

# *In vitro* Ruminant Fermentation Characteristics of Algal Protein Precipitate Supplemented in Lactation Dairy Diets in Continuous Cultures

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**ABSTRACT:** Identifying a sustainable source of dietary protein in lactation dairy diets is a sound approach to optimize N utilization efficiency. The algal protein precipitate (APP) generated from the wet lipid extraction procedure can be a valuable, unconventional protein source in lactation dairy diets. The present study was performed to investigate the effects of APP supplemented in high-forage (HF) or low-forage (LF) diet on *in vitro* ruminal fermentation in continuous cultures. Total volatile fatty acid (VFA) concentration was not affected due to forage concentration in the diets, whereas LEA supplementation increased total VFA concentration in HF diet ( $P < 0.05$ ). Decreasing forage in the diets as well as APP supplementation reduced culture ammonia-N concentration ( $P < 0.01$ ). Supplementing APP elevated C18:1 *trans*-11 concentration ( $P < 0.01$ ), regardless of forage concentration in the diets. However, methane production in the culture was not affected by dietary treatments. Supplementation of APP successfully replaced soybean meal and canola meal mixture, typical sources of protein supplement, without any negative effect on microbial fermentation profiles. Increased total VFA concentration coupled with decreased ammonia-N concentration in HF diet indicates that APP used in the present study exerted its potential effects in a diet-dependent manner.

**KEYWORDS:** algal protein precipitate, lipid-extracted algal biomass, protein supplement, lactation dairy diet, microbial fermentation, continuous culture

# International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

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Vol. 8, Issue 9, September 2019

## I. INTRODUCTION

Dietary sources of N in lactation diets are inefficiently utilized by dairy cows, as they excrete substantially more N in manure over milk, which increases milk production costs and environmental N excretion. In fact, in high-quality forage diets such as alfalfa hay-based lactation rations, most proteins are rapidly solubilized releasing between 56 and 65% of the N concentration in the rumen during microbial fermentation. Consequently, large losses of N occur (25–35%) as ammonia into urine [1]. Ruminant metabolism has been identified as the single most important factor contributing to the inefficient use of N in ruminants [2]. Thus, manipulation of rumen microbial fermentation is more feasible than modifying other metabolic processes to optimize dietary N utilization efficiency [3]. Therefore, identifying a sustainable source of dietary protein in lactation dairy diets is a sound approach to optimize N utilization efficiency.

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that convert sunlight and CO<sub>2</sub> to biomass that includes lipids, proteins, and carbohydrates [4]. Advantages of using microalgae for fuel production include ease of cultivation, use of water unsuitable for crop or human consumption and minimal competition for land resources with crops used for human food production [5]. In the biofuel production process, lipid is separated from the algal cell by various extraction methods. One of the major hurdles in reducing the cost of algal biodiesel is the need to de-water the algal biomass after harvesting the algae from the growth medium [6].

## II. RELATED WORK

Expensive costs for de-watering/drying, extraction and processing have limited commercial scale production of biodiesel from algal biomass. However, Sathish and Sims [7] recently developed the wet lipid extraction procedure (WLEP) that was capable of extracting 79% of transesterifiable lipids from wet algal biomass (84% moisture) via acid and base hydrolysis (90°C and ambient pressures), and 76% of those extracted lipids were isolated by further processing and converted to fatty acid (FA) methyl esters. Furthermore, the WLEP is capable of solubilizing and later precipitating a solid phase containing a high percentage of protein material (algae protein precipitate, APP) along with FA's APP [7]. In addition, the procedure generated side streams that serve as feedstocks for microbial conversion to additional bioproducts in addition to the APP material that can be used as a protein source in animal diets [7].

## III. OBJECTIVES

The aim of the present study was to investigate if the algal protein precipitate (APP) produced via the WLEP could replace typical protein supplements in lactation dairy diets by assessing *in vitro* ruminal fermentation profiles in continuous cultures. We hypothesized that supplementing APP would result in different patterns of microbial fermentation onto high- (HF) or low-forage (LF) diet due to dietary associative effects.

## IV. MATERIALS AND METHODS

Microalgae used for this study was obtained from the Logan City, Utah Wastewater Treatment Plant (Logan, UT, USA) and consisted of mixed naturally occurring cells composed primarily of *Chlorella* and *Scenedesmus* sp. [8,9]. The algal protein precipitate was generated as a protein supplement for this study using dilute acid and base hydrolysis of the algal biomass based on the WLEP. Chemical conditions allowed for the initial disruption of the algae cells under acidic conditions and partitioning of the protein fraction into the aqueous phase under basic

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Vol. 8, Issue 9, September 2019

conditions, followed by precipitation of the protein fraction under dilute acidic conditions. Hexanes were then used to partition the lipid fraction away from the precipitated protein fraction, which resulted in the production of the APP that contained the protein fraction as a main nutrient component.

## V. EXPERIMENTAL DESIGN, DIETS AND TREATMENTS

The design of the experiment was a  $2 \times 2$  factorial with four independent runs as replicates ( $n = 4$ ), and a fermentor in continuous cultures was considered an experimental unit. Diets based on alfalfa hay and corn silage as forage sources were formulated to maintain different dietary forage-to-concentrate (FC) ratios (60%:40% or 40%:60%, dry matter (DM) basis) in the HF or the LF diet, respectively (Table 1). Algal protein precipitate (APP) contained 925, 587, 3.5 and 68.7 g kg<sup>-1</sup> DM for organic matter, crude protein (CP), neutral detergent fibre and ether extract, respectively. Alfalfa hay had a chemical composition of 212, 377 and 274 g kg<sup>-1</sup> DM for CP, neutral detergent fibre and acid detergent fibre, respectively, whereas corn silage contained 84.0, 368 and 201 g kg<sup>-1</sup> DM for CP, neutral detergent fibre and acid detergent fibre, respectively. Four experimental treatments tested in this study were as follows: (1) HF without APP (HF-APP); (2) HF with APP (HF+APP); (3) LF without APP (LF-APP); and (4) LF with APP

**Table 1.** Ingredient and Chemical Composition of Experimental Diets

Item <sup>a</sup> (g kg <sup>-1</sup> dry matter (DM))	Diet <sup>b</sup>			
	HF		LF	
	-LE A	+LEA	-LEA	+LEA
<b>Ingredient</b>				
Alfalfa hay	377	367	229	232
Corn silage	227	241	174	176
Corn, steam-flaked	43.4	46.0	60.2	76.4
Corn, high-moisture	95.4	101	128	130
Corn distillers grains	76.5	56.8	95.5	97.0
SBMCM	62.9	-	88.3	-
LEA	-	56.1	-	59.1
Soy hulls	61.2	64.9	127	129
Beet pulp, shreds	30.6	40.5	71.6	72.7
Yeast culture	1.9	2.0	2.0	2.0
Sodium bicarbonate	4.2	4.4	4.4	4.4
Vitamin and mineral mix	19.7	20.9	20.5	20.8
<b>Chemical composition</b>				
Organic matter	954	953	951	950
Crude protein	146	150	148	140
Neutral detergent fibre	325	335	342	329
Acid detergent fibre	194	200	198	186
Ether extract	18.2	20.3	19.9	11.9
NFC	465	448	441	469
Net energy (MJ kg DM <sup>-1</sup> )	6.99	7.07	7.28	7.45

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Vol. 8, Issue 9, September 2019

<sup>a</sup> SBMCM, mixture of soybean meal and canola meal at 50%:50% in a DM basis; yeast culture, Diamond V XP<sup>®</sup> (Diamond V Mills Inc., Cedar Rapids, IA, USA); vitamin and mineral mix was formulated to contain (per kg DM): 226.7 mg of Se (from sodium selenite), 9,278.7 mg of Cu (from copper amino acid complex), 40,537.4 mg of Zn (from zinc amino acid complex), 38,653.4 mg of Mn (from manganese amino acid complex), 552.6 mg of Co (from cobalt carbonate), 1,234,585.2 IU of vitamin A, 152,808.1 IU of vitamin D, 3,815.1 IU of vitamin E and 295 mg of Rumensin<sup>®</sup> (Elanco Animal Health, Greenfield, IN, USA); NFC, nonfiber carbohydrates = 1000 – crude protein – neutral detergent fibre – ether extract – ash; net energy was based on tabular value.<sup>10</sup>

<sup>b</sup> HF–LEA = high-forage diet (HF; 60% forage:40% concentrate) without lipid-extracted algal biomass (LEA); HF+LEA = HF with LEA; LF–LEA = low-forage diet (LF; 40% forage:60% concentrate) without LEA; and LF+LEA = LF with LEA.

(LF+APP). Although we completely replaced 50:50 mixture of soybean meal and canola meal (SBMCM) with the APP in the HF+APP and the LF+APP, a similar CP content (146 g kg<sup>-1</sup> on average) across treatments was maintained (Table 1). The diets were formulated based on NRC [10] to meet the nutrient requirements of a dairy cow in early lactation to produce 38.0 kg of milk d<sup>-1</sup> with 35 g kg<sup>-1</sup> fat and 30 g kg<sup>-1</sup> protein. Before use in the fermentors, the diets were dried at 55°C for 48 h and ground through a 4.0 mm screen (standard model 4, Arthur Thomas Co., PA, USA).

## Donor Cows and Continuous Culture Operation

Whole ruminal contents were taken from two ruminally fistulated, lactating dairy cows fed the HF–APP. Care, handling and sampling of the donor animals were approved by the Institutional Animal Care and Use Committee of Utah State University (Logan, UT, USA).

Ruminal fluid was collected 4 h after the morning feeding (1100 h). Grab samples of ruminal contents were obtained from various locations within the rumen and composited. The ruminal contents were placed in sealed, preheated containers and transported to the lab, where the contents were strained through polyester screen (PeCAP, pore size 355 µm; B & SH Thompson, Ville Mont-Royal, QC, Canada). Each of four fermentors received approximately 700 mL of strained ruminal fluid under a stream of oxygen-free CO<sub>2</sub>. A dual-flow continuous culture system based on Teather and Sauer [11] was used, and it consisted of 1-L gas-tight fermentor vessels (Prism Research Glass, Inc., Research Triangle Park, NC, USA). A constant flow of CO<sub>2</sub>, delivered at 20 mL min<sup>-1</sup>, maintained anaerobic fermentation conditions. Over a 24-h period, artificial saliva [12] was delivered to each fermentor to yield a fractional dilution rate of 8.0% h<sup>-1</sup> (1.2 mL min<sup>-1</sup>) by precision pump (Model 323, Watson-Marlow Inc., Wilmington, MA, USA). The temperature of the cultures in the fermentors was maintained at 39°C by a circulating water bath.

A single run was composed of four fermentors that were inoculated simultaneously with ruminal contents obtained from the same cows. Each independent run lasted 11 days (8 days of treatment adaptation and 3 days of data and sample collection). The first 2 days of each run allowed for microbial adaptation to the fermentors with feeding the HF–APP to all fermentors. Starting day 3, experimental diets gradually replaced the HF–APP, and then by day 6, all fermentors received a full experimental diet. Therefore, all fermentors had an adaptation period with full experimental treatments (assigned diet) at least for 3 days. Each fermentor received 20 g d<sup>-1</sup> (DM basis) of the corresponding experimental diet in two equal portions being added to each fermentor at 0800 and 2000 h. Diets were manually fed to the fermentor through a feed port on the fermentor vessel. Data and samples were collected on day 9 to 11.

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## Sampling and chemical analysis

Experimental diets were analyzed for DM by oven drying at 55°C for 48 h (method 930.15), organic matter by ashing in a muffle furnace at 550°C for 5 h (method 985.01), and N (method 990.03) according to the Association of Official Analytical Chemists methods [13]. The neutral detergent fibre and acid detergent fibre, both inclusive of residual ash, were determined according to Hall *et al.* [14] with the method modified for use with an Ankom<sup>200</sup> Fiber Analyzer (Ankom Technology, Macedon, NY, USA). Heat stable  $\alpha$ -amylase and sodium sulfite were used in the neutral detergent fibre analysis. Experimental diets were analyzed for total FA content and FA profile according to the procedure of Sukhija and Palmquist [15] using a GLC (model 6890 series II; Hewlett Packard Co., Avandale, PA, USA) fitted with a flame ionization detector.

All data collection, sampling and analysis were independently performed in each run. Culture pH was recorded through a pH electrode connected to a pH meter (sympHony<sup>TM</sup> B10P benchtop meters, VWR International, Inc., Radnor, PA, USA) every hour for 12 h on d 9 and 10. Methane (CH<sub>4</sub>) samples were taken from the headspace gas of each fermentor at 0, 3, 6, 9 and 12 h after the morning feeding using a 10  $\mu$ L gastight syringe (Hamilton Co., Reno, NV, USA) and analyzed for methane with a GLC (model CP-3900, Varian, Walnut Creek, CA, USA). Daily CH<sub>4</sub> output (mmol d<sup>-1</sup>) was calculated as reported earlier [16] using the following equation: CH<sub>4</sub> concentration in fermentor headspace (mmol mL<sup>-1</sup>)  $\times$  CO<sub>2</sub> gas flow through the fermentor headspace (20 mL min<sup>-1</sup>)  $\times$  60 min  $\times$  24 h. Immediately after methane sampling, 5 mL of fermentor ruminal fluid was collected, filtered, added to 1 mL of 1% sulfuric acid, and retained for ammonia-N (NH<sub>3</sub>-N) determination. Another 5 mL of ruminal fluid taken at 3, 6 and 9 h was added to 1 mL of 25% meta-phosphoric acid, and samples were retained for volatile fatty acid (VFA) determination. These samples were stored at -40°C until analyses. Concentration of NH<sub>3</sub>-N was determined as described by Rhine *et al.* [17] using a microplate reader. Ruminal VFA were separated and quantified according to Holt *et al.* [18] using a GLC (model 6890 series II) with a capillary column (30 m  $\times$  0.32 mm i.d., 1  $\mu$ m phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA, USA) and flame-ionization detection.

On the final day of each run (day 11), the total volume of fermentor contents was collected and blended using a Waring blender (Waring Products Division, New Hartford, CT, USA) for 1 min. A total of 15 mL of blended ruminal fluid was collected and freeze-dried (FreeZone 12 L; Labconco Co., Kansas City, MO, USA) for FA analysis. The samples were methylated [19] and analyzed for long-chain FA by GLC (model CP-3380; Varian, Walnut Creek, CA, USA). Fatty acid methyl esters were separated on a 100 m  $\times$  0.25 mm  $\times$  0.2  $\mu$ m film thickness fused-silica capillary column (SP-2560, Supelco, Bellefonte, PA, USA). Verification of peak identity was established by comparison of peak retention times to known standards.

## Statistical analysis

The data in this study were analyzed using the mixed model procedures of SAS (version 9.4, SAS Institute, Inc., Cary, NC, USA). Data for VFA and ruminal FA profiles were analyzed using the following model:

$$Y_{ijkl} = \mu + R_j(F_j) + D_k + L_l + (DL)_{kl} + e_{ijkl}$$

where  $Y_{ijkl}$  = individual response variable measured;  $\mu$  = overall mean;  $R_j(F_j)$  = random effect of fermentor  $j$  within independent run  $i$ ;  $D_k$  = fixed effect of dietary FC  $k$  (HF *versus* LF;  $k = 1$  to  $2$ );  $L_l$  = fixed effect of APP supplementation ( $-APP$  *versus*  $+APP$ ;  $l = 1$  to  $2$ );  $(DL)_{kl}$  = interaction between dietary FC  $k$  and APP supplementation  $l$ ; and  $e_{ijkl}$  = residual error.

Denominator degrees of freedom were estimated using the Kenward-Roger option. The same mixed model was used for variables that were repeated in time (culture pH, NH<sub>3</sub>-N and CH<sub>4</sub>), but sampling time and a repeated statement were added to the model. One of three model structures was used depending on the finite-sample corrected Akaike's information criterion value for data that best fit the model. The structures were unstructured and compound symmetry, unstructured and first-order autoregressive, and unstructured and unstructured variance-covariance structure. Residual errors were used to test main effects and interactions. Differences were considered

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significant at  $P \leq 0.05$ , and trends were discussed at  $0.05 < P \leq 0.10$ . When the interaction between FC and LEA supplementation in the diet was  $P \leq 0.10$ , Bonferroni-adjusted  $P$ -values were used to assess the effects of APP within FC. Results are reported as least squares means.

## VI. RESULTS AND DISCUSSION

It is important to identify sources of supplemental protein that will accomplish optimal ruminant productive efficiency while minimizing feed cost and environmental concerns. Therefore, use of dietary CP is one of the main determinants affecting nutritive value of APP material for the ruminant industry. The research reported in this paper represents our initial attempt to characterize *in vitro* fermentation profiles of APP as an alternative protein source relative to SBMCM as a control using continuous cultures, and to the best of our knowledge, this is the first time to describe overall *in vitro* effects of APP produced through the WLEP.

### Experimental diets

Dietary concentrations of CP, neutral detergent fibre, acid detergent fibre and ether extract were similar across experimental diets, resulting in a similar concentration of non-fiber carbohydrates (Table 1). Due to relatively greater concentration of CP in APP (587 g kg<sup>-1</sup> DM) compared with SBMCM (474 g kg<sup>-1</sup> DM), reduced amounts of APP (57.6 g kg<sup>-1</sup> DM on average) were added to the HF-APP (56.1 g kg<sup>-1</sup> DM) as well as the LF-APP (59.1 g kg<sup>-1</sup> DM) to completely replace SBMCM (75.6 g kg<sup>-1</sup> DM on average). The FA profile of APP and the four experimental diets is reported in Table 2. Total lipid content in APP was low, as expected, indicating most of the lipids were removed during the extraction process. Consequently, APP did not contain any eicosapentaenoic acid (EPA; C20:5 n3) and docosahexaenoic acid (DHA; C22:6 n3), the major FA typically present in microalgae. Linoleic acid (C18:2) was

**Table 2.** Fatty Acid Profiles (g 100 g<sup>-1</sup> of Fatty Acid Methyl Esters) of Lipid-extracted Algal Biomass (LEA) and Experimental Diets

Fatty acid	Diet <sup>a</sup>				
	LEA	HF-LEA	HF+LEA	LF-LEA	LF+LEA
C14:0	3.48	1.86	2.15	2.07	1.63
C16:0	16.7	12.9	19.3	17.4	15.8
C18:0	4.60	3.94	4.14	3.90	3.05
C18:1 <i>cis</i> -9	11.6	13.7	12.8	10.8	17.0
C18:1 <i>trans</i> -11	5.58	0.00	3.49	3.29	2.24
C18:2 n6	7.14	28.5	25.1	23.7	29.5
C18:3 n3	6.52	5.77	7.34	6.92	4.97
C20:0	6.55	3.53	5.62	5.30	3.84
C22:0	3.30	1.89	1.75	1.65	1.72

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Vol. 8, Issue 9, September 2019

<sup>a</sup> HF–LEA = high-forage diet (HF; 60% forage:40% concentrate) without lipid-extracted algal biomass (LEA); HF+LEA = HF with LEA; LF–LEA = low-forage diet (LF; 40% forage:60% concentrate) without LEA; and LF+LEA = LF with LEA.

present in small amounts in APP compared to the experimental diets. The concentration of linolenic acid (C18:3) was quite similar in APP and the experimental diets, whereas APP had numerically greater concentration of C20:0 and

C22:0 FA. In general, the FA profile of experimental diets was relatively similar. Inclusion of forage in the diet resulted in greater concentrations of C18:2.

Nutrient profiles of microalgae vary considerably with the species used. Lum *et al.* [20] reported 60-730 g kg<sup>-1</sup> DM CP of various algae, with *Synechococcus* sp. being the greatest followed by *Arthrospira maxima* and *Chlorella vulgaris*. Similarly, Lodge-Ivey *et al.* [21] reported that CP concentration of traditional lipid extracted algal biomass (LEA) ranged from 115 to 452 g kg<sup>-1</sup> DM from *Nannochloropsis salina*, *Chlorella* sp. and diatoms. In addition to the source of organisms in algae, use of different combinations of processing methods may affect CP content of LEA. To extract lipids, microalgae are first de-watered, and then the concentrated biomass is subsequently processed to optimize the solvent extraction through cell disruption, particle size reduction and drying [22]. Common algal harvesting methods include sedimentation, centrifugation and flocculation [23]. The APP derived from the WLEP and tested in the current study contained a relatively great content of CP (587 g kg<sup>-1</sup> DM) due to the use of dilute solutions of acid and base that did not disrupt or hydrolyze the protein fraction, and the removal of hydrocarbon-based and insoluble algal biomass during the first acid hydrolysis step that would have diluted the final protein fraction with polysaccharides associated with the cell walls. This is a unique method of processing microalgal biomass in order to generate a protein rich fraction for utilization within ruminant feeds.

## Ruminal fermentation characteristics

Culture pH varied following feed provision, but the overall pattern of diurnal fluctuation of the culture pH was similar among treatments, with the highest pH values observed just before feed input and the lowest pH values 3-4 h after provision. Decreasing forage proportion in the diet decreased culture pH ( $P < 0.01$ ), and supplementing APP further decreased the culture pH ( $P = 0.05$ ). Yet, the pH drop was observed only in HF but not in LF diet, leading to an interaction between FC and APP ( $P = 0.05$ ). Nevertheless, all dietary treatments maintained the culture pH at least above 5.8 throughout incubation. Decreasing FC ratio in the diet typically results in a reduction in ruminal pH with an increase in VFA production. In the current study, we added more corn distillers grains, soy hulls, and beet pulp in addition to corn grains in LF diets to decrease the FC ratio. Traditionally, these nonforage fibre sources (NFFS) have been used as sources of concentrate, because many NFFS have relatively high net energy value and moderate CP contents [24]. Alternatively, NFFS can be successfully used as a source of fibre in rations for dairy cattle when forages are either of poor quality or in short supply, so feeding the NFFS has been very practical in U.S. dairy industry. Increasing dietary contents of the NFFS in LF diets in the current study could prevent negative effects on microbial fermentation due to their dilution effect on starch content and resultant prevention of decrease in culture pH from excessive starch fermentation which occurs by feeding typical LF diet. The culture pH averaged on an hourly basis was maintained above 6.0 in the current study. Calsamiglia *et al.* [25] reported that the effect of ruminal pH on neutral detergent fibre digestibility was relatively small above pH 6.0, but digestibility of neutral detergent fibre decreased sharply below this pH threshold. Therefore, the dietary effects on culture pH due to FC ratio, APP supplementation and their interaction effect would have biologically minor consequences on microbial physiology in the cultures.

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Total VFA concentration was not affected due to FC in the diet (Table 3), whereas APP supplementation increased total VFA concentration in HF but not in LF, resulting in a tendency toward FC × APP interaction ( $P = 0.10$ ). Neither

**Table 3.** Ruminal Fermentation Characteristics of Continuous Cultures

Item	Diet <sup>a</sup>				SE	Significance of effect <sup>b</sup>		
	HF		LF			FC	LEA	INT
	-LEA	+LEA	-LEA	+LEA				
Total VFA (mmol)	29.4 <sup>b</sup>	33.8 <sup>a</sup>	34.3	32.7	2.89	0.30	0.64	0.10
VFA (mol 100 mol <sup>-1</sup> )								
Acetate (A)	45.9	47.2	47.2	47.6	3.81	0.17	0.28	0.37
Propionate (P)	36.5	36.8	39.8	38.7	2.26	0.01	0.71	0.41
Butyrate*	14.5	11.4	10.3	11.3	1.42	0.02	0.21	0.02
Valerate <sup>†</sup>	1.82 <sup>b</sup>	2.29 <sup>a</sup>	1.52	1.33	0.323	< 0.01	0.61	0.07
Isobutyrate	0.01	0.01	0.01	0.02	0.009	0.28	0.29	0.68
Isovalerate	1.20	1.92	0.86	0.82	0.360	0.02	0.46	0.17
A:P	1.28	1.29	1.23	1.24	0.180	0.21	0.79	0.95
Ammonia-N (mg L <sup>-1</sup> )	80.4	53.6	65.4	31.0	4.03	< 0.01	< 0.01	0.31
Methane (mmol d <sup>-1</sup> )	14.9	12.7	14.8	15.6	1.17	0.26	0.58	0.23

<sup>a</sup> HF-LEA = high-forage diet (HF; 60% forage:40% concentrate) without lipid-extracted algal biomass (LEA); HF+LEA = HF with LEA; LF-LEA = low-forage diet (LF; 40% forage:60% concentrate) without LEA; and LF+LEA = LF with LEA.

<sup>b</sup> FC = forage-to-concentrate ratio in the diet (HF versus LF); LEA = supplementation of LEA (-LEA versus +LEA); and INT = interaction between FC and LEA.

\* Under HF diet, -LEA was greater than +LEA based on Bonferroni-adjusted  $P$ -value ( $P < 0.05$ ).

<sup>†</sup> Under HF diet, +LEA was greater than -LEA based on Bonferroni-adjusted  $P$ -value ( $P < 0.05$ ).

FC nor APP influenced acetate proportion, while feeding LF diet increased molar proportion of propionate. Offering LF diet decreased butyrate proportion, while APP supplementation decreased butyrate proportion only in HF diet, causing a FC × APP interaction ( $P = 0.02$ ). In contrast, molar proportion of valerate increased with APP supplementation only in HF diet, leading to a tendency toward FC × APP interaction ( $P = 0.07$ ). Molar proportion of isobutyrate and acetate-to-propionate ratio were similar across diets. Decreasing FC ratio as well as APP supplementation reduced culture NH<sub>3</sub>-N concentration ( $P < 0.01$ ). While VFA are evaluated as a measure of carbohydrate fermentation, NH<sub>3</sub>-N is a measure of proteolytic activity of rumen microorganisms on dietary protein and related to microbial protein synthesis.

In the current study, supplementing APP in HF but not in LF diet resulted in increased total VFA concentration, which corresponded to the decrease in culture pH. Additionally, APP supplementation decreased NH<sub>3</sub>-N concentration both in HF and LF diets. Chakraborty *et al.* [26] characterized the monosaccharide composition of *Chlorella* algal biomass and approximately 90% was glucose, while *Nannochloropsis* sp. is approximately 63% [27]. Consequently, additional glucose in APP compared with SBMCM may have contributed to the increased VFA concentration in HF diet, but its potential effect would disappear in LF diet due to additional glucose from corn grains and NFFS. In addition, this result was likely associated with different dietary concentration of alfalfa hay between the HF+APP and the LH+APP (367 versus 232 g kg<sup>-1</sup> DM, respectively).



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Ruminal microbes degrade alfalfa protein too rapidly, which results in inefficient utilization of feed N with high CP degradability of alfalfa hay in the rumen and limits optimal microbial protein synthesis [28]. Hence, the potential effects of APP may have been extensively exerted in HF diet with more alfalfa hay, showing an associative effect of CP fermentation in the culture. The increased VFA concentration due to APP supplementation may be associated with microbial protein synthesis. It is believed that energy is the most limiting factor in microbial growth [29], and consequently increased VFA concentration due to APP supplementation could contribute to the decreased  $\text{NH}_3\text{-N}$  concentration with enhanced microbial growth. Rumen bacteria can utilize more  $\text{NH}_3\text{-N}$  for microbial protein synthesis in the presence of readily available energy [10]. Meanwhile, the  $\text{NH}_3\text{-N}$  concentration in the current study would not be a limiting factor for microbial growth. Ruminal  $\text{NH}_3\text{-N}$  concentration of  $50 \text{ mg L}^{-1}$ , derived from *in vitro* continuous culture experiments by Satter and Slyter [30] is often accepted as the concentration required to meet N requirements and support maximal growth of ruminal microorganisms. Satter and Slyter<sup>30</sup> also observed that the limiting concentration of  $\text{NH}_3\text{-N}$  was closer to  $20 \text{ mg L}^{-1}$ . In the present study, the least concentration of the  $\text{NH}_3\text{-N}$  in cultures was  $31 \text{ mg L}^{-1}$  in the LF+APP.

Under typical cattle-feeding conditions, manipulation of ruminal protein degradation or the efficiency of N utilization in the rumen is the most effective strategy to reduce N losses [2]. Using data obtained from continuous culture studies, Bach *et al.* [29] reported that as efficiency of N utilization increases,  $\text{NH}_3\text{-N}$  accumulation in the fermentors decreases ( $R^2 = 0.78$ ). Thus, the reduction in the  $\text{NH}_3\text{-N}$  concentration through LEA supplementation can contribute to improving utilization of dietary N in ruminal fermentation and reducing N excretion. Reduction of  $\text{NH}_3\text{-N}$  in the rumen can be resulted from combined effects on a decrease in dietary protein degradation, a decrease in solubility of protein, and an inhibition of proteolytic bacteria or proteolytic enzymatic activity [31]. Therefore, further study needs to test biological value and ruminal degradability of APP.

Methane production in the culture was not affected by dietary treatments (Table 3). Due to minor effects on major VFA molar proportions and no difference on acetate-to-propionate ratio, no effect of dietary treatments on  $\text{CH}_4$  production was expected in the present study. Recent investigations have shown that dietary fat supplements, especially those containing unsaturated FA, may reduce ruminal methane emissions [32,33]. Both EPA and DHA are the major bioactive, unsaturated FA present in fish oil and marine algae [34]. Based on *in vitro* studies reporting 80% reduction of  $\text{CH}_4$  production [35,36], it has been proposed that these FA are particularly potent at reducing enteric  $\text{CH}_4$  emissions. The profile of polyunsaturated FA in the APP is affected by the lipid extraction procedure, and it is ineffective to extract microalgal lipid due to the relatively high moisture concentration and cellular elasticity [22]. Grima *et al.* [37] reported that the lipid extraction yields of dried algal biomass ranged from 50 to 93%, depending on the polarity of solvent systems. The APP used in the current study contained  $67.8 \text{ g kg}^{-1}$  DM of ether extract. This indicates an effective separation of lipids from the APP material during the hexane separation step, and consequently it had negligible concentrations of DHA and EPA in the APP, which caused no response on  $\text{CH}_4$  production in the cultures. Even Moate *et al.* [38] reported no reduction on  $\text{CH}_4$  emissions when dairy cows were fed a forage-based diet supplemented with algal meal up to  $0.375 \text{ kg d}^{-1}$  which supplied  $75 \text{ g cow}^{-1} \text{ d}^{-1}$  of DHA.

## Ruminal FA Metabolism

Effect of diet on culture FA composition is presented in Table 4. There was an interaction effect of diet on C18:0 ( $P = 0.04$ ); feeding APP decreased the production of C18:0 ( $P < 0.01$ ), and the effect was greater at the LF compared with the HF ( $P = 0.01$ ). High forage diets were associated with a greater C18:0 production when compared with LF

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**Table 4.** Fatty Acid Composition (g 100 g<sup>-1</sup> of Fatty Acid Methyl Esters) of Continuous Cultures

Fatty acid	Diet <sup>a</sup>				SE	Significance of effect <sup>b</sup>		
	HF		LF			FC	LEA	INT
	-LEA	+LEA	-LEA	+LEA				
C14:0	14.3	9.24	14.8	10.7	3.747	0.67	0.23	0.82
C16:0	17.7	21.1	16.4	19.2	1.06	0.01	< 0.01	0.46
C18:0*	15.5	4.48	10.3	3.85	0.878	0.01	< 0.01	0.04
C18:1 <i>t</i> 11	13.1	20.4	14.2	17.8	1.11	0.52	< 0.01	0.14
C18:1 <i>c</i> 9	15.5	18.1	16.2	18.7	1.55	0.62	0.10	0.94
C18:2 <i>n</i> 6	18.4	20.9	21.0	24.5	1.73	< 0.01	0.06	0.46
C18:3 <i>n</i> 3	3.46	3.23	2.83	2.71	0.318	0.09	0.58	0.87

<sup>a</sup> HF-LEA = high-forage diet (HF; 60% forage:40% concentrate) without lipid-extracted algal biomass (LEA); HF+LEA = HF with LEA; LF-LEA = low-forage diet (LF; 40% forage:60% concentrate) without LEA; and LF+LEA = LF with LEA.

<sup>b</sup> FC = forage-to-concentrate ratio in the diet (HF *versus* LF); LEA = supplementation of LEA (-LEA *versus* +LEA); and INT = interaction between FC and LEA.

\* Under HF and LF diets, -LEA was greater than +LEA based on Bonferroni-adjusted *P*-values (*P* < 0.05).

diet. Forage had no effect on the *trans*-18:1 or *cis*-18:1 isomers which were similar (*P* > 0.10) in the HF and LF diets. However, a greater amount of *cis*-18:2 was intact in diets with LF compared with HF diet (*P* < 0.01). The increased C18:0 and decreased C18:2 in HF diet is consistent with an increased degree of FA biohydrogenation in the HF diet. The most significant effect of APP was to markedly depress biohydrogenation; in the presence of APP, formation of C18:0 was reduced, and a greater proportion of C18:2 remained intact. Cultures receiving LEA had an increased proportion of the C18:1 *trans*-11 isomer and tendency (*P* = 0.10) for greater concentrations of the C18:1 *cis*-9 isomer. As expected, FA biohydrogenation was greater with HF compared with LF diet. Increasing forage level typically increases conversion of unsaturated FA to their more saturated end products. The decreased biohydrogenation with a decrease in the FC ratio has been associated primarily to a decline in pH. This might also be the reason for the further decline in FA biohydrogenation induced by the inclusion of APP. Both a decrease in FC ratio and inclusion of APP reduced pH of ruminal cultures. Because APP was included in small amounts in the diets and did not have any EPA or DHA, the effects on biohydrogenation were likely related to the 18:2 content provided mainly from the forages in the diets and the change in culture pH. It is interesting, however, that APP seemed to also increase *trans* C18:1 and *cis* C18:1, the intermediaries in the biohydrogenation process. In particular, C18:1 *trans*-11 from the ruminal biohydrogenation is a major precursor for the synthesis of C18:2 *cis*-9, *trans*-11 conjugated linoleic acid in milk fat via  $\Delta^9$ -desaturase in the mammary gland [39]. Thus, the APP tested in the current study has a potential to affect milk FA composition.

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## VII. CONCLUSIONS

Crude protein and FA have been suggested as two promising nutrients in microalgae to be used as value-added animal feeds. Supplementation of APP produced via the WLEP did not result in any negative impacts on *in vitro* ruminal fermentation profiles. Supplementing APP increased total VFA concentration coupled with decreased NH<sub>3</sub>-N concentration in HF diet, which partially supported our hypothesis by showing an associative effect of dietary nutrients on ruminal fermentation. Overall results in this study demonstrate that use of APP as a source of dietary protein supplement in dairy diets is promising, while a future direction in use of the APP to ruminants needs to consider comprehensive nutrient profiles of APP and its dynamic effects on rumen microbial ecosystem with target animals and their rations.

## VIII. ACKNOWLEDGEMENTS

This study was partially supported by the Sustainable Waste to Bioproducts Engineering Center (SWBEC), the Huntsman Environmental Research Center (HERC), the State of Utah Science Technology and Research (USTAR) Initiative, and the Utah Agricultural Experiment Station at Utah State University. We thank K. Neal at Utah State University and S. J. McLeod at North Carolina State University (Raleigh, North Carolina, USA) for their excellent assistance for the laboratory analyses.

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