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Chlorella pyrenoidosa supplementation increased the concentration of unsaturated fatty acids in the rumen fluid of cattle fed a low-quality tropical forage

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ABSTRACT - The objective of this study was to evaluate the effects of algae and cottonseed meal (CSM) supplementation on the fatty acid (FA) profile in the rumen fluid (RF) of steers fed a low-quality forage. Five *Bos indicus* crossbred steers, 187±7.5 kg liveweight (LW; mean±SD), were fed a low crude protein speargrass (Heteropogon contortus) hay as the basal diet alone or supplemented with either *Spirulina platensis, Chlorella pyrenoidosa*, *Dunaliella salina*, or CSM in Latin square design. The proportion of individual FA in the RF of steers varied in response to supplement, and these were most likely due to differences in the FA profile in supplements. Steers supplemented with *Chlorella pyrenoidosa* and CSM had a higher concentration of linoleic acid (C18:2n-6) in RF than unsupplemented steers or steers offered the other supplements, but there was no difference in the concentration in RF in steers supplemented with *Chlorella pyrenoidosa* and CSM. The concentration of linolenic acid (C18:3n-3) was higher in the RF of unsupplemented steers compared with supplemented steers. Steers receiving Chlorella pyrenoidosa supplementation showed an increase in total unsaturated FA in the RF compared with other supplemented and unsupplemented steers, which if transferred to meat, could have health related benefits to consumers. None of the supplements led to the formation of isomers known to inhibit fat synthesis.

Keywords: biohydrogenation, conjugated linoleic acid, microalgae, tropical grass

1. Introduction

In recent years, the biotechnology of fuel production has led to an increased use of algae, with a parallel interest in the potential use of de-oiled algae byproducts for animal feeds (Bryant et al., 2012). Algae are mainly targeted as feedstuffs for ruminants because of their fatty acid (FA) content and the health-related benefits to consumers of meat (Vahmani et al., 2013) and milk products (Papadopoulos et al., 2002). The protein and fat content vary considerably between algae, and this can have variable effects within the rumen (Panjaitan et al., 2015) and after absorption by the ruminant animal (Meale et al., 2014; Lamminen et al., 2019).

The hypothesis of this study was that a greater concentration of polyunsaturated FA in an algae species would be associated with a greater concentration of total unsaturated FA in the rumen fluid (RF) of ruminants consuming the algae. Therefore, supplementation with algae varying in FA profile, with

a high concentration of C18s FA, would result in different concentrations of conjugated linoleic acid isomers, reflecting the FA profile in RF and the extent of biohydrogenation thereof. The objective of this experiment was to evaluate the effects of algae and cottonseed meal (CSM) supplementation on the FA profile in the RF of cattle consuming a low-quality tropical forage.

2. Material and Methods

The experiment was conducted in Gatton, QLD, Australia (27.5551° S, 152.3369° E, and 94 m), and all procedures were performed in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, reviewed and approved by the institutional Animal Ethics Committee (SAS/236/10/MLA).

2.1. Experimental design, animals and nutritional treatments

The experiment was a 5×5 complete Latin square, incorporating five steers and five nutritional treatments. The experiment consisted of five 21-day experimental periods, with each experimental period consisting of 12 days in outdoor pens and nine days in metabolism crates. Each steer was exposed to a different nutritional treatment within each experimental period.

Five *Bos indicus* crossbred steers, 187±7.5 kg liveweight (LW; mean±SD) were randomly allocated to adjacent individual pens and allocated to one of five nutritional treatments. All steers were offered speargrass (*Heteropogon contortus*) hay *ad libitum* and were either unsupplemented (control) or supplemented with *Spirulina platensis* (Spirulina; Phytofoods Australia Pty. Ltd., Labrador, QLD, Australia), *Chlorella pyrenoidosa* (Chlorella; Phytofoods Australia Pty. Ltd.), *Dunaliella salina* (Dunaliella; Nutra-Kol, Perth, WA, Australia), or CSM (Riverina Stockfeeds, Murgon, QLD, Australia). Spirulina, Chlorella, and CSM were offered at 0.45 g N/(kg LW.day), whilst Duniella was offered at 4 g DM/(kg LW.day) [equivalent to the amount of Spirulina DM offered/(kg LW.day)]. The amount of *Spirulina platensis* supplied was similar to that which resulted in the maximum dry matter intake response in Panjaitan et al. (2015). *Chlorella pyrenoidosa* and CSM were offered at an equivalent amount of N [0.45 g N/(kg LW.day)], whereas *Dunaliella salina* was offered at an equivalent DM amount [4 g/(kg LW.day)]as that supplied by *Spirulina platensis* to accommodate its high ash content. Samples of feed offered were collected in duplicate for each run and bulked across the experiment (Table 1).

2.2. Feeding of hay and supplements

The speargrass hay was chopped to approximately 50 mm in length and offered *ad libitum* (previous day intake +10% as fed) at 08:00 h each day. Supplement allowances were adjusted based on steer liveweight measured on days 1 and 13 of each experimental run. Supplements were mixed with 300 g molasses and offered daily, prior to hay feeding, at 07:30 h, with the same amount of molasses supplied to control animals. Algae supplements were prepared by mixing the algae and molasses with water to a final concentration of approximately 30% algae (w/w), whereas CSM was offered with the molasses added on top.

Table 1 - Chemical composition of *Heteropogon contortus* (speargrass) hay, *Spirulina platensis* (Spirulina), *Chlorella pyrenoidosa* (Chlorella), *Dunaliella salina* (Dunaliella), and cottonseed meal (CSM)[1]

Parameter[2]	Speargrass	Spirulina	Chlorella	Dunaliella	CSM
Organic matter (g/kg DM)	910	743	798	213	764
Crude protein (g/kg DM)	24	648	548	62	422
Lipids (g/kg DM)	17	101	143	122	45
ADFom (g/kg DM)	418	0	0	0	143
aNDFom (g/kg DM)	695	63	4	0	199

DM - dry matter; ADFom - ash-free acid detergent fibre; aNDFom - ash-free neutral detergent fibre assayed with a heat stable amylase.
[1] Samples of feed offered were collected in duplicate for each run and bulked across the experiment. Results are for this single bulked sample across the entire experiment.

2.3. Sample collection and analytical procedures

Samples of hay and supplements were collected daily and bulked within each run. Rumen fluid was collected orally using a stomach tube attached to a hand pump 3 h after feeding on day 21 with unacidified duplicate sub-samples stored at −20 °C until analysis of the FA profile.

Hay and supplement samples were oven-dried to a constant weight at 60 °C and ground through a 1-mm screen (Retsch ZM 200; Haan, Germany). Residual moisture was determined at 105 °C using a drying oven (Industrial 200, Watson Victor Ltd.; Sydney, NSW, Australia) for 24 h and ash determined after incineration at 550 °C for 8 h in a muffle furnace (Modutemp Pty. Ltd.; Perth, WA, Australia). The N content of the hay and supplements was determined using a Nitrogen analyser (Kjeltec, 8400 FOSS; Hillerod, North Zealand, Denmark) based on the Kjeldahl method. The ash-free neutral detergent fibre (aNDFom) and ash-free acid detergent fibre (ADFom) were determined according to Van Soest et al. (1991) with the use of Gooch crucibles of grade 2 porosity because of the fine particle size of the microalgae. Briefly, for aNDFom analysis, approximately 0.5 g of sample was heated to boiling with 50 mL neutral detergent solution, 0.5 g Na_2SO_3, and 2 mL of heat stable alpha-amylase (17,400 Liquefon U/mL; FAA, ANKOM Technology; Macedon, USA), and then agitated for 1 h before transferring to a crucible. After draining the solution under vacuum, the samples were washed three times with hot water (the first rinse included a further application of 2 mL alpha-amylase) and twice with acetone. The ADFom was determined essentially the same way, except that the digestion was conducted in 50 mL of acid detergent solution, 2.45 g H_2SO_4, with no alpha-amylase added to the digestion or washes. Total lipid content was determined according to the method of Hara and Radin (1978). The FA content in hay, supplements, and RF was determined using a method based on Kramer et al. (1997) using modifications proposed by Sun and Gibbs (2012), which was described in full by Costa et al. (2019).

2.4. Statistical analysis

The statistical analyses for FA were carried out using the univariate procedure in General Linear Model of SPSS (SPSS for Windows, Version 17.0, SPSS Inc, Chicago, IL, USA), according to the following model:

$$Y_{ijk} = \mu + P_i + T_j + S_k + e_{ijk},$$

in which Y_{ijk} is the dependent variable, μ is overall mean, P_i is fixed effect of period (1 to 5), T_j is fixed effect of treatment (1 to 5), S_k is the random effect of steers (1 to 5), and e_{ijk} is the residual error. Tukey was used for treatment multiple comparisons. Significance was declared at $P<0.05$.

3. Results

Palmitic acid (C16:0) was present in the highest proportion FA in the speargrass hay and microalgae supplements but not in CSM, in which C18:2n-6 was the most abundant FA (Table 2). Speargrass and Dunaliella contained the highest proportion of total individual odd- and branched-chain FA, with a low proportion of C15:0anteiso evident in all feeds used. Cottonseed meal and Chlorella had the highest proportion of total unsaturated FA (TUFA) and total C18 (TC18s) FA, with high proportions of linoleic acid (C18:2n-6) in both supplements and a high proportion of alpha-linolenic (C18:3n-3) in Chlorella. The highest proportion of total saturated FA (TSFA) was present in Dunaliella, followed by Spirulina and speargrass hay, which were greater than Chlorella and CSM. The proportion of long-chain FA (LCFA) (*i.e.* >C20s) was low for all supplements evaluated in this experiment.

There was considerable variation in FA profiles in the RF among all treatments (Table 3). Palmitic acid (C16:0) was the most abundant FA in the RF of steers, with the highest proportion in the RF of steers fed Spirulina ($P<0.05$). Unsupplemented steers had the highest proportion of total odd- and branched-chain FA (TOBCFA) ($P<0.05$) in the RF and steers supplemented with Chlorella had the lowest proportion, with the microalgae supplemented steers intermediate. A similar pattern was evident for C15:0anteiso, which was present in a higher proportion in RF compared with the feeds

offered (P<0.05). The proportions of TUFA and TC18s were highest in the RF of steers supplemented with Chlorella (P<0.05) with no differences detected amongst the other treatments. The proportion of linoleic acid was similar in the RF of steers offered Chlorella and CSM and was higher than in the RF of steers fed the other treatments (P<0.05). Unsupplemented steers had a higher proportion of TSFA in RF than steers supplemented with Chlorella and CSM (P<0.05) but it was not different from that measured in the RF of steers supplemented with Spirulina and Dunaliella. Unsupplemented steers had the highest proportion of alpha-linolenic acid (C18:3n-3) in RF (P<0.05) with no differences in the RF of supplemented animals.

4. Discussion

This experiment compared the effect of supplementation with three species of microalgae and CSM on the profile of FA in the RF of growing steers fed speargrass, a low-quality tropical forage. It was hypothesized that the FA profile in the RF of steers supplemented with microalgae would be different from the profile of steers fed forage alone or supplemented with CSM, and that unsaturated FA intake, through supplementation, could lead to greater concentrations of unsaturated FA in RF. The results indicated that individual FA varied between supplements and that supplementation with Chlorella resulted in a high proportion of unsaturated FA in RF, in particular linoleic acid, which was present in the microalgae used as supplements in this experiment. Interestingly, the high proportion of linoleic acid in CSM did not lead to a higher TUFA in the RF of steers.

The protein *in vitro* degradation of Chlorella observed in Costa et al. (2016) was lower than the protein in other supplements. Lamminen et al. (2019) observed no differences in apparent total tract

Table 2 - Fatty acid profile of *Heteropogon contortus* (Speargrass) hay, *Spirulina platensis* (Spirulina), *Chlorella pyrenoidosa* (Chlorella), *Dunaliella salina* (Dunaliella), and cottonseed meal (CSM)[1]

Fatty acid	Speargrass	Spirulina	Chlorella	Dunaliella	CSM
	% of total fatty acids[2]				
C12:0	2.35	0.00	0.02	0.19	0.01
C14:0	2.11	0.11	0.25	1.90	0.36
C16:0	27.09	47.64	19.89	61.18	20.61
C18:0	4.77	0.76	1.73	2.20	2.47
C18:1*t*10+*t*11	0.00	0.02	0.00	0.04	0.00
C18:1*c*9	8.71	1.90	2.59	11.32	13.75
C18:1*c*11	0.00	0.20	0.49	0.00	0.00
C18:1*c*12	0.00	0.00	0.00	0.00	0.00
C18:2n-6	14.68	17.10	27.15	2.06	58.40
C18:3n-3	3.90	0.05	23.60	3.36	0.37
CLA*c*9,*t*11	1.01	0.00	0.10	0.33	0.01
C20:0	4.51	0.04	0.08	0.01	0.26
C20:1*c*11	0.15	0.00	0.02	0.09	0.04
C22:0	2.20	0.00	0.04	0.27	0.25
C22:2*c*13,*c*16	0.00	0.00	0.01	0.00	0.02
C22:4	1.34	0.00	0.00	0.00	0.00
C22:6	0.53	0.00	0.03	0.05	0.02
TOBCFA[3]	1.65	0.07	1.02	0.77	0.00
TSFA[3]	53.56	54.75	23.88	70.02	24.34
TUFA[3]	31.67	20.40	55.58	20.27	72.62
TC18s[3]	33.19	20.03	55.79	21.64	75.03

TOBCFA - total odd- and branched-chain fatty acids; TSFA - total saturated fatty acids; TUFA - total unsaturated fatty acids; TC18s - total fatty acids containing 18 carbon chains.
[1] Samples of feed offered were collected in duplicate for each run and bulked across the experiment. Results are for this single bulked sample across the entire experiment.
[2] Identifiable and quantifiable fatty acids presented only; values expressed as % of total fatty acids and some not presented in this table.
[3] Total of individual fatty acids listed in the table plus other identifiable fatty acids.

digestibility of organic matter and CP when soybean meal was replaced by Spirulina or Chlorella in a diet offered to lactating dairy cows. In their work, it was reported that ruminal microbial N outflow tended (P = 0.053) to be higher on the diet with Spirulina compared with the diet with Chlorella but with no significant differences between dietary treatments (Lamminen et al., 2019). Lower microbial activity could lead to less biohydrogenation in the rumen. Howerver, in corroboration, Costa et al. (2016) found no differences in microbial protein synthesis among steers supplemented with Spirulina, Chlorella, and CSM. Gulzari et al. (2019) reported lower protein digestibility in sheep supplemented with the macro-algae *Saccharina latissima* (55%) in comparison with the macro-algae *Porphyra* spp. (64%), and with soybean meal (66%), but the lower digestibility led to lower production of volatile FA, most likely linked to a lower microbial activity. Gulzari et al. (2019) suggested that phlorotannins present in some algae species may form complexes with protein and fibre, which could not only limit their degradability in the rumen, but also affect the post-ruminal absorption in the intestines. No speculation is made here to indicate that similar mechanisms would be related to the lower *in vitro* protein degradation of the microalgae Chlorella observed in Costa et al. (2016), although their results indicate that something within the composition of Chlorella affects the protein degradation and that could indirectly be affecting the biohydrogenation of FA in the RF of steers fed that algae. Regardless of the latter observation and, most importantly, microbial protein synthesis was not impaired by Chlorella supplementation in their work.

The proportion of TUFA was higher in the RF of steers fed Chlorella, and if transferred to meat, could induce health-related benefits to consumers. However, specifically the proportion of linolenic acid was higher in the RF of control steers, with most of the TUFA in the RF of steers supplemented with

Table 3 - Fatty acid profiles in the rumen fluid[1] of steers fed speargrass (*Heteropogon contortus*) hay (control) or supplemented with *Spirulina platensis* (Spirulina), *Chlorella pyrenoidosa* (Chlorella), *Dunaliella salina* (Dunaliella), and cottonseed meal (CSM)

Fatty acid	Control	Spirulina	Chlorella	Dunaliella	CSM	SEM
	% of total fatty acids[2]					
C12:0	1.05b	0.60a	0.25a	1.05b	0.45a	0.08
C14:0	3.70b	2.82ab	1.17a	3.78b	2.52ab	0.25
C16:0	27.86a	37.26b	25.77a	29.20a	28.58a	1.09
C18:0	9.84c	5.26a	9.02c	8.00bc	6.25ab	0.44
C18:1*t*10+*t*11	0.86ab	1.57ab	0.89ab	0.73a	2.16b	0.17
C18:1*c*9	6.60ab	4.00a	3.50a	6.71ab	7.52b	0.47
C18:1*c*11	0.80	0.93	0.69	0.77	2.10	0.26
C18:1*c*12	1.08	0.06	0.01	0.01	0.05	0.22
C18:2n-6	6.13a	9.25a	22.88b	5.16a	18.73b	1.66
C18:3n-3	15.40b	2.76a	3.27a	2.25a	1.60a	1.09
CLA*c*9,*t*11	1.17b	0.61a	0.24a	1.07b	0.62a	0.08
C20:0	1.13d	0.57ab	0.26a	0.95cd	0.66bc	0.07
C20:1*c*11	0.08	0.05	0.03	0.04	0.03	0.01
C22:0	0.58c	0.30ab	0.14a	0.49bc	0.42bc	0.04
C22:2*c*13,*c*16	1.17c	0.52ab	0.21a	0.89bc	0.51ab	0.08
C22:4	1.21b	0.54a	0.23a	1.14b	0.60a	0.09
C22:6	0.03	0.00	0.05	0.00	0.02	0.01
TOBCFA[3]	20.47d	9.73b	5.12a	16.18c	10.29b	1.17
TSFA[3]	63.45c	56.12bc	41.57a	58.52bc	48.02ab	1.59
TUFA[3]	25.93a	24.02a	47.33b	23.52a	33.24a	2.16
TC18s[3]	29.92a	29.51a	53.86b	26.45a	37.11a	2.38

TOBCFA - total odd- and branched-chain fatty acids; TSFA - total saturated fatty acids; TUFA - total unsaturated fatty acids; TC18s - total fatty acids containing 18 carbon chains.
Values are means with standard error of the mean (SEM).
Different letters across the rows indicate significant difference between treatments (P<0.05).
[1] Rumen fluid samples were collected by stomach tube 3 h after feeding in the morning.
[2] Identifiable and quantifiable fatty acids presented only.
[3] Total of individual fatty acids listed in the table plus other identifiable fatty acids.

Chlorella represented by linoleic acid. Linoleic acid is present in the lipids of the microalgae Spirulina and Chlorella (Otles and Pire, 2001), and C18:2 has been reported to be the main FA in CSM, almost double that of C16:0 (Mohamed et al., 1988). In the current experiment, the concentration of C18:2n-6 was greater for these three supplements, but a significantly greater concentration was only found in the RF of steers fed Chlorella and CSM.

The fresh water microalgae Spirulina and Chlorella and the marine microalgae Dunaliella are the most important commercially produced microalgae grown in open systems (Radmann et al., 2007). These algae species have long-chain unsaturated FA that, when offered to monogastric animals, could alter the FA composition of the meat (Raes et al., 2004), but when offered to cattle, the biohydrogenation process in the rumen could affect the original FA profile (Costa, 2018). Total unsaturated FA in various lipid sources were only partially protected from biohydrogenation, not directly translating to the final FA profile in the RF (Costa et al., 2017). In the current experiment, Chlorella supplementation was the only treatment that resulted in an increase in TUFA in the RF.

Other microalgae species are rich in longer chain polyunsaturated FA with 20 and 22 C. Meale et al. (2014) offered *Schizochytrium* sp, a marine alga rich in docosahexaenoic acid (DHA) (C 22:6n-3), to growing lambs and observed DHA increments on the FA profile of adipose tissue, positively altering carcass characteristics. Pickard et al. (2008) offered *Schizochytrium* sp to pregnant ewes in the final weeks of gestation, e.g. 10 to six weeks before birth, and reported that lambs from these ewes stood up faster after birth demonstrating a better vigour compared with lambs born from the ewes of the control treatment. The main FA of Spirulina were the already saturated palmitic acid 16:0 and the polyunsaturated FA (C18:1, C18:2, and C18:3) that could be completely hydrogenated to stearic and monoenoic acids in the rumen (Polan et al., 1964). In the current experiment, the unsaturated FA in Spirulina did not markedly increase the TUFA in the RF and Chlorella was the only treatment that led to increases in unsaturated FA, especially linoleic acid, the main polyunsaturated FA found in RF.

Spirulina and Dunaliella had the greatest concentrations of C16:0 (i.e. 47.6 and 61.2% of total FA, respectively), and this probably resulted in a significantly greater C16:0 proportion in the RF of steers supplemented with Spirulina (i.e. around 37% above the control treatment). Palmitic acid is also the most common saturated FA in plants (McDonald et al., 2002) and is found in greater concentration in the lipids of protozoa (Or-Rashid et al., 2007). However, there was no difference in the proportion of palmitic acid in the RF of steers supplemented with Chlorella, Dunaliella, CSM, or unsupplemented steers.

Meat from ruminants have a higher conjugated linoleic acid (CLA) content than meat from monogastric animals (Schimid et al., 2006). One of the main isomers of linoleic acid, CLA *c*9, *t*11, rumenic acid, was significantly lower in the RF of steers supplemented with Spirulina or Chlorella. Rumenic acid has been reported to have no effect on milk fat content or yield (Bauman et al., 2008) and is typically the dominant CLA in forage-based ruminant products (Kay et al., 2004), including the adipose tissue of ruminants, where it accounts for 75-90% of the CLA isomers (Bauman et al., 2008).

It appears that none of the supplements in the current experiment would produce a FA profile that could lead to high concentrations of isomers known to inhibit fat synthesis, since rumenic acid only decreased with inclusion of all supplements in the diet. Rumenic acid is an intermediate of C18:2n-6 biohydrogenation (Kay et al., 2004), and the relative differences viewed against the content in feeds were expected to be higher. The hydrogenation of linoleic acid is often incomplete, ranging from 70 up to 95% (Doreau and Ferlay, 1994). The high linoleic acid proportion observed in the RF of steers supplemented with Chlorella and CSM could be due to an escape from ruminal biohydrogenation, or due to a slow rate of this process. *In vitro* rates of disappearance have been reported ranging from 23.6 up to 44.6%/h (Jouany et al., 2007), and the RF from steers was collected approximately 3 h after feeding.

Various isomers result from ruminal biohydrogenation, and a portion of these CLA escape from the rumen and affect lipid metabolism in the mammary gland and subcutaneous and intramuscular fat (Bauman et al., 2011). More importantly, the *t*10 *c*12 isomer of CLA can markedly inhibit FA synthesis

in all three tissue types, although a much greater dose is required to inhibit body fat accretion (Smith et al., 2008) than a reduction in milk fat (Bauman et al., 2008). Bauman et al. (2008) reported that a dose of 2.5 g/day of *t*10 *c*12 CLA was required to achieve a 25% reduction in milk fat, and the maximum inhibition was 50%, and Smith et al. (2008) indicated that doses 20 times higher would be required to inhibit fat synthesis in tissues such as subcutaneous and intramuscular fat. The extent of formation of this specific isomer within the rumen of steers in the current experiment could not be accurately measured with the method utilised. In spite of that, the proportion of rumenic acid decreased in the RF of steers supplemented with Spirulina, Chlorella, and CSM when compared with the control treatment. If these treatments reduced the production of the main CLA isomer in the RF, possibly the other isomers would also decrease, and no effects in adipose fat synthesis could be expected in growing cattle fed these supplements.

The same pattern of CLA decrease was described by Noci et al. (2005), who reported that with the substitution of a pasture-based diet rich in linolenic (with an increased ruminal production of C18:1 *t*11) for a silage/concentrate-based diet rich in linoleic acid, there was a decrease in total CLA. The proportion of linoleic acid was significantly higher in the RF of steers supplemented with Chlorella and CSM. Lamminen et al. (2019) also reported a higher proportion of linoleic acid in RF of lactating dairy cows fed Chlorella in comparison with Spirulina diet. The proportion of linolenic was almost five times higher in the RF of unsupplemented steers compared with those supplemented with Chlorella. The FA profile of the tropical forage utilised in this experiment differs greatly from results seen for temperate forages. Chilliard et al. (2001) reported that temperate forages have 55-65% of their FA composed of linolenic acid, which was also observed in the work of Costa et al. (2019) using ryegrass (*Lolium perenne*). The speargrass hay in the current experiment had a much lower (4% of total FA) proportion of linolenic acid than reported in temperate pastures. O'Kelly and Reich (1976) analysed a variety of tropical grasses, and linolenic acid ranged from approximately 13% for speargrass up to 36% of total FA for *Dichanthium sericeum*. The lowest value observed for speargrass in their study was three times higher than the one observed in the current experiment; however, they analysed a fresh grass sample, whereas ours was a late-vegetative stage plant made into hay. Doreau and Poncet (2000) emphasised that hay processing tends to decrease the FA concentration, in particular the proportion of linolenic acid. Speargrass hay had 15% of FA comprised of linoleic acid. The ratio between linoleic and linolenic between tropical and temperate grasses is also different with temperate forages having a greater proportion of linolenic acid (Chilliard et al., 2001). The concentration of linoleic acid in speargrass hay utilised in the current experiment was almost four times greater than linolenic acid, and, interestingly, an inversion was observed in the RF of these steers, in which the concentration of linoleic acid was 2.5 times greater, indicating extensive ruminal biohydrogenation in this experiment.

Odd- and branched-chain fatty acids in the rumen are almost completely derived from bacterial synthesis (O'Kelly and Spiers, 1991; Kim et al., 2005). The proportion of TOBCFA in the RF of steers supplemented with CSM, Spirulina, and Chlorella was lower than unsupplemented and Dunaliella-supplemented steers. A higher proportion of the odd- and branched-chain FA C15:0anteiso was reported for acetate-producing bacteria (Vlaeminck et al., 2004). In this study, as the results observed for TOBCFA, steers supplemented with CSM, Spirulina, or Chlorella had a lower proportion of C15:0anteiso in the RF than unsupplemented steers. All microalgae had extremely low fibre content, and CSM also has a low ADFom and aNDFom contents relative to speargrass (Table 1).

The RF of steers supplemented with CSM and Chlorella had reduced proportion of TSFA in the RF, and Chlorella led to a corresponding increase in TUFA concentration, compared with the control. This is most likely a direct result from FA of dietary origin, since it appears that microbes are not capable of synthesizing polyunsaturated FA (O'Kelly and Spiers, 1991). Morais and Costa (2008) reported that Chlorella sp. had the greatest concentration of TUFA (72% of total lipids), whilst Spirulina had the greatest TSFA, i.e. 81.6% of total lipids, which agreed with the present results. The greater concentration of TUFA in the diet would increase the concentration of TUFA in milk or meat if they bypassed the rumen either unaffected by the rumen microbes or by incorporation into microbial FA with no structural changes, i.e. chain elongation or hydrogenation. Safflower oil increased the concentrations

of TUFA in rumen bacteria without altering their concentration of TSFA (O'Kelly and Spiers, 1991). It has been suggested that polyunsaturated FA inhibit the reduction of C18:1 to 18:0, giving rise to a high C18:1 *t* content (Bessa et al., 2007; Or-Rashid et al., 2008). A significant reduction of C18:0 was observed for CSM and Spirulina, but there were no significant increases in any of the C18:1 isomers. When supplements rich in polyunsaturated FA, such as Chlorella and CSM (i.e. 55.6 and 72.6 % of total FA, respectively), are utilised, there is a reduced yield of C18:0 (Bessa et al., 2007) and an accumulation of the intermediate C18:1 *t*11 (Chilliard et al., 2001). This same pattern, not observed here, has been observed for increasing levels of other algae used as supplement (Boeckaert et al., 2008; Or-Rashid et al., 2008), which can be associated with changes in the microbial community (Boeckaert et al., 2008). In the current experiment, significant reductions in C18:0 proportion were observed in RF of steers supplemented with CSM and Spirulina. However, no significant differences in accumulation of C18:1 *t*10 and C18:1 *t*11 were observed for any of the treatments, except for the Dunaliella supplement. The latter supplement, interestingly, caused a reduction of this intermediate in the RF, which was most likely related to the basal forage, considering that the intake of Dunaliella, high in Na, was very low (Costa et al., 2016).

Alpha-linolenic acid (C18:3n-3) was present in high concentrations in the Chlorella supplement and was significantly greater in the RF of steers supplemented with this source. Free FA, such as C18:3n-3 and C18:2n-6, are often incompletely biohydrogenated, converting into C18:0 and monounsaturated isomers (Scollan et al., 2001).

The concentration of LCFA was usually below 1% of total FA in the supplements and the RF of supplemented animals. Speargrass hay had a greater concentration of LCFA than the microalgae and CSM supplements used in the present study, and this was reflected in greater concentrations in the RF of unsupplemented steers, but they were still very low values. Some marine algae are rich sources of specific long chain FA, such as DHA C22:6n-3 in *Schizochytrium* sp. (as much as 39% of total FA; Jiang et al., 2004; Hauvermale et al., 2006) or in *Crypthecodinium cohnii* (68.9% of total FA; Pickard et al., 2008). However, the fresh algae Chlorella and Spirulina have much lower concentrations of those LCFA. In particular, C22:6 DHA was present in very low concentration in speargrass hay sample, i.e. 0.53% of total FA and close to zero for all supplements utilized in this study. Therefore, the results of the present experiment are more likely to reflect the general FA profile of the feeds than any ruminal function characteristic.

5. Conclusions

Chlorella pyrenoidosa supplementation results in a higher proportion of unsaturated fatty acids in rumen fluid of steers. Supplementation with microalgae sources and CSM decrease rumenic acid (C18:2 *c*9, *t*11), the main conjugated linoleic acid isomer, indicating that it is unlikely that any of the supplements tested would have differential effects on other isomers directly related to fat synthesis.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: D.F.A. Costa, S.P. Quigley, S.R. McLennan and D.P. Poppi. Data curation: D.F.A. Costa and S.J. Gibbs. Formal analysis: D.F.A. Costa, X.Q. Sun, S.J. Gibbs and D.P. Poppi. Funding acquisition: S.R. McLennan and D.P. Poppi. Investigation: D.F.A. Costa, S.P. Quigley, P. Isherwood, S.R. McLennan and D.P. Poppi. Methodology: D.F.A. Costa, S.P. Quigley, P. Isherwood, S.R. McLennan, X.Q. Sun and D.P. Poppi. Project administration: D.F.A. Costa, S.P. Quigley, S.R. McLennan and D.P. Poppi. Resources: D.P. Poppi. Supervision: S.P. Quigley, P. Isherwood, S.R. McLennan and D.P. Poppi. Visualization: S.R. McLennan, S.J. Gibbs and D.P. Poppi. Writing-original draft: D.F.A. Costa. Writing-review & editing: D.F.A. Costa, S.P. Quigley, P. Isherwood, S.R. McLennan, S.J. Gibbs and D.P. Poppi.

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