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Nutritional Evaluation of Whole and Lipid-Extracted Biomass of the Microalga *Scenedesmus* sp. AMDD for Animal Feeds: Simulated Ruminal Fermentation and *In Vitro* Monogastric Digestibility.

Running title: *In vitro* Digestion of *Scenedesmus* sp. AMDD

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Abstract

Background: *Scenedesmus* sp. AMDD (*S*-AMDD) has been the focus of several studies to assess its potential as a feedstock for biofuels and bioremediation, while the evaluation of its potential suitability as a novel animal feed ingredient has only just begun. In an initial study; *S*-AMDD demonstrated rapid growth rate and biomass productivity during exponential growth phase and the resulting biomass appeared to have good potential for animal nutrition based on its attractive proximate composition, favorable essential amino acid, fatty acid and elemental profiles and lack of contaminating heavy metals. However, the total carbohydrate and fibre fractions of whole-cell and lipid-extracted *S*-AMDD were relatively high which could limit their digestion, particularly when fed to monogastric animals. The difference in the capacity to digest and metabolically utilize diets rich in cellulosic material (e.g., fibre) is vast between various farmed animal species. As such, knowledge on the nutritional value of novel ingredients for ruminant animals can rarely be immediately extrapolated to monogastrics and vice versa. Simulated fermentation using rumen-derived digestive fluids or *in vitro* digestibility using purified monogastric-derived enzymes can provide valuable information. Although not fully conclusive, results from these types of rapid assays are generally inexpensive, require smaller amounts of sample; utilize fewer (or zero) experimental animals, avoid feed refusal issues associated with ingredient off-flavours or odours and can be effective tools for research and for routine industrial use.

Objective: The present study is the second in a series of projects designed to evaluate the nutritional value of *S*-AMDD for animal feed applications. The main objective was to generate novel digestibility data of whole-cell and lipid-extracted *S*-AMDD for both ruminant and monogastric animals including ruminal organic matter digestibility (OMD), apparent metabolizable energy (aME) content, methane (CH_4) production, dilute pepsin digestibility (DPD) and two-phase gastric/pancreatic digestibility of protein ($GPD_{Protein}$) and energy (GPD_{Energy}).

Methods: Simulated ruminal OMD, aME contents and CH_4 production of experimental test diets containing graded levels of whole-cell and lipid-extracted *S*-AMDD were estimated using a modified batch-culture *in vitro* fermentation system with total gas capture using lactating dairy cattle as rumen fluid donors. *In vitro* monogastric DPD and two-phase GPD were measured by incubation of whole-cell and lipid-extracted *S*-AMDD samples in porcine pepsin and porcine pancreatin, containing amylase, lipase and protease enzyme solutions.

Results: Simulated ruminal fermentations using lactating dairy cattle as rumen fluid donors indicate that both whole-cell and lipid-extracted *S*-AMDD have excellent potential for use in ruminant animal feeds. Dietary inclusion of whole-cell *S*-AMDD at 50% forage protein replacement (equivalent to 20% of the total diet) or lipid-extracted *S*-AMDD at 100% forage protein replacement (equivalent to 32% of the total diet) did not significantly affect OMD or aME content of the control diet. However, OMD was marginally comprised with 100% forage protein replacement with whole-cell *S*-AMDD (equivalent to 40% of the total diet) relative to the 25 and 50% replacement levels, although not significantly different from the control diet. Diets containing lipid-extracted *S*-AMDD significantly reduced CH_4 production by approximately 50% compared to the control diet (47% reduction) and those containing whole-cell *S*-AMDD (51% reduction). Since OMD and aME content of diets containing lipid-extracted *S*-AMDD were unaffected relative to the control diet and whole-cell *S*-AMDD-containing diets, it seems clear that lipid-extracted *S*-AMDD contains anti-methanogenic 'non-fatty acid' substances that have the ability to suppress rumen methanogenic bacteria without disturbing ruminal digestion. This area warrants further exploration *in vivo*, especially considering the large volume of algal feed that could be produced

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without occupying significant land resources. *In vitro* monogastric digestibility using porcine enzymes indicates that lipid-extracted *S*-AMDD has potential for use in monogastric animal feeds. Protein and energy digestibility of this product were moderately high (75-84% and 70%, respectively); which resulted in relatively high contents of DP (30%) and DE (14 MJ kg^{-1}). On the other hand, the digestibility of whole-cell *S*-AMDD was low at 52-61% and 50%, respectively resulting in lower levels of DP (15%) and DE (12 MJ kg^{-1}). Despite the encouraging results for lipid-extracted *S*-AMDD, the digestibility (particularly of energy) remains marginal for monogastric animals and requires improvement through additional cost-effective cell rupture technologies or the production algal protein concentrates.

Keywords: Microalgae, digestibility, ruminant, monogastric, protein, energy, methane

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1. INTRODUCTION

Microalgae are highly efficient at primary metabolism which transforms light, CO_2 and inorganic elements into nutrient-rich biomass [1]. Applied within the right technological and political framework, this capacity could play a significant role at reducing global temperature rise linked to anthropogenic carbon emissions by transforming the way we produce consumer products like food/feed, soil amendments, biomaterials and bioenergy [2-4]. Specifically, it has been suggested that microalgae in the *Scenedesmus* genus are more efficient at sequestering CO_2 into intracellular hydrocarbons than other similar green chlorophytic species; making them an attractive bio-energy feedstock [5-7]. On the other hand, *Scenedesmus* spp. are generally inferior with regards to high lipid accumulation potential compared to many other species [8]. In addition, while renewable energy such as algal-based biodiesel has been a major driver for technological innovations recently, it remains far from being economically viable [9-12]. Biorefineries that can valorize the whole algal crop is currently the most likely path towards a viable microalgae-based industry [13-17] and the animal nutrition and aquaculture sectors are highly promising areas to focus for generating revenues [18-21]. Depending on algal species/strain, cultivation conditions and post-harvest processing, whole-cell biomass and the residual cake after lipid-extraction may be highly attractive sources of essential dietary amino acids, fatty acids, sugars, vitamins, minerals, carotenoids, digestible energy and other health-promoting compounds well suited as feeds or feed additives for terrestrial livestock and aquatic animals [1,22]. It has been established that the protein fraction of many microalgae originally screened for biodiesel applications also have essential amino acid profiles more adequately balanced than many terrestrial plant-based crops in wide use in animal nutrition such as corn, soybean, canola and wheat [23-25]. As a result, microalgae-based products could offer a novel supply of valuable commodities for sustainable development of terrestrial livestock and aquaculture feed inputs [26].

Scenedesmus sp. AMDD (*S*-AMDD) is a proprietary chlorophytic (green) microalgae strain that has proven highly robust and productive at flask and PBR cultivation and under both batch and continuous culture in our laboratory. Like most microalgae species in the *Scenedesmus* genus, *S*-AMDD is non-motile and colonial; typically forming clumps of four cells or sometimes eight or more under environmentally stressful conditions [27,28]. In recent years, *S*-AMDD has been the focus of several studies to assess its potential as a feedstock for biofuels and bioremediation [29-36] while the evaluation of its potential suitability as a novel animal feed ingredient has only just begun [37,38]. In an initial study; *S*-AMDD demonstrated rapid growth rate and biomass productivity during exponential growth phase and the resulting biomass appeared to have good potential for animal nutrition based on its attractive proximate composition, favorable essential amino acid, fatty acid and elemental profiles and lack of contaminating heavy metals. However, the total carbohydrate (CHO) contents of whole-cell and lipid-extracted *S*-AMDD were relatively high (35-48% of DM). Perhaps more importantly, the CHO fraction was rich in fibre (74-77% of total CHO) with lower proportions of starch (23-26%) which could limit their digestion as feed ingredients, particularly when fed to monogastric animals, including fish.

The extent to which various animals digest the nutrients within novel ingredients varies due to their different feeding habits and digestive physiologies, which can be broadly classified as either ruminant or monogastric. In particular, the difference in their capacity to digest and metabolically utilize diets rich in cellulosic material (e.g., fibre) is vast. As such, knowledge on the nutritional value of novel ingredients for ruminant animals can rarely be immediately extrapolated to monogastrics and vice versa. Once the biochemical composition of a novel ingredient has been established, digestibility is often the most important aspect in its nutritional assessment [39]. This is because the extent of its chemical and enzymatic breakdown in the gut (digestion) is tantamount to the amount of substrate nutrients available for intestinal absorption and; ultimately have the potential to be used for anabolic purposes (e.g., tissue synthesis, repair and maintenance). Although measurement of digestibility *in vivo* provides the most accurate assessment, the methods required are time-consuming, expensive and often require a large number of experimental animals. As an alternative, simulated fermentation using rumen-derived digestive fluids or *in vitro* digestibility using purified monogastric-derived enzymes can provide valuable information. Although not fully conclusive, results from these types of rapid assays are generally inexpensive, require smaller amounts of sample; utilize fewer (or zero) experimental animals, avoid feed refusal issues associated with ingredient off-flavours or odours and can be effective tools for research and for routine industrial use. The present study is the second in a series of

projects designed to evaluate the nutritional value of *S*-AMDD isolated in Saskatchewan, Canada for animal feed applications. The main objective was to generate novel digestibility data of whole-cell and lipid-extracted *S*-AMDD for both ruminant and monogastric animals including ruminal OMD, aME content, CH_4 production, DPD and two-phase $GPD_{Protein}$ and GPD_{Energy}.

2. MATERIALS AND METHODS

2.1. Test ingredients

This study investigated a novel strain of the chlorophytic microalga *Scenedesmus* sp. AMDD (*S*-AMDD) that was isolated from a soil sample in Saskatchewan, Canada. Isolation conditions, 18S gene sequence identification, screening criteria, mass cultivation, harvesting and processing and biochemical characterization are fully described elsewhere [37]. For reference, the proximate and caloric content of whole-cell and lipid-extracted *S*-AMDD are presented in Table1.

2.2. Simulated ruminal fermentation

Simulated ruminal OMD, aME contents and CH_4 production of experimental test diets containing graded levels of whole-cell and lipid-extracted *S*-AMDD were estimated using a modified batch-culture *in vitro* fermentation system with total gas capture using lactating dairy cattle as rumen fluid donors [40]. Seven isonitrogenous (12.4% crude protein; CP, DM basis) dietary treatments (Table 2) were formulated using a constant inclusion level of medium grind corn (15% of the diet; equivalent to 10% of total CP) and three inclusion levels of *S*-AMDD products (Low, 23% of total CP; Medium, 45% of total CP; High, 90% of total CP) replacing grass and legume forage; 1 mm grind (Low, 67% of total CP; Medium, 45% of total CP; High, 0% of total CP) and nitrogen-free cellulose. These levels represented dietary *as-fed* ratios of forage (F) and *S*-AMDD algae (A) corresponding to Control (100F:0A), Low (75F:25A), Medium (50F:50A) and High (0F:100A). Mixed rumen fluid was obtained from two ruminally-fistulated mid-lactation Holstein-Friesian dairy cows fed a complete ration containing a 60:40 blend of grass and legume forage and a concentrate composed of barley grain (40.0%), solvent-extracted canola meal (21.1%), soybean meal (20.9%), medium grind corn (9.3%) and vitamin/mineral supplement (8.7%). Rumen fluid (pH 5.8±0.4) was collected by hand sampling various locations of the rumens, mixed and coarsely filtered to remove large particles before transporting to the laboratory in a warmed insulated container where it was further filtered through 3 layers of nylon followed by 16 layers of cheesecloth into an Erlenmeyer flask (purged with nitrogen gas to maintain anaerobiosis) in a heated water bath (39°C). For each treatment, 400 mg of test diet, 30 mL of warm (39°C) simulated saliva ($NaHCO_3$, 4.6 g L^{-1}; $NaH_2PO_4 \cdot H_2O$, 4.29 g L^{-1}; NaCl, 0.28 g L^{-1}; KCl, 0.358 g L^{-1}; $CaCl_2 \cdot 2H_2O$, 0.0176 g L^{-1}; $MgCl_2 \cdot 6H_2O$, 0.0365 g L^{-1}; $NH_2CONH_2CH_4N_2O$, 0.3 g L^{-1} in distilled water) [41] and 10 mL of filtered rumen fluid (39°C) were sequentially added to a capped 150 mL Luer lock syringe (5 replicates per treatment) and lightly lubricated plungers were inserted to provide expandable volumetric gas collection capacity. After 48 h of incubation, volume of headspace gas was measured and a sample was transferred to exutainers for gas analysis. Syringes were submersed in a crushed ice water bath to terminate fermentation and contents were transferred into 100 mL glass beakers, partially dried at 70°C and then fully dried for 12 h at 105°C. Dried residues were stored at -80°C for subsequent analysis. Procedural blanks were included to correct for potential background influence of the filtered rumen fluid.

Animals used in this study were housed and cared for in accordance with the Canadian Council on Animal Care [42]

2.3. *In vitro* monogastric digestibility

In vitro monogastric DPD was measured by incubation of 200 mg of test sample in 0.0002% porcine pepsin (P7000, Sigma-Aldrich) enzyme solution (1:10,000 w/v in 0.075 N HCl; pH 1.5) for 16 h at 39°C [43,44]. *In vitro* two-phase GPD was measured by incubation of 250 mg of test sample in porcine pepsin (P7000, Sigma-Aldrich) enzyme solution (25 mg mL^{-1} w/v in 0.2 N HCl, pH 1) for 2 h at 39°C (gastric phase) and then subsequent incubation in porcine pancreatin, containing amylase, lipase and protease (P1750, Sigma-Aldrich) enzyme solution (100 mg mL^{-1} w/v in 0.05 M Tris, 0.0115 M $CaCl_2$ buffer; pH 7) for 4 h at the same temperature (pancreatic phase) [45]. Both of these *in vitro* assays were slightly modified to account for the very small particle size of microalgae [46]. Assays were conducted with five replicates and procedural blanks were run in parallel to correct final *in vitro* digestibility calculations.

2.4. Analytical techniques

Proximate composition and caloric content of whole-cell and lipid-extracted *S*-AMDD used for digestibility studies is described in Tibbetts *et al.* [37]. Moisture, organic matter, crude protein and gross energy contents of undigested residues obtained from simulated ruminal fermentations and *in vitro* monogastric digestibility assays and methane contents of headspace gas samples obtained from *in vitro* ruminal fermentations were determined according to Tibbetts *et al.* [46].

Simulated ruminal OMD (%) was calculated as: ([g of OM in initial sample - g of OM in residue DM] ÷ [g of OM in initial sample] × 100%).

Simulated ruminal aME (MJ kg^{-1}) was calculated as: (MJ kg^{-1} in initial sample - MJ kg^{-1} in residue DM - MJ kg^{-1} enteric gas) ÷ (g forage DM fed).

In vitro monogastric DPD (%) and GPD (%) were calculated as: ([% protein or MJ kg^{-1} energy in initial sample - % protein or MJ kg^{-1} energy residue DM] ÷ [% protein or MJ kg^{-1} energy in initial sample] × 100%).

2.5. Statistical methods

Data are reported as mean±standard deviation. Statistical analyses were performed using one-way analysis of variance, ANOVA (SigmaStat® v.3.5) with a 5% level of probability ($P<0.05$) selected in advance to sufficiently demonstrate a statistically significant difference. Where significant differences were observed, treatment means were differentiated using pairwise comparisons using the Tukey test. Raw data was checked for normality and equal variance using the Kolmogorov-Smirnov test (SigmaStat® v.3.5).

3. RESULTS

3.1. Simulated ruminal fermentation

Simulated ruminal OMD, aME content and CH_4 production from 48 hour *in vitro* fermentation of diets containing varying levels of whole-cell and lipid-extracted *S*-AMDD are shown in Table 3. Ruminal OMD and aME content of a forage and grain based control diet (45% and 3.7 MJ kg^{-1}, respectively) was not significantly affected ($P\geq0.064$) by dietary supplementation with either whole-cell or lipid-extracted *S*-AMDD at any dietary inclusion level (equivalent to 25, 50 and 100% of forage protein replacement) with average OMD of 41% (range 37.7-44.4%) and aME content of 3.9 MJ kg^{-1} (range 3.7-4.0 MJ kg^{-1}). Ruminal CH_4 production of diets supplemented with varying levels of whole-cell *S*-AMDD (average 3.1 mol^{-10}, range 3.1-3.2 mol^{-10}) were statistically the same ($P\geq0.265$) as the control diet (2.9 mol^{-10}) and were unaffected ($P=0.698$) by dietary inclusion level. However, after lipid-extraction, CH_4 production was reduced significantly ($P<0.001$) to an average of 1.5 mol^{-10} (range 1.4-1.7 mol^{-10}) and unaffected ($P=0.066$) by dietary inclusion level.

3.2. *In vitro* monogastric digestibility

In vitro monogastric DPD and two-phase $GPD_{Protein}$ and GPD_{Energy} from whole-cell and lipid-extracted *S*-AMDD is shown in Table 4. Monogastric DPD and two-phase $GPD_{Protein}$ were relatively low for whole-cell *S*-AMDD (52-61%) but significantly improved ($P<0.001$) after lipid-extraction (75-84%). As a result of both higher protein content and protein digestibility, the digestible protein (DP) content of lipid-extracted *S*-AMDD is meaningful (30%) and significantly ($P<0.001$) exceeds that of whole-cell *S*-AMDD (15%). In a similar manner, monogastric two-phase GPD_{Energy} was low for whole-cell *S*-AMDD (50%) and significantly improved ($P<0.001$) after lipid-extraction (70%). Despite lower gross energy content, as a result of lipid removal, the higher energy digestibility resulted in a significantly higher ($P<0.001$) digestible energy (DE) content in lipid-extracted *S*-AMDD (14 MJ kg^{-1}) than whole-cell *S*-AMDD (12 MJ kg^{-1}).

4. DISCUSSION

As a newly identified microalga, the nutritional value of *S*-AMDD for food/feed is largely unknown. The first study in this series with whole-cell and lipid-extracted *S*-AMDD meals [37] have demonstrated that when harvested in exponential growth phase, *S*-AMDD products may have potential as a source of dietary protein (up to 44%) and energy (up to 23 MJ kg^{-1}). In addition, the protein fraction was composed of a well-balanced mixture of essential amino acids (EAA indices of 0.9-1.0); rich in first-limiting EAA lysine (5-6 g lysine 100 g^{-1} protein). As for whole-cell *S*-AMDD, the lipid fraction (11%) was high in PUFA (45-52% of total FAs); particularly n-3 PUFA (30-38% of total FAs) of which 18-23% was α-linolenic acid (ALA, 18:3n-3), and low in SFA (16%). However, the bioavailability of these essential nutrients in *S*-AMDD for commercially-important farmed ruminant or monogastric animals through *in vivo* feeding studies is completely unknown. A preliminary *in vitro* study using beef heifers as rumen fluid donors indicates that the DM digestibility of whole-cell *S*-AMDD was relatively high (65%) [38], and this result is similar to those recently reported *in vitro* for a typical grain and forage-based ruminant control diet (61-70%) [47]. While this preliminary work suggests that whole-cell *S*-AMDD could be easily digested and utilized by ruminants, additional work is required. Since the *in vitro* ruminal fermentation study with beef heifers tested only the single test ingredient by itself, it is possible that digestibility of *S*-AMDD in ruminants could be affected if included in different forms and if incorporated into a 'complete' test feed at more realistic dietary inclusion levels. Since the fibre fraction of chlorophytic microalgae is composed predominantly of cellulose [48], *in vitro* rumen fermentation assays that test only the algal test ingredient lack the other cellulosic materials typically provided by forages (e.g., hemicelluloses, pectin, lignin) and it is possible that some bacterial fermentative activity may have been inhibited. As such, the present *in vitro* study using lactating dairy cattle as rumen fluid donors provided the *S*-AMDD to the test diets at graded dietary inclusion levels (equivalent to 0 to 40% of the complete feed) and also in two different forms (whole-cell and lipid-extracted meals). Since dietary inclusion of whole-cell *S*-AMDD at 50% forage protein replacement (20% of the complete feed) or lipid-extracted *S*-AMDD at 100% forage protein replacement (32% of the complete feed) did not significantly affect OMD or aME content of the control diet it seems that, indeed, the digestibility of *S*-AMDD is high for ruminant animal feeds and contributes to the diet a higher level of dietary protein and digestible energy supplementation than a standard grain and forage based diet. A striking finding from this study was that while diets containing whole-cell *S*-AMDD did not differ in their CH_4 production compared to the control diet, CH_4 production by diets containing lipid-extracted *S*-AMDD was reduced by 47-51% compared to the control diet and diets containing whole-cell *S*-AMDD. Since OMD and aME content of diets containing whole-cell *S*-AMDD were statistically similar to the control diet, the lack of any effect on CH_4 production is not entirely surprising. On the other hand, the OMD and aME content of diets containing lipid-extracted *S*-AMDD were also statistically similar to the control diet, but the effect on CH_4 production was profound. It has been estimated that 16% of global warming-causing greenhouse gas (GHG) emissions are in the form of CH_4, of which up to 30% is as a direct result of enteric fermentation

from ruminant animal agriculture [49,50]. While previous studies have correlated the dietary intake of certain lipids, particularly medium- and long-chain fatty acids, with enteric CH_4 abatement [51-58], lipid-extracted *S*-AMDD is virtually devoid of these fatty acids. Based on this finding, it seems clear that lipid-extracted *S*-AMDD may contain unknown anti-methanogenic 'non-fatty acid' substances that have the ability to suppress rumen methanogenesis. The potential for CH_4 production from *S*-AMDD biomass has recently been studied, albeit with a largely different focus (e.g., for biogas production), where high CH_4 production is desired. Tartakovsky *et al.* [32] found that CH_4 production from *S*-AMDD in continuous flow anaerobic bioreactors was inhibited by high levels of hydrogen sulfide produced as a result of enhanced growth of sulfate-reducing bacteria. While this is viewed as a negative result for that particular application, it may be a highly encouraging finding for ruminant animal feeding applications where high CH_4 production is not desired and much effort is currently underway to find mitigation options. Microalgae in the *Scenedesmus* genus typically accumulate high total CHO levels, both as structural cell wall fibre (predominantly cellulose) and intracellular starch [7], and the *S*-AMDD samples in the present study are consistent with this (total CHO, 36-44%; starch, 9-11% and fibre 28-33%). Anele *et al.* [38] speculated that the high CHO fraction of freshwater microalgae could be partly responsible for suppression of *in vitro* CH_4 production by shifting ruminal fermentation away from acetate production; in favour of propionate, which provides an alternative hydrogen sink in a similar manner to that observed in ruminants fed a high grain-based diet [59]. The observed uncoupling between digestion and methanogenesis in the present study has been observed previously with macroalgae (seaweeds) [60]. These findings show potential for *S*-AMDD to inhibit methanogenesis, perhaps by targeting ruminal protozoa, without impairing feed utilization. This enteric CH_4 abatement potential of feeding *S*-AMDD to ruminant animals, together with the fact that the *S*-AMDD biomass is likely to be produced at large-scale using industrial point-source CO_2 as the primary carbon source for growth could, in combination, substantially reduce two major sources of industrial and agricultural greenhouse gas emissions and help the world meet its ambitious climate change targets [3].

While these *in vitro* data provide highly encouraging results for the potential utility of *S*-AMDD for ruminant animals, the digestibility of *S*-AMDD, either *in vitro* or *in vivo*, has never been examined for monogastric animals until this study. Of course, it is well-established that ruminant animals are more specialized fermenters of cellulosic materials than monogastric animals [61], so how well monogastrics are able to digest and metabolically utilize *S*-AMDD products is questionable given its relatively high contents of fibre (~75% of total CHO) relative to starch (~25% of total CHO). Its cell wall, in particular, is composed of a cellulose-based inner layer surrounded by an algaenan-based outer layer. In addition, microalgae in the *Scenedesmus* genus, including *S*-AMDD, grow in colonies or 'clumps' of four to eight cells (or possibly more) and these cell clumps are communally surrounded by a coating of mucilage [27,28]. Interestingly, this cell clumping characteristic is thought to have evolved as a defense mechanism against grazing predation [62]. With specific reference to the use of *S*-AMDD biomass for monogastric animal feeds, these recalcitrant cellulose, algaenan and mucilage layers are likely to make the biomass relatively hydrophobic; and the clumping characteristic reduces the overall surface to volume ratio of the cells; both of which could make *S*-AMDD biomass somewhat resistant to penetration by gastric and pancreatic juices and digestive enzymes in the monogastric alimentary tract. The highly significant increase in protein and energy digestibility of *S*-AMDD biomass after lipid-extraction may be the result of disintegration of the communal mucilage layer surrounding the algal clumps and partial rupture of individual cell walls as a result of the thermal treatment during the defatting process. While this is the first study to explore the monogastric digestibility of *S*-AMDD products, a limited number of studies have focused on other related *Scenedesmus* species for monogastric animals. *In vitro* protein digestibility (with pepsin) and *in vivo* protein digestibility (with rats) have provided highly inconsistent results for *S. quadricauda* and *S. obliquus* (11-75%) [63]. The dietary use of lipid-extracted *S. dimorphus* for rats was reported to be safe and effective up to an inclusion level of 10%, after which high ash and fibre levels reduced feed intake and growth [64], although digestibility was not reported. Whole-cell *S. almeriensis* and that of another unidentified *Scenedesmus* species were investigated for their potential to replace fish meal in juvenile feeds for gilthead sea bream (*Sparus aurata*) and Nile tilapia (*Oreochromis niloticus*) and the authors reported no negative effects on growth performance, nutrient utilization, product quality, digestive enzyme activity and intestinal histopathology at 16-20% dietary inclusions [65,66], but again, nutrient or energy digestibility was not reported. Using a three-enzyme (trypsin, peptidase, chymotrypsin) indirect pH-Drop assay, the *in vitro* protein digestibility of whole-cell and lipid-extracted *Acutodesmus dimorphus* (formerly *S. dimorphus*) was estimated to be moderately high (78%) for monogastric animals [23]. In the present study, the *in vitro* protein digestibility of whole-cell *S*-AMDD was relatively low (52%) and this in a similar range as previously reported for other freshwater chlorophytic whole-cell microalgae (49-78%) [46]. However, as mentioned the value in the present study was significantly increased after defatting (84%); which is consistent with findings for other marine and freshwater microalgae processed in a similar manner (78-97%) [23,46]. The improved *in vitro* digestibility of defatted material has been attributed to the mild thermal treatment associated with the lipid-extraction processing; which has the potential to both disrupt the rigid algal cell walls and subsequently unfold the secondary and tertiary structures of intracellular proteins exposing them to a higher level of digestive enzyme activity. In the same manner, the *in vitro* energy digestibility of whole-cell *S*-AMDD was relatively low (50%) in the present study; which is similar to previous reports for freshwater chlorophytic whole-cell microalgae (52-57%) [46]. Consistent with the protein digestibility results previously mentioned, the defatting processing significantly improved *in vitro* energy digestibility in both studies (61-70%). This resulted in digestible energy (DE) values for whole-cell and lipid-extracted *S*-AMDD of 12 and 14 MJ kg^{-1}, respectively. At these levels, *S*-AMDD biomass fits with relatively inexpensive lipid and CHO-rich plant-

based feed ingredients such as flax, canola and wheat germ (12-14 MJ kg^{-1}) but is inferior to higher-value protein ingredients commonly used in monogastric animal feeds such as processed oilseed and animal, crustacean and fish by-product meals (13-21 MJ kg^{-1}) [67-69]. Reported DE values of *Scenedesmus* biomass for monogastric animals are not available, but similar, albeit slightly lower, values (14-16 MJ kg^{-1}) have been reported for *Chlorella vulgaris* measured *in vitro* and *in vivo* with rats [42,44] and may be related to the lower lipid content of *Scenedesmus*.

5. CONCLUSION

Simulated ruminal fermentations using lactating dairy cattle as rumen fluid donors indicate that both whole-cell and lipid-extracted *S*-AMDD have excellent potential for use in ruminant animal feeds. Dietary inclusion of whole-cell *S*-AMDD at 50% forage protein replacement (equivalent to 20% of the total diet) or lipid-extracted *S*-AMDD at 100% forage protein replacement (equivalent to 32% of the total diet) did not significantly affect OMD or aME content of the control diet. However, OMD was marginally comprised with 100% forage protein replacement with whole-cell *S*-AMDD (equivalent to 40% of the total diet) relative to the 25 and 50% replacement levels, although not significantly different from the control diet. Diets containing lipid-extracted *S*-AMDD reduced CH_4 production by approximately 50% compared to the control diet (47% reduction) and those containing whole-cell *S*-AMDD (51% reduction). Since OMD and aME content of diets containing lipid-extracted *S*-AMDD were unaffected relative to the control diet and whole-cell *S*-AMDD-containing diets, it seems clear that lipid-extracted *S*-AMDD contains anti-methanogenic 'non-fatty acid' substances that have the ability to suppress rumen methanogenic bacteria without disturbing ruminal digestion. This finding supports our previous studies with other freshwater chlorophytic microalgae [38,46] and this area warrants further exploration *in vivo*, especially considering the large volume of algal feed that could be produced without occupying significant land resources. *In vitro* monogastric digestibility using porcine enzymes indicates that lipid-extracted *S*-AMDD has potential for use in monogastric animal feeds. Protein and energy digestibility of this product were moderately high (75-84% and 70%, respectively); which resulted in relatively high contents of DP (30%) and DE (14 MJ kg^{-1}). On the other hand, the digestibility of whole-cell *S*-AMDD was low at 52-61% and 50%, respectively resulting in lower levels of DP (15%) and DE (12 MJ kg^{-1}). Despite the encouraging results for lipid-extracted *S*-AMDD, the digestibility (particularly of energy) remains marginal for monogastric animals and requires improvement through additional cost-effective cell rupture technologies or the production algal protein concentrates.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Table 1. Proximate composition and caloric content of whole-cell and lipid-extracted *Scenedesmus* sp. AMDD used for *in vitro* digestibility studies (DW basis)[a].

	Whole-cell *S*-AMDD	Lipid-extracted *S*-AMDD	*P-value*
Ash (%)	3.0±0.1[ns]	3.1±0.1	*0.194*
Crude protein (%N×5.55)	33.1±1.5[a]	41.4±2.0[b]	*<0.001*
Esterifiable lipid (%)	11.4±0.9[a]	0.6±0.1[b]	*<0.001*
Carbohydrate (%)	36.3±1.3[a]	44.1±3.3[b]	*<0.001*
Starch (%)	8.7±0.6[a]	11.3±0.9[b]	*<0.001*
Fibre (%)	27.7±1.2[a]	32.8±2.4[b]	*<0.001*
Gross energy (MJ kg^{-1})	23.2±0.2[a]	20.0±0.3[b]	*<0.001*

[a] Values within the same row having different superscript letters are significantly different ($P<0.05$).

Table 2. Composition of dietary treatments used for *in vitro* ruminant digestibility studies of whole-cell and lipid-extracted *Scenedesmus* sp. AMDD (DW basis).

Dietary treatment	Contribution to dietary treatment (% of diet)[a]				Contribution to dietary crude protein (CP) (% of dietary CP)		
	Corn[b]	**Forage**[c]	**Cellulose**[d]	**Algae**[e]	**Corn**	**Forage**	**Algae**
Control (100F:0A)	15.0	75.0	10.0	-	9.73	90.27	-
Whole-cell *S*-AMDD							
Low (75F:25A)	15.0	56.25	18.875	9.875	9.69	67.45	22.86
Medium (50F:50A)	15.0	37.5	27.750	19.750	9.66	44.79	45.55
High (0F:100A)	15.0	-	44.500	39.500	9.58	-	90.42
Lipid-extracted *S*-AMDD							
Low (75F:25A)	15.0	56.25	20.858	7.892	9.70	67.46	22.84
Medium (50F:50A)	15.0	37.5	31.717	15.783	9.66	44.82	45.52
High (0F:100A)	15.0	-	53.425	31.575	9.59	-	90.41

[a] Total CP of all dietary treatments was 12.4±0.1% of DW.

[b] Corn, medium grind (7.95% CP).

[c] Grass/legume forage, 1 mm grind (14.75% CP).

[d] Nitrogen-free pure cellulose (CP-free).

[e] Whole-cell and lipid-extracted *S*-AMDD (CP according to Table 1).

Table 3. Organic matter digestibility (% OMD), apparent metabolizable energy (MJ kg^{-1} aME) content and methane production (mol^{-10} CH_4) from 48 hour *in vitro* fermentation of diets containing varying levels of whole-cell and lipid-extracted *Scenedesmus* sp. AMDD (n=15)[a].

	Dietary inclusion level (% of forage replacement)			
	Low (25%)	**Medium (50%)**	**High (100%)**	***P-value***
OMD				
Control diet	44.8±7.1[ns]	44.8±7.1[ns]	44.8±7.1[ns]	-
Whole-cell *S*-AMDD	44.4±8.3	41.7±7.4	37.7±5.2 *	*0.041*
Lipid-extracted *S*-AMDD	42.3±5.7	41.9±6.2	39.3±5.6	*0.336*
P-value	*0.655*	*0.658*	*0.064*	-
aME				
Control diet	3.7±0.4[ns]	3.7±0.4[ns]	3.7±0.4[ns]	-
Whole-cell *S*-AMDD	4.0±1.4	3.9±0.9	3.7±0.8	*0.591*
Lipid-extracted *S*-AMDD	4.0±0.4	3.9±0.9	4.0±1.0	*0.807*
P-value	*0.807*	*0.900*	*0.445*	-
CH_4				
Control diet	2.9±0.7[a]	2.9±0.7[a]	2.9±0.7[a]	-
Whole-cell *S*-AMDD	3.2±0.4[a]	3.1±0.5[a]	3.1±0.4[a]	*0.698*
Lipid-extracted *S*-AMDD	1.7±0.3[b]	1.5±0.3[b]	1.4±0.2[b]	*0.066*
P-value	*<0.001*	*<0.001*	*<0.001*	-

[a] Values within the same column having different superscript letters are significantly different (P<0.05).

* Indicates a significant difference between dietary inclusion levels (P<0.05).

Table 4. Dilute pepsin digestibility (DPD) and two-phase gastric/pancreatic digestibility (GPD) of whole-cell and lipid-extracted *Scenedesmus* sp. AMDD (n=15)[a].

	Whole-cell *S*-AMDD	**Lipid-extracted *S*-AMDD**	***P-value***
DPD (%)	61.4±2.1[a]	74.6±3.1[b]	*<0.001*
$GPD_{Protein}$ (%)	52.0±2.4[a]	84.3±1.6[b]	*<0.001*
GPD_{Energy} (%)	50.3±3.0[a]	70.3±5.0[b]	*<0.001*
DP (%)	14.8±0.9[a]	30.1±1.5[b]	*<0.001*
DE (MJ kg^{-1})	11.6±0.7[a]	14.0±1.0[b]	*<0.001*

[a] Values within the same row having different superscript letters are significantly different ($P<0.05$).

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