Freeze-dried *Nannochloropsis oceanica* biomass protects eicosapentaenoic acid (EPA) from metabolization in the rumen of lambs

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Eicosapentaenoic acid (EPA) from freeze-dried biomass of *Nannochloropsis oceanica* microalgae resists ruminal biohydrogenation in vitro, but in vivo demonstration is needed. Therefore, the present study was designed to test the rumen protective effects of *N. oceanica* in lambs. Twenty-eight lambs were assigned to one of four diets: Control (C); and C diets supplemented with: 1.2% *Nannochloropsis* sp. oil (O); 12.3% spray-dried *N. oceanica* (SD); or 9.2% *N. oceanica* (FD), to achieve 3 g EPA/kg dry matter. Lambs were slaughtered after 3 weeks and digestive contents and ruminal wall samples were collected. EPA concentration in the rumen of lambs fed FD was about 50% higher than lambs fed SD or O diets. Nevertheless, the high levels of EPA in cecum and faeces of animals fed *N. oceanica* biomass, independently of the drying method, suggests that EPA was not completely released and absorbed in the small intestine. Furthermore, supplementation with EPA sources also affected the ruminal biohydrogenation of C18 fatty acids, mitigating the shift from the t₁₀ biohydrogenation pathways to the t₁₁ pathways compared to the Control diet. Overall, our results demonstrate that FD *N. oceanica* biomass is a natural rumen-protected source of EPA to ruminants.

The health benefits associated with the consumption of omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA), particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are well known¹,². However, in ruminant edible fats their content is very low even when animals are supplemented with enriched n-3 LC-PUFA diets. The explanation for this finding relies on the ruminal microbiota intervention over the dietary lipids. Indeed, rumen microbes have the capacity to hydrolyse lipids and subsequently biohydrogenate the unsaturated fatty acids (FA). The biohydrogenation involves isomerization and hydrogenation of FA double bonds, forming a wide range of FA intermediates and saturated FA as end products, which will further alter the FA profile of ruminant edible products³.

Strategies to enhance the content of n-3 LC-PUFA in ruminant edible fat include the dietary supplementation with products derived from the marine food chain, as fish oil or microalgae, which are naturally enriched in EPA and DHA⁴,⁵. However, the efficiency is rather low as it is well established that both EPA and DHA undergo extensive biohydrogenation in the rumen⁴,⁵. Thus, rumen-protected marine-derived supplements could be the most effective way to increase the concentration of EPA and DHA in ruminant derived foods.

Indeed, several rumen protection technologies have been proposed but often with low efficacy and with application difficulties⁶. Furthermore, the bypass must allow post-ruminal release once it reaches the small intestine. Calcium salts have a disadvantage related to dissociation in lower pH, the limited amount of protectable polyunsaturated fatty acids (PUFA) and the need of free FA to create an ionic bond between the free carboxyl group of the FA and Ca ions. Formaldehyde is a carcinogenic compound; the reaction is untargeted, and it is an expensive
resource. Other methods such as fatty acyl amides, non-enzymatic browning, lipid composite gels, encapsulation within lipids and protein crosslinking, all have several disadvantages as many of these technologies use harmful products, are not cost-effective, or are lacking consistency regarding rumen protection efficiency. Thus, the identification of PUFA sources naturally protected from rumen metabolism is highly relevant and promising.

Consequently, using in vitro batch incubations with rumen inoculum, our team was able to identify that biomass of *Nannochloropsis oceanica* microalgae was a partially protected source of EPA. The protection against rumen biohydrogenation was moderate with spray-dried (SD) biomass and exceptionally high when freeze-dried (FD) biomass was used. Microalgae are usually included in diet formulations after dehydration of the slurry biomass using industrial-scale SD technology. Dehydration by freeze-drying seems better in preserving the structural components and nutritional properties than SD. Thus, the higher rumen-protection of EPA observed in vitro with FD biomass was probably due to better preservation of *N. oceanica* cell walls than with SD biomass. However, the better preservation of cell walls might limit the post-ruminal release and availability for the EPA’s absorption.

The objective of the present study was to evaluate FD *N. oceanica* as a natural rumen-protected source of EPA in vivo. We hypothesized that lambs fed a diet containing FD *N. oceanica* biomass would present a higher content of EPA through the main gastrointestinal tract compartments, comparing to those fed diets with SD biomass, or with *Nannochloropsis sp*. Oil (O).

**Results**

**Abundance of EPA in digestive tract and whole tract apparent digestibility.** The proportion of EPA in the rumen was higher (*P* < 0.01) in the FD treatment (Fig. 1a), reaching 3.5% of total FA and dimethyl acetics (DMA) (1.6 mg/g DM), compared to SD and O treatments, which reached 1.8% and 1.1% of total FA + DMA, respectively. In the abomasal digesta the highest proportion (*P* = 0.001) of EPA (Fig. 1b) was found in...
FD treatment (3.4% of total FA + DMA) followed by O and SD treatments that averaged 2% of total FA + DMA. The EPA in cecum digesta and faeces (Fig. 1c,d, respectively) also differed (P < 0.001) among treatments, however both FD and SD treatments equally registered the higher content compared to O treatment. The estimated whole tract EPA apparent digestibility was significantly different between diets (P = 0.002), being lowest in the FD diet (74.0% ± 2.31) fed lambs (Fig. 2a). The O treatment registered the highest EPA whole tract apparent digestibility (93.8% ± 0.38) and SD treatment presented an intermediate level (82.4% ± 1.02). The post-ruminal apparent digestibility also differed among diets (P = 0.012) being highest in O diet (65.8% ± 4.34) and lowest in SD diet (33.4% ± 9.31) (Fig. 2b), while FD did not differ from both O and SD diets. The estimated biohydrogenation of EPA (Fig. 2c) differed among the Nannochloropsis treatments (P < 0.001), increasing from 44.7% with FD diets to 69.8% with SD and 80.7% with O diet, respectively.

Scanning electron microscopy (SEM) of N. oceanica biomass. The microscopic evaluation of the surfaces of SD and FD biomass incorporated in the diets revealed two different patterns (Fig. 3a–h). The FD biomass (Fig. 3a) consisted of a cluster of perfectly individualized cells (although organized in plate-like structures) that maintained an overall spherical shape. In contrast, SD biomass (Fig. 3b) consisted of a set of large amorphous granules consisting of clustered microalgae cells that loss the spherical structure, and thus they could not be easily individualized. Apparently, cell wall integrity was maintained in FD biomass but not in the SD form. Both FD and SD presented a wrinkled and slightly collapsed cell wall, although more severe alterations were found in the SD that presented fractured granules (Fig. 3g), and irregular shaped holes and cracks (Fig. 3h).

Fatty acid composition of digesta contents. The total FA content, including DMA, and the partial FA sums in the rumen, abomasal and cecum digesta of lambs fed control (C) and Nannochloropsis supplemented diets are presented in Table 1. The complete FA and DMA profile are presented in the Supplementary Tables S1, S2 and S3.
Figure 3. Scanning Electron Microscopy Focused Ion Beam (SEM–FIB) of freeze-dried (FD) and spray-dried (SD) N. oceanica. Stabilization in carbon matrix and gold–palladium coating. (a) Granules correspond to individualized FD N. oceanica cells; (b) Granules correspond to clusters of more than one SD N. oceanica cell; (c) Individual FD N. oceanica cell; (d) SD N. oceanica cells cluster; (e) FD N. oceanica cell wall appearance detail; (f) SD N. oceanica cell walls appearance in detail; (g) SD N. oceanica fragmented granule; (h) SD N. oceanica granule surface in detail.
Table 1. Total FA and dimethyl acetals content (mg/g DM) and FA partial sums (% total FA + DMA) in the rumen, abomasum, and cecum. Values are means ± standard error of the mean. Means within a row with different letters are significantly different (P < 0.05).

Diet(s): C, control; O, Control plus 1.2% *Nannochloropsis* sp. oil; SD, control plus 12.3% spray-dried *Nannochloropsis oceanica*; FD, control plus 9.2% freeze-dried *N. oceanica*.

| Item | Diets | C | O | SD | FD | P-value |
|------|-------|---|---|----|----|---------|
| **Rumen** | | | | | | |
| Total FA + DMA | | 36.36±3.028 | 42.76±4.461 | 38.80±1.545 | 45.79±1.143 | 0.004 |
| SFA | | 49.28±5.569 | 38.62±2.274 | 52.47±3.900 | 44.01±1.243 | 0.027 |
| MUFA | | 28.60±4.662 | 30.40±3.042 | 25.76±2.648 | 30.35±0.586 | 0.415 |
| trans-MUFA | | 26.24±4.830 | 26.20±2.948 | 20.06±2.434 | 21.90±0.454 | 0.347 |
| PUFA | | 12.18±1.279 | 17.75±1.569 | 12.66±1.657 | 17.70±1.387 | 0.012 |
| DMA | | 4.87±0.328 | 3.96±0.171 | 3.82±0.284 | 3.44±0.307 | 0.030 |
| C16:1 FA | | 0.23±0.030 | 1.78±0.177 | 2.75±0.648 | 5.93±0.436 | <0.001 |
| RCFA | | 4.07±0.261 | 3.89±0.232 | 3.25±0.199 | 2.92±0.249 | 0.009 |
| C18 FA | | 66.62±0.601 | 52.15±3.302 | 47.82±0.397 | 43.81±0.1178 | <0.001 |
| C18 BI | | 29.52±4.817 | 31.41±3.182 | 24.18±2.541 | 26.20±0.556 | 0.316 |
| **Abomasum** | | | | | | |
| Total FA + DMA | | 42.43±6.529 | 33.74±4.053 | 49.24±3.165 | 47.32±5.112 | 0.043 |
| SFA | | 50.50±6.970 | 41.20±2.226 | 50.74±3.463 | 44.14±0.899 | 0.133 |
| MUFA | | 30.35±5.149 | 33.81±2.966 | 30.05±2.819 | 33.34±0.643 | 0.655 |
| trans-MUFA | | 19.26±4.351 | 23.01±3.199 | 17.12±2.526 | 16.81±0.644 | 0.303 |
| PUFA | | 11.44±1.672 | 16.75±1.592 | 11.82±0.586 | 16.08±1.090 | 0.003 |
| DMA | | 2.68±0.191 | 2.32±0.228 | 1.91±0.154 | 2.08±0.285 | 0.036 |
| C16:1 FA | | 0.48±0.047 | 2.75±0.278 | 3.75±0.508 | 6.96±0.438 | <0.001 |
| C18 FA | | 68.22±0.387 | 54.63±2.041 | 48.59±0.547 | 43.58±0.500 | <0.001 |
| C18 BI | | 21.08±4.310 | 25.60±3.320 | 20.51±2.528 | 19.24±0.946 | 0.336 |
| **Cecum** | | | | | | |
| Total FA + DMA | | 42.02±8.230 | 34.88±6.677 | 28.42±5.836 | 27.50±3.459 | 0.376 |
| SFA | | 58.81±6.640 | 48.39±2.335 | 56.41±2.962 | 55.62±1.083 | 0.055 |
| MUFA | | 22.38±4.454 | 26.85±2.943 | 17.58±2.217 | 18.32±0.747 | 0.048 |
| trans-MUFA | | 21.42±4.207 | 23.26±2.816 | 13.13±2.284 | 12.07±0.785 | 0.003 |
| PUFA | | 6.14±0.874 | 12.71±1.079 | 12.23±1.121 | 14.18±0.830 | <0.001 |
| DMA | | 4.26±1.354 | 4.91±1.413 | 5.25±0.886 | 5.01±0.608 | 0.943 |
| BCFA | | 6.79±1.013 | 6.34±0.838 | 7.91±1.369 | 6.31±0.370 | 0.706 |
| C16:1 FA | | 0.24±0.047 | 1.21±0.177 | 2.56±0.328 | 4.44±0.559 | <0.001 |
| C18 FA | | 57.37±2.984 | 47.98±3.806 | 34.06±4.010 | 30.86±1.361 | <0.001 |
| C18 BI | | 17.54±3.860 | 21.70±2.659 | 11.33±2.303 | 11.59±0.817 | 0.008 |
| Total C20 FA | | 1.65±0.363 | 5.69±0.461 | 8.56±0.520 | 8.79±0.354 | <0.001 |
| C20:1 FA | | 0.05±0.027 | 0.45±0.127 | 0.25±0.055 | 0.21±0.041 | 0.003 |
| C20:2 FA | | 0.61±0.579 | 1.13±0.285 | 0.81±0.249 | 0.60±0.183 | 0.479 |
| C20:3 FA | | n.d | 1.08±0.230 | 0.61±0.178 | 0.94±0.101 | 0.212 |
| C20:4 FA | | n.d | 0.33±0.068 | 0.12±0.016 | 0.23±0.056 | 0.012 |
Ruminal digesta. Average FA and DMA content was highest in the FD (45.8 mg/g DM, \(P = 0.001\)), but it did not differ from O treatment (42.8 mg/g DM). The lowest total FA content (36.4 mg/g DM) was observed in the C treatment, but it did not differ from the O and SD treatments (Table 1). The proportion of saturated fatty acids (SFA) was affected by treatments (\(P = 0.027\)), being highest in the SD and lowest in the O treatment (52.5% and 38.6% of total FA + DMA, respectively). Neither monounsaturated FA (MUFA) nor trans-MUFA proportions in the rumen differed among treatments (\(P = 0.415\) and \(P = 0.347\), respectively), as there were no differences in total C18 biohydrogenation intermediates (BI). On the contrary, PUFA were higher in the O and FD treatments (averaging 17.7% of total FA + DMA) compared to C and SD treatments (averaging 12.6% of total FA + DMA). The branched-chain fatty acids (BCFA) were higher in the rumen of lambs fed C and O and lower in those fed SD and FD diets. The highest proportion of the total C20 FA was found in the FD treatment (9.1% of total FA + DMA), followed by SD and O treatments (7.7% and 7.1% of total FA + DMA, respectively), and the C showed the lowest proportion (1.4% of total FA + DMA) (Table 1).

Abomasal digesta. Average FA and DMA content was lowest for animals fed O diet (33.7 mg/g DM), although not different from animals fed C diet (42.4 mg/g DM), while the highest content was observed in animal fed SD (49.2 mg/g DM), but it did not differ from those fed FD and C diets. Contrary to what was observed for the rumen contents, SFA did not differ among treatments (\(P = 0.133\)), but similarly, to what was observed in the rumen, the proportion of MUFA, trans-MUFA and C18 BI did not differ (\(P > 0.05\)) among treatments. The proportion of PUFA was higher (\(P = 0.003\)) in animals fed O and FD diets (averaging 16.4% of total FA + DMA) compared to C and SD diets, (averaging 11.6% of total FA + DMA). Similarly, to what was observed in the rumen, the total C18 FA differed among treatments (\(P < 0.001\)), being highest and lowest in the C (68.2%) and FD fed lambs (43.6%), respectively. The total DMA proportion was highest in C (2.7%) and lowest in SD treatments (1.9%) while O and FD presented intermediate proportions. The highest BCFA proportion was also observed in C diet (4.0% of total FA + DMA) but the lowest proportion was observed in FD diet (2.7% of total FA + DMA). The highest proportion of total C20 was observed in FD diet (9.8% of total FA), while in C diet it only reached 2.4% of total FA. There were also differences (\(P < 0.001\)) among treatments for the sums of C20:2, C20:3 and C20:4.

Caecal digesta. Average FA + DMA content did not differ (\(P = 0.376\)) among treatments (Table 1). Following the same trend that was verified in the ruminal content, the proportion of SFA showed a tendency (\(P = 0.055\)) for being higher in the cecum of C, SD, and FD lambs (averaging 57.0% of total FA + DMA) than in the O fed lambs (48.4% total FA + DMA). Contrary to what was observed in both rumen and abomasum, the proportion of MUFA and trans-MUFA in cecum differed among treatments (\(P < 0.05\)), being the lowest proportions found in FD and SD fed animals. Moreover, total C18 FA and C18 BI differed among treatments (\(P < 0.001\) and \(P = 0.008\), respectively) being highest in the C and O treatment and lowest in the SD and FD treatments. In the cecum, neither total DMA nor BCFA differed among treatments (\(P > 0.05\)). PUFA presented higher proportions (\(P < 0.001\)) in Nannochloropsis fed lambs (averaging about 13% of total FA) compared to C fed animals that only showed 6.1% of total FA. The proportions of total C20 FA were higher (\(P < 0.001\)) for FD and SD treatments (8.8 and 8.6% of total FA + DMA, respectively) compared to O treatment (5.7% of total FA + DMA).

Biohydrogenation of C18 PUFA in the rumen. Diets were not formulated considering the C18 FA, nevertheless the content of the C18 FA (g/kg DM) were similar among diets. Also, to better evaluate the effect of Nannochloropsis supplementation on the biohydrogenation of C18 PUFA in the rumen, the profile of C18 FA was expressed as a percentage of total C18 FA (Table 2). Although the total C18 FA (mg/g DM) only tended to differ (\(P = 0.080\)) across treatments, several C18 BI differed statistically among treatments. The major BI were the \(t_10\)-18:1 and \(t_11\)-18:1. The \(t_11\)-18:1 was higher with Nannochloropsis supplemented diets (averaging 14.1% of total C18 FA) than with the C diet. On the contrary, the \(t_10\)-18:1 tended (\(P = 0.057\)) to be highest in C and lowest in SD treatments. The ratio between \(t_10\)-18:1 and \(t_11\)-18:1 (\(t_10\)/\(t_{11}\)-18:1 ratio) was highest in the C treatment (7.6), although it did not differ from O treatment (6.5) due to one sample with a very high \(t_10\)/\(t_{11}\)-18:1 ratio (Fig. 4), and it was lowest in the SD and FD treatments (0.47 and 1.69, respectively).

Other rumen C18 BI differed (\(P < 0.05\)) among treatments, namely \(t_9\)-18:1, c16-18:1, and \(t_9\)c12-18:2 were highest in FD, and the \(t_12\)-18:1, c12-18:1, c13-18:1 and oxo-18:0 were highest in SD, whereas the C treatment presented the lowest proportions of these BI (Table 2). No differences among treatments were observed for 18:2n-6, but the 18:3n-3 was 43% higher with Nannochloropsis supplemented diets than with C diet (\(P = 0.001\)). In contrast, the 18:0 was about two folds higher with C diet (40% of total C18 FA) than with FD diet (21%), and the O and SD diets presented intermediate values, i.e., 22% and 35% of total C18 FA, respectively (Table 2).

Consistent with these results, the estimated biohydrogenation of 18:2n-6 did not differ (\(P = 0.133\)) among treatments (Table 2), whereas the biohydrogenation of 18:3n-3 was highest in C and lowest in SD group (\(P < 0.001\)). Also, the estimated biohydrogenation completeness differed (\(P = 0.01\)) among treatments, reaching the highest value with C (55.0%) and the lowest with O and FD treatments (averaging 30.3%), while the biohydrogenation completeness in SD did not differ from the other treatments.

Fermentation parameters, protozoa counting, and rumen mucosa evaluation. Rumen pH averaged approximately 6.0 ± 0.26 in animals consuming different diets and did not differ (\(P = 0.782\)) among them (Table 3). Total volatile fatty acids (VFA) concentration, which averaged 222 mmol/L, the molar proportions of linear chain VFA (2.0, 3.0, 4.0, 5.0 and 6.0) and the branched-chain VFA (iso-4:0 and iso-5:0) did not differ among treatments (\(P > 0.05\)).
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Most of the lambs were defaunated and only 3 presented countable rumen ciliate protozoa. Two lambs consuming SD diet had counts of $2.3 \times 10^6$ and $7.5 \times 10^5$ ciliate cells per mL and one animal consuming O diet had a count of $2.3 \times 10^6$ ciliate cells per mL. *Isotricha* genus was only found in one animal fed SD diet ($1.0 \times 10^5$ cells per mL). *Entodinium* sp. were found in three lambs, two from the SD group having $2.2 \times 10^6$ and $7.5 \times 10^5$ cells per mL and one from the O fed group having $1.3 \times 10^6$ cells per mL.

Regarding the evaluation of rumen mucosa integrity in what to concerns to the presence of parakeratotic lesions, none of the histometric indicators (i.e., papillae length, epithelium thickness, and *stratum corneum* thickness) differed among treatments ($P > 0.120$) as shown in Table 3. Image greyscale values from ruminal mucosa digital photograph processing did not differ among treatments ($P = 0.867$) as well.

### Discussion

In a previous in vitro study,$^7$ we found that EPA disappearance from *N. oceanica* dried biomass, after incubation with rumen fluid for 24 h, was reduced compared with non-esterified EPA (unprotected EPA) and that the EPA disappearance was 25% lower when *N. oceanica* biomass was FD than SD. We hypothesised that protection against biohydrogenation was somehow related to *N. oceanica* cell wall's structure and that the freeze-drying would better preserve the integrity of cell walls.$^7$ The present experiment was designed to confirm in vivo our previous findings related to the higher capacity of FD *N. oceanica* to decrease EPA disappearance on in vitro rumen batch incubations compared to SD *N. oceanica*. Thus, we designed the lamb diets to provide similar amounts of EPA, via free *Nannochloropsis* sp. oil or *N. oceanica* biomass, either SD or FD. However, after diet sampling, the final EPA content was slightly lower in FD diet compared to SD or O diets. This could be related with losses during handling, feed preparation or differences among slurry batches. Despite that, the results confirmed that

### Table 2. Total C18 fatty acid content (mg/g DM) and composition (% of total C18) in the rumen and biohydrogenation (BH) indicators. Values are means ± standard error of the mean. Means within a row with different letters are significantly different ($P < 0.05$). *Diets (or treatments):* C, control; O, Control plus 1.2% *Nannochloropsis* sp. oil; SD, control plus 12.3% spray-dried *Nannochloropsis oceanica*; FD, control plus 9.2% freeze-dried *N. oceanica*. *C18 FA,* Fatty acids with 18 carbon chain length. *C18 biohydrogenation completeness (%) that was estimated considering the maximum 18:0 in the rumen and assuming a complete biohydrogenation of the C18 FA from the diet. n.d = not detected.

| Item | Diets* | C | SD | O | FD | P-value |
|------|--------|---|----|---|----|---------|
| Total C18 FA | 24.26 ± 2.185 | 22.15 ± 2.41 | 18.57 ± 0.81 | 20.06 ± 0.49 | 0.080 |
| 18:0 | 39.86± 7.145 | 21.86± 2.751 | 35.28± 6.744 | 20.76± 1.889 | 0.034 |
| 16:0/18:1 | 1.32± 0.312 | 2.24± 0.305 | 2.00± 0.350 | 2.36± 0.171 | 0.056 |
| 19:1-18:1 | 0.61± 0.0221 | 1.43± 0.203 | 1.60± 0.252 | 1.69± 0.107 | 0.002 |
| 110-18:1 | 20.80± 6.187 | 18.64± 5.655 | 6.66± 2.089 | 12.4± 2.718 | 0.057 |
| 11-18:1 | 3.46± 0.1078 | 11.94± 3.550 | 15.48± 2.911 | 14.92± 3.168 | 0.001 |
| 12-18:1 | 1.12± 0.113 | 1.29± 0.253 | 2.17± 0.206 | 2.10± 0.315 | 0.001 |
| 113/14:0-9-18:1 | 10.83± 1.259 | 12.54± 1.041 | 12.05± 1.880 | 14.66± 1.018 | 0.143 |
| 115-18:1 | 0.84± 0.101 | 0.46± 0.167 | 1.22± 0.177 | 0.98± 0.183 | 0.036 |
| <c1-18:1 | 1.31± 0.248 | 2.05± 0.239 | 1.97± 0.169 | 2.64± 0.247 | 0.010 |
| <c2-18:1 | 0.52± 0.097 | 0.31± 0.110 | 1.10± 0.199 | 0.82± 0.064 | 0.012 |
| <c3-18:1 | 0.08± 0.015 | 0.09± 0.015 | 0.14± 0.016 | 0.13± 0.024 | 0.026 |
| n16/14-18:1 | 0.52± 0.099 | 0.29± 0.145 | 0.80± 0.161 | 0.53± 0.148 | 0.169 |
| <c15-18:1 | 0.41± 0.113 | 0.58± 0.127 | 0.24± 0.041 | 0.56± 0.168 | 0.038 |
| c16-18:1 | 0.13± 0.013 | 0.20± 0.020 | 0.19± 0.034 | 0.25± 0.037 | 0.003 |
| t-18:2 | 0.13± 0.033 | 0.36± 0.138 | 0.10± 0.018 | 0.13± 0.027 | 0.224 |
| 9,c12-18:2 | 0.14± 0.018 | 0.29± 0.092 | 0.29± 0.105 | 0.47± 0.112 | 0.006 |
| n11/15:10,15-18:2 | 0.66± 0.155 | 2.97± 0.742 | 0.55± 0.147 | 1.48± 0.339 | 0.007 |
| 18:2n-6 | 13.52± 1.681 | 15.56± 1.256 | 10.43± 2.532 | 15.84± 1.957 | 0.279 |
| <c9,11-CLA | n.d | 0.18± 0.022 | 0.40± 0.108 | 0.27± 0.061 | 0.102 |
| t-t-CLA | 0.26± 0.015 | 0.23± 0.036 | 0.37± 0.072 | 0.73± 0.267 | 0.119 |
| 18:3n-3 | 2.33± 0.215 | 3.52± 0.269 | 3.73± 0.114 | 3.58± 0.169 | 0.001 |
| oxo-18:0 | 0.97± 0.362 | 2.95± 0.660 | 3.23± 0.554 | 2.78± 0.670 | 0.006 |
| n10/9-11-18:2 ratio | 7.64± 2.594 | 6.46± 4.859 | 0.47± 0.124 | 1.69± 0.725 | 0.022 |

**BH indicators**

- **BH-18:2n-6** | 75.79± 3.101 | 74.14± 2.013 | 82.14± 4.334 | 71.03± 3.580 | 0.270 |
- **BH-18:3n-3** | 79.11± 1.768 | 72.97± 1.598 | 64.04± 1.002 | 70.27± 1.403 | <0.001 |
- **Completeness** | 55.03± 8.813 | 28.68± 2.438 | 46.97± 7.78 | 31.91± 2.11 | 0.018 |
the EPA from FD biomass was better protected from rumen biohydrogenation than SD biomass. The estimates of biohydrogenation extent (disappearance) of EPA in the rumen also confirmed that FD
*N. oceanica* was more protected from biohydrogenation, as EPA biohydrogenation of FD was only 45% compared to fairly high values found with SD (70%) and O (81%). Consistently the concentration of the EPA in the rumen of lambs fed FD was about 50% higher than lambs fed SD or O diets.

**Figure 4.** Dispersion of individual values of t10/t11-18:1 ratio in the rumen of lambs fed experimental diets. Diets are represented as follow: C (Control), O (*Nannochloropsis* sp. oil), SD (spray-dried *N. oceanica*) and FD (freeze-dried *N. oceanica*). Least square means are presented in each point.

**Table 3.** Rumen pH, volatile fatty acid (VFA) concentration and molar proportion, and mucosa variables of lambs fed experimental diets. Values are means ± standard error of the mean. Diets: C, control; O, Control plus 1.2% *Nannochloropsis* sp. oil; SD, control plus 12.3% spray-dried *Nannochloropsis oceanica*; FD, control plus 9.2% freeze-dried *N. oceanica*.

| Item                      | Diets          | C    | O    | SD   | FD   | P-value |
|---------------------------|----------------|------|------|------|------|---------|
| Rumen pH                  |                | 5.7 ± 0.27 | 6.0 ± 0.23 | 6.0 ± 0.26 | 5.8 ± 0.26 | 0.782   |
| Total VFA (mmol/L)        |                | 199 ± 31.6 | 246 ± 45.9 | 233 ± 63.8 | 211 ± 31.2 | 0.847   |
| VFA (mol/100 mol)         |                | 43.4 ± 3.32 | 47.9 ± 4.06 | 50.2 ± 2.10 | 50.5 ± 2.54 | 0.329   |
| 2:0                       |                | 26.3 ± 2.82 | 26.9 ± 2.0  | 23.6 ± 0.75 | 24.9 ± 1.97 | 0.370   |
| 3:0                       |                | 6.8 ± 2.09  | 6.0 ± 3.01  | 6.7 ± 1.55  | 5.6 ± 1.25  | 0.928   |
| 4:0                       |                | 14.3 ± 1.49 | 15.6 ± 2.24 | 13.3 ± 1.50 | 12.7 ± 0.79 | 0.564   |
| 5:0                       |                | 3.1 ± 1.17  | 2.0 ± 0.75  | 3.3 ± 0.56  | 2.4 ± 0.58  | 0.518   |
| 6:0                       |                | 4.7 ± 1.24  | 2.9 ± 0.60  | 2.5 ± 0.54  | 3.3 ± 0.58  | 0.417   |
| 7:0                       |                | 1.4 ± 0.53  | 0.9 ± 0.29  | 0.5 ± 0.09  | 0.7 ± 0.27  | 0.206   |
| Papillae length (mm)      |                | 5.30 ± 0.37 | 5.03 ± 0.33 | 5.76 ± 0.78 | 4.33 ± 0.30 | 0.125   |
| Epithelium thickness (µm) |                | 195 ± 20.3  | 173 ± 20.2  | 182 ± 18.8  | 167 ± 7.5   | 0.558   |
| Stratum corneum thickness (µm) | 53.6 ± 6.91 | 47.1 ± 3.87 | 52.7 ± 3.85 | 45.9 ± 2.39 | 0.410   |
| Grey scale value           |                | 59.3 ± 4.86 | 61.8 ± 3.56 | 61.7 ± 6.15 | 56.1 ± 6.20 | 0.867   |
void space in the middle. However, the Chlorella and Spirulina FD particles consisted in sheets of cells that were no longer spherical but adhered together in a linear fashion, which differed from what we presently found. The freeze-dried method can cause damage to cells, as intracellular water expands upon freezing, but this might be highly variable with the freezing conditions and with microalgae species. Nevertheless, the impact of the freeze-dried process on the cell wall structure seems to be less drastic compared with spray-dried as previously discussed. The changes on the surface of *N. oceanica* biomass morphology reported here evidenced that FD maintains a better overall cell structure (shape and integrity), while SD suffered more severe shape alterations, including the presence of cracks and holes. This integrity loss of SD *N. oceanica* biomass might have compromised the ability to keep the EPA inside the cell structures, exposing it to microbial metabolism. This could explain why rumen biohydrogenation of EPA with the SD treatment was much higher than FD's and similar to that found when free *Nannochloropsis* oil was used.

Ruminal biohydrogenation of EPA and DHA from several experiments with fish oil or marine microalgae averaged 80% as reviewed by Doreau and co-workers. This value is similar to that found for the biohydrogenation of EPA from *Nannochloropsis* oil, which supports the evidence of the effective protection against microbial attack in the rumen offered by FD *N. oceanica* biomass. Considering that the *Nannochloropsis* sp. contains relevant amounts of 9-16:1 and 20:4n-6, both were also partially protected from ruminal metabolization with the FD *N. oceanica*. So, the ruminal biohydrogenation of 20:4n-6 was lowest in FD group and highest in lambs fed *Nannochloropsis* free oil. The highest proportion of 20:2 and 20:3 intermediates, likely to be formed from biohydrogenation of EPA (20:5n-3) and 20:4n-6, was found in the O group. These results suggest that in oil, FA are more exposed to rumen microbes than in SD and FD *N. oceanica* biomass. Moreover, the formation of a large range of C20 intermediates from the ruminal biohydrogenation of unesterified EPA using deuterated (d5-20:5n-3) and non-deuterated forms was already demonstrated in vitro.

An efficient lipid-protection supplement needs to allow lipid release during abomasal passage and further digestion and absorption in the intestine. We did not evaluate microalgae cell disruption in the abomasum, but the highest proportion of EPA in the abomasal digesta was found in FD *N. oceanica* fed animals, similar to what was observed in the ruminal digesta. Also, the proportions of total SFA, MUFA and PUFA in abomasal digesta were in the same range to those found in the rumen content. Indeed, the FA composition of abomasal digesta were in the same range to those found in the rumen content. However, the high levels of EPA in both cecum and faeces of animals fed *N. oceanica* biomass, independently of the drying method, suggests that EPA was not completely released and absorbed in the small intestine.

Compared to the literature our estimates of post-ruminal apparent digestibility of EPA are quite low for SD (33%) and FD (51%) treatments but similar to previous reported values for O treatment (66%). In sheep the apparent intestinal digestibility of EPA supplied by fish oil ranged between 73 and 89%. Additionally, the acid environment at abomasum should help disruption of *N. oceanica* cell walls and promote intracellular lipids to get released. In fact, low pH solution was reported to increased porosity of cell wall and help lipid extraction from several microalgae, including *Nannochloropsis* sp. This microalgae cell disintegration will allow the digestion and absorption of other nutrients in the intestine. However, the high levels of EPA in both cecum and faeces of animals fed *N. oceanica* biomass, independently of the drying method, suggests that EPA was not completely released and absorbed in the small intestine.

Dietary supplementation of EPA or DHA-enriched products, especially from marine origin, affects the ruminal biohydrogenation of both 18:2n-6 and 18:3n-3 by disturbing the rumen microbial population and inhibiting the final biohydrogenation reductive step, resulting in the accumulation of *trans*-18:1 isomers and reduction of 18:0 in the rumen. Indeed, several studies reported that dietary supplementation of fish oil or microalgae lipid extracts decreased the proportion of 18:0 and increased the *trans*-18:1 in the rumen and in the duodenal flows. Consistently with the literature, the proportion of 18:0 in the rumen was highest in the C treatment and decreased in *Nannochloropsis* fed lambs (Supplementary Table S1), even when expressed in percentage of total C18 FA. However, in SD fed lambs, neither the 18:0 nor C18 biohydrogenation completeness in the rumen differed from the C group. In vitro incubations with unesterified DHA or DHA-microalgae suggested that the DHA is the active component that promotes incomplete biohydrogenation of C18 PUFA and this might also differed from the C was also present in the abomasal digesta were in the same range to those found in the rumen content. Indeed, the FA composition of abomasal digesta were in the same range to those found in the rumen content. However, the high levels of EPA in both cecum and faeces of animals fed *N. oceanica* biomass, independently of the drying method, suggests that EPA was not completely released and absorbed in the small intestine.

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Several individual C18 BI differed among treatments, with a particular interest in t11-18:1 and t10-18:1. The t11-18:1 is often the main *trans*-18:1 to accumulate in the rumen of animals fed forage-based diets but occasionally, a shift toward the formation of t10-18:1 (t10-shift, with t10/t11-18:1 ratio > 1) is observed, particularly in high-concentrate diets supplemented with vegetable oils and/or low rumen pH. In the present experiment, the t10-shift was evident (i.e., t10/t11-18:1 ratio ≈ 7.61) in C treatment. As there were no differences in rumen pH (≈ 5.9) among treatments and the basal diet was the same of the C, it was expected that t10-shift observed in the C was also present in the *Nannochloropsis* fed animals. But surprisingly, lower
t10/t11-18:1 ratios were observed in animals fed the *Nannochloropsis* dried biomasses compared to C diet, while *Nannochloropsis* sp. extracted oil did not differ among groups but showing a large individual variability. Large individual susceptibility of lambs and bulls to the t10-shift has been often observed, although not well understood. Lipid supplementation often favours the t10-shift, particularly when supplementing low-forage diets. Marine lipids suplementations have been identified as one of the triggers of milk fat depression in dairy cows, which in great part could be explained by the rumen t10-shift occurrence. Nevertheless, the effect of marine lipid is often determined by the composition of typical dairy or finishing diets. Data on supplementation of high-forage, with marine lipids are more scarce and so support that marine lipids are not relevant as inducer of the t10-shift. The notable decrease in the t10/t11-18:1 ratio with the inclusion of *N. oceanica* biomass was due to a large increase of t11-18:1 accumulation and tend to decrease the accumulation of t10-18:1, indicating that *N. oceanica* biomass was effective in deviating the C18 biohydrogenation pathways toward the t11-18:1 production. A potential explanation for such positive effect of *N. oceanica* biomass in mitigating the t10-shift could be linked to the additional vitamin E added in the *Nannochloropsis* diets. Indeed, vitamin E has been suggested to inhibit the t10-shift, but such preventive effect was not confirmed in beef cattle or lambs, as evaluated by trans-18:1 deposition in the tissues. Moreover, the O diet also included the same additional vitamin E content than SD and FD diets and did not offer such a clear mitigation of the t10-shift, suggesting a direct effect of *N. oceanica* biomass. The occurrence of the t10-shift in finishing ruminants is a major constraint of the strategies based on lipid supplementation designed to improve the nutritional value of meat, and this promising effect of *N. oceanica* biomass might constitute a clue towards a novel approach to reduce t10-shift occurrence in animals fed high-concentrated diets.

Other major C18 BI were affected by treatments as the coeluted peak of t11,c15-/t10,c15-18:2 that was higher in lambs fed O and FD diets than in those fed C and SD diets. Both t11,c15-18:2 and t10,c15-18:2 are intermediates from the low level in the rumen of animals fed SD might be explained by the lower biohydrogenation estimated for 18:3n-3 compared with the other diets. The low levels in C group might be explained by the high C18 completeness, as previously discussed. Indeed, supplementation with microalgae has been reported to favour the accumulation of t11,c15-18:2 in rumen fluid of cows and ewes. Other studies also reported that fish oil increases the ruminal outflow of t11,c15-18:2 due to incomplete biohydrogenation of 18:3n-3.

No differences among treatments were found on 18:2n-6 or in its estimated biohydrogenation. Thus, contrasting to what was observed for 18:3n-3, diets did not affect the initial steps of 18:2n-6 biohydrogenation. Similar results have been observed on the in vitro incubations with rumen fluid and SD or FD *N. oceanica*. Other authors also did not find effects of dietary microalgae inclusion on the concentration of 18:2n-6 in the rumen. Incorporation of supplements containing very long-chain PUFA, as those supplied by fish oil or microalgae, can affect rumen fermentative activity often increasing propionic acid molar proportion. In our experiment, none of diets containing *Nannochloropsis* sources affected the rumen fermentation parameters, probably because the amount incorporated in the diet was not high (1.2% of oil or microalgae biomass supplying equivalent amount of EPA). Nevertheless, other authors reported increases in VFA and proportionate in goats fed with dietary inclusions of *Nannochloropsis oculata* biomass as low as 0.5%. The effects of *Nannochloropsis* microalgae on rumen fermentation might be dependent on the type of basal diet as reported for *Nannochloropsis salina*, that induced larger effects on rumen fermentation in continuous flow fermenters when fed with forage basal diet and minor effect when fed with a concentrate basal diet. Thus, differences in experimental conditions, including animal species, basal diets, or even in intrinsic differences in the nutrient composition and cell wall structure of each *Nannochloropsis* species might result in different effects on rumen fermentation.

In south-western Europe, light lambs are usually finished for few weeks after weaning with high-energy diets and slaughter with up to 25 kg of liveweight. In these conditions a high incidence of rumen mucosa lesions including ruminal parakeratosis is observed and thus we routinely evaluate the rumen mucosa lesions in our lamb experiments. Parakeratosis of rumen mucosa is characterized by an accumulation of layers of keratinized, nucleated squamous epithelial cells and excessive sloughing of the epithelium, increased thickness of the stratum corneum and consequent dark brown coloration of the mucosa. In the present study, none of the histological parakeratosis indicators nor colour greyscale values differed among treatments, which indicates the absence of parakeratotic lesions. Despite neither the histometric parameters nor the pH differed among diets, a negative Pearson correlation between *stratum corneum* thickness and ruminal pH was found (r = -0.38, P = 0.043). The inclusion of 35–40% of dehydrated alfalfa in lamb diets could have contributed to the lack of differences, although rumen lesions in lambs have been reported using similar diets. These results indicate that dietary inclusion of *Nannochloropsis* biomass in lamb finishing diets does not influence the occurrence of ruminal wall lesions.

We conclude that the drying method applied to *N. oceanica* strongly influences powder architecture and cell wall integrity and consequently the degree of EPA protection against rumen microbes. Indeed, we confirmed that freeze-drying has an advantage over spray-drying in preserving *N. oceanica* cell wall. Thus, FD *N. oceanica* can constitute a better source of ruminal protected-EPA comparing to SD *N. oceanica*, once higher EPA levels...
were found in the rumen and abomasum, indicating a better escape against ruminal biohydrogenation. However, EPA was also found in cecum content and faeces, suggesting that its absorption at the small intestine was not totally efficient.

Moreover, the supplementation of high-concentrated diets with *Nannochloropsis* microalgae affected the biohydrogenation of C18 fatty acids. The most notable effect was the deviation from the $t_{10}$ biohydrogenation pathways to the $t_{11}$ pathways, resulting in the higher abundance of $t_{11}-18:1$ over $t_{10}-18:1$ in the rumen of lambs fed *N. oceanica*. Also, at this level of *Nannochloropsis* incorporation, no disturbances were found in fermentable parameters nor ruminal parakeratosis indicators. Further studies need to address if the ruminal microbiome was affected by the different treatments and if EPA was indeed successfully deposited in the lamb’s meat and edible fats.

Table 4. Ingredients, chemical composition, and fatty acid profile of the experimental diets. 1 Diets (or treatments): C, control; O, Control plus 1.2% *Nannochloropsis* sp. oil; SD, control plus 12.3% spray-dried *Nannochloropsis oceanica*; FD, control plus 9.2% freeze-dried *N. oceanica*. 2Premix composition: Vitamin A, 4,000,000 UI; Vitamin D3, 1,100,000 UI; Vitamin E, 7500 mg/kg; Vitamin B1, 250 mg/kg; Vitamin B2 250 mg/kg; Zinc, 35,000 mg/kg; Iron, 12,500 mg/kg; Manganese, 17,500 mg/kg; Iodine, 200 mg/kg; Cobalt 250 mg/kg; Selenium, 100 mg/kg; Magnesium oxide (excipient) 40,000 mg/kg. n.d. – not detected.

| Item                        | Diets 1 |                 |                 |                 |
|-----------------------------|---------|-----------------|-----------------|-----------------|
|                              | C       | O               | SD              | FD              |
| Ingredients (g/kg DM)        |         |                 |                 |                 |
| Barley                       | 390     | 385             | 342             | 354             |
| Soybean meal                 | 170     | 168             | 149             | 154             |
| Dehydrated alfalfa          | 400     | 395             | 351             | 363             |
| Freeze dried N. oceanica    | –       | –               | –               | 92              |
| Spray dried N. oceanica     | –       | –               | 123             | –               |
| Nannochloropsis sp. Oil     | –       | 12              | –               | –               |
| Calcium carbonate           | 13      | 13              | 11              | 12              |
| Sodium bicarbonate          | 20      | 20              | 18              | 18              |
| Marine salt                 | 4       | 4               | 4               | 4               |
| Premix 2                    | 3       | 3               | 3               | 3               |
| Vitamin E                   | –       | 1.67            | 1.67            | 1.67            |
| Chemical composition (g/kg DM) |         |                 |                 |                 |
| DM                          | 904     | 907             | 902             | 907             |
| Crude Protein               | 191     | 183             | 206             | 211             |
| Ether Extract               | 13.3    | 32.5            | 35.2            | 33.9            |
| NDF                         | 267     | 267             | 232             | 290             |
| ADF                         | 173     | 174             | 150             | 167             |
| ADL                         | 30.3    | 28.1            | 26.8            | 31.2            |
| Sugar                       | 73.2    | 69.4            | 65.5            | 67.9            |
| Starch                      | 277     | 279             | 247             | 227             |
| Crude Energy (kJ/100 g)     | –       | –               | –               | –               |
| Ash                         | 84.6    | 89.9            | 114.1           | 90.8            |
| Total Fatty acids (g/kg DM)  | 13.7    | 20.6            | 19.5            | 20.2            |
| FA profile (g/kg DM)         |         |                 |                 |                 |
| 14:0                        | n.d     | 0.26            | 0.47            | 0.46            |
| 16:0                        | 3.49    | 4.74            | 4.66            | 5.27            |
| 16:1c9                      | 0.07    | 1.06            | 1.99            | 1.66            |
| 17:0                        | 0.09    | 0.07            | n.d             | n.d             |
| 18:0                        | 0.56    | 0.61            | 0.49            | 0.67            |
| 18:1c9                      | 2.55    | 2.99            | 2.30            | 2.77            |
| 18:1c11                     | 0.10    | 0.19            | 0.14            | 0.14            |
| 18:2n-6                     | 5.62    | 6.49            | 5.50            | 5.88            |
| 18:3n-3                     | 1.22    | 1.40            | 0.98            | 1.30            |
| 20:4n-6 (EPA)               | n.d     | 0.53            | 0.72            | 0.64            |
| 20:5n-3 (EPA)               | n.d     | 2.14            | 2.20            | 1.39            |
| 22:0                        | n.d     | 0.12            | 0.07            | n.d             |

were found in the rumen and abomasum, indicating a better escape against ruminal biohydrogenation. However, EPA was also found in cecum content and faeces, suggesting that its absorption at the small intestine was not totally efficient.
Material and methods

Animals, diets, and management. The experimentation involving live animals was conducted under strict compliance with international guidelines (Directive 2010/63/EU) regulating the use of production animals in animal experimentation, at the INIAV-Santarém facilities. The INIAV-Santarém facilities are certified by the competent veterinary authority (DGAV) to conduct animal experimentation (Ref: 04211000/000/2013). The experimental animal procedures were approved by the Ethical and Animal Well-Being Commission (CEBEA) of the Faculty of Veterinary Medicine, University of Lisbon, Portugal (Protocol FVM/CEBEA 007/2016). Animal management, handling, transport, and sacrifice were conducted replicating approved standard commercial practices regarding animal welfare, except that animals were individually housed. The study was also carried out in compliance with the ARRIVE guidelines.

Twenty-eight Merino Branco ram lambs were reared with dams on extensive grazing until weaning at approximately 60 days of age. After that, lambs were transported to INIAV—Santarém facilities, and randomly allocated to individual pens (1.52 m²) with wood shaving beds, and with free access to clean water. Animals were allocated to one of 4 groups of 7 lambs each, and randomly allocated to diets, following a completely randomized design. Lamb’s initial live weight averaged 21.8 ± 4.4 kg.

The C diet consisted of pellets containing dehydrated lucerne, barley and soybean meal (Table 4) and no added sources of EPA. The other diets maintained the same ingredients and proportion of C diet plus: SD diet—123 g/kg of spray-dried N. oceanica biomass; FD diet—92 g/kg freeze-dried N. oceanica biomass; O diet—12 g/kg of Nannochloropsis sp. free oil. The amounts of microalgae biomass or oil added to diets was determined to supply identical quantities of EPA (= 3 g/kg DM). The analysis of the final diets demonstrated that FD diet contained less EPA than the SD diet (Table 4), probably due to losses during handling, feed production, or differences among slurry batches. Diets containing Nannochloropsis were supplemented with 1.67 g/kg DM tocopheryl acetate (3α700 Vitamine E, 500 mg/g). Control diet was not supplemented because the premix already contained vitamin E in levels close to the NRC47 requirements for growing-finishing lambs of 20–30 kg of body weight. Diets were not formulated to be isoproteic or isocaloric, but crude protein ranged from 191 to 211 g/kg DM.

The N. oceanica biomasses were produced at allmicroalgae industrial plant located in Pataias, Portugal. Cultures were autotrophically grown in Guillard’s F2 medium as previously described48. Around three months after inoculation, the microalgae biomass was harvested from the photobioreactors, concentrated in a membrane system, subjected to a short-term high temperature treatment and dried in an industrial spray dryer to obtain the spray-dried N. oceanica biomass; FD diet—92 g/kg freeze-dried N. oceanica biomass; O diet—12 g/kg of Nannochloropsis sp. free oil. The amounts of microalgae biomass or oil added to diets was determined to supply identical quantities of EPA (= 3 g/kg DM). The analysis of the final diets demonstrated that FD diet contained less EPA than the SD diet (Table 4), probably due to losses during handling, feed production, or differences among slurry batches. Diets containing Nannochloropsis were supplemented with 1.67 g/kg DM tocopheryl acetate (3α700 Vitamine E, 500 mg/g). Control diet was not supplemented because the premix already contained vitamin E in levels close to the NRC47 requirements for growing-finishing lambs of 20–30 kg of body weight. Diets were not formulated to be isoproteic or isocaloric, but crude protein ranged from 191 to 211 g/kg DM.

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During the adaptation period of 8 days, lambs were dewormed against gastrointestinal and pulmonary nematodes by dosing with Sinvermin (Lapsa—Portuguesa Pecuária Lda., Portugal) and coccidiosis by dosing with Vecoxan (Elanco GmbH—Germany) and vaccinated against enterotoxaemia with Miloxan (Merial Labs., Spain). After that, they went through a transition period of 6 days where they were given 1/3 of the pelleted experimental diet plus 2/3 of the basal ground feed ration, on the 1st and 2nd days and then the proportion of the experimental diet was regularly increased for the remaining days, until reaching 3/3. The experimental period started on the 14th day post-arrival when the diet was exclusively composed by the experimental pellets and lasted for 3 weeks. During the first two weeks of the experiment, 1.2 kg of feed was offered once a day (0900 am) and thereafter animals were fed ad libitum. Feed intake was measured during the entire experiment and averaged 1.19 ± 0.13 kg of DM/day.

Slaughter procedures and sampling. At the end of the trial period, lambs were weighed at 0830 am at the housing facilities, without previous fasting, and immediately transported (circa 1 km) to the experimental abattoir of the INIAV—Santarém. Immediately after the slaughter, the whole rumen, abomasum, and lower intestine (cecum) digestive contents were collected from each lamb and frozen at − 80 °C. After that, they were freeze-dried, milled, and re-stored at − 80 °C until FA analysis. Faeces were collected directly from the rectum, frozen at − 80 °C, freeze-dried, milled and re-stored at − 80 °C. Samples of the ruminal wall (5 × 5 cm) were collected after washing the ruminal mucosa with tap water and fixed in 10% buffered formalin for at least 24 h. After fixa-
with haematoxylin and eosin for routine microscopic examination. From the 2 fragments corresponding to the ruminal wall of one animal, the 5 better preserved papillae were selected for histometry analysis, which was performed using a BX 511 microscope (Olympus, Tokyo, Japan) and the images were digitally captured using a DP 11 camera (Olympus, Tokyo, Japan) under a magnification of 20 ×. The measurements were made using DP-Soft (Olympus) and Image 1.43 softwares (Image, Health National Institute of Mental, Bethesda).

**Rumen protozoa counting.** Protozoal densities were obtained individually by microscopic counting as previously described49. Ciliate cell numbers were determined in duplicate for each sample and the identification at the genus level was made based on protozoa morphology, according to others49.

**Chemical analysis.** Chemical analysis of diets was obtained as the average of the results of two pooled samples of each diet and analysed as previously described50. Acid-insoluble ash of feed and faeces, mainly consisting in silica, was determined gravimetrically after drying, ashing, boiling of ash in hydrochloric acid (HCl), filtering and washing of the hot hydrolysate, and re-ashing46. Ruminal volatile FA (VFA) were determined by gas chromatography with flame ionization detection (GC–FID) using a Shimadzu GC 2010-Plus (Shimadzu, Kyoto, Japan) equipped with a Nukol (30 m × 0.25 mm, 0.20 µm film thickness, Supelco, Bellefonte, PA, USA) capillary column and quantification was made using calibration curves according to others52.

Fatty acid methyl esters (FAME) of feed samples were prepared according to Sukhija and Palmquist53. Freeze-dried rumen, abomasum and lower intestine contents, and faeces samples were prepared by direct transesterification by reaction with sodium methoxide (0.5 M) in methanol at 50 °C for 15 min followed by addition of hydrogen chloride (1.25 M) in methanol at 80 °C for 20 min. Methyl nonadecanoate (1 mg/mL) was added as internal standard. Fatty acid methyl esters and DMA were analysed by GC–FID using a Shimadzu GC 2010-Plus (Shimadzu, Kyoto, Japan) equipped with an SP-2560 (100 m × 0.25 mm, 0.20 µm film thickness, Supelco, Bellefonte, PA, USA) capillary column. The chromatographic conditions were as follow: injector and detector temperatures were set at 250 °C and 280 °C, respectively; helium was used as the carrier gas at 1 mL/min at a constant flow; the initial oven temperature of 50 °C was held for 1 min, increased at 50 °C/min to 150 °C and held for 20 min, increased at 1 °C/min to 190 °C and then increased at 2 °C/min to 220 °C and held for 40 min. Identification of FAME and DMA was achieved by comparison of fatty acid retention times with those of commercial standards (FAME mix 37 components from Supelco Inc., Bellefont, PA, USA) and with published chromatograms54. Additional confirmation of FAME and DMA was achieved by electron impact mass spectrometry using a Shimadzu GC–MS QP2010 Plus (Shimadzu, Kyoto, Japan). The chromatographic column and the GC conditions were like the ones in the GC–FID analysis.

**Scanning electron microscopy (SEM) of N. oceanica biomass.** Spray-dried and FD microalgae samples were mounted on aluminium stubs with carbon tape and coated with an 8 nm thick palladium-gold film in a Quorum Q150T ES sputtering system. *N. oceanica* surface morphology was observed in a Carl Zeiss AURIGA Crossbeam SEM–FIB workstation, using an accelerating voltage of 5 keV with an aperture size of 30 microns.

**Calculations and statistical analysis.** The biohydrogenation estimates (disappearance, %) in the rumen for 18:2n-6, 18:3n-3, 20:4n-6 and 20:5n-3, were obtained using the diminishing abundance of these FA, proportional to the sum of C18 FA or of C20 FA, between diet and rumen, assuming that no losses of FA occur in the gastric compartments as shown below in the Eq. (1).

$$\text{Biohydrogenation}_{\text{UFA}}(\%) = \frac{([\text{UFA} - D] - [\text{UFA} - R])}{[\text{UFA} - D]} \times 100$$  \hspace{1cm} (1)$$

where [UFA-D] and [UFA-R] are the proportions of each dietary unsaturated FA (UFA) expressed as % of total C18 FA or as % of total C20 FA, respectively for C18 UFA or C20 UFA.

C18 biohydrogenation completeness (%) was estimated considering the maximum 18:0 in the rumen and abomasal digesta, assuming a complete biohydrogenation of the C18 FA from the diet12. The calculations exemplified for rumen are presented in the Eq. (2):

$$\text{C18 biohydrogenation completeness (})\% = \frac{[\text{SA} - R]}{[\text{MaxSA} - R]} \times 100$$  \hspace{1cm} (2)$$

The [SA-R] is the proportion of 18:0 in the rumen digesta and the [Max SA-R] is the maximum 18:0 proportion in the rumen, both expressed as % of total C18 FA, assuming that 100% of dietary unsaturated C18 FA biohydrogenated is converted to 18:0 and computed as shown in the Eq. (3).

$$\text{Max SA-R} = ([\text{AO-D} - \text{AO-R}] + ([\text{LA-D} - \text{LA-R}] + ([\text{LnA-D} - \text{LnA-R}] + [\text{SA-D}]$$  \hspace{1cm} (3)$$

Where: [SA-R], [AO-R], [LA-R], [LnA-R] are respectively the proportions of 18:0, 9-18:1, 18:2n-6 and 18:3n-3 in the rumen, and [SA-D], [AO-D], [LA-D], [LnA-D] the proportion of the same FA in the diets, expressed as % of total C18 FA.

The whole tract apparent digestibility (WTAD) of EPA, which included both biohydrogenation and post-ruminal digestion, was calculated using silica as the internal digestibility marker using marker and nutrients concentration ratios as described in Eq. (4):
Post ruminal digestibility (PRD) of EPA was estimated using both WTAD and biohydrogenation values, by computing the balance between the proportion of EPA escaping rumen metabolism (EPA_RE) and the proportion of EPA escaping the digestive tract as faecal excretion (EPA_FE). PRD calculations are presented in the Eq. (5).

\[
PRD (%) = \left( \frac{(EPA_{RE}) - (EPA_{FE})}{EPA_{RE}} \right) \times 100
\]  

where: EPA_{RE} (%) = 100 — % EPA biohydrogenation, and EPA_{FE} (%) = 100 — % EPA WTAD.

Chemical analysis (FAME and DMA), histometric data, ruminal pH and ruminal mucosa greyscale evaluation and FA data were analysed as a completely randomized experimental design using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC), using diet as a fixed factor and the animal as the experimental unit. When needed, the group option of the repeated statement was included in the model to accommodate the variance heterogeneity. Least square means (LSM) and standard error of the mean (SEM) are reported, and main effects and their interactions were considered significant at P < 0.05 and trends toward significance at 0.05 < P < 0.10. Due to an experimental incident the night before the slaughter, two animals receiving C diets accidentally had access to diets containing Nannochloropsis and consequently, those two animals were removed from the analysis.

**Data availability**
All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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S.P.A. and R.J.B.B. conceptualization and funding acquisition. A.C.M.V., R.J.B.B., S.P.A., S.A.H. and J.S.S. implemented the experiment. J.L.S. provided the microalgae. A.C.M.V., A.E.F., and J.S.S. conducted the animal experiment. A.C.M.V. and S.P.A. conducted laboratory analysis. M.P. monitored and assisted histopathological analyses. A.C.M.V., R.J.B.B., and S.P.A. performed data analysis. A.C.M.V., R.J.B.B., S.P.A. and S.A.H. interpretation of results. A.C.M.V. drafted the manuscript. S.P.A. and R.J.B.B. edited and revised the original draft. All authors revised and approved the final manuscript.

Competing interests
J.S. is employee of the company that provided the microalgae (Allmicroalgae). The remaining authors declare no competing interests.

Additional information
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