



In vitro evaluation of microalgae as a source of polyunsaturated fatty acids for ruminants

Carlos Navarro Marcos¹ · Ana Heras-Molina¹ · Trinidad de Evan² · María Dolores Carro² · Cintia Gómez-Serrano³ · Marta Terré⁴ · Sonia Pérez Garnelo⁵ · Susana Astiz Blanco⁵

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Abstract

Microalgae may be adequate sources of polyunsaturated fatty acids (PUFA) for ruminants, but information regarding their dry matter (DM) digestibility is still scarce. The *in vitro* digestibility of various dried PUFA-rich microalgae for ruminants (*Arthrospira platensis*, *Nannochloropsis gaditana*, *Scenedesmus almeriensis*, *Tetraselmis chuii*, and *Tisochrysis lutea*) was evaluated and the ruminal biohydrogenation of their PUFA assessed. All microalgae had high content of ash (> 78.1 g kg⁻¹ DM), total nitrogen (> 66.3 g kg⁻¹ DM) and ether extract (> 122 g kg⁻¹ DM). The highest levels of α -linolenic acid (C18:3*n*-3), eicosapentaenoic acid (C20:5*n*-3), and docosahexaenoic acid (C22:6*n*-3) were found in *S. almeriensis*, *N. gaditana*, and *T. lutea*, respectively. Most PUFA of microalgae were extensively biohydrogenated by ruminal microorganisms, but biohydrogenation of C20:5*n*-3 in *N. gaditana* and C22:6*n*-3 in *T. lutea* was lower, suggesting that a substantial amount of these PUFA can escape the rumen unaffected. The *in vitro* total DM digestibility (TDMD) of microalgae was high (from 538 to 970 g kg⁻¹ DM). The proportion of DM digested in the rumen in relation to TDMD ranged from 49.3 to 64.5%, indicating that a considerable DM digestion occurred in the intestine. Therefore, the microalgae *N. gaditana* and *T. lutea* are efficient nutritional sources of bypass C20:5*n*-3 and C22:6*n*-3 fatty acids, respectively, for ruminants.

Keywords Microalgae · Polyunsaturated fatty acids · *In vitro* digestibility · Rumen biohydrogenation · Nutritional supplements

Introduction

Supplementation of polyunsaturated fatty acids (PUFA) such as α -linolenic (C18:3*n*-3), eicosapentaenoic (C20:5*n*-3) and docosahexaenoic (C22:6*n*-3) acids in ruminant diets has been associated with improved health and reproductive performance of animals (Mattos et al. 2000; Marques et al. 2019) as well as with an increase in PUFA content in meat and milk that is beneficial for consumers health (Cosentino et al. 2021). Microalgae are good sources of PUFA and are also rich in protein, carbohydrates, and other lipids (Anele et al. 2016). Although microalgae are interesting sources of nutrients for ruminants, they are still rarely used in practice. Inclusion of unconventional feeds in ruminant diets, such as microalgae, requires knowledge on their nutritional value, but available information on the nutritional value of microalgae is still scarce. In addition, their chemical composition is highly variable, particularly regarding lipid and fatty acid (FA) content (Altomonte et al. 2018; Costa et al. 2022). Previous studies (Anele et al. 2016; Altomonte et al. 2018;

✉ Carlos Navarro Marcos
carlon05@ucm.es

✉ Susana Astiz Blanco
astiz.susana@inia.csic.es

¹ Departamento de Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain

² Departamento de Producción Agraria, Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas, Universidad Politécnica de Madrid, Madrid, Spain

³ Departamento de Ingeniería Química, Universidad de Almería, Almería, Spain

⁴ Departament de Producció de Remugants, Institut de Recerca i Tecnologia Agroalimentaris, Caldes de Montbui, Spain

⁵ Departamento de Reproducción Animal, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA-CSIC), Madrid, Spain

Lamminen 2021; Costa et al. 2022) have assessed the potential of various microalgae as a source of PUFA for ruminants, focusing on their chemical composition and effects on ruminal fermentation parameters when they were included in the diet. However, only limited values of digestibility of dry matter (DM) are available (Anele et al. 2016; Wild et al. 2019) despite DM digestibility (DMD) is a crucial parameter to define the feed nutritive value in ruminants.

Methods commonly used to estimate ruminal and intestinal digestibility in both *in situ* and *in vitro* assays require filter polyester bags that were designed for common feeds for ruminants and are not suitable for studies on microalgae due to their microscopic particle size (Lamminen 2021). Recently, ANKOM Technology (Fairport, NY, USA) released the new F58 filter polyester bags with smaller pore size than those currently used (ANKOM F57 bags) for fiber analysis and *in vitro* digestibility assays. The F58 filter bags have been previously used to accurately estimate the *in vitro* digestibility of forages and to study the nutritive value of feeds for ruminants (Valentine et al. 2019). Therefore, the objective of this research was to assess the *in vitro* ruminal (DMDr) and total (TDMD) digestibility of DM by employing F58 filter bags, and the ruminal biohydrogenation of PUFA of five microalgae which, according to the available literature, are rich in PUFA (*Arthrospira platensis*, *Nannochloropsis gaditana*, *Scenedesmus almeriensis*, *Tetraselmis chuii*, and *Tisochrysis lutea*) that may be used as a source of PUFA in ruminant diets.

Material and methods

Microalgae samples

Samples of dried *Arthrospira platensis*, *Nannochloropsis gaditana*, *Scenedesmus almeriensis*, *Tetraselmis chuii*, and *Tisochrysis lutea* were evaluated. *Nannochloropsis gaditana* and *S. almeriensis* samples were cultivated in 3 m³ reactors using agricultural fertilizers as culture medium and spray dried at −18 °C. *Arthrospira platensis*, *T. chuii* and *T. lutea* samples were acquired from commercial providers (Biorizon Biotech S.L., Almería, Spain; Necton - Companhia Portuguesa de Culturas Marinhas S.A., Olhão, Portugal).

In vitro measurements

Ruminal fluid from three adult rumen-fistulated Lacaune female sheep were used independently as source of ruminal microorganisms to obtain three replicates ($n = 3$) in the *in vitro* measurements. Sheep were fed a diet composed of 80% grass hay and 20% commercial high-cereal concentrate. Before the morning feeding, ruminal content (solids and fluid) from each sheep (about 300 g) was collected and

filtered through four layers of surgical gauze to obtain the ruminal fluid. The ruminal fluid of each sheep was independently mixed with pre-warmed (39 °C) buffer solution (Goering and Van Soest 1970) in a 1:4 ratio under a constant flow of CO₂ to obtain three different inocula.

Ruminal dry matter digestibility (DMDr)

The DMDr was assessed by weighting samples of 400 mg of each microalga into ANKOM F58 filter bags (ANKOM Technology, USA), using 9 bags/microalgae. The bags were incubated in three bottles, each containing 1 L of the inoculum from each sheep, which were placed in an ANKOM Daisy II incubator at 39 °C with continuous rotation for 12 h. The incubation time was set to 12 h to simulate the retention time of digesta in the rumen in high-producing dairy animals, taking also into account the faster ruminal passage rate of feedstuffs with small particles size (INRA 2018). Afterwards, bags were rinsed with cold water, dried at 60 °C for 48 h, and weighed again to calculate the DMDr by difference as follows:

$$\text{DMDr} = \frac{\text{Initial sample weight} - \text{Final sample weight}}{\text{Initial sample weight}} \times 100$$

Total and intestinal dry matter digestibility (TMDT)

The TDMD was assessed by weighing samples of each microalgae (800 mg) in 120-mL glass vials (9 vials per sample). Vials were filled with 80 mL of the inoculum (ruminal fluid and buffer mixture) prepared with the ruminal fluid from each sheep (3 vials per microalgae and inoculum), closed with rubber plugs and incubated at 39 °C for 12 h. Three blanks (vials without microalgae) were filled only with each inoculum to correct both for the endogenous DM added with the inoculum to calculate the *in vitro* true digestibility of the microalgae, and for the gas produced by the fermentation of the endogenous substrates. Additionally, 240 mL of each inoculum were collected in plastic containers before filling the vials to determine the initial contribution of FA from each inoculum.

After 12 h incubation the amount of gas produced in each vial was measured using a pressure transducer (DeltaOhm DTP704-2BGI, Herter Instruments SL, Spain) and a calibrated plastic syringe (Ruthe, Normax Marinha Grande, Portugal) and fermentation was immediately stopped by placing the vials in an iced-water bath. The residues in the vials were then pooled by microalgae and inoculum in previously weighed plastic containers and immediately frozen (−80 °C). Afterwards, containers were freeze-dried and weighed again to determine the amount of residue DM. Finally, the freeze-dried residues were used to estimate the TDMD following

the procedure of Marcos et al. (2019a). Briefly, 500 mg of DM of the freeze-dried residues were weighed in triplicate into F58 filter bags, which were incubated in ANKOM Daisy II bottles containing a solution of pepsin at 39 °C under constant rotation for 1 h. Bags were then rinsed with cold water and incubated again in a pancreatin solution at 39 °C for 24 h to simulate intestinal digestion. Bags were washed with cold water, dried at 60 °C for 48 h, and weighed to estimate the amount of undigested residue remaining. The TDMD was estimated by correcting the undigested residue with blanks and differentiating with the initial pooled weighted sample as described previously. For each microalgae, the intestinal digestibility of the DM undigested in the rumen (DMDi) was calculated as the difference between TDMD and DMDr.

Ruminal biohydrogenation of polyunsaturated fatty acids

The FA profile of the microalgae and of the vials' freeze-dried residues after 12 h of incubation and of each ruminal inoculum was analyzed to calculate the ruminal biohydrogenation of each FA (FAb) as follows:

$$FAb = \frac{\text{Initial FA} - \text{Final FA}}{\text{Initial FA}} \times 100$$

The initial FA were calculated as the sum of FA in the microalgae and that in the inoculum, whereas the final FA were the FA in the vial's freeze-dried residues after 12 h of incubation.

Chemical analyses

The DM (ID 934.01), ash (ID 048.13), and ether extract (EE; ID 954.02) content in the microalgae were analyzed according to AOAC methods (AOAC 2005). Total nitrogen (N) content was determined using the Dumas method using a TruSpec CN equipment (Leco Corp., USA). Fatty acids were determined by esterifying the lipid fraction as described by Garcés and Mancha (1993). After tempering, FA methyl esters were extracted and injected in a gas chromatography system (HP 6890 Series GC System, Hewlett Packard, Germany) equipped with a split injector, a flame ionization

detector, and a 30 m x 0.32 mm x 0.25 µm cross linked polyethylene glycol capillary column (Hewlett Packard). C15:0 was used as an internal standard for quantification (g kg⁻¹) of each FA. Total PUFA included C18:2*n*-6, C18:3*n*-3, C18:3*n*-6, C18:4*n*-3, C20:2, C20:3*n*-6, C20:4*n*-6, C20:5*n*-3, C22:4, C22:5*n*-3 and C22:6*n*-3. The whole procedure has been detailed by López-Bote et al. (1997).

Statistical analyses

The experiment was conducted as a completely randomized design. Data were analyzed using a mixed model with PROC MIXED of SAS (SAS 2017). Microalgae were considered a fixed effect, whereas the ruminal inoculum was considered random. Statistical significance was determined at $p < 0.05$. The Tukey's multiple comparison test was used to assess differences between means when a significant effect of microalgae was observed. Pearson correlation coefficients were used to assess correlations between gas production and DMD values (DMDr, DMDi, TDMD), and ruminal PUFA biohydrogenation using the PROC CORR of SAS (SAS 2017).

Results

Chemical composition

Chemical composition of microalgae is shown in Table 1. The average content of ash, N, and EE in the microalgae was 164, 82.1, and 174 g kg⁻¹ DM, respectively. Large differences were observed among microalgae species. The ash content of *N. gaditana*, *T. chuii*, and *T. lutea* was above 180 g kg⁻¹ DM, whereas *A. platensis* and *S. almeriensis* showed values below 80 g of ash per kg DM. Differences among microalgae in N and EE content were less marked than those for ash content, with N content ranging from 66.3 to 110 g kg⁻¹ DM and that of EE from 122 to 268 g kg⁻¹.

The content of C18:3*n*-3 ranged from 0.123 to 36.7 g kg⁻¹ DM in *A. platensis* and *S. almeriensis*, respectively. Both *N. gaditana* and *T. lutea* exhibited large quantities of

Table 1 Chemical composition in g kg⁻¹ of dry matter (DM) of microalgae, unless otherwise stated¹

Item	DM (g kg ⁻¹)	Ash	N	EE	C18:3 <i>n</i> -3	C20:5 <i>n</i> -3	C22:6 <i>n</i> -3	Total PUFA
<i>A. platensis</i>	902	78.1	110	159	0.123	0.100	0.087	16.6
<i>N. gaditana</i>	947	206	67.1	122	9.22	18.5	0.230	38.4
<i>S. almeriensis</i>	941	66.5	96.2	151	36.7	0.158	0.121	43.1
<i>T. chuii</i>	936	288	70.9	172	12.3	2.08	0.043	27.1
<i>T. lutea</i>	916	185	66.3	268	8.13	14.9	1.61	58.4

¹DM: dry matter; N: total nitrogen; EE: ether extract; C18:3*n*-3: α-linolenic acid; C20:5*n*-3: eicosapentaenoic acid; C22:6*n*-3: docosahexaenoic acid; PUFA: polyunsaturated fatty acids

C20:5n–3 compared to *A. platensis*, *S. almeriensis*, and *T. chuii*. The average content of C22:6n–3 in the microalgae was 0.42 g kg^{−1} DM, and the largest value was observed in *T. lutea* (1.61 g kg^{−1} DM). Total PUFA content was the highest in *T. lutea*, followed by *S. almeriensis* and *N. gaditana* (58.4, 43.1, and 38.4 g kg^{−1} DM, respectively).

In vitro measurements

Gas production after 12 h of *in vitro* incubation ranged between 65.5 and 18.8 mL g^{−1} DM (Table 2). *Arthrospira platensis* produced the highest volume of gas, while *T. lutea* produced the least, with the remaining microalgae exhibiting intermediate values ($p < 0.05$). The average value for DMDr was 452 g kg^{−1} DM, with *T. chuii* showing significantly lower DMDr, whereas *A. platensis* showed the highest value ($p < 0.05$). The mean values for DMDi and TDMD were 717 and 826 g kg^{−1} DM, respectively. Both DMDi and TDMD values for *T. chuii* were significantly lower than those of the other microalgae. Although some variation was observed among the remaining microalgae, their values were comparable, with significant differences ($p < 0.05$).

The lowest *in vitro* ruminal biohydrogenation rates of C18:3n–3 and C20:5n–3 were observed in *N. gaditana* (51.0 and 33.1 %, respectively; Table 3), whereas the highest were observed in *T. lutea* (92.9 and 97.2 %). An increase in the amount of C22:6n–3 after the *in vitro* incubation was evidenced in *T. lutea*, whereas the average biohydrogenation was 58.3 % in the other microalgae. Biohydrogenation of PUFA was greater ($p < 0.05$) in *A. platensis*, *S. almeriensis*, and *T. chuii* compared with *N. gaditana* and *T. lutea*.

No correlations between gas production and DMDr, DMDi or TDMD were observed ($p > 0.05$; Table 4). In contrast, gas production was negatively correlated with the biohydrogenation rates of C18:3n–3 and C20:5n–3 ($p < 0.05$). Positive correlations were found among DMDr, DMDi, and

Table 2 *In vitro* gas production (Gas; mL g^{−1} dry matter (DM)), ruminal dry matter digestibility (DMDr; g kg^{−1} DM), intestinal digestibility of the DM undigested in the rumen (DMDi; g kg^{−1} DM), and total DM digestibility (TDMD; g kg^{−1} DM) of microalgae samples ($n = 3$)

Item	Gas	DMDr	DMDi	TDMD
<i>A. platensis</i>	65.5a	587a	781a	910a
<i>N. gaditana</i>	41.9b	411c	723a	819b
<i>S. almeriensis</i>	23.2cd	514b	926a	970a
<i>T. chuii</i>	31.3c	265d	366b	538c
<i>T. lutea</i>	18.8d	483bc	791a	892ab
SEM ¹	1.89	13.9	32.2	15.8
<i>p</i> -Value	<0.001	<0.001	0.001	<0.001

¹standard error of the mean, a–d values with different superscript letters in the same column differ ($p < 0.05$).

Table 3 *In vitro* ruminal biohydrogenation (%) of α -linolenic (C18:3n–3), eicosapentaenoic (C20:5n–3), docosahexaenoic (C22:6n–3) acids, and total polyunsaturated fatty acids (PUFA) of microalgae samples after incubation with ruminal fluid from sheep for 12 h ($n = 3$)

Item	C18:3n–3	C20:5n–3	C22:6n–3	Total PUFA
<i>A. platensis</i>	63.9bc	40.2c	53.0a	76.1a
<i>N. gaditana</i>	51.0c	33.1c	70.2a	36.6b
<i>S. almeriensis</i>	89.6a	92.3ab	70.7a	73.3a
<i>T. chuii</i>	81.5ab	50.0bc	39.4a	80.0a
<i>T. lutea</i>	92.9a	97.2a	−472b	54.0b
SEM ¹	4.24	8.59	6.61	3.71
<i>p</i> -Value	<0.001	0.004	<0.001	<0.001

¹standard error of the mean, a–c values with different superscript letters in the same column differ ($p < 0.05$).

TDMD ($p < 0.05$). Additionally, DMDi and TDMD were positively correlated with C22:6n–3 biohydrogenation rate. No significant correlations were identified between DMDr and either individual fatty acids or total PUFA biohydrogenation rates ($p > 0.05$).

Discussion

Chemical composition values are within the range reported by Lamminen et al. (2021), although the ash content of *N. gaditana* and *T. chuii*, and the EE content of *A. platensis* were slightly greater in our samples than those reported in the literature. In addition, contents reported by Lamminen et al. (2021) of C20:5n–3 and C22:6n–3 for *T. lutea* were lower and higher, respectively, than those found in our study.

Table 4 Pearson correlation coefficients between *in vitro* gas production (Gas), ruminal dry matter (DM) digestibility (DMDr), intestinal digestibility of the DM undigested in the rumen (DMDi), and total DM digestibility (TDMD), and the *in vitro* ruminal biohydrogenation of α -linolenic (C18:3n–3), eicosapentaenoic (C20:5n–3), docosahexaenoic (C22:6n–3) acids, and total polyunsaturated fatty acids (PUFA) in microalgae samples. Only *p*-values < 0.05 are shown ($n = 15$).

Item	Gas	DMDr	DMDi	TDMD
Gas				
DMDr				
DMDi		0.76		
TDMD		0.84	0.97	
C18:3n–3	−0.72			
C20:5n–3	−0.74			
C22:6n–3 ¹			0.82	0.72
Total PUFA				

¹excluding *T. lutea* ($n = 12$).

The high EE and PUFA content observed in the microalgae evaluated suggests their potential use as a source of PUFA. Nonetheless, the high ash content, often associated with elevated levels of heavy metals such as copper, could pose a risk to animal health, thereby limiting their inclusion in animal diets (Campbell et al. 2020). Microalgae are generally recognized as a good source of protein, but the nutritional quality depends on their amino acid profile. Ruminant microorganisms are capable of utilizing both organic and inorganic nitrogen, and therefore, the high nitrogen content of these microalgae suggests they could serve as valuable nitrogen sources for ruminants. Significant variation in the chemical composition among microalgae species was observed, consistent with previous findings (Lamminen 2021; Costa et al. 2022). Moreover, production and environmental factors, such as N limitation, temperature or light can influence to the chemical composition and characteristics of microalgae (Lamminen 2021). Altogether, this highlights the importance of evaluating individual microalgae species prior to their inclusion in rations for ruminant animals.

Anele et al. (2016) determined the DMDr of various microalgae and reported greater values for *Nannochloropsis granulata* and *Scenedesmus* spp., as well as for other microalgae (654–797 g kg⁻¹ DM). However, results cannot be directly compared due to differences in the duration of the *in vitro* fermentations (24 h in the study of Anele et al. (2016) and 12 h in the present study). The values reported in this study indicate a moderate DMDr, comparable to that typically observed in forages, but the low gas production reported here indicate a low ruminal digestibility (Getachew et al. 2004). Gas produced during *in vitro* ruminal fermentations is proportionally associated to the carbohydrates and protein fermented in the rumen (Menke et al. 1979) and consequently, a positive correlation between gas production and DMDr is expected. During ruminal fermentation, ash and EE do not generate gas (Getachew et al. 1998). Therefore, the high ash and EE content in the microalgae, and consequently the lower protein and carbohydrate content, may partly explain the lower gas production and DMDr values observed. It is important to note that the remaining fraction after excluding EE and ash content do not necessarily correspond to protein or carbohydrate fractions, as other components, such as chlorophyll, non-protein nitrogen, and B vitamins, may contribute significantly to microalgae composition (Anele et al. 2016). Alternatively, long-chain unsaturated fatty acids may impair the *in vitro* ruminal fermentation (Maia et al. 2007), potentially explaining the observed results. However, other studies have reported no adverse effects of high EE and PUFA contents on the *in vitro* ruminal fermentation (Marcos et al. 2019b). Including large quantities of microalgae in ruminant diets may impair ruminal fermentation and reduce productivity. Ultimately, *in vivo* studies are needed to validate *in vitro* results and confirm

that no negative effects are observed when microalgae are included in rations for ruminants.

To our knowledge, there is no previous information regarding the *in vitro* DMDi or TDMD of microalgae but previous studies have suggested that the cell walls of certain microalgae may be highly degradable (Anele et al. 2016; Vizcaíno et al. 2016). The large DMDi and TDMD values determined for most microalgae studied in the current work agree with those statements and suggest that they are highly digestible in the intestine, despite the low DMDr of some of them. Ruminal digestion differs significantly from intestinal digestion. In the rumen, microorganisms degrade and ferment feed, while in the intestine, enzymatic digestion takes place. Complex carbohydrates and other components of the microalgal cell wall may be difficult for ruminal microorganisms to break down, whereas these structures are more susceptible to the acidic and enzymatic digestion that occurs post-ruminally (Alves et al. 2018). However, there were high variations in the fraction undigested in the rumen (bypass fraction), likely attributable to differences in cell wall composition among microalgae, which can influence the ruminal digestibility. Nonetheless, *T. chuii* showed values that deviated notably from values reported among the other microalgae. It is possible that the DMDr, DMDi, and TDMD of *T. chuii* had been underestimated in our study. Low digestibility in ruminant feeds is often associated to cell walls resistance to ruminal microorganisms or digestive enzymes, but the low pH in the intestinal digestibility assay can disrupt cell walls, leading to higher DMDi values. It could be hypothesized that *T. chuii* either could have clogged the pores of the F58 bags during *in vitro* assays or formed a gel-like substance that restricted the access to ruminal microorganisms and/or the digestive enzymes (pepsin and pancreatin).

Ruminal DM digestibility is often positively associated with FA biohydrogenation. In this study, no significant correlations were found between DMDr and FA biohydrogenation rates. However, a positive correlation between DMDr and total PUFA biohydrogenation was observed when *T. chuii* was excluded from the analysis ($r = 0.77$, $p = 0.006$, $n = 12$). Additionally, the negative correlations between gas production and the biohydrogenation rates of C18:3n-3 and C20:5n-3 may reflect the negative effect of long-chain unsaturated FA on DMDr (Maia et al. 2007), which could, in turn, influence ruminal biohydrogenation. In agreement, Anele et al. (2016) reported high variability in biohydrogenation rates of FA in microalgae, as well as large biohydrogenation rates of PUFA after 24 h of *in vitro* fermentation, which agree with the variability and large DMDr reported in that study. In addition, Anele et al. (2016) reported an increase of C20:5n-3 in *N. granulata* after *in vitro* fermentation. Long-chain FA in microalgae may be part of the cell wall, thus posing difficulties for their extraction (Blasio and Balzano 2021). Possibly, FA extraction from the dried *T. lutea* sample was incomplete

initially, but FA were more easily extracted after fermentation due to the degradative activity of ruminal microorganisms. This might help explain these results, although it has been reported that *T. lutea* has no distinct cell wall (Vizcaíno et al. 2016). Nevertheless, C22:6n–3 might be a component of another structure that complicates its extraction. Although the *in vitro* intestinal digestibility of PUFA was not measured, the large DMDi and TDMD values suggest that PUFA might be highly digestible, and that *N. gaditana* and *T. lutea* could be good sources of bypass FA acids, which agrees with Alves et al. (2018).

Conclusions

The dry matter of the microalgae was highly digestible, with a significant portion being digested in the intestine. Although polyunsaturated fatty acids were extensively biohydrogenated by ruminal microorganisms, a substantial amount of C20:5n–3 from *N. gaditana* and C22:6n–3 from *T. lutea* escaped the rumen unaffected. These findings suggest that *N. gaditana* and *T. lutea* are efficient nutritional sources of bypass C20:5n–3 and C22:6n–3, respectively, for ruminants. However, these findings require confirmation through *in vivo* studies.

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Data availability Data are available from the corresponding author upon reasonable request.

Declarations

Statement of informed consent, human/animal rights All experimental procedures were performed in compliance with relevant laws and institutional guidelines and have been approved by the Institutional Animal Care and Use Committee of the Comunidad Autónoma de Madrid of Spain (PROEX 212.2/22).

Competing interest The authors declare no competing interests.

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