UK, 2012.

[16] K.K. Lum, J. Kim, X.G. Lei, Dual potential of microalgae as a sustainable biofuel feedstock and animal feed, J. Anim. Sci. Biotechnol. 4 (53) (2013), https://doi.org/10.1186/2049-1891-4-53.

[17] Z. Salehian, H. Khalilvandi Behroozyar, R. Pirmohammadi, N. Ahmadifard, H. Almasi, Determining the nutritional value of protein of two microalgae species used in animal nutrition, J. Rumin. Res. 10 (2) (2022) 1–18, https://doi.org/10.22069/ejrr.2022.19617.1810. In Farsi.

[18] A. Sukenik, O. Zmora, Y. Carmeli, Biochemical quality of marine unicellular algae with special emphasis on lipid composition. II Nannochloropsis sp, Aquac 117 (3–4) (1993) 313–326, https://doi.org/10.1016/0044-8486(93)90328-V.

[19] I. Saadaoui, R. Rasheed, A. Aguilar, M. Cherif, H. Al Jabri, S. Sayadi, S.R. Manning, Microalgal-based feed: promising alternative feedstocks for livestock and poultry production: a review, J. Anim. Sci. Biotechnol. 12 (1) (2021) 76, https://doi.org/10.1186/s40104-021-00593-z.

[20] Z. Salehian, H. Khalilvandi Behroozyar, R. Pirmohammadi, N. Ahmadifard, H. Almasi, The optimal culture media for crude protein and polyunsaturated fatty acid production from *Isochrysis galbana* and *Nannochloropsis oculata* for livestock and aquatic species nutrition, J. Anim. Sci. Res. (Agricult. Sci). 34 (1) (2024) 117–127, https://doi.org/10.22034/as.2023.54279.1687.

[21] E. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. 37 (8) (1959) 911–917, https://doi.org/10.1139/o59-099.

[22] FASS, Guide for care and use of agricultural animals in research and teaching. Federation of Animal Science Societies Champaign, IL, third ed., 2010.

[23] NRC, Nutrient Requirements of Dairy Cattle, seventh ed., National Academy Press, Washington, DC USA, 2001.

[24] K.H. Menke, H. Steingass, Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid, Anim. Res. Dev. 28 (1988) 7–55.

[25] M.K. Theodorou, B.A. Williams, M.S. Dhanoa, A.B. McAllan, J. France, A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds, Anim. Feed Sci. Technol. 48 (3–4) (1994) 185–197, https://doi.org/10.1016/0377-8401(94)90171-6.

[26] J. France, J. Dijkstra, M.S. Dhanoa, S. Lopez, A. Bannink, Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed in vitro: derivation of models and other mathematical considerations, Br. J. Nutr. 83 (2) (2000) 143–150, https://doi.org/10.1017/S0007114500000180.

[27] A.K. Patra, D.N. Kamra, N. Agarwal, Effect of plant extracts on *in vitro* methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo, Anim. Feed Sci. Technol. 128 (3–4) (2006) 276–291, https://doi.org/10.1016/j.anifeedsci.2005.11.001.

[28] B.A. Dehority, Rumen Microbiology, Notingham university press. Notingham, United Kingdom, 2003.

[29] J. Folch, M. Lees, G. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem. 226 (1) (1957) 497–509, https://doi.org/10.1016/S0021-9258(18)64849-5.

[30] M. Blümmel, H. Steingas, K. Becker, The partitioning of *in vitro* fermentation products and its bearing for the prediction of voluntary feed intake, Proc. British Soc. Nutr. Physiol. 3 (123) (1994) (Abstract).

[31] G. Broderick, J.H. Kang, Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media, J. Dairy Sci. 63 (1) (1980) 64–75, https://doi.org/10.3168/jds.S0022-0302(80)82888-8.

[32] K. Tajima, R.I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, Y. Benno, Dietdependent shifts in the bacterial population of the rumen revealed with real-time PCR, J. Appl. Environ. Microbiol. 67 (6) (2001) 2766–2774, https://doi.org/10.1128/AEM.67.6.2766-2774.2001.

[33] S.L. Yang, D.P. Bu, J.Q. Wang, Z.Y. Hu, D. Li, H.Y. Wei, L.Y. Zhou, J.J. Loor, Soybean oil and linseed oil supplementation affect profiles of ruminal microorganisms in dairy cows, Animal 3 (11) (2009) 1562–1569, https://doi.org/10.1017/S1751731109990462.

[34] S.E. Denman, C.S. McSweeney, Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen, FEMS Microbiol. Ecol. 58 (3) (2006) 572–582, https://doi.org/10.1111/j.1574-6941.2006.00190.x.

[35] G. Goel, H.P.S. Makkar, K. Becker, Changes in microbial community structure, methanogenesis and rumen fermentation in response to saponin-rich fractions from different plant materials, J. Appl. Microbiol. 105 (3) (2008) 770–777, https://doi.org/10.1111/j.1365-2672.2008.03818.x.

[36] SAS Institute Inc, Statistical Analysis System (SAS) User's Guide, SAS Institute, Cary, N.C USA, 2002.

[37] D.A. Marrez, A. Cieślak, R. Gawad, H.M. Ebeid, M. Chrenková, M. Gao, Y.R. Yanza, M. El-Sherbiny, M. Szumacher-Strabel, Effect of freshwater microalgae *Nannochloropsis limnetica* on the rumen fermentation *in vitro*, J. Anim. Feed Sci. 26 (2017) 359–364, https://doi.org/10.22358/jafs/81275/2017.

[38] E. Sucu, Effects of microalgae species on *in vitro* rumen fermentation pattern and methane production, Ann. Anim. Sci. 20 (1) (2020) 207–218, https://doi.org/10.2478/aoas-2019-0061.

[39] V. Fievez, F. Dohme, M. Danneels, K. Raes, D. Demeyer, Fish oils as potent rumen methane inhibitors and associated effects on rumen fermentation *in vitro* and *in vivo*, Anim. Feed Sci. Technol. 104 (1–4) (2003) 41–58, https://doi.org/10.1016/S0377-8401(02)00330-9.

[40] V. Grossi, P. Blokker, J.S. Sinninghe Damsté, Anaerobic biodegradation of lipids of the marine microalga *Nannochloropsis salina*, Org. Geochem. 32 (6) (2001) 795–808, https://doi.org/10.1016/S0146-6380(01)00040-7.

[41] K.Y. Show, D.J. Lee, J.H. Tay, T.M. Lee, J.S. Chang, Microalgal drying and cell disruption–recent advances, Bioresour. Technol. 184 (2015) 258–266, https://doi.org/10.1016/j.biortech.2014.10.139.

[42] C. Liu, X.H. Li, Y.X. Chen, Z.H. Cheng, Q.H. Duan, Q.H. Meng, X.P. Tao, B. Shang, H.M. Dong, Age-related response of rumen microbiota to mineral salt and effects of their interactions on enteric methane emissions in cattle, Microb. Ecol. 73 (3) (2017) 590–601, https://doi.org/10.1007/s00248-016-0888-4.

[43] P. Spolaore, C. Joannis-Cassan, E. Duran, A. Isambert, Commercial applications of microalgae: a review, J. Biosci. Bioeng. 101 (2) (2006) 87–96, https://doi.org/10.1263/jbb.101.87.

[44] M. Blümmel, H.P.S. Makkar, K. Becker, *In vitro* gas production: a technique revisited, J. Anim. Physiol. Anim. Nutr. 77 (1–5) (1997) 24–34, https://doi.org/10.1079/BJN19970089.

[45] S.M.A. Sallam, I.C.S. Bueno, P. Brigide, P.B. Godoy, D.M.S.S. Vitti, A.L. Abdalla, Efficacy of eucalyptus oil on *in vitro* ruminal fermentation and methane production, Méditerranée 85 (2009) 267–272. http://om.ciheam.org/article.php?IDPDF=801016.

[46] H. Zhu, V. Fievez, S. Mao, W. He, W. Zhu, Dose and time response of ruminally infused algae on rumen fermentation characteristics, biohydrogenation and Butyrivibrio group bacteria in goats, J. Anim. Sci. Biotechnol. 7 (22) (2016), https://doi.org/10.1186/s40104-016-0080-1.

[47] S.O. Richard Williams, C.M. Hannah, J.L. Jacobs, W.J. Wales, P.J. Moate, Volatile Fatty Acids in ruminal fluid can be used to predict methane yield of dairy cows, Animals 9 (12) (2019), https://doi.org/10.3390/ani9121006.

[48] B.S. Oldick, J.L. Firkins, Effects of degree of fat saturation on fiber digestion and microbial protein synthesis when diets are fed twelve times daily, J. Anim. Sci. 78 (9) (2000) 2412–2420, https://doi.org/10.2527/2000.7892412x.

[49] F. Dohme, A. Machmüller, B.L. Estermann, P. Pfister, A. Wasserfallen, M. Kreuzer, The role of the rumen ciliate protozoa for the methane suppression caused by coconut oil, Lett. Appl. Microbiol. 29 (3) (1999) 187–192, https://doi.org/10.1046/j.1365-2672.1999.00614.x.

[50] H. Khalilvandi-Behroozyar, M. Dehghan- Banadaky, M. Ghaffarzadeh, K. Rezayazdi, Effects of different fatty acid supplements on rumen fermentation parameters and microbial population in vitro condition. Iran, J. Appl. Anim. Sci. 47 (1) (2016) 1–17, https://doi.org/10.22059/ijas.2016.58191 (In Farsi).

[51] L. Dewanckele, B. Vlaeminck, E. Hernandez-Sanabria, A. Ruiz-González, S. Debruyne, x J. Debruyne, V. Fievez, Rumen biohydrogenation and microbial community changes upon early life supplementation of 22:6n-3 enriched microalgae to goats, Microbiol. 27 (9) (2018) 573, https://doi.org/10.3389/fmicb.2018.00573.

[52] M.R.G. Maia, L.C. Chaudhary, L. Figueres, R.J. Wallace, Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen, Antonie Leeuwenhoek 91 (4) (2007) 303–314, https://doi.org/10.1007/s10482-006-9118-2.

[53] I. Altomonte, F. Salari, R. Licitra, M. Martin, Use of microalgae in ruminant nutrition and implications on milk quality: a review, Livest. Sci. 214 (2018) 25–35, https://doi.org/10.1016/j.livsci.2018.05.006.