Variability of \textit{in vitro} ruminal fermentation and nutritional value of cell-disrupted and nondisrupted microalgae for ruminants

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Abstract
The objective of this study was to investigate ruminal fermentation and the nutritional value of different microalgae products (MAP) for ruminants, including inter- and intra-genera variability. Furthermore, the effect of mechanical cell disruption was also evaluated. Cell-disrupted and nondisrupted MAP of four genera were investigated using the Hohenheim Gas Test. The investigations included characterization of gas production (GP), production of volatile fatty acids (VFA) and methane, organic matter digestibility, and energetic value as well as utilizable crude protein at the duodenum and ruminally undegradable crude protein (RUP). Furthermore, a three-step enzymatic in vitro system was used to estimate intestinal digestibility of RUP (IDP). Ruminal fermentation was low for all investigated microalgae genera, as indicated by overall low GP, low production of VFA, and low ruminal protein degradation. Nevertheless, all microalgae genera were characterized by high RUP concentrations (236–407 g/kg dry matter; passage rate = 8% hr\(^{-1}\)), indicating that microalgae might be a promising protein source for high-performing ruminants. Low IDP (26%–49% of RUP) considerably contradicted this potential. Mechanical cell disruption in general enhanced the extent of ruminal fermentation of MAP but, as RUP was decreased and IDP was hardly affected, mechanical cell disruption appears not to be necessary when microalgae are intended for application as a protein source for ruminants. Because of the high variability in the characteristics of the nutritional value, general means are inappropriate to characterize the nutritional value of MAP. In conclusion, suitability of microalgae as a protein source for ruminants might be limited because of low IDP, although further studies are necessary to prove these findings in vivo.

KEYWORDS

cell disruption, digestibility, feeding value, microalgae, ruminal degradation, ruminants

1 | INTRODUCTION

The world population is predicted to rise from 7.6 billion in 2017 to 9.8 billion in 2050 (United Nations, 2017). Income growth, especially in developing countries, is expected to lead to a rising demand for meat and milk products (FAO, 2017).

The concurrent decline of arable land per capita (Bruinsma, 2011) additionally strengthens the essential need for the improvement of feed utilization and the establishment of alternative feed resources that do not compete with the production of food or can be produced independently from arable land.
Microalgae are a diverse group of photosynthetic, unicellular or simple multicellular organisms, occurring in seawater and in freshwater. Cultivation of microalgae can be undertaken on marginal or nonarable land (Schuhmann & Schenk, 2013), providing the opportunity to repurpose idle land for the production of food and feed. Some microalgae species have promising nutritional properties such as very high crude protein (CP) concentrations, up to 70% of dry matter (Becker, 2007), or the occurrence of omega-3 fatty acids (Ryckeboes, Bruneel, Muylaert, & Foubert, 2012), for which they are regarded as alternative feed resources. The nutritional value of a feedstuff is determined by its nutrient composition and the utilization of the nutrients by the animal. Detailed information on the nutrient composition and utilization of a feedstuff is required for a nutrient supply meeting the animal’s requirements.

The nutrient utilization in ruminants is mainly determined by microbial fermentation in the rumen. Data on the nutritional value of microalgae are scarce. Most of the previous research in ruminants concerns the application of docosahexaenoic acid-rich microalgae for the alteration of fatty acid profiles of milk (e.g., Boeckaert et al., 2008; Glover et al., 2012) or the inhibition of ruminal methanogenesis (Boeckaert, Mestdagh, Vlaeminck, Clayton, & Fievez, 2006; Elghandour et al., 2017). Only a few studies have investigated nutrient utilization of microalgae in ruminants. In addition, nutrient composition of microalgae products (MAP) is highly variable between and within microalgae genera (Wild, Steingaß, & Rodehutscord, 2018) but there are hardly any studies investigating whether the nutrient utilization by the animal is also variable. The availability of microalgae nutrients to animals can be limited by the presence of robust cell walls or other cell coverings made of cellulose (Domozych et al., 2012; Popper & Tuohy, 2010), silicates (Popper & Tuohy, 2010; Tesson, Gaillard, & Martin-Jézéquel, 2009), or the insoluble and nonhydrolyzable biopolymer algaenan (Allard & Templier, 2000; Scholz et al., 2014) formed by some microalgae. In vitro studies for nonruminant animals (Cavonius, Albers, & Undeland, 2016; Hedenskog, Enebo, Vendlová, & Prokes, 1969; Wild et al., 2018) and few in vivo studies with rats (Janczyk, Franke, & Souffrant, 2007; Janczyk, Wolf, & Souffrant, 2005) or fish (Tibbetts, Mann, & Dumas, 2017) have shown that cell disruption can increase the nutrient digestibility of microalgae. Nevertheless, there are no investigations studying the effects of cell disruption on ruminal fermentation or the nutritional value of microalgae for ruminants.

Therefore, the objective of this study was to investigate the ruminal fermentation and nutritional value of MAP for ruminants, including the inter- and intra-genera variability. It was hypothesized that cell disruption affects ruminal fermentation and hence the nutritional value of microalgae for ruminants.

2 | MATERIALS AND METHODS

2.1 | Sample material, sample processing, and chemical analyses

Sixteen commercially available MAP of different origin were investigated. The MAP were declared as Arthrospira (n = 2), Chlorella (n = 8), Nannochloropsis (n = 4) or Phaeodactylum (n = 2) and were nondisrupted whole microalgae biomasses. They were delivered as slurry or as powder. The MAP delivered as slurry were lyophilized (DELTAM 1–24 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and ground to powder with a vibrating disk mill (Pulverisette 9, Fritsch GmbH, Idar-Oberstein, Germany). Until further processing, all MAP were stored at approximately −30°C vacuum-packed as powder. A subset of each sample was treated with a stirred ball (Dyno Mill KDL A, Willy A. Bachofen AG—Maschinenfabrik, Muttenz, Switzerland) to disrupt cells as described by Wild et al. (2018). This publication also provides comprehensive data on the chemical composition and the in vitro crude protein digestibility for pigs of the MAP included in this study.

2.2 | Animals and diet

Two ruminally-fistulated late-lactating Jersey cows served as donor animals for the in vitro experiments using the Hohenheim Gas Test method. Cows were offered a total mixed ration composed of 24% maize silage, 24% grass silage, 23% concentrate mixture, 16% hay, 8% rapeseed meal, 3% barley straw, 1% mineral mixture, and 1% limestone (by dry matter, DM). The concentrate mixture was composed of 25% rapeseed cake, 23% maize, 20% barley, 20% field beans and 12% pea. Cows were housed in groups and had ad libitum access to feed and water.

2.3 | Experiment 1: In vitro gas production, energy value, and digestibility of organic matter

The Hohenheim Gas Test was used according to the method of Menke and Steingass (1988). In brief, approximately 200 mg DM of each MAP was weighed into 100 ml glass syringes, which were sealed airtight with greased plungers and had been prewarmed. A buffered mineral solution was prepared and maintained under continuous stirring and flushing with CO₂ at 39°C. Rumen fluid was collected from two cows prior to the morning feeding, mixed, and filtered through two layers of cheesecloth. The filtered rumen fluid was subsequently added to the reduced buffer solution under constant stirring. Thirty milliliters of the rumen fluid-buffer solution was dispensed
Experiment 3: Utilizable crude protein at the duodenum and ruminally undegradable crude protein

The Extended Hohenheim Gas Test method (Raab, Cafantar, Jilg, & Menke, 1983) with the modifications described by Steingass, Nibbe, Südekum, Lebzien, and Spiekers (2001) was used to estimate utilizable crude protein at the duodenum (uCP) and ruminally undegradable crude protein (RUP). The incubations were carried out as described for Experiment 1 with the following modifications: Each of a total of eight runs comprised one incubation over 8 hr and one incubation over 48 hr. Both incubations contained 50 glass syringes with the same microalgae samples as well as four blanks and three standard concentrate samples with known GP and uCP concentration. The cell-disrupted and the nondisrupted material of the MAP were distributed to the eight runs according to a fully randomized block design. Each run contained no or one replicate of each microalgae sample. Approximately 130 mg DM of each MAP was weighed into the syringes with and without the addition of 130 mg of a carbohydrate mixture (50% corn starch, 30%


dOM (\%) = 14.88 + 0.8893 GP_{24} + 0.0448 CP + 0.0651 CA

ME (MJ/kg DM) = 1.68 + 0.1418 GP_{24} + 0.0073 CP + 0.0217 EE − 0.0028 CA

where GP_{24} is the gas production (ml 200 mg\(^{-1}\) DM) after 24 hr of incubation, CP is the crude protein, EE is the ether extract and CA is the crude ash concentration in g/kg DM.
cellulose, 20% sucrose). After 8 and 48 hr, GP was recorded and the microbial fermentation was stopped immediately by putting syringes on ice. NH$_3$-N was measured by steam distillation with subsequent titration (Vapodest 50, C. Gerhardt GmbH & Co. KG, Königswinter, Germany). For this, the complete incubation residue was transferred to digestion flasks and 15 ml of phosphate buffer (90 g Na$_2$HPO$_4$·12 H$_2$O L$^{-1}$, adjusted to pH 11.0 using sodium hydroxide) was added. Distilled NH$_3$ was trapped in 3% boric acid and titrated with 0.05 M HCl. For the 8 and 48 hr incubations the uCP concentration was calculated as follows for the syringes without carbohydrate addition:

$$uCP\ (g/kg\ DM) = \left(\frac{(N_{MAP} - (NH_3-N_{MAP} - NH_3-N_{blank})}{\text{initial weight}}\right) \times 6.25 \times 1,000$$

where $N_{MAP}$ is the amount of N added by the MAP (mg), NH$_3$-N$_{MAP}$ and NH$_3$-N$_{blank}$ are the NH$_3$-N concentrations of MAP and blank incubation residues (mg), respectively, and initial weight is the exact amount of MAP initially incubated into glass syringes (mg DM).

For the calculation of RUP, a linear regression was fitted to the GP and NH$_3$-N values of samples with and without carbohydrate mixture addition (Raab et al., 1983). Rumen degradable N (RDN, mg) was calculated by subtracting the NH$_3$-N concentrations of the blanks from the y-intercept. The amount of ruminally undegradable N (RUN, mg) was the difference between the amount of N added by the MAP and RDN. The concentration of RUN (%) was the amount of RUN (mg) relative to the amount of N added by the MAP. The RUP (g/kg DM) was the CP concentration multiplied by concentration of RUN.

Effective uCP and effective RUP were estimated for assumed ruminal passage rates (k) of 2% hr$^{-1}$, 5% hr$^{-1}$, and 8% hr$^{-1}$ by plotting uCP and RUP values (y) against the natural logarithm of the incubation time (x) in a linear regression model and calculating the function values of ln (50), ln (20), and ln (12.5), respectively, using PROC MIXED of SAS. The effective uCP was differentiated into the effective RUP and microbial protein (MP), and MP was calculated as the difference between effective uCP and effective RUP.

### 2.6  |  Experiment 4: Intestinal digestibility of ruminally undegraded crude protein

Intestinal digestibility of RUP (IDP) was determined using a three-step enzymatic in vitro method (Irshaid, 2007), including modifications to ensure applicability for MAP. In brief, the true protein concentration of each MAP was determined using copper hydroxide as the precipitating agent (VDLUFA, 1976). Ruminal protein degradation was simulated in this assay by the application of a *Streptomyces griseus* protease (Licitra et al., 1998). For the cell-disrupted and nondisrupted material of each MAP, three subsequent experimental runs were performed. For each run, 1.5 g of each sample was weighed into Erlenmeyer flasks in triplicate, 120 ml of borate-phosphate buffer (pH 6.7–6.8) was added, and flasks were incubated in a water bath at 39°C for 1 hr under continuous stirring. Then, the protease solution (Borate-phosphate buffer and 1.0 U/ml *Streptomyces griseus* protease; Type XIV, ≥ 3.5 units/mg solid, P5147, Sigma-Aldrich, St. Louis, MO, USA) was added in an amount corresponding to 41 U/g true protein and incubation was continued under continuous stirring for 18 hr. After 18 hr of incubation, the entire content of the Erlenmeyer flasks was transferred to a 250 ml polycarbonate centrifuge vessel (Nalgene™, Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged for 25 min at 15,000 g, discarding the supernatant. Then, the pellets were rinsed with 200 ml of distilled water and centrifuged at 15,000 g for 15 min, discarding the supernatant. Washing and centrifugation of the pellets were repeated twice. The three residues of one run were pooled after lyophilization (DELTAg 1–24 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and analyzed for N concentration by Kjeldahl digestion (VDLUFA, 1976). Pooled residues were weighed into 100 ml Erlenmeyer flasks in an amount corresponding to 15 mg N. Ten milliliters of 0.1 M HCl at pH 1.9 containing 1 g/L of pepsin (P7012, Sigma-Aldrich, St. Louis, MO, USA) was added to the samples and they were incubated for 1 hr at 38°C under continuous stirring. After the addition of 0.5 ml of 1.0 M NaOH, 13.5 ml of a pancreatic solution (68.05 g/L KH$_2$PO$_4$, 50 mg/L thymol, and 3 g/L pancreatin; P7545, Sigma-Aldrich, St. Louis, MO, USA; adjusted to pH 7.8) was added. Incubation was continued for 24 hr and was then stopped by the addition of 3 ml 100% trichloroacetic acid. Samples were allowed to stand under room temperature and continuous stirring for 15 min. Afterward, an aliquot of each sample was pipetted into centrifuge vessels and was centrifuged for 25 min at 15,000 g. Supernatant was pipetted off and used for analysis of the soluble protein by the Kjeldahl method (VDLUFA, 1976). The IDP was calculated as the amount of soluble N relative to the amount of N incubated with pepsin and pancreatin.

### 2.7  |  Statistical analysis

Data were subjected to a two-factorial analysis of variance using PROC MIXED of SAS. The model was:

$$Y_{xy} = \mu + MAG_x + CD_y + (MAG_x \times CD_y) + e_{xy},$$

where $Y_{xy}$ is the estimate for the observed trait, $\mu$ is the overall mean, MAG$_x$ is the fixed effect of the microalgae
genera (Arthrospira, Chlorella, Nannochloropsis, Phaeodactylum), CD, is the fixed effect of the cell disruption (cell-disrupted, nondisrupted), MAG × CD is the interaction of the fixed effects microalgae genera and cell disruption and $e_{xy}$ is the residual error. Model assumptions were checked on the residuals. Differences between least square means were tested using t test and significance was declared at $p < 0.05$. One Chlorella product with very high ether extract and very low crude protein concentration was omitted from all statistical analyses related to uCP and RUP because of unrealistic estimates for these traits. Pearson correlation coefficients were calculated using PROC CORR of SAS.

3 | RESULTS

In the nondisrupted MAP, GP after 24 hr ranged between 12.1 and 24.5 ml 200 mg$^{-1}$ DM and pGP ranged from 12.6 to 26.4 ml 200 mg$^{-1}$ DM in Phaeodactylum and Arthrospira, respectively (Table 1). The interaction effect MAG × CD was significant ($p < 0.001$) for GP after 24 hr and pGP and cell disruption increased both traits in Chlorella, Nannochloropsis, and Phaeodactylum products but not in Arthrospira. The interaction effect was also significant for the rate constant of GP ($p < 0.001$) and cell disruption decreased the rate constant of GP in Arthrospira and Phaeodactylum and increased it in Chlorella and Nannochloropsis. Variability within genera was considerable for the pGP and the rate constant of GP, expressed by high ranges in all genera. Similar to pGP, the interaction effect was significant ($p < 0.001$) for dOM and cell disruption led to a significant increase of dOM in Chlorella, Nannochloropsis, and Phaeodactylum and to a significant decrease in Arthrospira. The interaction effect was significant for ME ($p < 0.001$). In the nondisrupted MAP, ME was highest in Arthrospira (11.3 MJ/kg DM), while in the cell-disrupted MAP it was highest in Chlorella (12.7 MJ/kg DM). Phaeodactylum had lowest mean ME concentration, independent of cell disruption.

The interaction effect MAG × CD was significant ($p < 0.001$) for all traits related to methane production (Table 2). Mean CH$_4$-volume of the nondisrupted MAP ranged between 2.4 and 5.6 ml 180 mg$^{-1}$ DM in Phaeodactylum and Arthrospira, respectively. Cell disruption led to a significant increase of CH$_4$-volume in Chlorella, Nannochloropsis, and Phaeodactylum and did not affect CH$_4$-volume of Arthrospira products. The CH$_4$-concentration in GP of the nondisrupted MAP ranged from 20.5% to 25.2% in Phaeodactylum and Arthrospira, respectively. Cell disruption significantly decreased CH$_4$-concentration in GP in

**TABLE 1** Gas production (GP) after 24 hr of incubation, potential GP (pGP), rate constant of GP, digestibility of organic matter (dOM) and metabolizable energy (ME) of four microalgae genera. Mean, range, pooled standard error (SEM) and results of two-factorial analysis of variance (ANOVA)

| Cell disruption (CD) | GP after 24 hr (ml 200 mg$^{-1}$ DM) | pGP (ml 200 mg$^{-1}$ DM) | Rate constant of GP (% hr$^{-1}$) | dOM (%) | ME (MJ/kg DM) |
|---------------------|------------------------------------|--------------------------|---------------------------------|---------|----------------|
|                     | n-cd | cd | n-cd | cd | n-cd | cd | n-cd | cd | n-cd | cd |
| Arthrospira (n = 2) | 24.5$^a$ | 22.2$^b$ | 26.4$^a$ | 24.8$^{ab}$ | 14.2$^c$ | 12.3$^{d*}$ | 72$^a$ | 70$^{b*}$ | 11.3$^a$ | 11.0$^{b*}$ |
| Chlorella (n = 8)   | 17.2$^{ab}$ | 28.6$^{a*}$ | 24.0$^b$ | 32.8$^{b*}$ | 8.9$^d$ | 13.5$^{c*}$ | 58$^b$ | 67$^{a*}$ | 10.8$^{ab}$ | 12.7$^{a*}$ |
| Nannochloropsis (n = 4) | 14.9$^c$ | 18.6$^{a*}$ | 16.6$^c$ | 18.7$^{c*}$ | 15.3$^b$ | 27.5$^{b*}$ | 57$^b$ | 60$^{a*}$ | 10.6$^b$ | 11.1$^{b}$ |
| Phaeodactylum (n = 2) | 12.1$^d$ | 16.9$^{a*}$ | 12.6$^d$ | 17.3$^{d*}$ | 35.3$^c$ | 23.6$^{c*}$ | 57$^b$ | 61$^{a*}$ | 8.7$^c$ | 9.2$^{a*}$ |
| Pooled SEM          | 1.01  | 0.38  | 0.38  | 0.98  | 0.21  |

p-Values (ANOVA)

- MAG <0.001
- CD <0.001
- MAG × CD <0.001

*Indicates a significant effect of cell disruption for tagged microalgae genera (nondisrupted vs. cell-disrupted biomass of one microalgae genera) within one trait ($p < 0.05$) in case of a significant interaction effect.

Notes. cd: cell-disrupted microalgae biomass (stirred ball mill); n-cd: nondisrupted microalgae biomass.

Different superscripts within one column indicate significant mean differences ($p < 0.05$) in case of a significant interaction effect.
**Nannochloropsis**, but did not affect \(\text{CH}_4\)-concentration in GP in the other microalgae genera.

In the nondisrupted MAP, total VFA varied between 11.4 and 22.4 mmol/L in *Phaeodactylum* and *Arthrospira*, respectively. Cell disruption significantly increased mean values of total VFA in all investigated microalgae genera (Table 3). The predominantly produced VFA in all microalgae genera, and regardless of cell disruption, was acetate, followed by propionate and butyrate. The interaction effect \(\text{MAG} \times \text{CD}\) was significant \((p < 0.05)\) for acetate, propionate, and butyrate but was not significant for the other VFA. Acetate proportions were decreased by cell disruption in *Nannochloropsis* and not affected in the other microalgae genera. Proportions of propionate were decreased by cell disruption in *Nannochloropsis* and not affected in the other microalgae genera. Proportions of propionate were unaffected in *Arthrospira* and *Chlorella* products, increased in *Nannochloropsis* and decreased in *Phaeodactylum*. Proportions of butyrate were decreased by cell disruption in *Chlorella* but cell disruption did not significantly affect proportions of butyrate in the other microalgae genera. Proportions of branched-chain fatty acids and valerate were particularly high in some cases and the proportions were variable between genera. The effect CD was significant for isobutyrate and isovalerate \((p < 0.05)\) and cell disruption led to an increase of these VFA in all microalgae genera, while proportions of valerate were unaffected by cell disruption \((p = 0.245)\).

Crude protein concentration was not affected by cell disruption \((p = 0.869)\) but varied between MA genera (Table 4). The interaction effect was significant \((p < 0.001)\) for uCP and RUP after 8 and 48 hr of incubation but the effect of cell disruption was not consistent across the different incubation periods for each trait. After 8 hr of incubation, cell disruption increased uCP in *Arthrospira*, decreased uCP in *Chlorella*, and did not significantly affect uCP in *Nannochloropsis* and *Phaeodactylum*. In contrast, after 48 hr of incubation, uCP was unaffected in *Arthrospira* and significantly decreased in the other microalgae genera. RUP after 8 hr of incubation was decreased by cell disruption in *Chlorella, Nannochloropsis*, and *Phaeodactylum* but not in *Arthrospira*. Cell disruption decreased RUP after 48 hr of incubation in *Chlorella* and *Nannochloropsis* and *Phaeodactylum* but did not have a significant effect in *Arthrospira*. Compared to uCP and RUP values after 8 hr of incubation, both traits declined after 48 hr of incubation in all genera and independently from cell disruption, but the extent of the decline was variable between microalgae genera. The IDP of the nondisrupted MAP varied between 27% (*Arthrospira*) and 43% of RUP (*Chlorella* and *Nannochloropsis*). The interaction effect was significant \((p = 0.011)\) for IDP and cell disruption increased IDP in *Phaeodactylum* but did not have an effect on IDP in the other microalgae genera (Table 4).

![Table 2](image)

**Table 2** Gas production (GP) and methane production after 24 hr of incubation of four microalgae genera. Mean, range, pooled standard error (SEM) and results of two-factorial analysis of variance (ANOVA)

Notes. cd: cell-disrupted microalgae biomass (stirred ball mill); n-cd: nondisrupted microalgae biomass.

Different superscripts within one column indicate significant mean differences \((p \leq 0.05)\) in case of a significant interaction effect.

*Indicates a significant effect of cell disruption for tagged microalgae genera (nondisrupted vs. cell-disrupted biomass of one microalgae genera) within one trait \((p \leq 0.05)\) in case of a significant interaction effect.
### Table 3

Volatile fatty acids (VFA) after 24 hr of incubation of four microalgae genera (% of total VFA unless otherwise stated). Mean, range, pooled standard error (SEM) and results of two-factorial analysis of variance (ANOVA).

| Cell disruption (CD) | Acetate | Propionate | Isobutyrate | Butyrate | Isovalerate | Valerate | Total VFA [mmol/L] |
|---------------------|---------|------------|-------------|----------|-------------|----------|-------------------|
| MAG                 |         |            |             |          |             |          |                   |
| Arthrospira (n = 2) | 51.9b   | 19.2b      | 4.7         | 10.2b    | 9.5         | 4.5      | 22.4a            |
|                     | 50–53   | 18–20      | 4.3–5.3     | 10–11    | 8.3–11      | 3.9–5.2  | 21–24            |
|                     | 51–52   | 18–21      | 4.7–5.1     | 10–10    | 8.6–10      | 4.1–5.1  | 23–24            |
| Chlorella (n = 8)   | 57.2a   | 21.1ab     | 2.2         | 12.3a    | 3.4         | 3.8      | 15.6b            |
|                     | 49–64   | 13–41      | 0.6–3.4     | 8.3–22   | 0.7–5.3     | 2.0–4.9  | 10–23            |
|                     | 50–59   | 16–38      | 0.5–3.8     | 8.7–16   | 0.7–6.4     | 2.1–4.8  | 11–31            |
| Nannochloropsis (n = 4) | 52.2b | 20.8ab     | 2.6         | 12.3a    | 5.1         | 7.0      | 12.3c            |
|                     | 47–54   | 19–22      | 1.8–3.7     | 11–15    | 3.4–7.4     | 3.6–8.3  | 10–14            |
|                     | 44–51   | 21–33      | 1.5–3.6     | 12–15    | 3.1–7.2     | 3.5–7.5  | 16–20            |
| Phaeodactylum (n = 2) | 53.2b  | 22.2a      | 3.1         | 10.3b    | 6.2         | 4.9      | 11.4c            |
|                     | 52–54   | 20–24      | 2.8–3.5     | 7.6–13   | 6.2–7.8     | 4.2–5.5  | 9.6–13           |
|                     | 54–55   | 19–19      | 3.4–3.9     | 10–12    | 6.2–7.8     | 4.5–5.2  | 14–15            |
| Pooled SEM          | 0.68    | 0.88       | 0.15        | 0.47     | 0.32        | 0.24     | 0.50             |

*p*-Values (ANOVA)

| MAG     | CD | MAG × CD |
|---------|----|----------|
| <0.001  | 0.006 | 0.004 |
| <0.001  | 0.232 | <0.001 |
| <0.001  | 0.007 | 0.140 |
| <0.001  | 0.900 | 0.027 |
| <0.001  | 0.033 | 0.280 |
| <0.001  | 0.245 | 0.156 |
| <0.001  | 0.001 | <0.001 |

Notes. cd: cell-disrupted microalgae biomass (stirred ball mill); n-cd: nondisrupted microalgae biomass.

*Indicates a significant effect of cell disruption for tagged microalgae genera (nondisrupted vs. cell-disrupted biomass of one microalgae genera) within one trait (*p* ≤ 0.05) in case of a significant interaction effect.

Different superscripts within one column indicate significant mean differences (*p* ≤ 0.05) in case of a significant interaction effect.
| Cell disruption (CD) | Microalgae genera (MAG) |  |  | RUP [% of CP] |  |  |  |  | IDP [% of RUP] |  |
|---------------------|------------------------|---|---|----------------|---|---|---|---|----------------|---|
|                     |                        |  |  | 8 hr | 48 hr | 8 hr | 48 hr | 8 hr | 48 hr | 8 hr | 48 hr | 8 hr | 48 hr | 8 hr | 48 hr | 8 hr | 48 hr | 8 hr | 48 hr |
|                     |                        |  |  | n-cd | cd | n-cd | cd | n-cd | cd | n-cd | cd | n-cd | cd | n-cd | cd | n-cd | cd | n-cd | cd |
|                     | Arthrospira (n = 2)    |  |  | 692  | 690 | 477a | 505** | 300b | 287a | 60b | 64b | 40d | 38c | 27b | 26c |
|                     |                        |  |  | 650–733 | 651–729 | 468–479 | 491–517 | 292–310 | 262–313 | 58–64 | 61–71 | 38–44 | 32–45 | 26–27 | 22–29 |
|                     | Chlorella (n = 7)      |  |  | 567  | 562 | 470a | 457b* | 296b | 216** | 76a | 71** | 52c | 35* | 43b | 44b |
|                     |                        |  |  | 516–629 | 517–623 | 413–498 | 421–489 | 182–245 | 160–279 | 69–84 | 62–79 | 25–73 | 23–45 | 26–53 | 25–52 |
|                     | Nannochloropsis (n = 4)|  |  | 432  | 431 | 357b | 344c | 272b | 248** | 76a | 69* | 61a | 57a | 43b | 41b |
|                     |                        |  |  | 353–500 | 350–500 | 302–415 | 282–391 | 214–321 | 230–268 | 60–82 | 69–71 | 54–69 | 52–64 | 28–57 | 35–49 |
|                     | Phaeodactylum (n = 2)  |  |  | 451  | 446 | 298c | 289d | 260b | 205** | 58b | 54** | 56b | 43** | 40b | 49** |
|                     |                        |  |  | 433–470 | 429–462 | 296–297 | 283–297 | 252–263 | 196–204 | 59–60 | 52–58 | 56–57 | 41–45 | 38–43 | 49–49 |
| Pooled SEM          | 29.4                   |  |  | 6.99 | 8.40 | 2.64 | 1.74 | 2.11 |

*MAG* × *CD* values (ANOVA)

| MAG | CD | MAG × CD |
|-----|----|----------|
|   <0.001 | 0.869 | 0.999 |

**Notes.** cd: cell-disrupted microalgae biomass (stirred ball mill); n-cd: nondisrupted microalgae biomass.

Different superscripts within one column indicate significant mean differences (*p* ≤ 0.05) in case of a significant interaction effect.

*Data on crude protein concentration for nondisrupted microalgae biomass has been first published in Wild et al. (2018).*

*Indicates a significant effect of cell disruption for tagged microalgae genera (nondisrupted vs. cell-disrupted biomass of one microalgae genus) within one trait (*p* ≤ 0.05) in case of a significant interaction effect.
Effective uCP and RUP (Figure 1) were highest in cell-disrupted *Arthrospira* products at a passage rate of 8% hr$^{-1}$ (effective uCP: 449 g/kg DM; effective RUP: 407 g/kg DM) and were lowest in cell-disrupted *Phaeodactylum* at a passage rate of 2% hr$^{-1}$ (effective uCP: 196 g/kg DM; effective RUP: 187 g/kg DM). The proportion of MP in uCP was below 15% for the cell-disrupted and nondisrupted sample material of all investigated microalgae genera and at all passage rates.

4 | DISCUSSION

4.1 | Ruminal fermentation characteristics

The GP and the total production of VFA were on a generally low level for all investigated microalgae genera, which was accompanied by high RUP levels, indicating low ruminal fermentation. Substrates yielding energy for microbial growth are mainly dietary polysaccharides like cellulose, hemicellulose, pectin, and starch while the relevance of protein as an energy source for the growth of rumen microbiota is limited (Owens & Basalan, 2016). The starch concentration of the investigated MAP was low and a characterization of nonstarch polysaccharides could not be carried out for the investigated MAP due to technical difficulties (Wild et al., 2018) but was probably similarly low. In addition, Han and McCormick (2014) proposed that microalgae carbohydrates are less fermentable than carbohydrates of soybean meal. Therefore, the synthesis of microbial protein was very low in the present study (Figure 1). A maximum of 21% of the ruminally degraded crude protein (cell-disrupted *Chlorella* at a passage rate of...
8% hr\(^{-1}\)) was reincorporated in MP. A limitation of MP synthesis because of nitrogen deficiency appears unlikely as the amount of rumen degradable CP exceeded that of MP by far. It is more likely that microbial yield was limited because of energy deficiency. Lodge-Ivey, Tracey, and Salazar (2014) investigated the effects of a complete replacement of soybean meal in forage or concentrate-based diets by lipid-extracted \textit{Chlorella} or \textit{Nannochloropsis} products in a continuous rumen fermentation system. Lipid-extracted \textit{Nannochloropsis} products consistently decreased microbial efficiency compared to soybean meal, while microbial efficiency was increased with some lipid-extracted \textit{Chlorella} products and decreased with others. This suggests a low utilization of microalgae protein for ruminal MP synthesis. However, further studies are needed to investigate whether the utilization of microalgae protein for MP can be increased when microbial growth is not limited by energy deficiency.

### 4.2 Ruminal protein degradation and protein value

At higher performance levels, the importance of RUP for the protein supply of dairy cows increases (Stern et al., 1994) as the synthesis of MP is limited. Therefore, feedstuffs high in RUP are particularly needed for high-performing cows. At present, the most important protein supplements for ruminant nutrition are rapeseed and soybean meal, with typical CP concentrations between 40% and 50% of DM and proportions of RUP in CP between 25% and 45% depending on the assumed passage rate (National Academies of Sciences, Engineering, and Medicine, 2016; National Research Council, 2001). In comparison with these values, CP and RUP were high in the investigated MAP. Therefore, microalgae protein naturally appears to have a high resistance against ruminal microbial degradation, in particular, considering that the microalgae protein was native and not specifically thermally treated to achieve higher RUP levels. This is partly in accordance with the results of Costa, Quigley, Isherwood, McLennan, and Poppi (2016), who determined the ruminal in vitro protein degradability of several microalgae. In vitro protein degradability of \textit{Chlorella pyrenoidosa}, \textit{Nannochloropsis}, and \textit{Schizochytrium} sp. was lower than or on a similar level to that of soybean meal, while only \textit{Dunaliella salina} and \textit{Spirulina platensis} had higher in vitro protein degradability than soybean meal (Costa et al., 2016). Susceptibility of protein to microbial protein degradation in the rumen is determined by protein solubility and the tertiary and quaternary structure of the protein. Furthermore, it is affected by interactions with other nutrients and depends on the predominant microbial population (Bach, Calsamiglia, & Stern, 2005). Susceptibility of microalgae protein to ruminal protein degradation might be particularly restricted by the presence of rigid cell walls. Nevertheless, even in the cell-disrupted MAP ruminal CP degradation was low compared to common protein-rich feedstuffs, so that the presence of cell walls may not be the sole cause for the restricted ruminal CP degradation of the investigated MAP. It is interesting that RUP after 48 hr of incubation (% of CP) was negatively related to the CP concentration of the MAP ($r = -0.77$), indicating that ruminal CP degradability increases with higher CP concentrations of microalgae. We are not aware of any studies investigating the protein solubility of microalgae in the rumen or the protein structure of microalgae, therefore the cause of the low ruminal degradation of microalgae protein cannot be clarified yet. Nevertheless, donor animals of rumen fluid were never exposed to microalgae as a feedstuff and therefore the microbial population was not adapted to microalgae protein. Therefore, it might be that ruminal CP degradation increases when animals are fed microalgae long-term but this requires further investigation.

The IDP is of crucial importance for the comprehensive evaluation of the protein value as it provides information on the availability of protein for the animal, especially with rising contribution of RUP (Calsamiglia & Stern, 1995). To the best of our knowledge, this is the first study investigating the IDP of microalgae. Hippenstiel, Kivitz, Benninghoff, and Südekum (2015) determined the IDP of several common protein sources (e.g., soybean meal, rapeseed meal) using the same method. For soybean meal, they found a range between 700 and 880 g IDP per kg CP and for rapeseed meal a range between 500 and 820 g IDP per kg CP. Compared to these feedstuffs, the IDP of microalgae was notably low, thus restricting the value of microalgae as a protein source for ruminants. Further investigations are necessary to evaluate the importance of these findings in vivo and whether it is possible to enhance the IDP by the cultivation conditions or processing of microalgae. In addition, amino acid composition of the RUP should be considered in further investigations, as it might have importance in high-performing dairy cows.

Protein degradation proceeds with longer retention in the rumen and accordingly, RUP values of investigated MAP were not constant but declined with increasing incubation time or decreasing passage rates. The corresponding decline of uCP was probably additionally enhanced by the lysis of rumen microbes over time, even though the contribution of MP to uCP was low. The more abrupt decline of uCP and RUP in the cell-disrupted MAP than in the nondisrupted ones is not surprising as cell disruption likely increased the availability of protein to rumen microbiota and hence enhanced ruminal protein degradation. Variable decline rates between different microalgae genera might be related to different protein characteristics and cell
structures. It is interesting that the change of RUP with the incubation time was very low for nondisrupted *Phaeodactylum*. The RUP declined only marginally, indicating that the ruminal protein degradation of *Phaeodactylum* barely advanced with longer retention times. This finding is in accordance with the GP kinetics, where an early and low plateau was reached at a concomitant high rate constant of GP. Han and McCormick (2014) investigated in vitro gas accumulation of de-oiled microalgal residues. They found a distinct pattern of GP (a steep rise of GP approaching the asymptote after 5 hr) with the marine diatom *Thalassiosira weissflogii* compared to other tested microalgal and soybean meal and suggested that this was caused by unique characteristics of this species related to evolution and growth environment. As this finding is very similar to the herein presented results for the marine diatom *Phaeodactylum*, it is possible that this noticeable rumen fermentation is related to specific characteristics of marine diatoms.

### 4.3 Digestibility of organic matter and energetic value

Several studies investigated the effect of microalgae supplementation on diet total tract digestibility (Costa et al., 2016; Lamminen et al., 2017; Lodge-Ivey et al., 2014). The effects were variable depending on the considered microalgae, the extent of supplementation, and the feedstuff that was substituted by microalgae, suggesting variable suitability of microalgae as feedstuffs for ruminants. In contrast, data on the digestibility of individual microalgal species in ruminants are scarce. Anele, Yang, McGinn, Tibbetts, and McAllister (2016) determined dry matter digestibility of five nondisrupted microalgae samples with a rumen batch culture system. Dry matter digestibility of *Chlorella vulgaris* and *Nannochloropsis granulata* were 76% and 72%, respectively, which is higher than the dOM value estimated in this study (58% and 57% for *Chlorella* and *Nannochloropsis*, respectively). In vitro dry matter digestibility after 24 hr of incubation of a mechanically de-oiled, milled microalgal coproduct was 59% (van Emon, Loy, & Hansen, 2015). Differences between the results of the current study and previous findings may be related to characteristics of the investigated microalgae as well as to different methodologies. Nevertheless, the variability in dOM between and within microalgae genera observed in the present study was related to different characteristics of the MAP as the same method was applied for all samples.

Compared to common protein-rich feedstuffs like soybean meal or rapeseed meal, the dOM and energy values of investigated MAP were relatively low irrespective of cell disruption (DLG, c2006–2010), indicating that the considered MAP are protein supplements rather than energy sources. The low energy value of the investigated MAP might be related to the probable low quantities of fermentable carbohydrates and minor importance of protein degradation for energy supply. The low energy concentration of microalgae is in accordance with the results of Tibbetts, MacPherson, McGinn, and Fredeen (2016), who found a general downward trend in apparent metabolizable energy when whole algal biomass and lipid-extracted microalgal biomasses were included in a batch-culture in vitro ruminal fermentation system as a forage replacement.

### 4.4 Production of volatile fatty acids and methane

Typically, the proportion of acetate in rumen fluid varies between 55% and 70% of total VFA, that of propionate between 20% and 25%, and that of butyrate between 10% and 20% (Fuller, 2004), while the proportions of branched-chain fatty acids and valerate are usually <5%. The proportions of acetate propionate and butyrate did not observably shift from this usual pattern in the present study or when several microalgae were supplemented in the diets for cows (Moate et al., 2013), steers (Costa et al., 2016; Drewery, Sawyer, Pinchak, & Wickersham, 2014), or goats (Kholif et al., 2017b; Lv, Mao, & Zhu, 2016; Zhu, Fievez, Mao, He, & Zhu, 2016) or in vitro (Lodge-Ivey et al., 2014). It is interesting that the proportions of branched-chain fatty acids and valerate were particularly high in some cases. Increased concentrations of branched-chain fatty acids in the rumen fluid of cattle receiving microalgae have been reported before (Costa et al., 2016; Drewery et al., 2014; Lamminen et al., 2017; Panjaitan, Quigley, McLennan, & Poppi, 2010; Panjaitan, Quigley, McLennan, Swain, & Poppi, 2015). Lamminen et al. (2017) suggested that high concentrations of branched-chain fatty acids in rumen fluid may be related to the increased intake of branched-chain amino acids when microalgae are supplemented into ruminant diets. Furthermore, they hypothesized that a high degradability of CP in microalgae might have promoted the availability of branched-chain amino acids for the synthesis of branched-chain fatty acids, but this assumption is strongly contradicted by the low ruminal CP degradation found in the current study. The isobutyrate and isovalerate concentrations in the incubation residue were highly correlated with the concentrations of isoleucine (isobutyrate: \( r = 0.86 \) and isovalerate \( r = 0.88 \)), leucine (isobutyrate: \( r = 0.80 \) and isovalerate \( r = 0.78 \)), and valine (isobutyrate: \( r = 0.83 \) and isovalerate \( r = 0.80 \)) in the MAP examined by the present study. This strengthens the assumption that high proportions of branched-chain fatty acids might be related to higher supply of branched-chain amino acids with the supplementation of microalgae.
Microalgae have been discussed as potential inhibitors of methane production because of the occurrence of eicosapentaenoic or docosahexaenoic acids (Boeckaert et al., 2006; Fievez, Boeckaert, Vlaeminck, Mestdagh, & Demeyer, 2007) in some species. Several authors have reported a reduction of ruminal methanogenesis in vitro (Boeckaert et al., 2006; Fievez et al., 2007; Ungerfeld, Rust, Burnett, Yokoyama, & Wang, 2005) or in vivo (Elghandour et al., 2017) when microalgae rich in polyunsaturated fatty acids (docosahexaenoic acid, hexadecatrienoic acid) were supplemented. Nevertheless, Kholif et al. (2017a) observed an increase in methane production and Tsiplakou et al. (2016) observed an increase in methane-producing bacteria and protozoa with the supplementation of protein-rich Chlorella vulgaris, indicating that not all microalgae are likely to have methane reducing properties. Data on methane production in this study showed a similar trend. The CH₄-volume was negatively correlated with the ether extract \( r = -0.50 \) and the eicosapentaenoic acid concentration \( r = -0.51 \) of the MAP, while CP concentration \( r = 0.70 \) was positively correlated with CH₄-volume. Amino acids are converted to ammonia and α-keto acids during deamination and subsequently produce \( \text{H}^+ \) ions, which contribute to the \( \text{CH}_4 \)-pool in the rumen (Hossain, Sherasia, Phondha, Patel, & Garg, 2017). Therefore, the degradation of protein and assimilation of microbial protein can result in either a net production or consumption of hydrogen, as microbial protein synthesis utilizes reducing equivalents (Knapp, Laur, Vadas, Weiss, & Tricarico, 2014). Vanegas, González, and Carro (2017) suggested that an excess of rumen degradable protein might increase methane emission, especially when microbial protein synthesis is limited by energy deficiency. Thus, the higher CH₄-volume and concentration with higher CP concentrations observed herein might be related to excess of rumen degradable protein, which is supported by a strong negative relationship between RUP and CH₄-volume \( r = -0.75 \) for RUP after 48 hr of incubation).

### 4.6 Effects of cell disruption

For the most part, cell disruption increased traits related to the extent of ruminal fermentation (e.g., GP, dOM, total VFA, and ruminal protein degradation) with Chlorella, Nannochloropsis, and Phaeodactylum products, while it did not increase, or even decreased, the extent of ruminal fermentation with Arthrospira products. The observed increase in ruminal fermentation may be related to the destruction of ruminally undegradable cell wall compounds and the higher accessibility of rumen microbiota to fermentable, intracellular compounds which are expected to be released by cell disruption. These results are in accordance with those of previous studies on nonruminant animals which predominantly found an increased nutrient digestibility of microalgae when a cell disruption treatment was applied (Cavonius et al., 2016; Hedenskog et al., 1969; Janczyk et al., 2005, 2007; Tibbetts et al., 2017; Wild et al., 2018). Arthrospira has a thin and fragile cell wall made up of layers of fibrils and peptidoglycan (van Eylkenenburg, 1977), which is expected to be easily degraded by rumen microbiota. Therefore, no effect of cell disruption on ruminal fermentation of Arthrospira was expected. It is interesting that cell disruption appeared to actually decrease the ruminal fermentation of Arthrospira products (e.g., GP, dOM, uCP, and RUP after 8 hr of incubation). It is possible that cell disruption released compounds that may inhibit ruminal fermentation or lead to the formation of complexes or agglomeration and hence decreased the accessibility of microbiota to fermentable compounds. More precise, these compounds may be
cyanotoxins (Roy-Lachapelle, Solliec, Bouchard, & Sauvé, 2017) or gamma-linolenic acid (Wild et al., 2018), which are not present in the other investigated microalgae genera. Nevertheless, the effect of cell disruption to decrease ruminal fermentation of Arthrospira products was relatively low and not consistent for all investigated traits. Therefore, despite its statistical significance, it cannot be ruled out that this effect is due to the range of uncertainty of the respective method. Variation in the extent of the effect of cell disruption between microalgae genera may be explained by different cell wall structures and the composition or variable amounts of intracellular fermentable compounds and hence a variable amount of additionally released fermentable compounds. In contrast to the extent of fermentation, the fermentation pattern (proportions of VFA) was barely affected by cell disruption, indicating that access of rumen microbiota to fermentable compounds is increased but the types of degraded compounds remained similar.

5 | CONCLUSIONS

In conclusion, ruminal fermentation of different MAP was low. Limited protein fermentation caused very high concentrations of RUP, but IDP was very low. Further investigations will be necessary to evaluate these findings in vivo. Mechanical cell disruption mostly enhanced the extent of ruminal fermentation of MAP, but as RUP was decreased and IDP was hardly affected by cell disruption, it appears not to be necessary when microalgae are intended for use as a protein source for ruminants. Because of the high variability in the nutritional value characteristics for ruminants, general means are inappropriate to characterize the nutritional value of MAP. Further studies are necessary to achieve either a standardization of microalgae biomass or the possibility of easy prediction of the nutritional value of microalgae.

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