Investigations on Nutritional Characteristics of Microalgae with Emphasis on Ruminants

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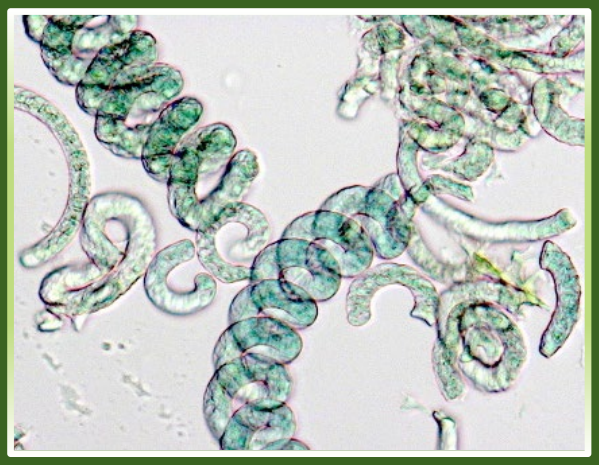

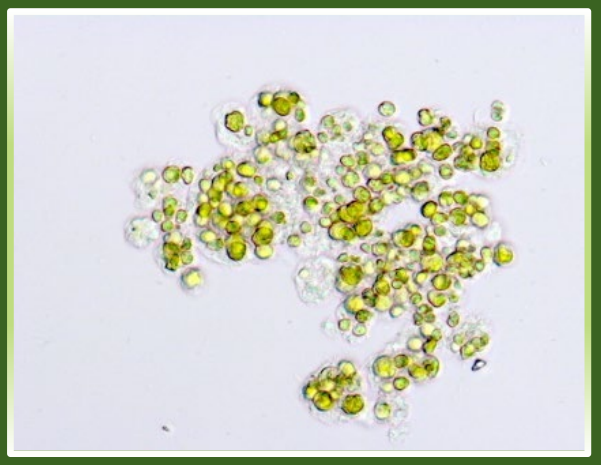

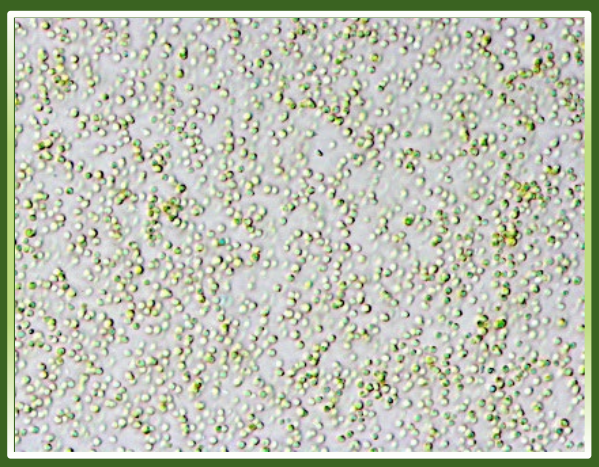

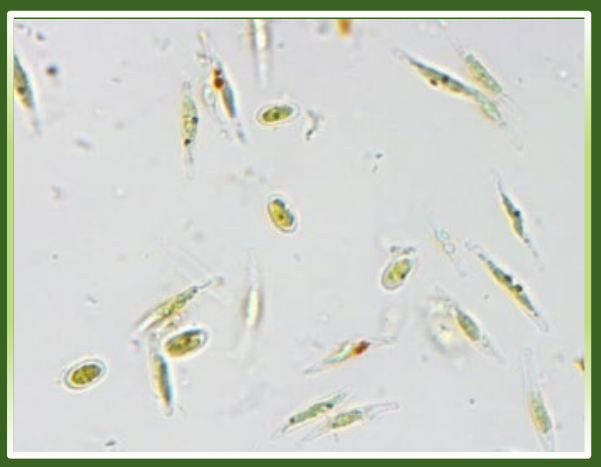

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INVESTIGATIONS ON NUTRITIONAL CHARACTERISTICS OF MICROALGAE WITH EMPHASIS ON RUMINANTS

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The abbreviations defined by the international system for units and the symbols for the chemical elements of the periodic table of elements are not listed. Abbreviations exclusively used in tables and in the CHAPTERS 5, 6, and 7 are not listed because they are explained there.

AA	Amino acids
ADF	Acid detergent fibre
CLA	Conjugated linoleic acids
CP	Crude protein
DHA	Docosahexaenoic acid
DM	Dry matter
DMI	Dry matter intake
dOM	Digestibility of organic matter
EE	Ether extract
eHGT	Extended Hohenheim Gas Test
EIV	Three-step enzymatic *in vitro* method
EPA	Eicosapentaenoic acid
GP	Gas production
HGT	Hohenheim Gas Test
IDP	Intestinal digestibility of ruminally undegraded crude protein
IVPD	*In vitro* crude protein digestibility
mCP	Microbial crude protein
ME	Metabolisable energy
NDF	Neutral detergent fibre
NEL	Net energy lactation
NPN	Non-protein nitrogen
PEAR	Post-extraction microalgae residues
pGP	Potential gas production
PUFA	Polyunsaturated fatty acids
r	Pearson correlation coefficient

r^2	Coefficient of determination
RUP	Ruminally undegradable crude protein
$RUPe_{HGT}$	Ruminally undegradable crude protein determined by the Extended Hohenheim Gas Test method
RUP_{EIV}	Ruminally undegradable crude protein determined by the three-step enzymatic *in vitro* method
TP	True protein
uCP	Utilizable crude protein at the duodenum
VFA	Volatile fatty acids

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL 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, particularly in developing countries, is expected to lead to a transition towards a higher consumption of meat and milk products (FAO, 2017). Taken together, this will lead to a continuous increase in the demand of meat and dairy products. Estimates suggest that growth rates of per capita meat consumption will slow down compared to previous decades, because of approaching a saturation level in developed countries and a deceleration of growth rates in countries like China and Brazil that dominated past increases. However, the overall increase in world meat consumption will be tremendous: 256 million tonnes in 2005/2007 will increase by 1.3 % annually, which will cause a total increase in total meat consumption by approximately 200 million tonnes until 2050 (Alexandratos & Bruinsma, 2012). The concurrent decline of arable land per capita (Bruinsma, 2011) additionally strengthens the essential need for the improvement of utilization of feed resources, as well as the establishment of alternative feed resources which do not compete with the production of food or can be produced independently from arable land.

The term "microalgae" summarizes a diverse group of plant-like, photosynthetic, unicellular or simple multicellular organisms. Microalgae do not belong to a single monophyletic group and the term includes Eukaryotes as well as Prokaryotes. Microalgae commonly refers to aquatic organisms (fresh and sea water). Nevertheless, microalgae as a group occur ubiquitous, whereas individual species have specific habitats, such as water, soils, rocks, snow, plants, and even animals (Andersen, 2013). Microalgae can be cultivated on non-arable land, providing the opportunity of feed production on current idle areas, without further stressing the competition with food production. Additionally, photoautotrophic cultivation of microalgae can be operated with CO_2 as sole carbon source and light as sole energy source and is thus not consuming valuable resources which can be used for other nutritional purposes. Recent estimations suggest that there are 200,000 up to several millions of microalgae species (Singh & Saxena, 2015), with more than 40,000 species already described (Hu *et al.,* 2008), but only a few have been analysed in details or are industrially utilized. This vast number of species and their diversity in terms of composition reveals manifold potential applications of microalgae in the food and feed sector. For example, *Chlorella* and *Arthrospira* are discussed as novel protein sources as they can have a crude protein (CP) concentration of up to 70 % of dry matter (DM) with an amino acid (AA) composition resembling that of soy protein (Becker, 2007) or of animal protein (Khatun *et al.,* 1998). Other genera, such as *Nannochloropsis*, *Phaeodactylum* and

Schizochytrium are promising commercial sources of omega-3 fatty acids (Ryckebosch *et al.,* 2012). Other applications such as astaxanthin from *Haematococcus*, phycocyanin from *Arthrospira* or β-carotene from *Dunaliella* as colourants have been commercialised (Enzing *et al.,* 2014).

The outlined nutritional properties of microalgae have led to strong interest of researchers in different areas and microalgae have been the research subject of numerous studies. Nevertheless, despite that earliest research on the nutritional characteristics of microalgae date back to the 1950s (Combs, 1952; Fink & Herold, 1956, 1957, 1958), research on this topic is still in its infancy. Most of the previous research in farm animals concerns the application of docosahexaenoic acid (DHA)-rich, heterotrophic microalgae for the enrichment of animal products (Boeckaert *et al.,* 2008; Fredriksson *et al.,* 2006; Phelps *et al.,* 2016a, 2016b), but systematic data on nutrient digestibility and nutrient utilization of photoautotrophic microalgae are rare, in particular for ruminant animals. Furthermore, the high diversity of species and the high capacity of microalgae to adapt to environmental and cultivation conditions is an important issue to deal with, because findings on nutritional properties cannot be easily generalised for different microalgae species or possibly not even for the same species. Moreover, many microalgae species develop robust cell walls or cell coverings that might restrict their nutrient digestibility. Nevertheless, investigations on the effects of cell disruption methods on nutrient digestibility and the nutritional value of microalgae for farm animals are scarce and this issue was not yet investigated in ruminant animals.

The objective of the present doctoral thesis was to evaluate the suitability of microalgae as animal feed. The conducted experiments aimed to systematically determine nutritional characteristics of microalgae for farm animals, with emphasis on ruminants. The experiments investigated different factors affecting the nutritional value of microalgae, including the intra- and inter-genera variability, cell disruption and cultivation conditions. The thesis aims to evaluate the importance of these factors in terms of suitability of microalgae as feedstuffs and to highlight possible relationships between them.

CHAPTER 2

LITERATURE OVERVIEW

2. LITERATURE OVERVIEW

2.1. Overview on the investigated microalgae genera

The following section shall give a brief overview on the properties of the microalgae genera that have been subject of the present thesis (*Arthrospira*, *Chlorella*, *Nannochloropsis* and *Phaeodactylum*). It outlines briefly taxonomy, morphology and important compositional properties of these microalgae. Moreover, many microalgae develop cell walls or cell coverings which are very diverse in terms of structure and composition. The occurrence of these cell walls or cell coverings might have nutritional importance, since they can be robust and rigid, and might therefore limit accessibility of nutrients for the animal. The following section therefore emphasizes the structure and composition of cell walls or cell coverings of the microalgae that have been the subject of the present thesis. Literature results on the effects of cell disruption on nutrient accessibility of microalgae will be reviewed in CHAPTER 2.2.

Arthrospira

Arthrospira is a multicellular, filamentous cyanobacterium (blue green-algae). It occurs in alkaline waters (pH 11) of tropical and subtropical regions with high concentrations of carbonates and bicarbonates (Belay, 2013; Tomaselli, 1997). Taxonomic classification of *Arthrospira* and *Spirulina* has led to confusion over the last decades, because of the existence of two designations (Tomaselli, 1997). Nevertheless, nowadays it is generally accepted that *Arthrospira* and *Spirulina* are two distinct genera. It has to be kept in mind that the term "Spirulina" is commonly used as the commercial name for *Arthrospira* species (Belay, 2013; Sili *et al.,* 2012), resulting in a lack of transparency and uncertainties regarding the correct taxonomic classification, even in scientific publications. Sili *et al.* (2012) proposed to take this issue into account by writing Spirulina for commercial materials, i.e., not using italics. Nevertheless, since the true species designation can usually not be clarified from the information given in literature, the term applied by the respective authors will be quoted throughout the present thesis. *Arthrospira* is approved as a feedstuff under its commercial designation "Spirulina" in the EU (The European Parliament and Council, 2009). *Arthrospira* has high CP concentrations between 45 and 70 % of DM (Becker, 2007, 2013) and is therefore regarded as an alternative protein source in animal nutrition.

The cellular structure of *Arthrospira* is typical for that of Prokaryotes, lacking a morphologically limited nucleus and plastids and is enveloped by gram-negative cell wall. *Arthrospira* cells are cylindrical, shorter than broad, with a cell diameter from about 6 to 12 µm.

They are arranged as trichomes in an open left-hand helix. Trichome length and width varies from 50 to 500 µm and from 3 to 4 µm, respectively (Vo *et al.,* 2015). The helix pitch and the helix diameter vary from 12 to 72 µm and from about 30 to 70 µm, respectively (Tomaselli, 1997). The trichomes are divided into the cells by transverse, three layered cross-walls (van Eykelenburg, 1977). Helicity is characteristically for the genus *Arthrospira*, but helix morphology varies considerably between and even within species (Belay, 2013). For example, environmental factors like temperature and irradiance, the supply of nutrients, as well as the harvest regime have been shown to affect the helix morphology of *Arthrospira* (Belay, 1997; Jeeji Bai, 1985; Jeeji Bai & Seshadri, 1980; van Eykelenburg, 1979). *Arthrospira* has a thin cell wall that is thought not to represent a barrier to proteolytic digestion enzymes (Becker, 2013). It is four-layered, with a total thickness of about 40 to 60 nm, enveloped in a fibrillar, net-like structured sheath (Tomaselli, 1997), and it is made up of peptidoglycan (van Eykelenburg, 1977). The ultrastructure of *Arthrospira* cell walls was apparently not affected by light intensity and temperature (van Eykelenburg, 1977). Furthermore, *Arthrospira* cells contain several cellular inclusions, such as thylakoids, cyanophycin granules, polyglucan granules, carboxysomes, and gas vesicles that are typical for cyanobacteria (Belay, 2013).

Chlorella

Chlorella are eukaryotic green microalgae, belonging to the phylum Chlorophyta and the class Trebouxiophyceae. *Chlorella* was first described by Beijerinck (1890) and *Chlorella vulgaris* is the type species. *Chlorella* occur in freshwater, seawater, and soil (Liu & Hu, 2013). *Chlorella* has gained interest as a feed source because of its high CP concentration of up to 60 % of dry mass, and it is regarded as valuable source of carotenoids for the pigmentation of egg yolk and fish flesh (Safi *et al.,* 2014b). *Chlorella* is approved as a feed source in the EU (The European Parliament and Council, 2009).

Cells of *Chlorella* are spherical or ellipsoidal with a cell diameter ranging from 2 to 10 µm. Its chloroplast is cup-shaped and located peripherally in the cytoplasm. Mitochondria are closely associated with the chloroplasts, and the nucleus is situated near the cytoplasmic membrane (Liu & Hu, 2013). Most of the *Chlorella* species contain pyrenoid centrally located in the chloroplast and enveloped by a starch sheath (Bertagnolli & Nadakavukaren, 1970). *Chlorella* forms a rigid cell wall, but its structure (Yamada & Sakaguchi, 1982) and composition (Abo-Shady *et al.,* 1993; Blumreisinger *et al.,* 1983; Takeda, 1988a, 1988b, 1993, 1995; Takeda & Hirokawa, 1984) varies considerably between species and strains and can therefore be used as taxonomic marker (Takeda, 1993). Additionally, it changes during cell

growth (Takeda & Hirokawa, 1978; Yamamoto *et al.,* 2004; Yamamoto *et al.,* 2005). In terms of cell wall structure, some *Chlorella* species possess a mono-layered, microfibrillar cell wall, while others have a two layered cell wall, whose inner layer is always microfibrillar, but the outer layer can be mono- or trilaminar (Yamada & Sakaguchi, 1982). Furthermore, data on cell wall thickness are very variable with values ranging between 20 and 130 nm (Corre *et al.,* 1996; Gerken *et al.,* 2013; Martínez *et al.,* 1991; Yamamoto *et al.,* 2005; Yap *et al.,* 2016). The cell wall is either characterized by glucose-mannose or glucosamine polymers (Takeda, 1991, 1993), of which glucosamine forms a chitin-like glycan layer (Kapaun & Reisser, 1995). Additionally, some *Chlorella* species such as *Chlorella emersonii* or *Chlorella minutissima* possess a trilaminar cell wall, containing algaenan which is described as an insoluble and non-hydrolysable biopolymer (Allard & Templier, 2000). Furthermore, cell walls of *Chlorella protothecoides* were reported to contain sporopollenin (He *et al.,* 2016), an extremely tough biopolymer that is resistant many kinds of chemical treatments (acid and alkaline hydrolysis, acetolysis) and to enzymatic degradation (Ueno, 2009; Xiong *et al.,* 1997). The sugars of the cell wall are a variable mixture of arabinose, fucose, galactose, glucose, mannose, rhamnose, xylose, and uronic acids (Abo-Shady *et al.,* 1993; Blumreisinger *et al.,* 1983; Takeda, 1988a, 1988b, 1991, 1993; Takeda & Hirokawa, 1984) and neutral sugars can make up 24 to 74 % of the cell wall constituents (Blumreisinger *et al.,* 1983). Safi *et al.* (2013) pointed out that disruption of cell walls of *Chlorella vulgaris* is required for complete protein extraction, indicating that cell walls of *Chlorella* might limit nutrient accessibility for the animal.

Nannochloropsis

The genus *Nannochloropsis* comprises six species of yellow-green, unicellular microalgae (Zhang *et al.,* 2015) and was first described by Hibberd (1981). *Nannochloropsis* belongs the phylum Ochrophyta and the class Eustigmatophyceae, occurring in fresh, brackish, and sea water (Adamczyk *et al.,* 2016; Beacham *et al.,* 2014; Zhang *et al.,* 2015). Among the six *Nannochloropsis* species, *Nannochloropsis gaditana* is the only species, which is approved as a feed source in the EU (The European Parliament and Council, 2009). *Nannochloropsis* has gained interest as a potential feedstuff because of its high concentration of eicosapentaenoic acid (EPA; 2 to 3 % of DM), which is accompanied by CP concentrations between 20 and 40 % of DM (Anele *et al.,* 2016; Matos *et al.,* 2016; Rebolloso-Fuentes *et al.,* 2001a; Tibbetts *et al.,* 2015b). Furthermore, it is regarded as source of pigments, comprising chlorophyll *a* as dominant pigment and carotenoids such as violaxanthin, vaucheraxanthin, canthaxanthin, and beta-carotene (Faé Neto *et al.,* 2018; Lubián *et al.,* 2000; Rebolloso-Fuentes *et al.,* 2001a).

Nannochloropsis forms spherical to ovoid single cells with a diameter of 2 to 6 µm (Andersen *et al.,* 1998; Beacham *et al.,* 2014; Gwo *et al.,* 2005). Cells of *Nannochloropsis salina* tend to be cylindrical in shape, with a cell length of 2 to 8 µm (Andersen *et al.,* 1998). Cell walls of *Nannochloropsis* have an outer algaenan-based layer and an inner cellulose-based layer which is connected to the plasma membrane with struts. Algaenan in the outer layer is associated with a trilaminar sheath and algaenan was described as comprising highly saturated, long-chain aliphatic compounds that are cross-linked by ether bonds (Scholz *et al.,* 2014). Furthermore, Scholz *et al.* (2014) described that the cell wall mostly consists of carbohydrates (~ 80 %) with glucose being the dominant sugar (> 90 %) (Vieler *et al.,* 2012), and around 15 % of the cell wall material is likely comprised of algaenan (Scholz *et al.,* 2014). Cell wall thickness of *Nannochloropsis* varies amongst the different species but also within one species amongst different sub-strains between 63 and 119 nm (Beacham *et al.,* 2014). These complex cell wall characteristics of *Nannochloropsis* and its thickness, in combination with the very low cell size make them difficult to disrupt (Alhattab *et al.,* 2018).

Phaeodactylum

Phaeodactylum tricornutum is a pleiomorphic diatom, occurring in brackish water. It was first described by Bohlin (1897) and its polymorphic nature was revealed by Wilson (1946) as *Nitzschia closterium*. *Phaeodactylum* belongs to the class of Bacillariophyceae and has three interconvertible morphotypes (oval, fusiform and triradiate), but the conditions that promote the development of the morphotypes are not well understood (Martin-Jézéquel & Tesson, 2012; Tesson *et al.,* 2009a). Oval cells are 7.5 to 16 µm long and 2.5 to 6.5 µm wide, while fusiform cell are 15 to 27 µm long and their width ranges between 3 to 5 µm. The length of the arms of the triradiate cells as measured from the valve centre has been described to range between 6 and 15 µm (Johansen, 1991). Currently, *Phaeodactylum* is not approved as a feed source in the EU (The European Parliament and Council, 2009). *Phaeodactylum* is discussed as alternative source for EPA (2 to 3 % of DM), it has CP concentrations between 30 and 40 % (Cartens *et al.,* 1996; Matos *et al.,* 2016; Rebolloso-Fuentes *et al.,* 2001b; Tibbetts *et al.,* 2015b), and it is a potential source of carotenoids, in particular fucoxanthin (Kim *et al.,* 2012).

Cell walls of diatoms are characterized by silicified shells, termed frustules. They are composed of two overlapping theca formed of a valve and a set of girdle bands, called hypotheca and epitheca (Tesson *et al.,* 2009b). Nevertheless, unlike other diatoms, cell walls of *Phaeodactylum* are poorly silicified and are mainly composed of organic compounds (Borowitzka & Volcani, 1978; Reimann & Volcani, 1967). All morphotypes develop a three-

layered structure, but silicification of the cell wall is dependent on the morphotype. While the oval form synthesizes a silica valve with pores and a central raphe, the fusiform and the triradiate morphotypes only possess silica bands that are located at the epitheca on the junctions of the two valves (Tesson *et al.,* 2009a). The three layers of the cell wall have been reported to have a thickness of 7, 5 and 3 nm from outside to inside (Reimann & Volcani, 1967). The majority of the low amount of silicia in *Phaeodactylum* is weakly polymerised silicate. For all morphotypes, polysaccharides mostly replace the structural part of the cell walls compared to other diatoms (Tesson *et al.,* 2009b). The major component of *Phaeodactylum* cell walls are sulphated glucuronomannans (Le Costaouëc *et al.,* 2017; Tesson *et al.,* 2009a; 2009b; Willis *et al.,* 2013). The main storage product of *Phaeodactylum* is chrysolaminarin, a 1,3-β-D-glucan that is located in the vacuoles (Chiovitti *et al.,* 2004; Martin-Jézéquel & Tesson, 2012).

2.2. Effects of cell disruption on nutrient digestibility and accessibility

Various cell disruption methods for microalgae exist, which were particularly investigated for enhancement of nutrient extraction from microalgae. Cell disruption can be achieved by mechanical disruption methods that utilize shear forces (e.g., bead/ball milling, high-pressure homogenization), and by utilizing wave (e.g., ultra-sonication, microwave), current (pulsed electric field) or thermal (e.g., steam explosion, freeze drying, autoclave) energy. Furthermore, chemical treatments (e.g., acids, solvents) or enzymatic lysis have been utilized for cell wall disruption or cell wall degradation of microalgae (Bharte & Desai, 2018; Lee *et al.,* 2017). Few data are available how these methods affect nutrient digestibility of microalgae in farm animals, but there are some investigations in rats, fish and *in vitro* data which show that digestibility or accessibility of microalgae nutrients is affected by cell disruption.

For *Chlorella vulgaris* it has been shown that ultrasound treatment or high pressure homogenization can significantly increase CP and AA digestibility in rats (Janczyk *et al.,* 2005; Janczyk *et al.,* 2007) and Atlantic salmon (Tibbetts *et al.,* 2017). Becker *et al.* (1976) reported that drum-drying increased digestibility coefficients measured in rats compared to sun-dried or sun-dried and cooked *Scenedesmus* and suggested that superior digestibility of drum-dried microalgae was due to rupture of cell wall during drying. Furthermore, an increase of *in vitro* digestibility in *Scenedesmus* algae was observed after cell disruption treatment with a ball mill (Hedenskog *et al.,* 1969). Bead milling did also enhance nutrient digestibility of *Nannochloropsis* in Nile tilapia and *in vitro* nutrient accessibility (e.g., nitrogen solubility, fat extractability) (Teuling *et al.,* 2019). Furthermore, Teuling *et al.* (2019) reported that nutrient digestibility of *Nannochloropsis* in Nile tilapia and *in vitro* nutrient accessibility were affected

by variable physical treatments (pasteurisation, freeze-drying, freezing-thawing), although these methods were not as effective as bead milling. Lemahieu *et al.* (2016) investigated the effect of high pressure homogenization on omega-3 polyunsaturated fatty acids (PUFA) enrichment from the microalgae *Isochrysis galbana* and *Nannochloropsis oculata* in the eggs of laying hens. For *Nannochloropsis oculata*, enrichment efficiency of omega-3 PUFA in the eggs was increased by 52 %, but for *Isochrysis galbana* no effect of cell disruption on omega-3 PUFA enrichment in the eggs was observed. The authors concluded that the absence of the effect of cell disruption with *Isochrysis galbana* was because this microalgae species does not contain a rigid cell wall or only a cell membrane, but rigid *Nannochloropsis oculata* cell walls hampered accessibility of omega-3 PUFA (Lemahieu *et al.,* 2016). Bioaccessibility of lutein and β-carotene from the microalgae *Chlamydomonas reinhardtii* was not increased by ultra-sonication, while it significantly increased bioaccessibility of these carotenoids from *Chlorella vulgaris* (Gille *et al.,* 2015). These observations indicate that cell disruption is not necessary for all microalgae species. Low nutrient accessibility of non-disrupted *Nannochloropsis oculata* was also observed by Cavonius *et al.* (2016), who investigated the degree of protein hydrolysis and liberation of free fatty acids as measures for protein and lipid accessibility in a multi stage *in vitro* system. Degree of protein hydrolysis and liberation of free fatty acids of non-disrupted *Nannochloropsis* was close to zero but could be considerably enhanced by cell disruption with a ball mill. Cavonius *et al.* (2016) concluded that cell disruption is urgently necessary to make protein and lipids of *Nannochloropsis* accessible.

In some cases, application of cell disruption methods decreased nutrient accessibility from microalgae. Electroporation of the *Chlorella vulgaris* did decrease CP and AA digestibility in rats (Janczyk *et al.,* 2005; Janczyk *et al.,* 2007). Reduced CP digestibility was also observed for high pressure homogenization in another study with rats (Komaki *et al.,* 1998). Furthermore, stewing of the microalgae *Spirulina maxima* reduced its digestibility in rats (Clément *et al.,* 1967). That indicates that there are cell disruption methods which do not lead to an enhanced nutrient accessibility with all microalgae species.

Cell wall degradation with enzymes was described as a method to enhance accessibility of microalgae nutrients by increasing permeability of algal cell walls (Gerken *et al.,* 2013). For example, treatment of dried microalgae *Galdieria sulphuraria* with a mixture of polysaccharidases effectively degraded cell walls as demonstrated by an increased protein hydrolysis (Graziani *et al.,* 2013). Dietary supplementation of glucanase and pectinase to a diet for laying hens containing 1 % of *Nannochloropsis* did slightly affect egg yolk pigmentation

compared to a diet not containing supplemented enzymes, but did not have an effect on enrichment of eggs with omega-3 fatty acids (Nitsan *et al.,* 1999).

In conclusion, it appears that cell disruption treatments generally enhance digestibility of microalgae nutrients. This is likely because of the higher accessibility of nutrients after the disruption of the cells and it seems that this independent from the respective animal species. Nevertheless, extent of the effects is expected to be dependent on the treated microalgae species and for species that lack a rigid cell wall it might not be necessary (e.g., *Chlamydomonas reinhardtii*).

2.3. Relevance of microalgae in ruminant nutrition

The following chapter will give an overview on previous research on the application of microalgae in the nutrition of ruminant animals. The chapter includes an overview on the application of microalgae in lactating ruminants (cows, goats and sheep) and in beef cattle. Furthermore, an overview on general effects of microalgae supplementation on ruminal fermentation is given, including *in vivo* and *in vitro* studies. Because of incomplete development of the forestomach system in young ruminants (calves, kids and lambs), effects of microalgae supplementation are expected to be distinctly different from effects in adult ruminants. Nevertheless, review of literature results on the effects of microalgae supplementation in young ruminants is beyond the scope of the present thesis, as the emphasis is on the impact of microalgae supplementation on ruminal processes.

2.3.1. Application of microalgae in lactating ruminants

Recently, Altomonte *et al.* (2018) reviewed the implications of microalgae supplementation to ruminant diets with emphasis on milk production. According to the authors, microalgae have been used in dairy ruminant nutrition as a source of energy, protein or natural antioxidants, with the majority of studies concerning the enrichment of milk with omega-3 fatty acids provided by heterotrophic, DHA-rich microalgae *Schizochytrium* (cf. TABLE 1). Besides that, investigated microalgae in nutrition of dairy ruminants were *Aurantiochytrium limacinum*, several *Chlorella* species, *Prototheca moriformis*, and *Spirulina platensis*. *Chlorella* species and *Spirulina* have been used as an alternative protein source (Lamminen *et al.,* 2017), or for alteration of milk fatty acid profiles (Póti *et al.,* 2015; Tsiplakou *et al.,* 2017a; 2018). *Aurantiochytrium limacinum* was used for enrichment of milk with DHA (Moran *et al.,* 2017; 2018) and *Prototheca moriformis* was used as a substitute for corn (da Silva *et al.,* 2016). The investigated microalgae doses varied between few grams (Kholif *et al.,* 2017b) and

approximately one kilogram per day, the latter corresponding to 5 % of DM intake (DMI) (Franklin *et al.,* 1999). Duration of microalgae supplementation lasted between 7 and 125 days (cf. TABLE 1).

Effects on dry matter intake

Effects of microalgae supplementation in lactating ruminants are difficult to compare, because of high variation in the dose of microalgae supplementation, the applied microalgae species, the diet composition, the duration of microalgae supplementation and the feedstuff which was substituted by microalgae. Nevertheless, several authors consistently observed a reduction in DMI (cf. TABLE 1) when microalgae were fed to lactating ruminants. The extent of the reduction in DMI varied between 10 and 46 % compared with the respective control group (Boeckaert *et al.,* 2008; Moate *et al.,* 2013; Papadopoulos *et al.,* 2002). Additionally, Lamminen *et al.* (2017) observed an incomplete intake of concentrates that contained microalgae, but no effect on total DMI, as cows compensated for the lower concentrate intake with a higher intake of silage. Several authors suggested that reduction of DMI was caused by low palatability of microalgae (Franklin *et al.,* 1999; Lamminen *et al.,* 2017; Papadopoulos *et al.,* 2002). Besides of the taste and the smell of microalgae, their physical structure might also be a cause for their low acceptability in lactating ruminants, especially when they were provided as fine powder (Altomonte *et al.,* 2018; Lamminen *et al.,* 2017). Hintz *et al.* (1966) suggested that this issue might be overcome by pelleting the diet. A further explanation for the reduced DMI of dairy ruminants receiving microalgae might be the disturbance of ruminal fermentation through high concentrations of PUFA contained in some microalgae species (Boeckaert *et al.,* 2008). High lipid concentrations in ruminant diets may greatly disturb ruminal fermentation, causing in particular reduced digestibility of structural carbohydrates (Jenkins, 1993). However, Franklin *et al.* (1999) fed rumen-protected and non-protected *Schizochytrium* microalgae to cows and observed a reduction in DMI for both treatment groups compared to a control group, which did not receive microalgae. Protection of microalgae against ruminal biohydrogenation should prevent disturbance of ruminal fermentation. Thus, the results of Franklin *et al.* (1999) indicate that it is unlikely that the disturbance of ruminal fermentation by high PUFA concentrations of microalgae is the sole cause for DMI reduction in dairy ruminants receiving microalgae.

Effects on milk yield

Most of the studies investigating the effects of microalgae supplementation on milk yield did consistently not observe an effect (cf. TABLE 1), in some cases even despite observing a reduction in DMI (Franklin *et al.,* 1999; Moate *et al.,* 2013; Papadopoulos *et al.,* 2002;

Reynolds *et al.,* 2006). Franklin *et al.* (1999) and Papadopoulos *et al.* (2002) attributed this to an increased efficiency of milk production (energy corrected milk yield/DMI), because of direct incorporation of microalgae derived fatty acids into milk. Nevertheless, Moate *et al.* (2013) did not observe an increased efficiency of milk production, but microalgae supplementation was accompanied by a reduced milk fat yield, indicating that cows compensated lower energy intake by lower *de novo* synthesis of milk fat. Nevertheless, Reynolds *et al.* (2006) observed a 43 % reduction in milk yield when they supplemented 25 g *Schizochytrium*/kg diet DM to an alfalfa haylage-based diet in ewes. In a study with cows, the supplementation of 43 g *Schizochytrium*/kg DMI through rumen fistula resulted in a 44 % reduction of milk yield (Boeckaert *et al.,* 2008). Vanbergue *et al.* (2018b) observed a 9 % reduction in milk yield when feeding 340 g *Schizochytrium* per day to cows and the reduction was even more severe (18 % reduction) when 156 g *Schizochytrium* per day were combined with the feeding of a sieved, extruded linseed product. In contrast to that, supplementation of 200 g *Spirulina platensis* per day increased milk yield of cows by 21 % (Kulpys *et al.,* 2009). Supplementation of 5 and 10 g *Chlorella vulgaris* per day increased milk yield of goats by 10 and 12 % (Kholif *et al.,* 2017b). Kulpys *et al.* (2009) suggested that improved milk production was related to the chemical composition of the applied microalgae that has positive impact on rumen microbiota and the ruminal fermentation processes. Kholif *et al.* (2017b) attributed increased milk yield associated with microalgae supplementation to an increased ruminal fermentation with higher proportions of propionate in rumen fluid. They suggested that the higher propionate concentration might have been utilized as precursor in gluconeogenesis and lactose synthesis and therefore improved milk yield. Moran and co-workers investigated the effect of *Aurantiochytrium limacinum* supplementation on milk production in dairy cows over an 84-day period under commercial conditions in two experiments. The authors observed an improved persistency of the milk lactation curve and higher milk yields in the final phase of the experiments (day 78-84). They concluded that long-term supplementation of microalgae to diets of lactating ruminants might have positive long-term effects on milk yield (Moran *et al.,* 2017; 2018).

Effects on milk composition

Supplementation of microalgae to diets of lactating ruminants often resulted in a reduction of milk fat concentration and consequently lower milk fat yields in cows (Angulo *et al.,* 2012; Franklin *et al.,* 1999; Glover *et al.,* 2012; Moate *et al.,* 2013; Moran *et al.,* 2017; Offer *et al.,* 2001; Póti *et al.,* 2015; Vahmani *et al.,* 2013; Vanbergue *et al.,* 2018b), goats (Kholif *et al.,* 2017b) and sheep (Bichi *et al.,* 2013; Toral *et al.,* 2010). Additionally, Boeckaert *et al.* (2008)

observed a time-depended reduction of milk fat concentration. Starting from 4.79 %, milk fat concentration levelled off at 2.25 % after 13 days of supplementation of 10 g *Schizochytrium*/ kg DMI. A generally accepted hypothesis regarding milk fat depression associated with supplementation of plant- or marine-derived PUFA is that specific intermediates from the ruminal biohydrogenation of long-chain PUFA exert anti-lipogenic effects (Shingfield & Griinari, 2007). Trans-10, cis-12 conjugated linoleic acid (CLA) has been shown unequivocally to inhibit milk fat synthesis (Shingfield & Griinari, 2007), but also trans-9, cis-11 CLA and cis-10, trans-12 CLA have been demonstrated to induce reduction of milk fat concentrations (Perfield *et al.,* 2007; Sæbø *et al.,* 2005). Nevertheless, the causal intermediates for milk fat depression associated with microalgae supplementation are not fully known yet. Moate *et al.* (2013) attributed only minor importance to the formation of trans-10, cis-12 CLA and suggested that other intermediates were involved, when feeding an algal meal rich in DHA to dairy cows. Toral *et al.* (2010) suggested a joint action of trans-9, cis 11 CLA and trans-10 C18:1 as inhibitors of milk fat synthesis. In accordance to that, Boeckaert *et al.* (2008) found increased concentrations of trans-9, cis-11 CLA, trans-10 C18:1, and trans-11 C18:1, as well as increased concentration of trans-11, cis-15 C18:2 and cis-9, trans 11 CLA in milk associated with decreased milk fat concentration when microalgae were supplemented to diets of cows. Angulo *et al.* (2012) attributed a milk fat depression observed in cows fed marine microalgae to a joint down-regulation in the mammary gland of lipogenic gene expression (stearoyl-CoA desaturase, fatty acid synthase) and of the sterol regulatory element binding transcription factor 1 and they hypothesised that this was mediated by trans-10, cis-12 CLA and long-chain omega-3 PUFA. Furthermore, the authors suggested that other intermediates such trans C18:1 isomers (in particular trans-10 C18:1 and trans-11 C18:1) or trans-7, cis-9 CLA were involved (Angulo *et al.,* 2012). Reduced expression of the sterol regulatory element binding transcription factor 1 and an increased milk fat concentration of trans-10 C18:1 associated with milk fat depression induced by microalgae feeding was also observed by Vahmani *et al.* (2014), but this was not accompanied by an increase of trans-10, cis-12 CLA. The importance of trans-10 C18:1 in milk fat depression associated with microalgae supplementation was also highlighted by Offer *et al.* (2001) and by Vanbergue *et al.* (2018b), who found a fourfold and a twenty-ninefold increase of this fatty acid in milk of cows exhibiting reduced milk fat concentrations, respectively. Furthermore, trans C18:1 isomers were generally increased in milk fat, when a milk fat depression was observed with microalgae feeding in several studies (Angulo *et al.,* 2012; Bichi *et al.,* 2013; Boeckaert *et al.,* 2008; Toral *et al.,* 2010; Vahmani *et al.,* 2013). Additionally, several authors suggested that milk fat depression

associated with the feeding of DHA-rich microalgae was related to the effects of a reduction of C18:0 and cis-9 C18:1 synthesis and its consequences for milk fat fluidity (Bichi *et al.,* 2013; Boeckaert *et al.,* 2008; Toral *et al.,* 2010). The outlined reductions in milk fat concentration and associated changes in milk fatty acids were mostly observed with the addition of DHA-rich microalgae (e.g., *Schizochytrium*). Nevertheless, there are also contrasting results, where authors reported the absence of effects on milk fat concentration, in some cases even despite that similar or higher concentration of DHA-rich microalgae were provided (da Silva *et al.,* 2016; Kulpys *et al.,* 2009; Lamminen *et al.,* 2017; Moran *et al.,* 2018; Papadopoulos *et al.,* 2002; Reynolds *et al.,* 2006; Stamey *et al.,* 2012; Tsiplakou *et al.,* 2017a; 2018). In few cases even an increase in milk fat concentration was observed (Papadopoulos *et al.,* 2002; Póti *et al.,* 2015). Moran *et al.* (2018) suggested that the absence of milk fat depression, despite an increase in trans-11 C18:1, in their study was related to the high forage proportions. High concentrate proportions in the diet tend to decrease milk fat concentration and this effect becomes even more severe in diets that also contain PUFA-rich plant oils or lipids (Shingfield & Griinari, 2007). Accordingly, Moran *et al.* (2018) concluded that negative impact of microalgae-derived long-chain PUFA on milk fat synthesis might be reduced or negated if fibre content is high enough to maintain rumen function and an optimal pH. In agreement with that, Papadopoulos *et al.* (2002) suggested that the increased milk fat concentration observed with the feeding of DHA-rich microalgae to sheep was related to an increased ratio of forage to concentrate. The higher proportions of forage intake resulted from reduced intake of microalgae containing concentrates and might have caused a higher ruminal production of acetate as a precursor for *de novo* milk fat synthesis.

In their review, Altomonte *et al.* (2018) compiled data on the effects of microalgae supplementation on ruminant milk fatty acid composition. They summarized that greatest changes were related to increases in long-chain PUFA and omega-3 fatty acids and to accompanying decreases in saturated fatty acids. Indeed, supplementation of DHA-rich microalgae such as *Schizochytrium* or *Aurantiochytrium* has consistently changed milk fatty profiles to higher concentrations of omega-3 fatty acids in cows, sheep and goats (cf. TABLE 1). Nevertheless, increases of omega-3 fatty acid concentration in milk ranged between 19 % (Moran *et al.,* 2018; Póti *et al.,* 2015) and more than 100 % (Boeckaert *et al.,* 2008; Moate *et al.,* 2013), depending on inclusion level of microalgae and the supplemented diet. The omega-3 fatty acid with the major increases was DHA in cows (Angulo *et al.,* 2012; Bichi *et al.,* 2013; Boeckaert *et al.,* 2008; Franklin *et al.,* 1999; Glover *et al.,* 2012; Moate *et al.,* 2013; Moran *et*

al., 2017; Moran *et al.,* 2018; Stamey *et al.,* 2012; Vahmani *et al.,* 2013; Vanbergue *et al.,* 2018b) and in sheep (Bichi *et al.,* 2013; Papadopoulos *et al.,* 2002; Reynolds *et al.,* 2006; Toral *et al.,* 2010). In their review, Altomonte *et al.* (2018) reported that increases of DHA concentration in milk of cows varied between 100 and more than 1,000 % and that highest increases in sheep were 660 % compared to the respective control group. The effects on the concentration of EPA in milk fatty acids associated with microalgae supplementation were less pronounced, but still considerably high. In cows, increases ranged between 17 and 112 % and in sheep, increases ranged from 50 to more than 100 % (Altomonte *et al.,* 2018). The transfer efficiency of DHA in milk fat ranged between 1 and 18.1 % (Moran *et al.,* 2017; Stamey *et al.,* 2012) and was dependent on the type of the supplement (microalgae oil, microalgae biomass), the dosage of the supplement and the basal diet composition (Boeckaert *et al.,* 2008; Franklin *et al.,* 1999; Glover *et al.,* 2012; Moate *et al.,* 2013; Moran *et al.,* 2017; Moran *et al.,* 2018; Offer *et al.,* 2001; Stamey *et al.,* 2012). Franklin *et al.* (1999) observed an almost doubled transfer efficiency of DHA in milk fat as a result of coating *Schizochytrium* microalgae with xylose (from 8.4 to 16.7 % DHA transfer efficiency). The authors suggested that the rumen protection of microalgae with xylose prevents ruminal biohydrogenation of DHA and therefore increased transfer efficiency (Franklin *et al.,* 1999). Enrichment with DHA was also observed in butter produced from milk of cows receiving a DHA-rich microalgae, but oxidative stability was reduced with microalgae supplementation, although reduced oxidation stability was not observed in the milk from which the butter was produced (Glover *et al.,* 2012). Additionally, milk fat globule size was decreased, and concentrations of free fatty acids as well as spontaneous lipolysis were increased in milk of cows fed *Schizochytrium*, indicating detrimental effects on milk processing and sensory properties (Vanbergue *et al.,* 2018b). Indeed, production of butter from this milk was impossible and cheese processing and sensory characteristics were altered, although the effects on cheese properties were not evaluated as detrimental by the authors (Vanbergue *et al.,* 2018a).

Most of the studies investigating the effects of microalgae supplementation on milk protein concentration did not observe a significant effect (cf. TABLE 1). Significant changes in milk protein concentration were only observed in sheep (Papadopoulos *et al.,* 2002; Reynolds *et al.,* 2006; Toral *et al.,* 2010) and in cows, when microalgae supplementation was combined with the addition of a sieved extruded linseed product (Vanbergue *et al.,* 2018b), but not in goats. While Papadopoulos *et al.* (2002), Reynolds *et al.* (2006) and Vanbergue *et al.* (2018b) observed an increase, Toral *et al.* (2010) found a decrease in milk protein concentration. The

decrease was attributed to a reduced AA availability due to supplementation of microalgae and sunflower to the diet of lactating ewes (Toral *et al.,* 2010). Although Lamminen *et al.* (2017) did not observe a significant effect on milk protein concentration, they observed a tendency to decreased milk protein yield which was not related to reduced milk yields in cows, when rapeseed meal was substituted by *Spirulina platensis*. They suggested that milk protein yield might be limited in diets containing microalgae because of insufficient histidine supply or an imbalanced AA profile (Lamminen *et al.,* 2017). Reynolds *et al.* (2006) suggested that increases in milk protein concentration in their study were due to a concentration effect because of decreased milk yield. Papadopoulos *et al.* (2002) explained that addition of lipids to ruminant diets usually reduce milk protein concentration because of associated increases in milk yield. The authors therefore attributed the lack of reduction in milk protein concentration to unaffected milk yield.

As for milk protein concentration, the supplementation of microalgae to diets of lactating ruminants did mostly not affect milk lactose concentration (cf. TABLE 1). Nevertheless, Reynolds *et al.* (2006) and Papadopoulos *et al.* (2002) observed a reduction of milk lactose concentration in ewes, while Kholif *et al.* (2017b) and Moate *et al.* (2013) observed an increase in milk lactose concentration in goats and sheep, respectively. Increases in milk lactose concentration of goats were attributed to increased ruminal production of propionate, which might have been utilized as precursor for gluconeogenesis and lactose synthesis (Kholif *et al.,* 2017b).

To summarize, it is difficult to draw a general conclusion on the effects of microalgae supplementation in lactating ruminants. That is due to the high variation in the supplemented microalgae amounts and species, the differences in the substituted components and in the composition of the basal diets, and the variation in the duration of microalgae supplementation. Supplementation of omega-3 fatty acid-rich microalgae recurrently enhanced milk fatty acid profiles with omega-3 fatty acids. However, microalgae supplementation appears to might have negative impact in terms of DMI, milk fat production and milk processing properties. Therefore, in order to prevent negative effects of microalgae supplementation in lactating ruminants, systematic studies will be necessary in order to clarify the causal metabolic processes.

TABLE 1: Effects of microalgae supplementation on dry matter intake, milk yield and milk composition of lactating ruminants

Microalgae (MA)	Microalgae dose	Days of treatment	Animal species	Effects on:		Effects on milk composition‡				Authors
				DMI†	MY†	Fat	ω3 FA	Protein	Lactose	
Aurantiochytrium limacinum	6 g MA/kg DMI	84	Cow	n.s.	n.s.	−	+	n.s.	n.s.	Moran *et al.* (2017)
Aurantiochytrium limacinum	100 g MA/d	84	Cow	n.s.	n.s.	n.s.	+	n.s.	n.s.	Moran *et al.* (2018)
Chlorella kessleri	10 g MA/kg DMI	17	Goat	n.s.	n.a.	+	+	n.s.	n.s.	Póti *et al.* (2015)
Spirulina platensis	7.4 g MA/kg DMI		Cow	n.s.		−	+	n.s.	n.s.	
Chlorella pyrenoidosa	5 g MA/kg DM	30	Goat	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	Tsiplakou *et al.* (2017b)
Chlorella vulgaris	5 g MA/d	84	Goat	+	+	−	n.a.	n.s.	+	Kholif *et al.* (2017b)
	10 g MA/d			+	+	−		n.s.	+	
Chlorella vulgaris	5 g MA/kg DM	30	Goat	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	Tsiplakou *et al.* (2018)
Chlorella vulgaris* & *Spirulina platensis	10 g of both MA/kg DM	21	Cow	n.s.	n.s.	n.s.	n.a.	n.s.	n.s.	Lamminen *et al.* (2017)
	21 g of both MA/kg DM			n.s.	n.s.	n.s.		n.s.	n.s.	
	23 g Spirulina/kg DM			n.s.	n.s.	n.s.		n.s.	n.s.	
	44 g Spirulina/kg DM			n.s.	n.s.	n.s.		n.s.	n.s.	
DHA-rich MA, rumen protected, species unknown	200 g MA/d added to:	28	Cow							Glover *et al.* (2012)
	Pasture plus concentrate diet			n.s.	n.s.	−	+	n.s.	n.s.	
	Silage based diet			n.s.	n.s.	−	+	n.s.	n.s.	
Prototheca moriformis, de-oiled, 53:47 mixture with soy hulls	92 g MA/kg DM MA mixture replaced 34% of corn	21	Cow	n.s.	n.s.	n.s.	n.a.	n.s.	n.s.	da Silva *et al.* (2016)

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TABLE 1: Continued

Microalgae (MA)	Microalgae dose	Days of treatment	Animal species	Effects on:		Effects on milk composition‡				Authors
				DMI†	MY†	Fat	ω3 FA	Protein	Lactose	
***Schizochytrium* sp.**, commercial product	4 g MA/kg DM combined with the addition of: 27 g/kg DM linseed oil 27 g/kg DM sunflower oil	70	Cow	n.s. n.s.	n.s. n.s.	− −	+ n.s.	n.s. n.s.	n.s. n.s.	Angulo *et al.* (2012)
***Schizochytrium* sp.**, commercial product	8 g MA/kg DM	54	Sheep	n.s.	n.s.	−	+	n.s.	n.s.	Bichi *et al.* (2013b)
***Schizochytrium* sp.**, commercial product	43 g MA/kg DMI	21	Cow	−	−	n.s.	+	n.s.	n.s.	Boeckaert *et al.* (2008)
***Schizochytrium* sp.**,	910 g unprotected MA/d 910 g rumen protected MA/d	42	Cow	− −	n.s. n.s.	− −	+ +	n.s. n.s.	n.a.	Franklin *et al.* (1999)
***Schizochytrium* sp.**, commercial product	125 g MA/d 250 g MA/d 375 g MA/d	7 days gradual adaption, 16 days treatment	Cow	n.s. n.s. −	n.s. n.s. n.s.	− − −	+ + +	n.s. n.s. n.s.	n.s. n.s. +	Moate *et al.* (2013)
***Schizochytrium* sp.**, commercial product	17 g MA intake/d 28 g MA intake/d 52 g MA intake/d	42	Sheep	− − −	n.s. n.s. n.s.	n.s. n.s. +	+ + +	+ + +	n.s. n.s. −	Papadopoulos *et al.* (2002)
***Schizochytrium* sp.**, rumen protected	150 g MA biomass/d 300 g MA biomass/d 194 g MA oil/d	7	Cow	n.s. n.s. n.s.	n.s. n.s. n.s.	n.s. n.s. n.s.	+ + +	n.s. n.s. n.s.	n.a.	Stamey *et al.* (2012)

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TABLE 1: Continued

Microalgae (MA)	Microalgae dose	Days of treatment	Animal species	Effects on:		Effects on milk composition‡				Authors
				DMI†	MY†	Fat	ω3 FA	Protein	Lactose	
***Schizochytrium* sp.**, commercial product	25 g MA/kg DM added to:	21, gradual adaption over the first 3 or 5 days of treatment	Sheep							Reynolds *et al.* (2006)
	Corn silage based diet			n.s.	n.s.	n.s.	+	n.s.	n.s.	
	Alfalfa meal based diet			n.s.	n.s.	n.s.	+	n.s.	n.s.	
	25 g MA/kg DM added to:									
	Corn silage based diet			–	n.s.	n.s.	+	n.s.	n.s.	
	Alfalfa haylage based diet			–	–	n.s.	+	+	–	
	38 g MA/kg DM added to:									
	Corn silage based diet			n.s.	n.s.	n.s.	+	n.s.	n.s.	
***Schizochytrium* sp.**, commercial product	8 g MA/kg DM	28	Sheep	n.s.	n.s.	–	+	–	n.a.	Toral *et al.* (2010)
	16 g MA/kg DM			n.s.	n.s.	–	+	–		
	24 g MA/kg DM			n.s.	n.s.	–	+	–		
***Schizochytrium* sp.**, rumen protected	Provision of 200 g total lipids/d	125	Cow	n.s.	n.s.	–	+	n.s.	n.s.	Vahmani *et al.* (2013)
***Schizochytrium* sp.**, commercial product	156 g MA/d combined with extruded linseed	70	Cow	–	–	–	+	+	n.s.	Vanbergue *et al.* (2018a; 2018b)
	340g MA/d			–	–	–	+	n.s.	n.s.	
Spirulina platensis	200 g MA/d	90	Cow	n.a.	+	n.s.	n.a.	n.s.	n.s.	Kulpys *et al.* (2009)

Adapted from Altomonte *et al.* (2018)

n.s.: No significant effect **+:** Significant increase **–:** Significant decrease **n.a.:** No information available

DM: Dry matter **DMI:** Dry matter intake **MY:** Milk yield **ω3 FA:** omega-3 fatty acids

†**:** Effects on DMI and MY refers to comparisons with the respective control group over the entire experimental period.

‡**:** Effects on milk fat, ω3-FA, protein and lactose refers to milk composition (g/kg DM or %) in comparison with the respective control group over the total experiment period.

2.3.2. Application of microalgae in beef cattle

In beef cattle, microalgae were applied mostly as an alternative source of protein. The studies included whole-cell microalgae biomasses (Costa *et al.,* 2016; Panjaitan *et al.,* 2010; Panjaitan *et al.,* 2015), as well as in several cases post-extraction microalgae residues (PEAR) from biofuel production (Tibbetts *et al.,* 2015a). The PEAR were supplemented alone or in some cases in mixture with soyhulls (Stokes *et al.,* 2016; van Emon *et al.,* 2015). In few other studies, DHA-rich microalgae *Schizochytrium* (Phelps *et al.,* 2016a, 2016b; Rodriguez-Herrera *et al.,* 2018) and *Aurantiochytrium* (Carvalho *et al.,* 2018) were utilized for enrichment of meat with omega-3 fatty acids. Besides that, investigated microalgae were *Chlorella* species, *Dunaliella salina* and *Spirulina platensis* (Costa *et al.,* 2016; Panjaitan *et al.,* 2015). In some experiments, supplemented microalgae were not included in the diet fed, but were administered by infusion *via* rumen cannula (Drewery *et al.,* 2014; McCann *et al.,* 2014; Panjaitan *et al.,* 2015) or were included in the drinking water (Panjaitan *et al.,* 2010). Dosage of microalgae in beef cattle diets ranged from 50 g DM per day (Phelps *et al.,* 2016a, 2016b) up to approximately 2.5 kg DM per day, the latter corresponding to 18 % of DMI (Stokes *et al.,* 2016). Duration of supplementation lasted between 35 and 108 days (Carvalho *et al.,* 2018; Morrill *et al.,* 2017a; 2017b).

Effects on dry matter intake

Literature results on the effects of microalgae supplementation on DMI of beef cattle are not consistent. While Carvalho *et al.* (2018) observed a 10 % reduced DMI with the supplementation of 100 g *Aurantiochytrium limacinum* per day, other authors (cf. TABLE 2) observed increases in DMI up to 68 % compared to the respective control group (Costa *et al.,* 2016). Stokes *et al.* (2016) and van Emon *et al.* (2015) suggested that microalgae supplementation might have increased palatability of diets, but this suggestion is in contrast with the hypothesis described for dairy ruminants (cf. CHAPTER 2.3.1). Morrill *et al.* (2017a) attributed increased DMI with the supplementation of 1 kg of a *Chlorella* PEAR per day to the lesser energy concentration of the diet. The authors suggested that steers compensated the lower energy concentration by increased DMI. Furthermore, Morrill *et al.* (2017a) explained that changes in DMI might have impact on the digestibility of the diet, because of changes in ruminal passage rate and retention time, and thus the available time for microbial degradation. Indeed, the authors observed a 16 % increase in DMI which was accompanied by a decrease in DM digestibility of a diet containing PEAR by approximately 14 % (Morrill *et al.,* 2017a). Nevertheless, it cannot be clarified whether the reduction of DM digestibility was a result of increased DMI or whether DMI was increased to compensate for low DM digestibility.

Panjaitan *et al.* (2015) compared the effects of *Spirulina platensis* and urea-sulphur supplementation on DMI, digestibility of organic matter (dOM), ruminal retention time and microbial protein synthesis in steers fed a low protein hay. The effects on all traits were greater for the *Spirulina* supplementation than for the urea-sulphur supplementation, in particular for DMI. The authors suggested that the observed increase in DMI was related to an increase of ruminal passage rate, caused by the supplementation of *Spirulina platensis* (Panjaitan *et al.,* 2015). Carvalho *et al.* (2018) suggested that decreased DMI observed in their study with steers fed *Aurantiochytrium limacinum* might be related to impairment of ruminal fermentation, palatability of the diet, gut motility, or to the release of gut hormones that control satiety.

Effects on growth, feed efficiency and digestibility

Data on the effects of microalgae supplementation on growth of beef cattle are scarce, with only five studies reporting data on average daily gain (cf. TABLE 2), of which one observed a significant increase (Costa *et al.,* 2016). Feed efficiency of steers was not affected by the feeding of 100 g *Aurantiochytrium limacinum* per day (Carvalho *et al.,* 2018), but linearly decreased when increasing amounts of an unspecified PEAR mixed with soyhulls were fed to steers (Stokes *et al.,* 2016; van Emon *et al.,* 2015). In accordance with that, digestibility of DM was reduced when 1 kg of a *Chlorella*-derived PEAR was fed per day to steers (Morrill *et al.,* 2017a). Reduced feed efficiency as well as reduced DM digestibility were suggested to be related to increased passage rates (Morrill *et al.,* 2017a; van Emon *et al.,* 2015). Stokes *et al.* (2016) also observed a reduced feed efficiency in steers fed an unspecified PEAR mixed with soyhulls over 103 days, but effects of microalgae supplementation were dependent on the duration of supplementation: While in the first 74 days of the experiment, steers fed a corn-based diet had a higher feed efficiency than steers fed diets containing 14 to 42 % of a PEAR-soyhull mixture, this effect reversed in the last 28 days of the experiment. However, steers were also fed a β-agonist in the last 28 days of the trial so that it could not be clarified whether this effect was related to microalgae supplementation, the β-agonist or synergistic effects of both (Stokes *et al.,* 2016). Panjaitan *et al.* (2015) and Costa *et al.* (2016) supplemented S*pirulina platensis* to cattle consuming pasture low in CP concentration or a diet based on low CP spear grass hay and consistently observed an increase in dOM or DM digestibility. In accordance with that, Drewery *et al.* (2014) observed a quadratic increase in dOM when a *Chlorella*-derived PEAR were administered *via* rumen cannula in steers which had *ad libitum* access to oat straw.

TABLE 2: Effects of microalgae supplementation on dry matter intake (DMI), average daily gain (ADG), feed efficiency (FE), digestibility of dry or organic matter (dDM, dOM), carcass characteristics, sensory properties and fatty acid composition of meat in beef cattle

Microalgae (MA)	MA dose	Days of treatment	Animals	Effects of supplementation on:						Authors
				DMI†	ADG †	dDM, dOM or FE†	Carcass characteristics Meat quality	Sensory properties, Oxidation stability	ω3 FA in meat	
Aurantiochytrium limacinum	100 g MA/d	108	Steers	−	n.s.	n.s. (FE)	HCW, MS, quality grade: n.s.	Oxidation stability: n.s.	+	Carvalho *et al.* (2018)
Chlorella pyrenoidosa, ***Dunaliella salina,*** ***Spirulina platensis***	4.7, 4.0, 4.0 g MA/kg BW per day	21	Steers	+ n.s. +	n.a.	n.s. n.s. n.s. (dDM)	n.a.	n.a.	n.a.	Costa *et al.* (2016), Experiment 2
PEAR (*Chlorella* sp.)	1 kg MA/d	35	Steers	+	n.a.	− (dDM, dOM)	HCW: n.s. MS, quality grade: +	Overall flavour, tenderness, overall liking : n.s.	n.s.	Morrill *et al.* (2017b; 2017a)
PEAR, species unknown; 43:57 mixture with soyhulls	140, 280 or 420 g MA/kg DM	103	Steers	+ (Lin.)	n.s.	− (FE, Lin.)	HCW, MS, quality grade: n.s.	n.s.	n.s.	Stokes *et al.* (2016)
PEAR, species unknown; 43:57 mixture with soyhulls	150, 300 or 450 g MA/kg DM	55	Steers	+ (Lin.)	n.s.	– (FE, Lin.)	n.a.	n.a.	n.a.	van Emon *et al.* (2015)

Continues on next page

TABLE 2: Continued

Microalgae (MA)	MA dose	Days of treatment	Animals	Effects of supplementation on:						Authors
				DMI†	ADG †	dDM, dOM or FE†	Carcass characteristics Meat quality	Sensory properties, Oxidation stability	ω3 FA in meat	
Schizochytrium limacinum	50, 100 or 150 g MA/d	89	Heifers	n.a.	n.a.	n.a.	n.a.	OF: + (Quad.) Colour stability: − (Quad.)	+ (Quad.)	Phelps *et al.* (2016a, 2016b)
Schizochytrium limacinum	150 or 300 g MA/d	95	Heifers	n.a.	n.s.	n.a.	HCW, carcass conformation, fat class: n.s.	OF, tenderness: + Shear force: −	+	Rodriguez-Herrera *et al.* (2018)
Spirulina platensis	Provision of 0.08 up to 0.48 g N/kg BW per day	77	Steers	+ (Lin.)	+ (Lin.)	+ (dDM, Lin.)	n.a.	n.a.	n.a.	Costa *et al.* (2016), Experiment 3
Spirulina platensis	0.5, 1.4, 2.,5 and 6.1 g MA/kg BW per day	30	Steers	+ (Lin.)	n.a.	+ (dOM, Lin.)	n.a.	n.a.	n.a.	Panjaitan *et al.* (2015)

n.s.: No significant effect **+:** Significant increase **−:** Significant decrease **n.a.:** No information available

+ (Quad.) or − (Quad.): Quadratic increase or decrease with increasing MA dose; **+ (Lin.) or − (Lin.):** Linear increase or decrease with increasing MA dose

† Effects on DMI, ADG, FE, dDM and dOM refers to comparisons with the respective control groups (without MA supplementation) over the entire experimental period.

PEAR: Post extraction microalgae residue; **BW:** Body weight; **HCW:** Hot carcass weight; **MS:** Marbling score; **OF**: Off-flavour intensity; **ω3 FA:** omega-3 fatty acids

Effects on carcass characteristics, meat quality, sensory properties and enrichment of meat with omega-3 fatty acids

Studies investigating the effects of microalgae supplementation on carcass characteristics and meat quality in beef cattle mostly did not observe significant effects (cf. TABLE 2). Hot carcass weight was not affected by the supplementation of *Aurantiochytrium limacinum*, *Chlorella*-derived PEAR, unspecified PEAR or *Schizochytrium limacinum* in several studies (Carvalho *et al.,* 2018; Morrill *et al.,* 2017a; Rodriguez-Herrera *et al.,* 2018; Stokes *et al.,* 2016). Marbling score and quality grade have been increased in steers receiving a *Chlorella* PEAR (Morrill *et al.,* 2017a), but not in heifers or with the supplementation of *Aurantiochytrium limacinum* or *Schizochytrium limacinum* (Carvalho *et al.,* 2018; Rodriguez-Herrera *et al.,* 2018; Stokes *et al.,* 2016). Morrill *et al.* (2017a) suggested that increased marbling score and thus higher quality score in steers feed a *Chlorella* PEAR might have been due to altered ruminal fermentation and a resulting higher provision of marbling precursors. Morrill *et al.* (2017b) and Rodriguez-Herrera *et al.* (2018) observed reduced shear forces and thus a higher tenderness of meat from steers and heifers fed a *Chlorella*-derived PEAR or *Schizochytrium limacinum*.

Supplementation of DHA-rich microalgae *Aurantiochytrium limacinum* or *Schizochytrium limacinum* resulted in a significant increase of omega-3 fatty acid, in particular DHA, in meat of heifers and steers (Carvalho *et al.,* 2018; Phelps *et al.,* 2016a, 2016b; Rodriguez-Herrera *et al.,* 2018). Increases of total omega-3 fatty acid concentration in meat of beef cattle ranged between 17 % and 128 % (Carvalho *et al.,* 2018; Phelps *et al.,* 2016b) and increases of DHA concentration varied between 187 and 850 % (Phelps *et al.,* 2016b; Rodriguez-Herrera *et al.,* 2018) compared to the respective control, suggesting that DHA-rich microalgae are suitable sources for the enrichment of meat with omega-3 fatty acids. Nevertheless, in the studies of Phelps *et al.* (2016a, 2016b) and Rodriguez-Herrera *et al.* (2018) increases of omega-3 fatty acid concentration in meat were accompanied by the development of off-flavours and a reduced colour stability. It appears noteworthy that similar observations were also made in lambs (Nute *et al.,* 2007) and in non-ruminant animals (Meadus *et al.,* 2010; Ribeiro *et al.,* 2013; Ribeiro *et al.,* 2014; Rymer *et al.,* 2010) fed diets containing microalgae. Furthermore, Phelps *et al.* (2016a) observed a quadratic increase of concentration of thiobarbituric acid reactive substances in longissimus lumborum steak of heifers with increasing supplementation of *Schizochytrium limacinum*, indicating a reduced oxidation stability. Additionally, Carvalho *et al.* (2018) observed a tendency to increased concentrations of thiobarbituric acid reactive substances in meat of steers fed 100 g *Aurantiochytrium limacinum* daily after 21 days of meat

aging, indicating that shelf stability might be compromised. Nevertheless, there are also contrasting results, reporting that overall flavour and liking of meat were not impaired by the feeding of a *Chlorella*-derived PEAR to steers (Morrill *et al.,* 2017b). Decreased colour stability, off-flavours and increased concentrations of thiobarbituric acid reactive substances were attributed to higher susceptibility of meat to oxidation, because of the high concentrations of long-chain omega-3 PUFA (Phelps *et al.,* 2016a, 2016a; Rodriguez-Herrera *et al.,* 2018).

As for lactating ruminants, it is difficult to draw a general conclusion on the effects of microalgae supplementation in beef cattle. In contrast to lactating ruminants, mostly no detrimental effects on DMI were observed, but it appears that diet digestibility might be negatively affected. Furthermore, provision of omega-3 fatty acid rich microalgae to beef cattle resulted in an enrichment of these fatty acids in the meat, but it seems that this might compromise sensory properties and shelf life of the obtained meat.

2.3.3. Effects of microalgae supplementation on ruminal fermentation

Ruminal fermentation yields volatile fatty acids (VFA) as main end products, mainly acetate, propionate and butyrate. Acetate is associated with the fermentation of structural carbohydrates such as cellulose or hemicellulose and propionate is associated with concentrates (i.e., starch). Branched-chain fatty acids (isobutyrate and isovalerate) originate from ruminal degradation of branched-chain AA (valine, isoleucine, leucine), while valerate is produced from the degradation of carbohydrates and the AA proline, arginine, lysine and methionine (Andries *et al.,* 1987). 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 less than 5 %. The proportions of acetate, propionate and butyrate did in most of the cases not observably deviate from this usual pattern when several microalgae species were provided to cows (Moate *et al.,* 2013), steers (Costa *et al.,* 2016; Drewery *et al.,* 2014), or goats (Kholif *et al.,* 2017b; Zhu *et al.,* 2016) or when investigated *in vitro* (Lodge-Ivey *et al.,* 2014), suggesting that microalgae supplementation does not affect ruminal processes in an unphysiological manner. Nevertheless, in several cases, concentrations of branched-chain fatty acids and valerate were increased in the rumen fluid of cattle receiving microalgae (Costa *et al.,* 2016; Drewery *et al.,* 2014; Lamminen *et al.,* 2017; Panjaitan *et al.,* 2010; Panjaitan *et al.,* 2015). In the studies of Lamminen *et al.* (2017) and Costa *et al.* (2016) this was accompanied by increases of ruminal ammonia concentration. Lamminen *et al.* (2017) suggested that increased concentrations of branched-chain fatty acids might be related to an increased intake of

branched-chain AA when microalgae are added into diets for ruminants. They furthermore hypothesized that a high ruminal CP degradability of microalgae might promote availability of branched-chain AA for the synthesis of branched-chain fatty acids. Furthermore, in few cases when microalgae biomasses rich in ether extract (EE) and DHA or an oil rich in hexadecatrienoic acid were incubated with rumen fluid and hay as substrate, clear shifts towards propionate (up to 54 % of total VFA) at the expense of acetate were observed (Fievez *et al.,* 2007; Ungerfeld *et al.,* 2005). These extreme shifts were observed when microalgae were used in an attempt to reduce ruminal methanogenesis and were accompanied by a considerable impairment of total VFA production and ruminal nutrient degradation.

The potential of microalgae as inhibitors of ruminal methane production was also investigated by other authors, both *in vitro* (Anele *et al.,* 2016; Boeckaert *et al.,* 2006; Elghandour *et al.,* 2017; Gomaa *et al.,* 2018; Kholif *et al.,* 2017a; Marrez *et al.,* 2017) and *in vivo* (Moate *et al.,* 2013), particularly utilizing microalgae species rich in long-chain PUFA such as *Chaetoceros*, *Crypthecodinium*, *Nannochloropsis*, or *Schizochytrium.* While most of the *in vitro* investigations with microalgae containing long chain PUFA have shown an inhibitory effect of microalgae on ruminal methanogenesis (Anele *et al.,* 2016; Elghandour *et al.,* 2017; Fievez *et al.,* 2007; Gomaa *et al.,* 2018; Ungerfeld *et al.,* 2005) this could not be proven *in vivo* (Moate *et al.,* 2013). Additionally, in the study of Kholif *et al.* (2017a), the protein-rich microalgae *Chlorella vulgaris* led to increases in ruminal methane production. Tsiplakou *et al.* (2017b) observed an increase in methane-producing bacteria and protozoa with the supplementation of protein-rich *Chlorella vulgaris* to diets of goats. Furthermore, the latter authors observed reductions in cellulolytic and increases in proteolytic bacteria, which were accompanied with respective changes in ruminal enzyme activities (cellulase, protease) (Tsiplakou *et al.,* 2017b). Tibbetts *et al.* (2016) investigated the *in vitro* ruminal digestion of the four microalgae species *Chlorella vulgaris*, *Micractinium reisseri, Nannochloris bacillaris* and *Tetracystis* sp. at different inclusion levels as a forage replacement (25 – 100 % of forage replacement). They investigated whole microalgal biomasses, as well as lipid extracted biomasses for their potential as ruminal methane inhibitors. While none of the whole microalgal biomasses inhibited methane production at any inclusion level, all of the lipid-extracted biomasses reduced methane production by more than 50 % at all inclusion levels. However, this was accompanied by a reduction of dOM of diets containing the highest level of *Chlorella*, *Nannochloris* and *Tetracystis*. Concerning the results for ruminal methane reduction, Tibbetts & Fredeen (2017) made the same observation for whole and lipid-extracted *Scenedesmus* sp.,

but in this study dOM of the diets was not impaired by microalgae inclusion. The authors concluded that the investigated lipid-extracted microalgae contain substances other than fatty acids or lipids that inhibit ruminal methanogenesis (Tibbetts *et al.,* 2016; Tibbetts & Fredeen, 2017).

In vitro gas production (GP) kinetics of microalgae incubated with rumen fluid were investigated few times, including studies in which microalgae were incubated alone (Anele *et al.,* 2016; Elghandour *et al.,* 2017; Han & McCormick, 2014; Kholif *et al.,* 2017a) and studies in which microalgae were included in diets (Elghandour *et al.,* 2017; Gomaa *et al.,* 2018; Kholif *et al.,* 2017a). Extent and dynamics of GP varied considerably between investigated microalgae species (Anele *et al.,* 2016; Han & McCormick, 2014) and were also dependent on the inclusion level and the basal diet (Gomaa *et al.,* 2018; Kholif *et al.,* 2017a), as well as dependent from the animal species of the donor animals of the rumen fluid (Elghandour *et al.,* 2017). Anele *et al.* (2016) and Han & McCormick (2014) consistently observed that *Chlorella vulgaris* had highest cumulative GP amongst several microalgae species. Nevertheless, Han & McCormick (2014) found that accumulated GP of microalgae was clearly lower than that of soybean meal and suggested that the carbohydrate fraction of microalgae is less rumen fermentable than those of soybean meal.

To conclude from what is available in the literature, it appears that general conclusions on the impact of microalgae supplementation in ruminants are not possible. This is because of the high variation in terms of applied microalgae species, microalgae dosages, supplemented diets and substituted components, which makes the results difficult to compare. Additionally, most of the previous research in ruminants concerned the application of DHA-rich, heterotrophic microalgae for the enrichment of milk and meat, but systematic data on nutrient digestibility and nutrient utilization of photoautotrophic microalgae are rare. Nevertheless, systematic data on the intrinsic properties of microalgae and its consequences for their nutritional characteristics would be necessary in order clarify the reasons for the high deviation of the so far obtained results.

CHAPTER 3

OVERVIEW AND OBJECTIVES OF THE INCLUDED MANUSCRIPTS

3. OVERVIEW AND OBJECTIVES OF THE INCLUDED MANUSCRIPTS

The doctoral project was done within the framework of the Research Area Microalgae of the Bioeconomy Research Program Baden-Württemberg that aimed to evaluate the potential of microalgae for the use in the food and feed sector. Within in the research consortium it was decided to focus the investigations on the protein-rich microalgae genera *Arthrospira* and *Chlorella* that are approved as feedstuffs in the EU. Furthermore, the EPA-rich genera *Nannochloropsis* and *Phaeodactylum* were chosen for the investigations because they hold potential as sources of PUFA. The overall objective of the present thesis was to evaluate the suitability of microalgae as feedstuffs for farm animals, with emphasis on ruminant animals.

Aiming to generate a comprehensive and reliable database on the nutrient composition of microalgae, several commercial microalgae biomasses of four genera (*Arthrospira*, *Chlorella*, *Nannochloropsis*, *and Phaeodactylum*) were analysed for their nutrient composition utilizing well established methods for feed evaluation. Literature results indicated that cultivation conditions affect the nutrient composition of microalgae. Thus, it was hypothesized that nutrient profiles of commercial microalgae biomasses vary greatly between, but also within microalgae genera. The resultant objective of Manuscript 1 was to evaluate whether it is appropriate to characterize microalgae for feed and food applications with general mean values of nutrient concentration.

Manuscript 2 aimed to systematically determine characteristics of the nutritional value of microalgae for ruminant animals utilizing different *in vitro* methods. Since the nutrient utilization in ruminants is mainly determined by microbial fermentation in the rumen, the conducted experiments focussed on the investigation of ruminal fermentation processes of microalgae and their consequences for the nutritional value, but also considered post-ruminal protein digestion. The nutrient profiles presented in Manuscript 1 revealed a considerable variability between, but also within microalgae genera. Thus, the question arose whether these differences were also reflected in the availability and the degradability of nutrients. Consequently, one of the objectives of Manuscript 2 was to investigate the inter- and intra-genera variability of ruminal fermentation and characteristics of the nutritional value for ruminants.

It was known from literature that several microalgae possess robust cell walls or cell coverings that might limit availability of nutrients to the animal. For non-ruminant animals, few studies investigated the effects of cell disruption on nutrient digestibility and have shown that

cell disruption can increase nutrient digestibility. Nevertheless, data for non-ruminant animals were limited to few microalgae species and no study investigated this issue in ruminants. Therefore, a common objective of Manuscript 1 and Manuscript 2 was to investigate whether cell disruption affects nutritional characteristics of microalgae. In Manuscript 1, effects of cell disruption on the *in vitro* CP digestibility for pigs were investigated and in Manuscript 2 several *in vitro* methods were utilized to investigate whether cell disruption affect the nutritional value of microalgae for ruminants. Furthermore, it was evaluated whether effects of cell disruption vary between and within microalgae genera for pigs (Manuscript 1) and for ruminants (Manuscript 2).

The high variability of nutrient composition and nutritional characteristics of microalgae observed in Manuscript 1 and Manuscript 2 might be a problem when microalgae are used in commercial feed mills, where standardised raw materials are preferred. Nevertheless, this might also provide the opportunity to shift microalgae composition to a desired pattern by varying the cultivation conditions and hence producing a microalgal biomass fitted to the specific requirements of feed production. However, the effects of varying cultivation conditions on the nutritional value of microalgae for farm animals have not been investigated yet. Accordingly, the objective of Manuscript 3 was to investigate the effects of cultivation conditions (nitrogen and CO_2 supply, and environmental factors) on the nutritional value of microalgae for ruminants. For this, the microalgae *Chlorella vulgaris* was cultivated under varying conditions and the generated biomasses were analysed for their nutrient composition and their nutritional value for ruminants using different *in vitro* methods.

CHAPTER 4

GENERAL DISCUSSION

4. GENERAL DISCUSSION

4.1. Critical reflection on applied methods

To evaluate the nutritional value of microalgae, several microalgae biomasses were analysed utilizing established chemical-analytical methods for feed evaluation and different *in vitro* methods. Development of these methods did not specifically target the analysis of microalgae, hence they are not well suited to investigate microalgae in some cases. The following sections therefore discuss the suitability of these methods for microalgae.

4.1.1. Chemical-analytical characterization of microalgae

Carbohydrates

For the characterization of the carbohydrate fraction of the investigated microalgae, it was attempted to apply the official methods of the Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA) (1976) for determination of neutral (NDF, method 6.5.1) and acid (ADF, method 6.5.2) detergent fibre and additionally enzymatic determination of starch.

Concerning the determination of ADF and NDF, several methodical issues arose which were related to the small cell size of microalgae. For example, cells of *Chlorella* have a diameter between 2 and 10 µm and cells of *Nannochloropsis* between 2 and 6 µm (cf. CHAPTER 2.1). Even though *Arthrospira* forms trichomes which can reach a length of up to 500 µm, this structure is easily broken during drying, which was observed during microscopical examination of the samples, so that it can be expected that particle size was actually lower. Determination of ADF and NDF both include filtration steps utilizing filter bags with a pore size of 30 µm. Since this pore size is obviously larger than cell size using these standard filter bags, it was not possible to achieve a reliable filtration since whole cells can be washed-out during filtration procedure. It was therefore attempted to utilize filter materials with lower pore sizes (4 – 7 µm), but in this case filtration was not possible because the residues that occurred after treatment with acid and neutral detergent solutions were jellylike and clogged pores of the filters. Accordingly, filtration residues could not be accurately separated from the filtrates and it was not possible to achieve reliable and reproducible results. It was therefore attempted to determine ADF and NDF by replacing filtration with centrifugation. Nevertheless, even fourfold repeated centrifugation for 15 minutes at 15,000 × *g* did not achieve a stable centrifugation pellet from which the supernatant could have been separated accurately. Since all of these modifications of the original method did not allow a trustworthy and repeatable determination of NDF and ADF,

it was concluded that these methods are not appropriate for characterization of the carbohydrate fraction of microalgae and therefore cannot be recommended for application on microalgae. Actually, this conclusion is not really surprising when remembering the originally targeted application of these methods, namely the analysis of fibrous feedstuffs, particularly forages (van Soest, 1963; van Soest & Wine, 1967). Unsuitability of methods logically also applies for all methods that are based on NDF and ADF determination, for example the chemical fractionation of CP according to the Cornell Net Carbohydrate and Protein System (Licitra *et al.,* 1996). Hence, this method has not been applied in this work.

The concentration of starch was determined enzymatically using a commercially available starch analysis assay (Boehringer Mannheim, 1994). This assay includes acid hydrolysis followed by enzymatic hydrolysis using the enzyme amyloglucosidase, cleaving α-1,4 and α-1,6-glucan bonds of polysaccharides. Microalgae contain polysaccharides other than starch that have glucose as monosaccharide (de Jesus Raposo *et al.,* 2013; 2014; Sui *et al.,* 2012) and α-1,4 or α-1,6-glucan bonds (e.g., floridean starch, glycogen) (Brody & Vatter, 1959; de Philippis *et al.,* 1992) or with a physical behaviour like starch but a distinct glycosidic structure (e.g., chrysolaminarin in *Phaeodactylum*) (Goo *et al.,* 2013; Gügi *et al.,* 2015). Such polysaccharides might have been degraded to glucose by acid hydrolysis or amyloglucosidase or both and hence might have mimicked starch in the applied assay. Consequently, it cannot be distinguished between α-linked glucose and true starch. Therefore, the term "α-linked glucose" is used in this thesis when results of the respective assay are discussed, as this is what the assay detects. Laurens *et al.* (2012) compared the results of two commercially available starch assays applied on microalgae biomasses in a round-robin test. One assay included enzymatic hydrolysis by the enzyme amyloglucosidase and the other assay additionally included an α-amylase. They observed lower values when only applying the amyloglucosidase and concluded that an amylase hydrolysis in addition to the amyloglucosidase is necessary for determination of starch concentration of microalgae biomasses. Consequently, it cannot be ruled out that the assay applied herein underestimated concentration of α-linked glucose.

It was not in the scope of the present thesis to develop new methods for characterization of carbohydrate fraction of microalgae. However, it appears noteworthy that development of such methods would be necessary for a comprehensive evaluation of microalgae for feed purposes. As proposed by Bernaerts *et al.* (2018) for analysis of microalgae for food applications, this might include determination of three fractions, namely storage polysaccharides, cell wall polysaccharides and exocellular polysaccharides. Nevertheless, the approach proposed by

Bernaerts *et al.* (2018) appears to be too complex for routine use in feed evaluation and has the drawback that the determined fractions are not linked to feed evaluation systems. Nevertheless, to the best knowledge of the author, there are currently no methods available for the characterization of the carbohydrate fraction of microalgae that would meet these criteria.

Protein

The use of nitrogen-to-protein conversion factors is a rapid and practical way for determination of total protein concentration of feed materials. The traditional factor of 6.25 that is used for CP determination (N × 6.25) is based on the assumptions that all nitrogen present in the analysed material is protein and that all proteins contain 16 % nitrogen (Jones, 1931). Contrasting these assumptions, microalgae can contain considerable amounts of non-protein nitrogen (NPN) compounds, which can make up more than 50 % of total nitrogen (Templeton & Laurens, 2015). Thus, protein concentration might be overestimated when applying the general nitrogen-to-protein conversion factor of 6.25 to microalgae (Lee & Picard, 1982). In accordance with that, mean NPN concentrations of 10, 16, 11, and 25 % of CP were observed for *Arthrospira, Chlorella*, *Nannochloropsis* and *Phaeodactylum* samples used in Manuscript 1. Consequently, application of the nitrogen-to-protein conversion factor led to an overestimation of the protein concentration when compared to sum of analysed AA (FIGURE 1 a). A detailed characterization of the NPN fraction of the investigated samples was beyond the scope of the investigations for the present thesis. However, it is very likely that, at least in some samples, NPN fraction comprised free AA, since the sum of AA exceeded true protein (TP) concentration in some samples. Occurrence of free AA would be consistent with literature results (Al-Amoudi & Flynn, 1989; Dortch *et al.,* 1984; Flynn *et al.,* 1992).

In order to account for the NPN compounds in microalgae, Lourenço and co-workers developed specific conversion factors for microalgae (Lourenço *et al.,* 1998; Lourenço *et al.,* 2004). They suggested species-specific conversion factors for some species and a general conversion factor of 4.78 for species not yet studied (Lourenço *et al.,* 2004). This suggestion is generally accepted for application on microalgae by now. Application of the nitrogen-to-protein conversion factor of 4.78 to the microalgae investigated for the present thesis led to a systematic underestimation of protein concentration when compared to the sum of analysed AA (FIGURE 1 b). The suggested application of species-specific factors led a closer agreement with the sum of AA, but highest consistence was found with TP determination according to Barnstein (FIGURE 1 c, d).

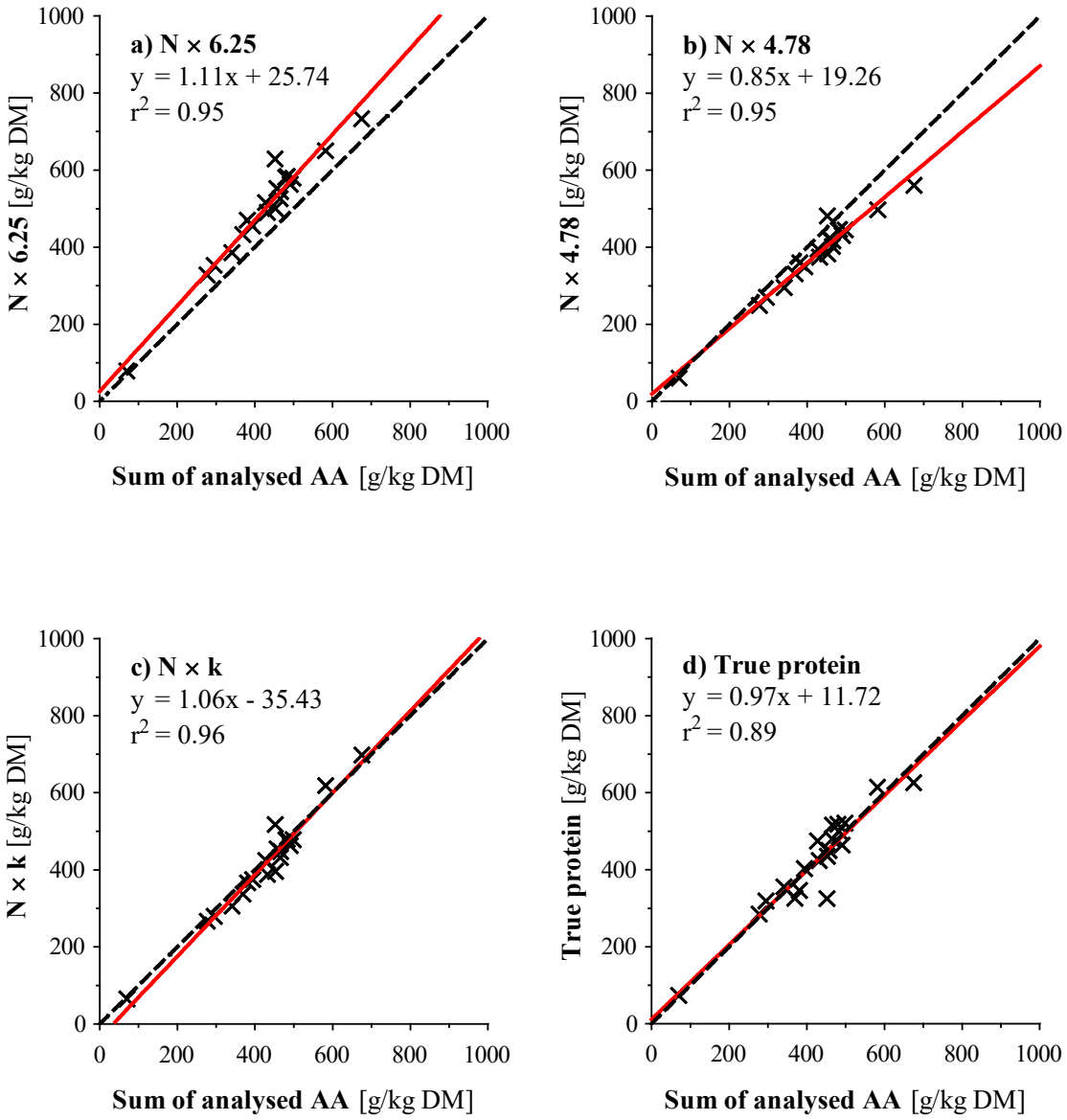

FIGURE 1: Linear regression between the sum of analysed amino acids (AA) and the concentration of protein determined using different nitrogen-to-protein conversion factors and determination of true protein concentration according to Barnstein

N × 6.25: Crude protein; N × 4.78: General conversion factor suggested by Lourenço *et al.* (2004); N × k: Species-specific conversion factors (Lopez *et al.,* 2010; Lourenço *et al.,* 2004; Tibbetts *et al.,* 2015c). Data obtained from Manuscript 1 (only data on disrupted samples included) and Manuscript 3; n = 20. Red line: Respective estimated regression line; dashed line: bisectrix (illustrates perfect accordance).

The TP determination had a lower accuracy of estimation than determination of protein concentration with species-specific conversion factors (cf. coefficient of determination, r^2). Nevertheless, that resulted mainly from a *Chlorella* sample with very high NPN concentration (48 % of CP). Excluding this sample from data evaluation increases accuracy of estimation to the same level achieved when applying the nitrogen-to-protein conversion factors (i.e., $r^2 = 0.95$). In this particular sample, the AA arginine and glutamic acid plus glutamine accounted for 60 % of total AA. It is likely that these AA were free AA to a large extent, since sum of AA exceeded TP concentration, and arginine, glutamic acid, and glutamine were found to be the most important free AA in microalgae (Al-Amoudi & Flynn, 1989; Derrien *et al.,* 1998). Accordingly, determination of TP concentration appears to enable consideration for the occurrence of free AA and additionally the estimation of NPN concentration when performed in addition to determination of total N concentration by the Kjeldahl method. It therefore appears to be a suitable method for protein determination of microalgae biomasses when data on AA is not available.

Because of the systematic overestimation of protein concentration when applying the factor 6.25, CP determination is not suitable for application on microalgae, which is in accordance with previous findings (Lee & Picard, 1982; Lourenço *et al.,* 2004; Templeton & Laurens, 2015). Other researchers (Tibbetts *et al.,* 2015c; Tibbetts *et al.,* 2015a) found a good agreement with protein determination based on AA data when applying the factor of 4.78 proposed by Lourenço *et al.* (2004), but this was not the case for the samples investigated in the present thesis. It might be that these contrasting results are related to the different methodologies of nitrogen determination. The development of the nitrogen-to-protein conversion factor of 4.78 was based on elemental analysis *via* combustion method and Tibbetts and co-workers also applied this method, while nitrogen determination for the samples of the present thesis was based on the Kjeldahl method. Lopez *et al.* (2010) showed that nitrogen determination with these two methods yields distinct results for microalgae and therefore the authors suggested to use distinct nitrogen-to-protein conversion factors depending on the method used for nitrogen determination. Thus, based on the findings of the present thesis, the nitrogen-to-protein conversion factor 4.78 appears unsuitable for application on microalgae, at least when nitrogen determination is based on the Kjeldahl method.

Application of species-specific nitrogen-to-protein conversion factors allowed a very precise estimation of protein concentration and therefore enables a rapid and reliable estimation of protein concentration of microalgae for feeding purposes. Nevertheless, it has to be kept in mind

that even a species-specific factor may be inaccurate because of high variability of NPN concentration of microalgae (cf. Manuscript 1).

Lipids

For characterization of the lipid fraction of investigated microalgae biomasses, EE determination and analysis of fatty acid composition were performed (Manuscript 1 and Manuscript 3). Determination of the lipid concentration of microalgae with methods applying gravimetric solvent extraction like EE determination often overestimate the true lipid concentration, because it may also include the extraction of non-lipid compounds (e.g., chlorophyll or other pigments) (Laurens *et al.,* 2012). Contrary, summation of individual fatty acids might underestimate lipid concentration because of incomplete lipid extraction from the microalgae cells, especially when no cell disruption treatment was applied before analysis. Analysis of fatty acid composition is obviously more accurate, when following the definition of lipids as "fatty acids and their derivates", however it is clearly more cost-, labour- and time-intensive (Laurens *et al.,* 2012). Consequently, with both methods being feasible for application on microalgae, method choice may be in this case dependent on the required degree of accuracy.

In conclusion, the application of established chemical-analytical methods for evaluation microalgae is not feasible in all cases. This particularly applies to standard methods for characterization of the polysaccharide fraction that include filtration steps and is related to the cell size and physical properties of microalgae. Therefore, the development of alternative methods for the comprehensive characterization of this fraction is necessary. For determination of lipid and protein concentration feasible methods are available, but in case of protein it appears necessary to complement standard determination of nitrogen by the determination of TP or AA in order to account for NPN compounds.

4.1.2. *In vitro* assays

Hohenheim Gas Test

The Hohenheim Gas Test method (HGT) first described by Menke *et al.* (1979) was used to investigate several aspects of ruminal fermentation of microalgae. It provides a high degree of standardisation and allows to address a wide range of nutritional aspects in ruminants, such as fermentation kinetics of feedstuffs or formation of fermentation end products (e.g., VFA and methane). Furthermore, it allows prediction of energetic value and digestibly of feedstuffs with high accuracy (Getachew *et al.,* 2005). Additionally, HGT provides the crucial benefit that it is a closed batch-system so that particle losses cannot occur (Cone *et al.,* 2002; Mertens, 2005)

and therefore reflects ruminal fermentation of all organic compounds of the investigated feed sample. It therefore appears to have a high suitability for the investigation of ruminal fermentation of microalgae, although it does not allow direct predictions of fermentation characteristics under *in vivo* conditions. Nevertheless, some methodical issues arose which were related to specific properties of microalgae.

The regression equations proposed by Menke & Steingass (1988) were used to estimate dOM, metabolisable energy (ME) and net energy (NEL) from compositional data and GP for the investigated microalgae (Manuscript 2 and Manuscript 3). Although microalgae were not included in the data base used to calculate the regression equations, their application on microalgae appears reasonable since the considered range of nutrient profiles of the feedstuffs investigated by Menke & Steingass (1988) were mostly congruent to those of the investigated microalgae. It was decided to apply the equations for "all feedstuffs" since microalgae could not be clearly assigned to one of the groups defined by Menke & Steingass (1988), neither by composition nor by structural properties. Regression equations for application in microalgae samples were chosen based on the following considerations: Because of the methodical issues with the determination of ADF and NDF concentration described in CHAPTER 4.1.1, equations that include ADF and NDF were not considered. Additionally, equations that included concentration of nitrogen-free extracts were not used, because it could not be calculated due to lack of crude fibre data. Beyond that, equations that included quadratic consideration of EE concentration were also not used because of the resulting overestimation of energetic value in EE-rich feedstuffs. From the remaining equations those were chosen for estimation of dOM and energy concentrations that comprised most of the analysed nutrients and achieved highest accuracy of estimation (r^2). Based on that, equation 17 f was chosen for estimation of ME and NEL, and 43 f for estimation of dOM (Menke & Steingass, 1988). With the exception of EE-rich microalgae samples, realistic estimates for dOM and ME could be achieved (cf. Manuscript 2 and Manuscript 3). However, since the regression equations were not validated for microalgae, it might be that true dOM and ME of microalgae differ from the herein obtained results. Consequently, the obtained data allow to rank the different microalgae samples among each other and give a first impression of their nutritional value. Conclusions on the absolute dOM and ME of microalgae are not possible as this would require a validation of the regression equations for microalgae first.

For samples with high EE concentrations, application of the before mentioned regression equations led to implausible estimates for dOM and ME. For instance, for a *Chlorella protothecoides* sample containing 549 g EE/kg DM, a ME concentration of 17.1 MJ/kg DM (non-disrupted) was estimated, while dOM was estimated to be only 39 % (Manuscript 2). Although not that pronounced, a similar observation was made for a sample cultivated under CO_2 deficient conditions included in Manuscript 3, containing 341 g EE/kg DM (dOM: 49.8 %, ME: 14.3 MJ/kg DM). In both samples EE was the major component, clearly exceeding the highest EE concentration of the feedstuffs included in the investigation of Menke & Steingass (1988) that was 198 g EE/kg DM. On the other hand, a plausible estimate for ME (11.5 MJ/kg DM) and dOM (52 %) could be achieved for a *Nannochloropsis* sample with a EE concentration of 252 g/kg DM but CP being the dominating component (350 g/kg DM). Therefore, it seems that the application of the regression equations is not well suited for microalgae when EE is the major component or there might be an upper limit for EE concentration to which applicability of the regression equations is given. That might be particularly the case with microalgae that accumulate lipids under stress conditions. In case of lipid accumulation under nutrient starvation, it might be of relevance that microalgae cells often store lipids in so called lipid droplets that are distinct from membrane lipids in terms of fatty acid composition and associated cell structures (Goold *et al.,* 2015).

The potential GP (pGP) and the rate constant of GP were calculated by fitting an exponential equation to the GP data in order to describe fermentation kinetics of the investigated microalgae samples (Manuscript 2 and Manuscript 3). For the majority of the samples a high goodness of fit was achieved ($r^2 \geq 0.85$; e.g., FIGURE 2 a, b) and asymptote of GP was approached within 72 hours, meaning that applicability of the chosen exponential equation for description of ruminal fermentation kinetics of microalgae was generally given. Nevertheless, in some non-disrupted *Chlorella* samples included in Manuscript 2, the asymptote of GP was not approached within 72 hours incubation (cf. FIGURE 2 b), indicating a very slow ruminal fermentation that was not completed before incubation was stopped. For these samples it is likely that the estimated pGP was an overestimation. It would therefore be interesting to include prolonged incubation periods to achieve a more accurate estimation of pGP for these samples.

In contrast to the before mentioned slow approach of the asymptote in some microalgae samples, a fast approach of the asymptote was observed in some of the *Nannochloropsis* and *Phaeodactylum* samples, as well as in EE-rich *Chlorella* samples (e.g., FIGURE 2 c, d). The approach of the asymptote indicates the cessation of ruminal fermentation. Cessation of ruminal

fermentation is either related to a depletion of ruminally fermentable compounds or to an impairment of ruminal microbiota. Consequently, the observed fast approach of the asymptote either reflects a fast fermentation rate or the inhibition of rumen microbiota after a certain time period. In the latter case, a small amount of gas might have been produced before rumen microbiota was completely inhibited.

As illustrated for a *Phaeodactylum* sample in FIGURE 2 (d), cumulative GP decreased steadily after 12 hours of incubation. This was because the additional GP of the incubated blanks exceeded the additional GP of the sample, resulting in a calculated decrease of cumulative GP of the sample. Hence, for this particular sample, applicability of the chosen exponential equation was therefore not given. Not that pronounced but still present, similar observation was made in some but not all *Nannochloropsis* and *Phaeodactylum* samples (e.g., FIGURE 2 c), but still providing an acceptable goodness of fit (r^2 of *Nannochloropsis* and *Phaeodactylum* samples between 0.59 and 0.88, excluding the sample illustrated in FIGURE 2 d). Han & McCormick (2014) found a similar gas accumulation pattern (a steep rise of GP approaching the asymptote after five hours) for marine diatom *Thalassiosira weissflogii* and suggested that this may reflect unique characteristics of marine microalgae species that are related to their evolution and growth environment. *Nannochloropsis* and *Phaeodactylum* are microalgae that are rich in PUFA, particularly EPA (Manuscript 1). High lipid concentrations and particularly PUFA are known to have negative impact on ruminal fermentation which is thought to be caused by a direct toxic effect of PUFA on rumen bacteria or the formation of a hydrophobic coating that prevents adhesion to feed particles. The direct toxic effect is proposed to be related to the incorporation of PUFA in bacterial membranes and a consequent change in their fluidness and permeability (De Beni Arrigoni *et al.,* 2016; Jenkins, 1993). For the microalgae products that were investigated in Manuscript 2, the rate constant of GP was significantly correlated with the EPA concentration (Pearson correlation coefficient (r) = 0.70; $p < 0.001$). Hence, fast approach of the asymptote with these samples may reflect the impairment of ruminal fermentation by high amounts of PUFA. Since GP was not stopped immediately after the start of the incubation it appears that full development of this effect takes some time or it might have been delayed until cell wall and membranes were degraded and lipids were released. This would mean that high rate constant of GP and early approach of the asymptote in these samples rather reflects the disturbance of ruminal fermentation than a fast fermentation rate and a depletion of ruminal fermentable compounds. Impairment of ruminal fermentation by PUFA from microalgae was also suggested by Boeckaert *et al.* (2008).

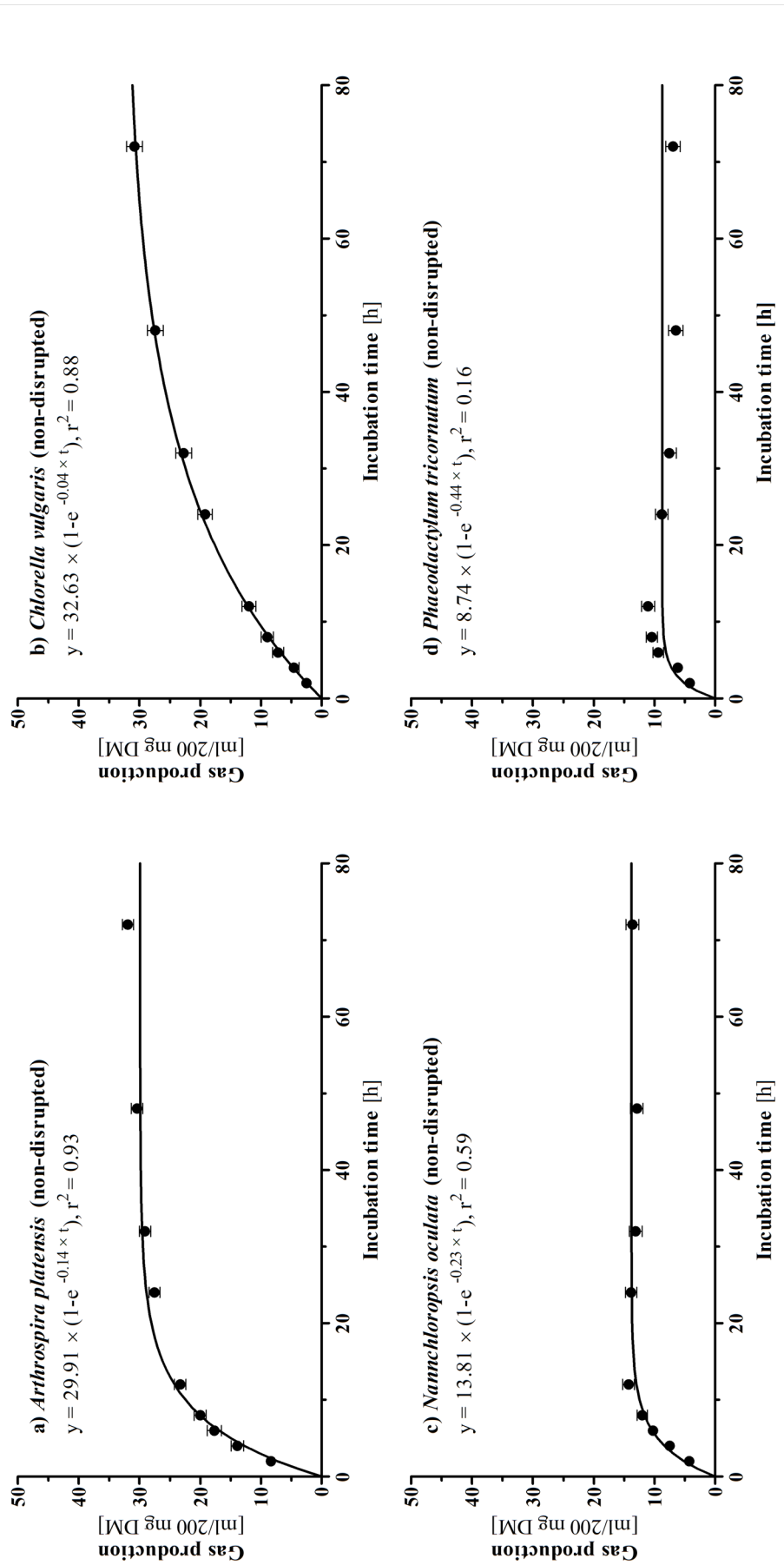

FIGURE 2: Comparison of calculated exponential gas production (GP) kinetic curves with mean GP values measured after different incubation periods for selected microalgae samples

Mean and standard error; n = 6 experimental replicates derived from subsequent *in vitro* incubations using Hohenheim Gas Test. Data obtained from Manuscript 2.

Impairment of ruminal fermentation by microalgae derived lipids is also supported by the data concerning ruminal CP degradation that was obtained using the Extended Hohenheim Gas Test method (eHGT). Generally, the investigated microalgae had high concentrations of utilizable CP at the duodenum (uCP) and ruminally undegradable CP (RUP) (cf. Manuscript 2 and Manuscript 3). With longer incubation times or slower calculated passage rates, RUP values and consequently uCP values declined because of the progressive ruminal degradation. Nevertheless, the extent of the decline varied between the investigated microalgae genera and was considerably lower in the PUFA-rich genera *Nannochloropsis* and *Phaeodactylum*: When comparing RUP values at a passage rate of 8 %/h with RUP values at a passage rate of 2 %/h, mean declines in RUP values were 28 %, 29 %, 17 % and 5 % for non-disrupted *Arthrospira*, *Chlorella*, *Nannochloropsis* and *Phaeodactylum*, respectively (data obtained from Manuscript 2; cf. FIGURE 3 a). This low decline indicates that ruminal CP degradation of particularly *Phaeodactylum* barely advanced with longer retention time in the rumen. A similar observation was made for two *Chlorella* samples with high EE concentration (549 and 341 g EE/kg DM; cf. FIGURE 3 b). As explained before, high EE concentration and particularly high concentrations of PUFA can interfere with microbial fermentation and it is known that also ruminal CP degradation can be impaired by the provision of high amounts of lipids (De Beni Arrigoni *et al.,* 2016; Jenkins, 1993). Therefore, it is likely that ruminal CP degradation was impaired by high EE concentration in case of the EE-rich *Chlorella* samples and by high amounts of PUFA in the *Phaeodactylum* samples. This suggestion is strengthened by a negative correlation of EE concentration with ruminal CP degradability (passage rate: 5 %/h; $r = -0.54$; $p < 0.001$). Furthermore, the change in RUP over the incubation time can be described by the slope of a linear regression between RUP values determined at different incubation time points (incubation time logarithmised). The slope of this linear regression was significantly correlated with the EE concentration ($r = 0.58$; $p < 0.001$), in which EE and EPA-rich samples had a slope close to zero, thus a low change in RUP concentration depending on the incubation period. Therefore, it appears that the cessation of ruminal CP degradation observed with EE and PUFA-rich microalgae rather reflects the inhibition of the ruminal fermentation than the depletion of potentially ruminally degradable CP.

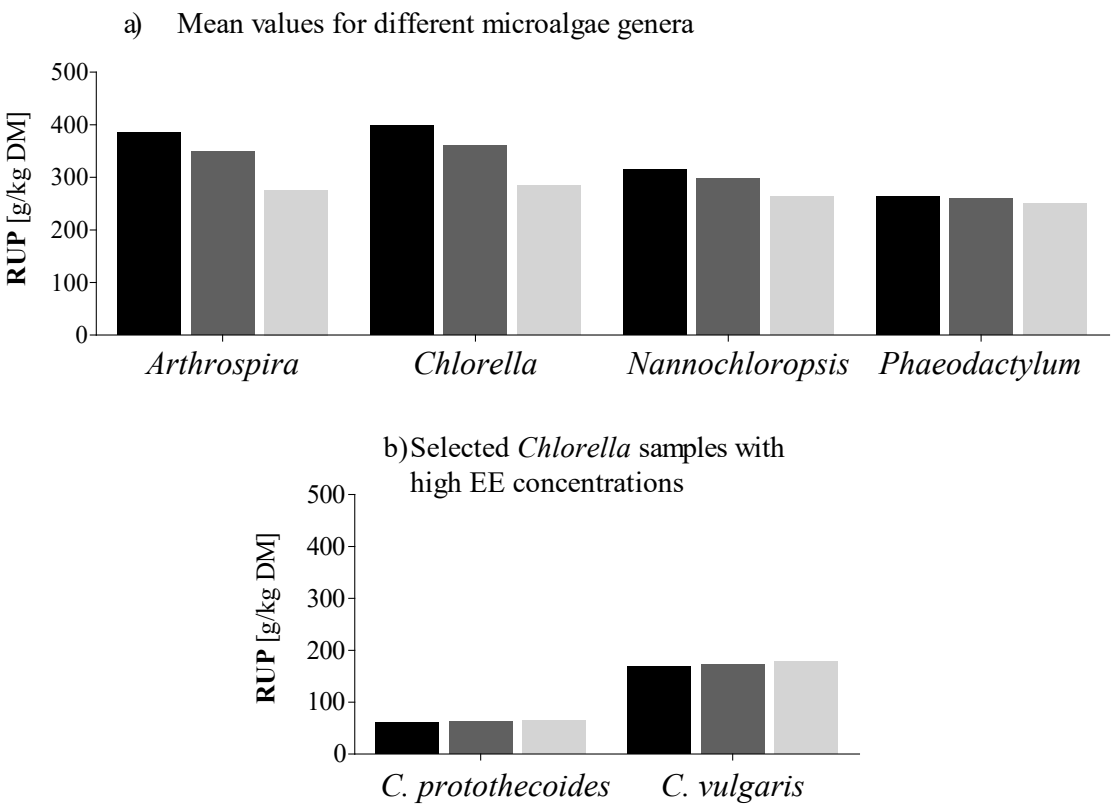

FIGURE 3: Concentration of ruminally undegraded crude protein (RUP) for different genera (a) and for selected *Chlorella* samples with high ether extract (EE) concentration (b) depending on the ruminal passage rate (■: 8%/h; ■: 5 %/h; ■: 2 %/h)

Mean values for the different microalgae genera and data on the *Chlorella protothecoides* sample (549 g EE/kg DM) were obtained from Manuscript 2 (non-disrupted samples). Data on the *Chlorella vulgaris* sample (341 g EE/kg DM) was obtained from Manuscript 3.

In conclusion it appears that the suitability of the HGT method for application on microalgae is generally given. It allows comparisons between different microalgae samples and gives a first impression on important nutritional characteristics. Nevertheless, conclusions on absolute dOM and ME values cannot be drawn, as a validation of the regression equations for microalgae is needed first. Moreover, several issues arose with the application of the HGT method on lipid-rich microalgae samples. In terms of estimation of dOM and energy values, application of the established regression equations on lipid-rich samples appeared to lead to a misjudgement of the actual dOM and energy concentration. Furthermore, lipid-rich microalgae showed fermentation characteristics distinctly different from common feedstuffs so that obtained data have to be interpreted with caution. It seems that these unusual fermentation characteristics reflected the impairment of the microbial fermentation in the rumen. This provides evidence that such biomasses might cause problems when they are used as feedstuffs for ruminants and might explain the adverse effects of microalgae supplementation that were observed in some *in vivo* experiments (e.g., reduced DMI, milk fat depression, impairment of sensory and processing properties of milk and meat; cf. CHAPTER 2.3).

Determination of intestinal digestibility of ruminally undegraded crude protein

The *in vitro* investigations using the eHGT (Raab *et al.,* 1983) revealed overall high concentrations of RUP in the investigated microalgae samples (Manuscript 2 and Manuscript 3). Consequently, microalgae provide great potential as protein sources for high performing ruminants, as the importance of RUP for protein supply of ruminants increases with higher performance levels (Stern *et al.,* 1994) because of the limited synthesis of microbial CP (mCP). Nevertheless, the amount of RUP alone is not meaningful for the evaluation of the protein value of a feedstuff as it does not reflect availability of protein, or more precise the absorbability of AA, at the small intestine. Consequently, comprehensive evaluation of the protein value of a feedstuff for ruminants should include determination of intestinal digestibility of RUP (IDP) and ruminally undegraded AA, particularly when considering that intestinal digestion varies among feedstuffs (Stern *et al.,* 1985) and that its importance increases with higher contributions of RUP in the diet (Calsamiglia & Stern, 1995). Intestinal digestibility of RUP or ruminally undegraded AA can be determined *in vivo* or with several *in situ* or *in vitro* approaches. *In vivo* determination of intestinal digestibility of RUP or ruminally undegraded AA is difficult, time consuming and expensive so that the *in situ* mobile bag technique or *in vitro* methods such as the three-step *in situ-in vitro* procedure proposed by Calsamiglia & Stern (1995) are more frequently used (Calsamiglia *et al.,* 2010).

Concerning the applicability for determination of IDP of microalgae, both of the mentioned methods have the drawback of involving incubation with nylon bags from which microalgae cells are expected to be rapidly washed-out. To test the hypothesis of rapid wash-out losses from nylon bags, one sample each of the microalgae species *Arthrospira platensis, Chlorella vulgaris, Nannochloropsis oculata* and *Phaeodactylum tricornutum* was weighed in nylon bags with pore size of 20, 30 and 50 µm and put into water for 10 minutes. Although bags were only carefully moved, DM losses were between 5 and 18 %, illustrating that ruminal incubation of microalgae in nylon bags is not feasible, because unpredictable wash-out losses would occur. Considering these observations and the methodical problems that occurred associated with the filtration for ADF and NDF determination (cf. CHAPTER 4.1.1), it was decided not to use any method involving ruminal incubation in nylon bags. Instead, IDP of the investigated microalgae samples was determined using a three-step enzymatic *in vitro* method (EIV) proposed by Irshaid (2007) (Manuscript 2 and Manuscript 3). In this procedure, the ruminal incubation of nylon bags proposed in the three-step *in situ-in vitro* method of Calsamiglia & Stern (1995) was replaced by enzymatic digestion through a protease derived from *Streptomyces griseus* to simulate ruminal CP degradation. IDP values of variable feedstuffs determined using this method were in good accordance with IDP values determined with the *in situ* mobile bag technique and with the three-step *in situ-in vitro* method (Irshaid, 2007). Furthermore, Irshaid (2007) explained that it does not need any involvement of animals and provides a high degree of standardisation. Consequently, it provides great potential for the estimation of IDP of microalgae although it has to be kept in mind that is does not reflect intestinal protein digestion under *in vivo* conditions and that this method was not validated for application on microalgae.

Mean IDP values for non-disrupted *Arthrospira*, *Chlorella*, *Nannochloropsis* and *Phaeodactylum* were 27, 43, 43, and 40 % (Manuscript 2) and 45 % for the *Chlorella vulgaris* samples investigated for Manuscript 3. A high repeatability could be achieved for estimation of IDP and percentage of RUP in CP (residual CP after enzymatic digestion through a protease derived from *Streptomyces griseus)*, with the mean standard deviation for the three experimental replicates of one sample being 2.9 and 1.6 %, respectively. To the best knowledge of the author, there are no previous studies investigating IDP of microalga. Nevertheless, compared to data on common protein sources, the IDP of microalgae appears to be relatively low. For example, Calsamiglia & Stern (1995) determined IDP values of around 90 % for soybean meal determined with the three-step *in situ-in vitro* method. Woods *et al.* (2003) reported IDP values of 71, 98 and 83 % for rapeseed meal, soybean meal and cottonseed meal

determined with the *in situ* mobile bag technique. Furthermore, Böttger & Südekum (2017) determined IDP of blend, maize and wheat distillers dried grains with solubles with the EIV method and reported mean values of 70, 83 and 71 %, respectively.

The relatively low IDP values of the investigated microalgae might be related to the fact that the applied protease treatment achieved a relatively high degradation of CP, which might have been higher than what is achievable in the rumen. When compared to RUP values of microalgae determined using eHGT (RUP_{eHGT}), the RUP values determined after protease treatment using EIV method (RUP_{EIV}) were considerably lower and this was independent from the considered ruminal passage rate in eHGT (FIGURE 4). This indicates that obtained RUP_{EIV} values might underestimate the true RUP concentration. Additionally, RUP_{EIV} showed only a slight relationship with RUP_{eHGT} at a passage rate of 5 and 8 %/h (r was 0.58 and 0.39, respectively; $p \leq 0.05$; FIGURE 4 b, c). Therefore, it appears that RUP_{EIV} does not properly reflect ruminal CP degradation of microalgae, at least at higher passage rates. Compared to that, RUP_{eHGT} at a passage rate of 2 %/h showed a relatively good relationship with RUP_{EIV} ($r = 0.78$; $p < 0.001$; FIGURE 4 a), but RUP_{EIV} was still lower than RUP_{eHGT} at a passage rate of 2 %/h. It might be that RUP_{EIV} rather reflects ruminal CP degradation of microalgae when passage would be assumed to be even lower, or in other words, when an unlimited ruminal CP degradation would be assumed so that the obtained RUP_{EIV} represents the microalgae protein totally undegradable in the rumen.

High estimated ruminal CP degradation by the EIV method is in contrast to the results of Edmunds *et al.* (2012) who compared RUP_{EIV} of forages with RUP values obtained by ruminal *in situ* incubation. They found a good agreement of the two methods, particularly when higher passage rates were assumed, but the authors applied a lower dosage of the *Streptomyces griseus* protease (24 U/g TP) combined with a longer incubation period (24 hours). It therefore might be that the applied dosage of 41 U/g TP (Manuscript 2 and Manuscript 3) was chosen too high for microalgae. Therefore, it would be interesting to test further dosages of the protease and RUP_{eHGT} might be used as reference values. Additionally, it could be of interest to test variable incubation periods that would represent variable ruminal passage rates. This would be interesting since it cannot be assumed that IDP of a feedstuff is constant, but it will be dependent on the extent of the preceding ruminal CP degradation, which is dependent on the time available for microbial fermentation.

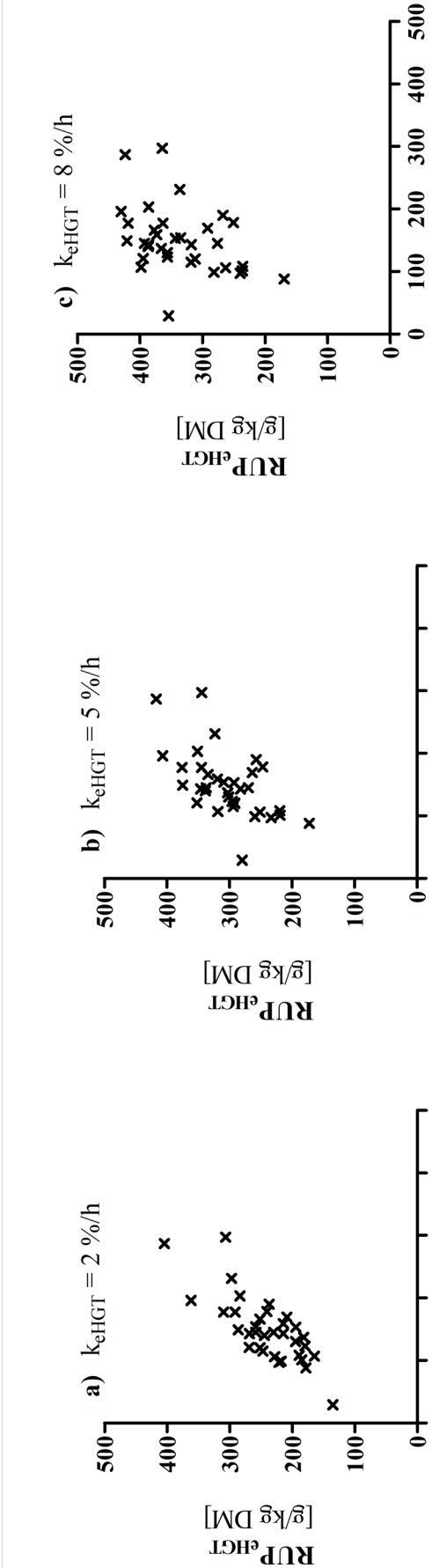

FIGURE 4: Relationship between ruminally undegraded crude protein estimated with the Extended Hohenheim Gas Test (RUP_{eHGT}) at different passage rates (a: k = 2 %/h, b: k = 5 %/h, c: k = 8%/h) and estimated with an enzymatic three-step *in vitro* method (RUP_{EIV})

r: Pearson correlation coefficient. r^2: Coefficient of determination. Data obtained from Manuscript 2 (non-disrupted and disrupted samples included) and Manuscript 3; n = 34.

Steingass *et al.* (2013) suggested that the protein resistant to ruminal degradation has also a low digestibility in the small intestine, or in other words, that ruminal and intestinal proteases degrade protein fractions with similar properties. Assuming that ruminal CP degradation was overestimated by the EIV method indicates that the true IDP of microalgae might be higher than values determined herein. This suggestion is strengthened by the fact that the ruminal *in vitro* CP digestibility determined with the EIV method was in good agreement with the *in vitro* CP digestibility (IVPD) determined with the method according to Boisen & Fernández (1995) (FIGURE 5 a). Determination of IVPD involves very similar digestion with pepsin and pancreatin as applied in the EIV method. High accordance of the determined values therefore indicates that applied *Streptomyces griseus* protease degrades similar CP fractions than the applied pepsin and pancreatin. Consequently, it is not surprising that subsequent treatment of the residuals obtained by incubation with *Streptomyces griseus* with pepsin and pancreatin only achieved a relatively low additional digestion (cf. FIGURE 5 b).

An overestimation of the RUP concentration by the EIV method could be related to the fact that the method is not suitable to simulate complexity of microbial fermentation and its interaction with other nutrients. For example, EE concentration and particularly high amounts of PUFA appeared to interfere with ruminal fermentation in general and also with ruminal CP degradation (cf. CHAPTER 4.1.2, Hohenheim Gas Test). Other microalgae constituents that might have impact on the microbial fermentation in the rumen could be carbohydrates or NPN compounds. Microalgae cell size and cell wall characteristics might also have an impact on microbial fermentation and will be discussed in CHAPTER 4.2. The discrepancy between RUP_{EIV} and RUP_{eHGT} values might therefore be related to inhibitory effects of microalgae nutrients on microbial fermentation in the rumen that are not simulated by the EIV method. Therefore, it would be interesting to investigate IDP of microalgae with a method that allows preceding simulation of microbial fermentation in the rumen and interaction of microbiota with microalgae-derived nutrients. As outlined before, it appears not feasible to apply ruminal *in situ* incubation of microalgae because of unpredictable wash-out losses from the bags. Instead, it could be tested to incubate microalgae with buffered rumen fluid in a closed batch system and to investigate the incubation residue for its intestinal digestibility by incubation with pepsin and pancreatin. Since it would be hardly possible to achieve a separation of microalgae and rumen microbiota and thus investigation of RUP alone, such approaches would rather reflect the intestinal digestibility of uCP than the IDP. However, for microalgae this approach appears feasible to reflect the intestinal digestion of dietary protein as contribution of mCP on uCP was very low (cf. Manuscript 2 and Manuscript 3).

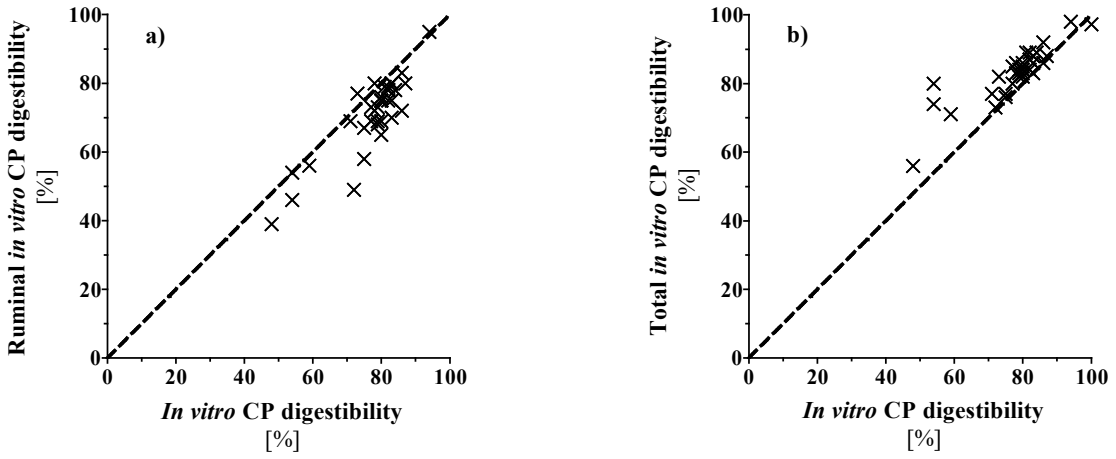

FIGURE 5: Comparison of *in vitro* crude protein (CP) digestibility (method according to Boisen & Fernández (1995)) with ruminal (a) and total (b) *in vitro* CP digestibility (enzymatic three-step *in vitro* method according to Irshaid (2007))

a) Ruminal *in vitro* CP digestibility refers to CP digestibility by *Streptomyces griseus* protease treatment; b) Total *in vitro* CP digestibility refers to CP digestibility by *Streptomyces griseus* protease treatment and subsequent pepsin and pancreatin treatment. Data obtained from Manuscript 2 and Manuscript 3, n = 36 (non-disrupted and disrupted samples included). Dashed line: bisectrix (illustrates perfect accordance).

A further aspect that might be particularly relevant for the IDP of microalgae is their residence time in the rumen. This determines the extent of ruminal CP degradation and hence the amount of substrate that it potentially available for intestinal digestion. Details on possible passage kinetics of microalgae will be discussed later in CHAPTER 4.2.4. Nevertheless, briefly summarized, it is very likely that microalgae have a fast passage rate, because of their small cell size and their fine, powdery texture that is lacking any physical structure. Considering that, it is possible that ruminal CP degradation of microalgae is low, because of only short time for microbial fermentation. That would mean that high percentages of the dietary CP of microalgae pass the rumen undegraded and are potentially available for intestinal digestion. Consequently, it might be that *in vivo* IDP of microalgae is actually higher. Further research is needed to investigate passage kinetics of microalgae in order to clarify this issue.

To summarize, ruminal CP degradation of microalgae probably was overestimated by the application of the *Streptomyces griseus* protease. This might be related to a too high dosage of the protease or interactions of rumen microbiota with inhibiting substances not being simulated by the EIV method. It appears that determined IDP values reflect the intestinal digestibility of

dietary CP that is totally undegradable in the rumen. Thus, assuming that CP degradation of microalgae is not complete under *in vivo* conditions, this might indicate that true IDP of microalgae was underestimated in the present work. Consequently, further research is needed to develop a method that allows simulation of rumen fermentation processes of microalgae and that allows to account for the expected dependence of IDP on ruminal passage rate.

4.2. Chemical and morphological characteristics of microalgae with impact on ruminal fermentation and nutritional characteristics

The experiments using the HGT method revealed relatively low ruminal fermentation of the investigated microalgae, indicated by an overall low level of GP, production of VFA and low ruminal CP degradation. Additionally, considerable variation existed in the nutritional and fermentation characteristics among and within microalgae genera (Manuscript 2 and Manuscript 3). The following sections are intended to give an overview on chemical and morphological characteristics of microalgae that might have an impact on ruminal fermentation characteristics.

4.2.1. Amino acids, proteins and non-protein nitrogen

The investigated microalgae samples were characterized by overall high concentrations of CP. Since ruminal CP degradation was rather low, uCP and RUP were relatively high compared to common feedstuffs (cf. Manuscript 2 and Manuscript 3). Ruminal CP degradation is influenced by several factors, of which some are related to the animal and others to intrinsic properties of the feed (Tamminga, 1979). Regardless of the animal factors and interactions of the protein with other associated nutrients, the most important determinants of ruminal CP degradation are the protein structure and the protein solubility that determine susceptibility and accessibility by rumen microbiota (Bach *et al.,* 2005).

To the best knowledge of the author there are no studies available that investigated solubility of microalgae protein in ruminal fluid, but some information is available on water-solubility of microalgae protein. Safi *et al.* (2014a) reported that percentage of water-soluble protein in total protein of untreated *Arthrospira platensis*, *Chlorella vulgaris*, *Nannochloropsis oculata* were 19, 10, and 8 %, and that it could be enhanced by high pressure homogenization to 78, 53, and 52 %, respectively. Enhancement of solubility by cell disruption was related to the distinct cell wall characteristics of the microalgae (Safi *et al.,* 2014a). In a further study Safi *et al.* (2013) reported percentages of water-soluble protein in total protein of 70, 43, and 33 % for the same microalgae species, respectively. Additionally, in a series of experiments Grossmann (2018)

found distinct solubility profiles amongst several microalgae species. These results suggest that solubility of microalgae protein is rather limited, varies between species and is related to cell wall characteristics of the respective microalgae, which might explain the observed variability amongst microalgae genera and the low ruminal CP degradation in general. Regarding the protein structure, Grossmann (2018) and Schwenzfeier *et al.* (2011) suggested the occurrence of glycoproteins in microalgae, which might explain variability of ruminal CP degradation as well as low CP degradation of the investigated microalgae in general. This suggestion is based on the variability of glycosylation in terms of glycan attachment and glycan's structure and the fact that glycosylation protects proteins against proteolytic degradation by the formation of a steric hindrance around the peptide backbone of the AA adjacent to the site of glycosylation (Solá & Griebenow, 2009). It would therefore be interesting to study glycosylation of microalgae proteins, structure of microalgae proteins in general, and whether it is linked to ruminal degradation characteristics.

The CP concentrations of investigated microalgae were negatively related to the RUP concentration (% of CP) at a passage rate of 5 %/h ($r = -0.59$; $p < 0.001$) indicating that ruminal CP degradability increases with higher CP concentrations of microalgae. The AA composition of the protein on the other hand appeared not to affect ruminal fermentation processes and particularly ruminal CP degradability because of the nearly complete absence of significant correlations between the AA composition of the protein and the estimates of ruminal CP degradation (for almost all AA and estimates of ruminal CP degradation r was between – 0.40 and 0.40 and $p \geq 0.05$). This was presumably related to the high similarity of the AA profiles, within and even between the investigated microalgae genera. Thus it is unlikely that the AA composition of the microalgae caused the variation in the ruminal fermentation characteristics.

As a consequence of the high CP level and the relatively low ruminal CP degradation, the RUP and uCP levels of the investigated microalgae biomasses were relatively high when compared to common protein-rich feedstuffs (cf. Manuscript 2 and Manuscript 3). Nevertheless, as explained in CHAPTER 4.1.2, the amount of RUP alone is not meaningful for the evaluation of the protein value of a feedstuff as it does not consider intestinal digestion processes. Thus, IDP or intestinal digestibility of AA should be determined to allow conclusions on the actual amount of CP or AA that is intestinally digestible. Amount of intestinal digestible RUP may be calculated by multiplying IDP data with RUP_{eHGT} or RUP_{EIV} concentrations. Based on the considerations made in CHAPTER 4.1.2, this approach has some limitations that have to be kept in mind when interpreting the resulting data. 1) The determined IDP might be

an underestimation of true IDP (resulting from the presumable overestimation of ruminal CP degradation by the EIV method); 2) The determined IDP values represent a constant, but IDP is dependent on the preceding ruminal CP degradation and thus the ruminal passage rate; 3) RUP_{eHGT} and RUP_{EIV} were not closely related, thus intestinal digestibility of RUP_{eHGT} and RUP_{EIV} might differ. Mean amount of intestinal digestible RUP_{eHGT} at a passage rate of 8 %/h was 102, 153, 134 and 108 g/kg DM for *Arthrospira*, *Chlorella*, *Nannochloropsis* and *Phaeodactylum* respectively and values declined with decreasing passage rates (TABLE 3). In comparison, amounts of intestinal digestible RUP at a passage rate of 8/h of sunflower, rapeseed, soybean and cottonseed meals were 55, 125, 192, and 128 g/kg, respectively (Woods *et al.,* 2003). Thus, the initially discussed superior protein value of microalgae appears to disappear when considering RUP and IDP together. It is interesting that when assuming lower passage rates the amount of intestinal digestible RUP of microalgae was higher than that of the common protein sources, indicating that microalgae proteins might be more resistant to ruminal degradation. Nevertheless, considering the before mentioned limitations, further research is necessary to verify the IDP values and to determine IDP of microalgae dependent on the ruminal passage rate. Furthermore, as non-ruminants, ruminants have a requirement for specific AA and not for protein *per se*. Thus, the investigation of the AA composition of microalgae RUP and the digestibility of the individual AA would be of interest and should be part of future research as it finally defines the supply of AA available for the animal.

The investigated microalgae samples had NPN concentrations ranging between 5 and 48 % of CP and the variation was related to differences within and between the microalgae genera (Manuscript 1 and Manuscript 3). A significant correlation of NPN with CP degradability at a passage rate of 5 %/h ($r = 0.46$, $p < 0.01$) was observed, indicating that microalgae rich in NPN compounds might contain less RUP. Besides that, no consistent and strong relationships of the NPN concentration with ruminal fermentation characteristics were observed for the investigated microalgae. However, the composition of the NPN fraction might have an impact on ruminal fermentation as well. Utilization by rumen microbiota is variable among different NPN compounds and also dependent from other factors, for instance the energy supplied to rumen microbiota (National Research Council, 1976; Tamminga, 1986).

TABLE 3: Concentration of intestinal digestible ruminally undegradable crude protein (RUP) determined with an enzymatic three-step *in vitro* method (EIV) and with the Extended Hohenheim Gas Test (eHGT) at different passage rates (g/kg DM)

	EIV		**Extended Hohenheim Gas Test**					
			k_{eHGT} = 2 %/h		**k_{eHGT} = 5 %/h**		**k_{eHGT} = 8 %/h**	
	nd	**cd**	**nd**	**cd**	**nd**	**cd**	**nd**	**cd**
Arthrospira	46 39 – 53	38 33 – 42	73 73 – 74	65 63 – 67	93 91 – 94	91 82 – 99	102 100 – 105	104 92 – 115
Chlorella	68 7 – 135	48 6 – 78	110 27 – 190	77 24 – 111	138 26 – 202	116 23 – 149	153 25 – 213	137 23 – 185
Nannochloropsis	94 58 – 133	50 37 – 76	113 72 – 171	99 79 – 127	127 91 – 185	112 87 – 152	134 101 – 193	119 91 – 165
Phaeodactylum	66 55 – 77	51 49 – 53	100 97 – 104	91 90 – 93	104 102 – 106	107 107 – 108	106 104 – 108	115 115 – 116
Sunflower meal			29		44		55	
Rapeseed meal			72		104		125	
Soybean meal			83		147		192	
Cottonseed meal			70		105		128	

Concentration of intestinal digestible RUP was calculated by multiplying intestinal digestibility of RUP determined with the EIV method with the respective concentration of RUP.

Data on microalgae represent the mean and range for the respective microalgae genera and were obtained from Manuscript 2.

nd: non-disrupted microalgae biomass, cd: cell disrupted microalgae biomass

Data on sunflower, rapeseed, soybean and cottonseed meal were obtained from Woods *et al.* (2003) (determined with the *in situ* mobile bag technique).

Characterization of the composition of the NPN fraction was not in the scope of the present thesis. Based on what is known from literature, microalgae comprise free AA (Al-Amoudi & Flynn, 1989; Dortch *et al.,* 1984; Flynn *et al.,* 1992), intracellular inorganic nitrogenous compounds, such as nitrate, nitrite, and ammonium (Dortch, 1982; Dortch *et al.,* 1984; Lourenço *et al.,* 1998; Lourenço *et al.,* 2004), as well as nucleic acids (Dortch *et al.,* 1984; Lourenço *et al.,* 1998; Lourenço *et al.,* 2004). Furthermore they contain nitrogenous pigments, such as chlorophylls (Lourenço *et al.,* 1998; Lourenço *et al.,* 2004). Contribution of the different NPN compounds to total NPN varies amongst microalgae and also within genera, as assimilation of nitrogenous compounds is affected by factors such as the amount and source of N supplied (Al-Amoudi & Flynn, 1989; Dortch, 1982; Dortch *et al.,* 1984; Flynn *et al.,* 1992; Lourenço *et al.,* 2004), and it changes during the life cycle of microalgae cells (Lourenço *et al.,* 1998; Lourenço *et al.,* 2004). Hence, composition of the NPN fraction of the investigated microalgae likely was variable, but discussing impacts on ruminal fermentation processes is impossible based on the available data. It would therefore be interesting to further characterize the NPN fraction of microalgae and investigate whether there are relationships with ruminal fermentation characteristics.

4.2.2. Lipids

Lipids are one of the main constituents of microalgae and are very diverse in terms of fatty acid composition and lipid classes. Non-polar lipids in microalgae include triacylglycerols, sterols, free (non-esterified) fatty acids and hydrocarbons. Polar lipids include phospholipids, glycolipids and betaine lipids. While phospholipids, glycolipids and sterols act as essential structural components of biological membranes, triacylglycerols and hydrocarbons are storage products of microalgae (Guschina & Harwood, 2013; Khozin-Goldberg, 2016). Lipid class composition as well as fatty acid composition of total lipids and also of individual lipid classes varies considerably amongst microalgae (Yao *et al.,* 2015). They are affected by several factors such as temperature, light intensity, salinity, pH and the supply of nutrients (Guschina & Harwood, 2013). Accordingly, the investigated microalgae samples varied considerably in terms of EE concentration and fatty acid composition, both between and also within the genera (Manuscript 1 and Manuscript 3). Since the fatty acid profiles of the investigated microalgae are discussed in depth in Manuscript 1 and Manuscript 3, they will not be discussed herein and the reader is referred to these chapters. Nevertheless, it appears noteworthy in this context that microalgae are capable of the synthesis of very high lipid concentrations, mainly in the form of triacylglycerols, that can reach up to 50 % of cell dry weight. Furthermore, long-chain PUFA

with chain lengths of 20 to 22 carbons and a high number of double bonds (up to six) are abundant in some microalgae, which is distinctly different from lipids in higher plants, where straight-chain C16 and C18 fatty acids with a lower degree of unsaturation (up to three double bonds) are most common (Guschina & Harwood, 2013; Khozin-Goldberg, 2016).

As previously mentioned, high lipid concentrations and particularly PUFA have negative impact on ruminal fermentation (cf. CHAPTER 4.1.2). As a consequence, ruminal degradation of structural carbohydrates but also of CP can be reduced by high dietary PUFA concentrations and this is accompanied by reduced production of methane, hydrogen, and VFA, including a lower acetate to propionate ratio (Jenkins, 1993). In accordance, high concentrations of EE and of PUFA in microalgae appeared to be related to several aspects of ruminal fermentation characteristics such as the kinetics of GP, the CP degradation and the methane production (cf. CHAPTER 4.1.2, Manuscript 2 and Manuscript 3). This was indicated by unusual GP kinetics of EE and PUFA-rich samples (cf. CHAPTER 4.1.2) and significant ($p < 0.001$) negative correlations of EE concentration with methane concentration in the produced gas (r = – 0.69), the acetate to propionate ratio (r = – 0.60), the total VFA production (r = – 0.57), and the ruminal CP degradability (r = – 0.54; at a passage rate of 5 %/h). Similar observations were also made previously. For example, Fievez *et al.* (2007) and Ungerfeld *et al.* (2005) incubated hay together with a DHA-rich microalgae biomass or an oil rich in hexadecatrienoic acid, which was derived from *Chaetoceros* microalgae, in rumen fluid, respectively. They consistently observed a shift towards propionate (up to 54 % of total VFA) at the expense of acetate which was accompanied by the impairment of total VFA production, ruminal nutrient degradation and the reduction of ruminal methanogenesis (Fievez *et al.,* 2007; Ungerfeld *et al.,* 2005). Additionally, reduction of DMI observed in dairy cows receiving PUFA-rich microalgae has been ascribed to the disturbance of ruminal fermentation by microalgae derived PUFA (Angulo *et al.,* 2012; Boeckaert *et al.,* 2008; Vanbergue *et al.,* 2018b). Consequently, application of EE and particularly PUFA-rich microalgae in the nutrition of ruminants must consider the potential negative impacts on ruminal fermentation. Protection of microalgae derived lipids or microalgae cells as a whole from ruminal biohydrogenation might help to overcome this issue. Nevertheless, further investigations are necessary to evaluate the potential of rumen protection of microalgae since the results of Franklin *et al.* (1999) indicate that it does not completely rule out negative impacts, at least on DMI.

Despite of the negative impact of high EE concentrations on ruminal fermentation, the synthesis of mCP appeared to be highest with the microalgae samples with the highest EE

concentrations. For an EE-rich *Chlorella protothecoides* sample (549 g EE/kg DM, non-disrupted) and a *Chlorella vulgaris* sample cultivated under CO_2 limited conditions (341 g/EE/kg DM, Manuscript 3), the mCP concentration at a passage rate of 8 %/h were 35 and 19 % of uCP, respectively. This is considerably higher than the mCP of the other investigated samples (4 – 12 % of CP at a passage rate of 8 %; non-disrupted samples). The effects of lipids on rumen microbiota vary for different microbial species and fatty acids (Enjalbert *et al.,* 2017). For instance, it is known that the addition of lipids can lead to an increase of the efficiency of mCP synthesis because of reduced numbers of ruminal protozoa that are predators of ruminal bacteria (De Beni Arrigoni *et al.,* 2016). Therefore, it might be that only specific microbial groups (e.g., protozoa) were affected by the high EE concentrations, but overall mCP synthesis was not reduced. Nevertheless, characterization of the microbiota associated with the incubation of microalgae in ruminal fluid was not in the scope of the present thesis. It would therefore be interesting to investigate microbiota associated with the ruminal incubation of microalgae in future studies.

4.2.3. Carbohydrates

Carbohydrates in microalgae include storage and cell wall related polysaccharides, the latter comprising cell wall polysaccharides and exocellular polysaccharides. Storage polysaccharides in microalgae include starch, floridean starch, glycogen and chrysolaminarin and their main function is energy storage. Cell wall polysaccharides and exocellular polysaccharides have structural function in the cell (Bernaerts *et al.,* 2018). Exocellular polysaccharides can be tightly adhered to the cell wall or released to the surrounding environment and act as physical barrier for protection of the cells (Rossi & de Philippis, 2016). Monosaccharide composition of total carbohydrates and individual carbohydrate classes is diverse amongst and within microalgae (Abo-Shady *et al.,* 1993; Bernaerts *et al.,* 2018; Takeda, 1988a, 1988b, 1991; Takeda & Hirokawa, 1978; Templeton *et al.,* 2012). Nevertheless, data on microalgae carbohydrates related to functional properties are scarce. The few publications in this field mostly reported either total carbohydrate concentrations or monosaccharide composition of total carbohydrates, both not allowing to draw conclusions on the structure of the original polysaccharides and thus their function (Bernaerts *et al.,* 2018; Templeton *et al.,* 2012) or on possible implications on nutritional properties.

Within the frame of Manuscript 1 it was attempted to characterize the carbohydrate fraction by the determination of ADF, NDF and starch, but reliable determination was not possible for ADF and NDF because of methodical problems (cf. CHAPTER 4.1.1) and the concentrations of

α-linked glucose were generally low (< 10 %, cf. Manuscript 1 and Manuscript 3). Consequently, suggestions on the composition of carbohydrate fraction and the presence of specific carbohydrates are rather speculative. Nevertheless, the overall relatively low level of the fermentation characteristics of the investigated microalgae (e.g., GP, total VFA, mCP) suggests that amount of carbohydrates was low, that their ruminal fermentability was low, or both. In accordance with that, Han & McCormick (2014) suggested that carbohydrates of microalgae were less fermentable than those of soybean meal. Bernaerts *et al.* (2018) observed relatively low percentages of the polysaccharide fraction in several commercially available microalgae biomasses, particularly of the storage polysaccharides. They reported a total carbohydrate concentration (sum of storage, cell wall and exocellular polysaccharide) of 185, 116, 97, and 105 g/kg DM for *Arthrospira platensis*, *Chlorella vulgaris*, *Nannochloropsis* sp., and *Phaeodactylum tricornutum*, respectively, in which the concentration of storage polysaccharides was below 10 % of DM in all samples. The authors explained that accumulation of carbohydrates is generally low during exponential growth phase of microalgae and enhances in the stationary phase when nutrients are exhausted or when microalgae are cultivated under nutrient depleted conditions. They suggested that low amounts of storage polysaccharides in the commercial biomass was related to optimized cultivation media and harvest in the late exponential phase in order to achieve a biomass optimized in terms of protein and lipid concentration and composition (Bernaerts *et al.,* 2018). The same could apply for the commercial microalgae biomasses investigated in frame of Manuscript 1 and Manuscript 2, and for the samples investigated in frame of Manuscript 3 that were cultivated under saturated conditions (Control, Outdoor). For the microalgae biomasses that were cultivated under nitrogen and CO_2 deficient conditions within the scope of Manuscript 3, their low level of α-linked glucose indicates that the used *Chlorella vulgaris* strain was oleaginous, thus accumulating lipids instead of carbohydrates under stress conditions. Thus, future research in context of the utilization of microalgae in the nutrition of ruminants could strive for optimization of cultivation conditions and harvest regime for higher carbohydrate yields. Additionally, targeted selection of microalgae species or specific strains that accumulate polysaccharides instead of lipids under stress conditions could enable the production of microalgae biomasses that have a higher fermentability and this might help to improve utilization of ruminal degradable CP for mCP synthesis. Both should not only consider the simple accumulation of carbohydrates but also their structure and possible implications for nutritional properties. The base for such efforts would be the development of methods for the

characterization of the different carbohydrate fractions of microalgae that are feasible for routine use and that can be associated with feed evaluation characteristics.

For all of the investigated microalgae samples, the utilization of the ruminally degraded CP for mCP synthesis was relatively low (12 – 25 % of ruminally degraded CP at a passage rate of 8 %/h). As outlined in Manuscript 2, this was likely related to an energy deficiency because of the presumably low amounts and low fermentability of microalgae carbohydrates. Consequently, avoidance of an energy limitation by the addition of a carbohydrate source might enhance utilization of ruminally degraded CP from microalgae for mCP synthesis. In the experiments using the eHGT method (Manuscript 2 and Manuscript 3), a carbohydrate source was added and thereby energy was supplied to the microbes. Efficiency of mCP synthesis could be calculated as mg N/mg degraded carbohydrates, when GP and NH_3-N concentration in the incubations with and without carbohydrate addition are compared. However, the efficiency calculated this way is specific for the carbohydrate source (i.e., the carbohydrate mixture), but not for the protein source (i.e., the microalgae) used, so that estimation of the efficiency of mCP synthesis from microalgae protein not restricted by energy availability is not possible. However, the data provide some evidence that protein utilization from microalgae protein by rumen microbiota can be enhanced when fermentable carbohydrates are supplied. When calculating uCP from the samples with carbohydrate addition, uCP was at the same level or even higher than CP, indicating that mCP synthesis can be enhanced by the addition a carbohydrate source (FIGURE 6). Based on the assumptions that uCP comprises RUP and mCP and that the RUP concentration is unlikely enhanced by the provision of energy, the enhancement of uCP in the samples with carbohydrate addition compared to uCP in the samples without carbohydrate addition likely originates from additionally produced mCP. This provides some evidence that ruminally degraded CP from microalgae can potentially be incorporated in mCP to a larger extent when energy is not a limiting factor. Nevertheless, further investigations will be necessary to investigate whether this is also the case when microalgae are combined with common feedstuffs and whether interactions occur that might have impact on the synthesis of mCP.

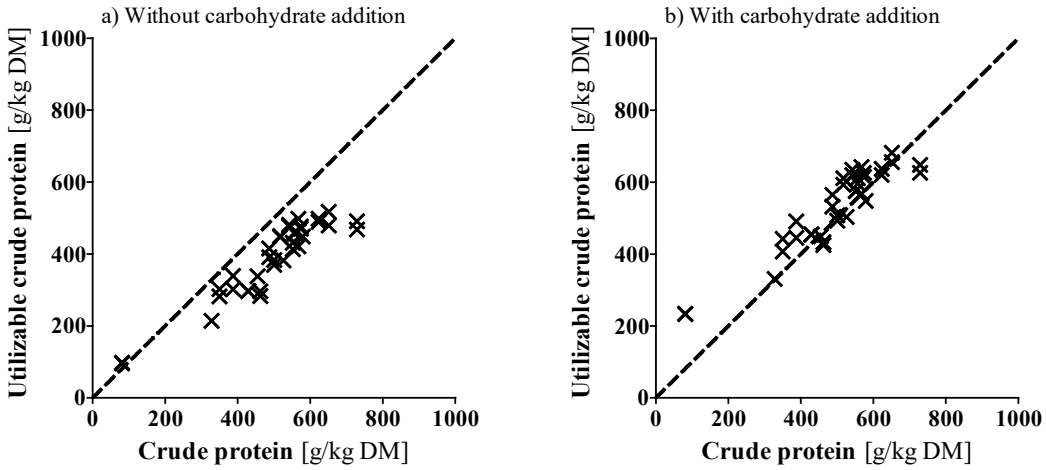

FIGURE 6: Comparison of utilizable crude protein concentration with crude protein concentration calculated with and without carbohydrate addition

Concentration of utilizable crude protein after 8 hours of incubation. Data obtained from Manuscript 2 (non-disrupted and cell disrupted samples included) and Manuscript 3; n = 36). Dashed line: bisectrix (illustrates perfect accordance).

It might also be that the enzyme activity of the rumen microbiota was not well adjusted to the microalgae ingredients, as the donor animals of the ruminal fluid were never exposed to microalgae as feedstuffs. Taxonomic composition of rumen microbiota (Ellison *et al.,* 2014; Tajima *et al.,* 2001), as well as enzymes synthesized by the microbiota (Wolff *et al.,* 2017), shifts and adapts to the provided diet. In accordance with that, Tsiplakou *et al.* (2017b) observed reductions in cellulolytic and increases in proteolytic bacteria, which were accompanied with respective changes in ruminal enzyme activities (cellulase, protease) in goats receiving a diet containing *Chlorella vulgaris* for 30 days. Thus, it is possible that rumen microbiota can adapt to microalgae when they are provided long-term and hence ruminal fermentation of microalgae might be increased. Thus, it would be interesting to investigate whether ruminal fermentation of microalgae increases when microalgae are fed long-term. In this context, the investigation of the rumen microbiota composition as well as enzyme activities would be of interest.

4.2.4. Microalgae cell size

Microalgae are in micrometre size and mostly they are single-celled organisms. For instance, cell diameter of *Chlorella* ranges between 2 to 10 µm (Liu & Hu, 2013) or that of *Nannochloropsis* cells between 2 to 6 µm (Andersen *et al.,* 1998; Beacham *et al.,* 2014; Gwo *et al.,* 2005). With that, they are only marginally larger than most of the rumen bacteria that are within the range of 0.4 to 1.0 µm in diameter and 1 to 3 µm length (Hungate, 1966), and even smaller than protozoa that range in size from 10 × 20 to 120 × 200 µm (Nagaraja, 2016). Furthermore, microalgae biomasses are very fine powders that lack a physical structure and easily suspend in fluids. Therefore, when taken up by a ruminant, it appears that microalgae will rather be associated with the fluid phase in the rumen than with the solid phase.

Ruminal microorganisms can roughly be divided in three distinct subpopulations: 1) microorganisms that are associated with the rumen fluid; 2) microorganisms that are loosely attached to the solid phase, and 3) microorganisms that are closely bound to the solid fraction (Cheng & McAllister, 1997). The subpopulation of the fluid associated microbes comprises microorganisms that have been detached from the solid phase and microorganisms that subsist on feed components that are soluble in the rumen fluid and it is assumed that these microorganisms have only a minor role in the degradation of insoluble feed particles (McAllister *et al.,* 1994). For solid associated microbes, attachment to feed particles is required for degradation of the feed components. When properly attached, solid associated microbes appear to have competitive advantages against fluid associated microorganisms because they are close to the site of digestion, thus receiving a large proportion of released nutrients, and their residence time in the rumen is longer because their passage is dependent on the associated feed particles, thus allowing a high reaction time between microbial enzymes and substrates (Wang & McAllister, 2002). Fluid associated microbes on the other hand must continuously seek out for new substrate and it appears that their enzymes are rapidly inactivated because of low enzyme activity of cell-free ruminal fluid (McAllister *et al.,* 1994). Furthermore, McAllister *et al.* (1994) compiled data of several studies indicating that particle associated microbial populations are responsible for the majority of ruminal feed degradation, in particular of structural polysaccharides and protein. This assumption was based on the higher enzyme activity in the solid phase of the rumen (e.g., hemicellulase, cellulase, amylase, protease), and the virtually absence of proteolytic activity in cell-free rumen fluid. The association of the different microbial subpopulations in the rumen and their ability to attach to microalgae cells might be of relevance for the ruminal fermentation of microalgae.

Considering the low cell size of microalgae, it appears questionable whether proper attachment of solid associated microorganisms to cell surface of microalgae can be achieved. It might be that, because of the relatively small area available for attachment of ruminal microbes on microalgae cells, a firm connection between microalgal and rumen microbial cells is not possible. This could mean that enzymes exerted by rumen microbes cannot reach the site of digestion targeted or are simply washed-off. Although smaller particle sizes usually enhance microbial fermentation because of an increased surface area to volume ratio and thus an increase of the surface area available for microbial attachment and enzymatic attack (Bowman & Firkins, 1993), there might be a critical lower limit of particle size for proper attachment of rumen microbiota. Furthermore, it is likely that only a relatively small number of rumen microbes can attach to one single microalgae cell, because of limited surface area when compared to feed particles of common feedstuffs. Since the synergistic effects of a wide range of hydrolytic enzymes derived by numerous microbial species are required to degrade complex cell wall components (McAllister *et al.,* 1994), this might obstruct the ruminal degradation of microalgae. Additionally, attachment of rumen microorganisms to microalgae cells might be hindered by the presence of complex exocellular polysaccharides synthesized by many microalgae species that appear as mucous surrounding the microalgae cells (Rossi & de Philippis, 2016). For several microalgae species, including *Arthrospira* and *Phaeodactylum*, exocellular polysaccharides have been shown to have anti-adhesive properties against disease-associated bacteria (Guzman-Murillo & Ascencio, 2000; Loke *et al.,* 2007) and this might also apply to ruminal bacteria. Thus, assuming that firm attachment of solid associated microbes to microalgae cells is difficult and that fluid associated microbes have only a minor contribution on ruminal degradation, this could explain the overall low ruminal fermentation of the microalgae investigated in the HGT experiments (Manuscript 2 and Manuscript 3). It would therefore be interesting to investigate by microscopical examination whether ruminal microorganisms are able to attach to microalgal cells.

Beyond the possible impacts on the attachment of rumen microorganisms to microalgae cells, their low cell size might be additionally relevant in the context of ruminal retention time. It has been reviewed that the passage of feed particles is inversely related to their size (Huhtanen *et al.,* 2006; Kennedy, 2005), suggesting that microalgae might have a fast rate of passage through the rumen. Furthermore, fibrous particles with a low density are buoyant and can be entangled in the rumen fibre mat, while small dense particles readily sink in the rumen fluid and are flushed from the rumen to the omasum rapidly (Owens & Basalan, 2016). Since

microalgae biomasses are very fine, have a high density, and are lacking any fibrous structure, it appears unlikely that they are trapped in the fibre mat, thus probability of rapid passage of microalgae from the rumen is high. On the other hand, particles with a very high density like metal or sand and with a high tendency for sedimentation settle to the ground of the rumen and may be retained in the rumen (Owens & Basalan, 2016). It was not yet studied whether microalgae cells tend to settle in the rumen or whether they are rapidly flushed to omasum, although information on the passage behaviour of microalgae in the rumen appears to be necessary for a comprehensive evaluation of their suitability as feedstuff for ruminants. As outlined before, it appears likely that microalgae biomasses are associated with the ruminal fluid phase that was reported to have a mean ruminal retention time of less than 10 hours (Mambrini & Peyraud, 1997). Hence, the available time for microbial fermentation of microalgae might be low so that the extent of ruminal fermentation of microalgae under *in vivo* conditions might be distinctly different from *in vitro* measurements. Moreover, this could mean that microalgae cells pass the rumen, at least partially, undegraded. Based on the considerations outlined in CHAPTER 4.1.2 this could indicate a high amount of nutrients that are potentially available for intestinal digestion. On the other hand, assuming that passage through the small intestine is also fast or that intestinal digestibility is low, high percentages of microalgae nutrients might be unutilized. Therefore, it would be interesting to study both ruminal and intestinal passage behaviour of microalgae.

4.2.5. Cell walls and cell disruption

The development of rigid cell walls or cell coverings is a common property of several microalgae species, but they are highly variable in terms of structure and composition. They can be made up of for example cellulose (Domozych *et al.,* 2012; Popper & Tuohy, 2010), silicates (Popper & Tuohy, 2010; Tesson *et al.,* 2009a), or algaenan that is described as an insoluble and non-hydrolysable biopolymer (Allard & Templier, 2000; Scholz *et al.,* 2014). Since the nutritionally valuable compounds are usually stored inside the microalgae cells, cell walls represent a natural barrier that have to be overcome to achieve accessibility of intracellular nutrients of microalgae (Baudelet *et al.,* 2017; Bernaerts *et al.,* 2018). For example, in *Chlorella vulgaris* 50 % of the protein are located inside the cell, 20 % are cell wall-bound and only 30 % are released to the outside of the cell (Berliner, 1986). Some studies investigated the effects of variable cell wall disruption methods on digestibility and extractability of microalgae nutrients in non-ruminants (*in vivo* and *in vitro*) and have mostly observed an enhancing effect. Enhancement of digestibility and extractability of nutrients by cell disruption is likely related

to the destruction of indigestible cell wall compounds and the resultant higher accessibility of valuable intracellular compounds which are expected to be released by cell disruption. Nevertheless, extent of the effects is apparently dependent on the cell wall disruption method and the microalgae species (cf. CHAPTER 2.2.). Effects of cell disruption on nutritional characteristics in ruminants have not been investigated before, but based on the information available from non-ruminants, it was expected that they might vary between and within microalgae genera. As it is assumed that differences in the effects of cell disruption are particularly associated with difference in cell wall structure and composition, it appears necessary to discuss the observed effects of cell disruption based on the information available on microalgae cell walls.

Cell disruption with a ball mill had a significant effect in most of the nutritional characteristics that were investigated in frame of the experiments for Manuscript 2, but extent of the effects varied between and within microalgae genera. Amongst the investigated microalgae genera, the effects of cell disruption were lowest in *Arthrospira* and highest in *Chlorella* in almost all of the investigated nutritional characteristics (cf. FIGURE 7). For *Arthrospira,* it was expected that cell disruption has only a minor effect since it possesses a thin and fragile cell wall that is made up of fibril layers and peptidoglycan (van Eykelenburg, 1977) and was expected to be easily degraded by rumen microbiota. Indeed, compared to the other investigated microalgae genera, the effects of cell disruption were relatively low, but it was interesting that direction of the effects was mostly contrary compared to the other genera. It might be that in case of *Arthrospira* 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. Such compounds might be cyanotoxins (Roy-Lachapelle *et al.,* 2017) or gamma-linolenic acid (cf. Manuscript 1) that are not present in the other investigated microalgae genera. Furthermore, it might be that particle size reduction resulting from the cell disruption treatment has caused the reduced ruminal fermentation of *Arthrospira,* because it might have hampered proper attachment of rumen microbiota to *Arthrospira* cells (cf. CHAPTER 4.2.4). It might be that this was only of importance for *Arthrospira* since these microalgae have the largest cells amongst the investigated genera and even when non-disrupted, the cell size of the other genera might have fallen below a critical cell size for proper attachment of rumen microbiota. Nevertheless, the effects of cell disruption on ruminal fermentation of *Arthrospira* samples were relatively low so that the observed effects might be in the range of uncertainty of the respective method.

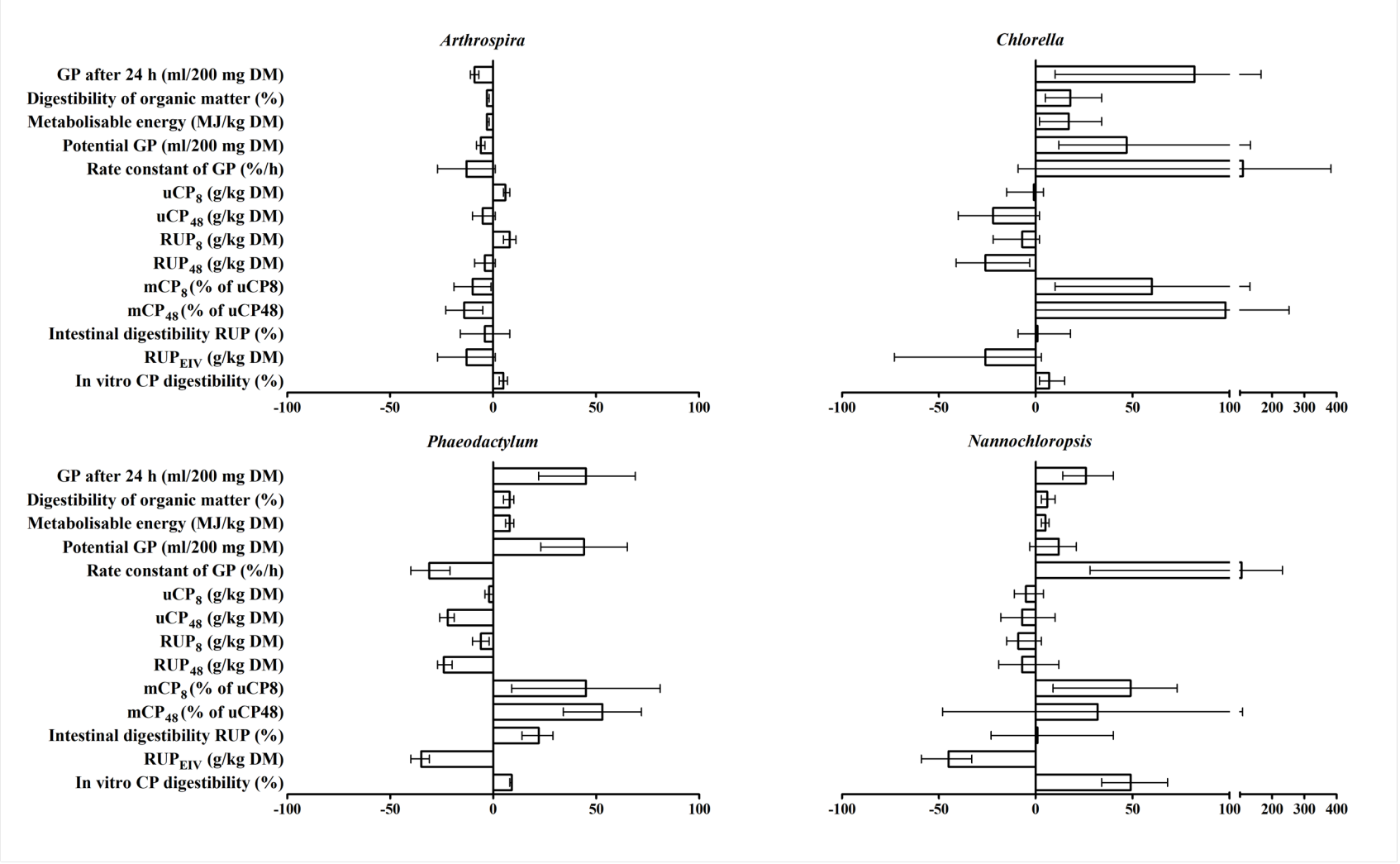

FIGURE 7: Relative effects (%) of cell disruption on nutritional characteristics in four microalgae genera

Values represent the mean changes and the range of the changes between non-disrupted and cell disrupted samples for the respective microalgae genera (referred to non-disrupted samples); Measuring units of the respective nutritional characteristics in brackets. Data obtained from Manuscript 1 and 2; n = 32. GP: Gas production; uCP_8, uCP_{48}: Utilizable crude protein (CP) at the duodenum after 8 or 48 h of incubation; RUP_8, RUP_{48}: Ruminally undegraded CP after 8 or 48 h of incubation; mCP_8, mCP_{48}: Microbial CP after 8 or 48 h of incubation; RUP_{EIV}: RUP determined with the EIV method.

Chlorella is known to possess a multi-layered rigid cell wall (cf. CHAPTER 2.1) that comprise substances like a chitin-like glycan or algaenan (Allard & Templier, 2000; Kapaun & Reisser, 1995). These compounds appear to be hardly digested even by rumen microbiota, thus the effects of cell disruption on nutritional characteristics were high in *Chlorella* samples, because accessibility of nutrients to rumen microbiota was enhanced by the destruction of the cell walls. Furthermore, it appears that variability of the effect related to cell disruption was highest in *Chlorella* samples, which is indicated by overall highest ranges (cf. FIGURE 7). Several authors described that cell wall structure and composition are highly variable between specific *Chlorella* species and strains (Abo-Shady *et al.,* 1993; Bernaerts *et al.,* 2018; Blumreisinger *et al.,* 1983; Takeda, 1988a, 1988b, 1993, 1995; Takeda & Hirokawa, 1984) and that it changes during growth of the cells (Takeda & Hirokawa, 1978; Yamamoto *et al.,* 2004; Yamamoto *et al.,* 2005). Therefore, it might be that high variability observed for *Chlorella* in terms of relative changes related to cell disruption resulted from differences in the cell walls. It is interesting that highest effects of cell disruption were mostly observed for a protein-rich *Chlorella protothecoides* sample that was heterotrophically produced. For a EE-rich sample of the same species the effects of cell disruption were relatively low. *Chlorella protothecoides* cell walls contain sporopollenin (He *et al.,* 2016), an extremely tough biopolymer that is resistant against many kinds of chemical treatments (acid and alkaline hydrolysis, acetolysis) and to enzymatic degradation (Ueno, 2009; Xiong *et al.,* 1997). High impact of cell disruption on nutritional characteristics of the protein-rich *Chlorella protothecoides* sample might therefore be related to a certain degree of mechanical destruction of sporopollenin containing cell walls that could not be degraded by rumen microbiota. It might be that the sporopollenin was only present in the protein-rich *Chlorella protothecoides* sample, but not in the lipid-rich sample so that the cell walls of the latter did not represent a barrier. On the other hand, it is likely that the observed effects do not only reflect differences in the cell wall composition and structure, but also a variation in the compounds that are released by the destruction of the microalgae cells. For example, in case of the EE-rich *Chlorella protothecoides* sample, cell disruption likewise released a high amount of lipids. As described previously, high amounts of lipids interfere with ruminal fermentation (cf. CHAPTER 4.2.2). Consequently, it might be that accessibility of intracellular compounds was enhanced in the mentioned sample, but this might be overlaid by the interference of the released lipids with ruminal fermentation processes, resulting in an apparently low effect of the cell disruption treatment. The same could also apply to *Nannochloropsis* that is also known to possess a very rigid cell wall composed of cellulose and algaenan (Scholz *et al.,* 2014), and contains high amounts of EPA (cf. Manuscript 1). Release

of intracellular EPA and thus inhibition of rumen microbiota might have compensated for additionally released potentially degradable compounds. Interestingly, relatively high effects of cell disruption in *Nannochloropsis* were observed for the traits that are based on enzymatic digestion (IVPD and RUP_{EIV}), indicating that cell disruption enhanced accessibility of nutrients in *Nannochloropsis*. Both, IVPD and RUP_{EIV} do not to reflect any inhibitory effects on ruminal microbiota. Therefore, this strengthens the assumption that accessibility of nutrients to rumen microbiota was indeed enhanced by cell disruption, but the effects were not observable in the traits based on ruminal incubations as it was negated by interference of ruminal fermentation by EPA.

Variability in cell wall structure and composition may not only affect accessibility of intracellular nutrients to rumen microbiota, but also susceptibility of microalgae cells to the cell disruption treatment. Grossmann *et al.* (2018) and Safi *et al.* (2014a) reported that the resistance of several microalgae species against mechanical cell disruption varied amongst species. For instance, 12, 9, 6, and 3 passes through a high pressure homogenizer (180 MPa at 22°C) were needed to achieve the disruption of 99.9 % of the microalgae cells of *Nannochloropsis oceanica*, *Chlorella vulgaris*, *Chlorella sorokiniana* and *Phaeodactylum tricornutum*, respectively (Grossmann *et al.,* 2018). The cell disruption treatment applied to the microalgae biomasses investigated in frame of Manuscript 2 was the same for all included microalgae genera. It was checked microscopically whether cells were apparently fractured but success of the cell disruption treatment was not quantified. Consequently, it cannot be ruled out that disruption of cells was incomplete. Thus, the variation of the effect induced by the cell disruption treatment amongst and within the microalgae genera might be related to a variable amount of cells that were not ruptured. Furthermore, the absence of effects might also indicate that cells were not ruptured at all or that cell disruption treatment did not achieve the release of fermentable compounds, although that appears rather unlikely when considering the microscopical observations.

Generally, cell disruption treatments are used to enhance accessibility of intracellular nutrients of microalgae. Nevertheless, this might be undesirable when microalgae are intended to be used as protein source for high performing ruminants. For these animals, feedstuffs rich in RUP are needed to meet requirements of protein, or more precisely AA, available for absorption at the small intestine. However, cell disruption with a ball mill decreased concentrations of RUP in most of the investigated microalgae samples (cf. Manuscript 2, FIGURE 7), presumably by enhancing ruminal CP degradation. Furthermore, IDP was mostly

not affected by the mentioned cell disruption treatment. Consequently, the overall effect of mechanical cell disruption on the protein value of the investigated microalgae was rather negative since the concentration of intestinal digestible RUP was generally decreased (cf. TABLE 3). Therefore, cell disruption is not recommended when microalgae are intended to be used as protein sources for ruminants and other processing methods are needed that achieve an increase in the concentration of intestinal digestible RUP.

4.3. Conclusions and perspectives for future research

Commercial microalgae biomasses from the genera *Arthrospira*, *Chlorella*, *Nannochloropsis* and *Phaeodactylum* showed a high variation of nutrient composition, ruminal fermentation and nutritional characteristics for ruminants, both amongst and within genera. The variation likely resulted from varying cultivation conditions used in different cultivation facilities. Thus, general mean values are inappropriate to characterize the nutritional value of microalgae. Hence, to the extent possible, it should be strived for a standardisation of cultivation conditions. Moreover, future research should include the development of methods that allow an easy and reliable prediction of the nutritional value of microalgae. As a starting point, it would be necessary to investigate relationships between the cultivation process and nutritional characteristics for a wide range of microalgae species and cultivation conditions.

The investigated microalgae genera were characterized by overall high concentrations of RUP. This resulted from initially high concentrations of CP and a low ruminal CP fermentation. The low ruminal CP fermentation might be related to low amounts of fermentable carbohydrates, interference of microalgae PUFA with ruminal microbiota, a low protein solubility or structure of microalgae proteins. Furthermore, the low cell size of microalgae might have hindered attachment of ruminal microbiota to the cells and thus might have limited their degradation. The overall high level of RUP lets microalgae appear to be a valuable protein source for ruminants, particularly for high performing animals. Nevertheless, the generally low IDP, which was determined for microalgae in this thesis for the first time, contradicts this potential. However, comparative data analysis of RUP_{EIV} and RUP_{eHGT} provided evidence that true IDP might have been underestimated. Therefore, further research is needed to verify the obtained results. This should include the development of a method that allows simulation of interaction of microalgae nutrients with rumen microbiota and that accounts for the expected dependence of IDP on the ruminal passage rate. For that, investigations concerning the ruminal and also the intestinal passage behaviour of microalgae should be conducted, in order to allow a realistic simulation of the *in vivo* digestion of microalgae. Additionally, the investigation of

the AA composition of microalgae RUP and the digestibility of the individual AA would be of interest and should be part of future research as it finally defines the supply of AA available for the animal.

The methods for determination of ADF and NDF for the characterization of the polysaccharide fraction are inappropriate for application on microalgae and their application cannot be recommended for microalgae. Thus, further research is necessary to develop methods that allow characterization of the different carbohydrate fractions of microalgae (storage, cell wall and exocellular polysaccharides) and are feasible for routine use. The development of such methods could help to identify microalgae species or specific strains that are capable of the accumulation of higher amounts of fermentable polysaccharides or to optimize cultivation conditions and harvest regimes in terms of carbohydrate yields. This might enable the production of microalgae biomasses that have a higher ruminal fermentability.

Cell disruption with a ball mill affected ruminal fermentation of microalgae and thus their nutritional value. Cell disruption enhanced ruminal fermentation for most of the samples, presumably by the disruption of the microalgae cell walls and a resulting enhancement of the accessibility of intracellular nutrients by rumen microbiota. The extent of the effects associated with cell disruption varied amongst and also within the investigated microalgae genera. This variation appeared to be related to differences in cell wall composition and structure of the investigated samples. In terms of the protein value for ruminants, the applied cell disruption treatment had a rather negative impact. Since RUP concentration was decreased and IDP was overall unaffected, the amount of intestinal digestible RUP was decreased by cell disruption with a ball mill. Therefore, mechanical cell disruption with a ball mill cannot be recommended when microalgae are intended to be used as protein source for ruminants. Future research should therefore strive for the identification or development of processing methods that achieve an increase in the amount of intestinal digestible RUP.

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CHAPTER 5

INCLUDED MANUSCRIPTS

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5.1. Manuscript 1

VARIABILITY IN NUTRIENT COMPOSITION AND *IN VITRO* CRUDE PROTEIN DIGESTIBILITY OF 16 MICROALGAE PRODUCTS

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Variability in nutrient composition and in vitro crude protein digestibility of 16 microalgae products

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Abstract

The chemical composition of 16 microalgae products of four genera, *Arthrospira* (*n* = 2), *Chlorella* (*n* = 8), *Nannochloropsis* (*n* = 4) and *Phaeodactylum* (*n* = 2), was assayed to evaluate the intra- and inter-genera variation of nutrient profiles of commercial microalgae products. Crude protein was the main component in all genera, followed by ether extract and crude ash. Mean crude protein concentrations were 690, 502, 431 and 446 g/kg dry matter, and mean ether extract concentrations were 63, 157, 188 and 113 g/kg dry matter for *Arthrospira*, *Chlorella*, *Nannochloropsis* and *Phaeodactylum* respectively. However, there was considerable inter- and intra-genera variation. The concentration of α-linked glucose was low (0–143 g/kg dry matter). There was high variation between and within genera in the crude ash concentration (22–237 g/kg dry matter), which was also observed for the mineral composition. In contrast to the crude protein concentration, the amino acid composition of the protein (g amino acid/16 g N) was less variable. The investigated samples possessed high concentrations of Glx, Asx and Leu, and low concentrations of Cys and Met. The mean concentration of non-protein nitrogen compounds was highest in *Phaeodactylum* (110 g/kg dry matter) and lowest in *Nannochloropsis* (47 g/kg dry matter) products, and as with proximate nutrients, high variability between and within genera was observed. In vitro crude protein digestibility varied between 54% (non-cell-disrupted *Nannochloropsis*) and 84% (cell-disrupted *Chlorella*). Inositol phosphate isomers were not detectable in any sample (concentration <1 μmol/g dry matter). The predominant fatty acids were C16:0 in *Arthrospira* products, C18:2 n-6+ C19:1 t7 and C18:3 n-3 in *Chlorella* products, and C20:5 n-3 in *Nannochloropsis* and *Phaeodactylum* products; however, the relative proportions of fatty acids varied within genera. Commercially available microalgae products appear to be valuable alternative food and feed products. However, because of the high variability in nutrient profiles, attention should be given to the analytical characterization of the products.

KEYWORDS

amino acids, fatty acids, in vitro digestibility, inositol phosphate, microalgae, minerals

1 | INTRODUCTION

The predicted rise in the world's population from about seven billion to more than nine billion people within the next decades will lead to a 50% increase in the demand for meat and dairy products and concurrently, the area of arable land is shrinking (Bruinsma, 2011). To meet this rising demand, it will be necessary to further improve the utilization of food and feed products and to establish alternative resources, which should preferably be produced independently from arable land.

Microalgae are a heterogeneous assemblage of unicellular photosynthetic organisms, including Eukaryotes and Prokaryotes. They can be cultivated in areas that are not suitable for agriculture (Schuhmann & Schenk, 2013) and some of them have promising nutritional properties for which they are considered as alternative feed and food resources. Genera like *Chlorella* or *Arthrospira* are discussed as novel protein sources as they can have crude protein (CP) concentration of up to 70% of dry matter with an amino acid (AA) composition resembling that of soy protein (Becker, 2007) or of animal protein (Khatun, Kamal, Huque, Chowdhury, & Nahar, 1998). Other genera, such as *Nannochloropsis* or *Phaeodactylum*, are regarded as potential commercial sources of omega-3 fatty acids (Ryckebosch, Bruneel, Muylaert, & Foubert, 2012). In addition, some genera can be rich sources of vitamins (Brown, Jeffrey, Volkman, & Dunstan, 1997; Fabregas & Herrero, 1990), carotenoids (Brown & Jeffrey, 1992) and minerals (Fabregas & Herrero, 1986). Due to these properties, microalgae may be a promising food and feed resource. Nevertheless, for their efficient use in human and animal nutrition, reliable information regarding their nutritional value is required. Cultivation conditions (e.g., light, media composition, temperature, CO_2 supply and pH) affect the relative proportions of proximate nutrients, and to a lesser extent, the composition of chemical fractions (Volkman & Brown, 2006). Therefore, varying cultivation conditions may lead to variability in chemical composition and consequently, to variability in the nutritional value of microalgae.

Thus, it was hypothesized that the nutrient profiles of commercial microalgae biomasses vary greatly between and within genera. To verify this hypothesis, 16 commercially available microalgae products (MAP) of four genera were assayed for their chemical composition, including analyses of proximate nutrients, AA, other nitrogenous compounds, fatty acids, α-linked glucose, energy, minerals and inositol phosphates.

2 | MATERIAL AND METHODS

2.1 | Microalgae products and sample preparation

Sixteen MAP of different origin were investigated, including MAP declared as *Arthrospira (n = 2)*, *Chlorella (n = 8)*, *Nannochloropsis (n = 4)* and *Phaeodactylum (n = 2)*. The products were non-cell-disrupted, whole algae biomasses, which were purchased from different commercial producers and thus cultivation conditions were largely unknown (Table 1). They may contain impurities of other microalgae species, micro-organisms or inorganic compounds (e.g., residues of the cultivation medium). They were delivered as powder or as slurry. When delivered as slurry, MAP were lyophilized (DELTA 1-24 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and then ground to powder with a vibrating disc mill (Pulverisette 9, Fritsch GmbH, Idar-Oberstein, Germany). All MAP were vacuum-packed and then stored as powder at about −30°C until further processing.

TABLE 1 Information on microalgae products[a]

Sample	Cultivation	Drying method
A. platensis 1	Autotrophic	Spray drying
A. platensis 2	Autotrophic	Spray drying
C. vulgaris 1	Autotrophic	Unknown
C. vulgaris 2	Mixotrophic	Spray drying
C. vulgaris 3	Mixotrophic	Spray drying
C. vulgaris 4	Autotrophic	Spray drying
C. vulgaris 5	Autotrophic	Spray drying
C. protothecoides 1	Heterotrophic	Spray drying
C. protothecoides 2	Heterotrophic	Spray drying
C. sorokiniana	Heterotrophic	Spray drying
N. oceanica 1	Mixotrophic	Spray drying
N. oceanica 2	Mixotrophic	Spray drying
N. oculata 1	Autotrophic	Lyophilization
N. oculata 2	Autotrophic	Spray drying
P. tricornutum 1	Autotrophic	Lyophilization
P. tricornutum 2	Autotrophic	Lyophilization

Note. [a]According to the suppliers.

The MAP were treated with a stirred ball mill (Dyno Mill KDL A, Willy A. Bachofen AG – Maschinenfabrik, Muttenz, Switzerland) to disrupt cells. For this, a subset of the microalgae material was suspended in distilled water at a dilution of 1:9. The microalgae suspension was pumped through a stirred ball mill at a pumping capacity of about 50 ml/min, where the grinding chamber was filled with glass beads (diameter 0.5 mm) to 80% capacity. The rotational speed was 3,200 rpm and two runs were conducted for each MAP. The success of cell disruption was evaluated via microscopy. The treated MAP were frozen, lyophilized and ground as previously described. Treated MAP were vacuum-packed and stored at about −30°C until being analysed.

2.2 | Chemical analyses of microalgae products

All analyses except in vitro crude protein digestibility (IVPD) were performed in duplicate. Official methods of the Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA, 1976) were used to assay concentrations of crude ash (CA, method 8.1), CP as nitrogen (N) × 6.25 (method 4.1.1), true protein (TP, method 4.4.1) and ether extract (EE, method 5.1.1 B). Non-protein nitrogen (NPN) was calculated as the difference between CP

and TP. In addition to the CP concentration, the protein concentration was calculated by multiplying the N concentration with a generally accepted nitrogen-to-protein conversion factor for microalgae of 4.78 (Lourenço, Barbarino, Lavín, Lanfer Marquez, & Aidar, 2004) or species-specific nitrogen-to-protein conversion factors (Lopez et al., 2010; Lourenço et al., 2004; Tibbetts, Whitney, et al., 2015).

Analyses of minerals were performed using the non-cell-disrupted MAP, to avoid potential contamination during processing. The concentration of calcium (Ca), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), sodium (Na), sulphur (S) and zinc (Zn) were determined using inductively coupled plasma optical emission spectrometry ((VDLUFA, 2011), method 2.2.2.6). Arsenic (As), cadmium (Cd), copper (Cu), iodine (I), lead (Pb) and selenium (Se) were determined using inductively coupled plasma mass spectrometry ((VDLUFA, 2011), method 2.2.2.5) and mercury (Hg) was determined using cold vapour atomic absorption spectrometry ((VDLUFA, 2011), method 2.2.2.9).

A bomb calorimeter (C 200; Ika-Werke GmbH & Co. KG, Staufen, Germany) was used to determine gross energy using benzoic acid as a standard. The concentration of α-linked glucose was determined enzymatically. The samples were successively treated with hydrochloric acid (8 M) and dimethyl sulfoxide, and subsequently incubated at 60°C for 30 min in a shaking water bath. Then, samples were cooled to room temperature, water was added and the pH value of the samples was adjusted to 4–5 by adding sodium hydroxide. The sample solution was allowed to settle for 30 min and the supernatant was used for analyses. Soluble polysaccharides were hydrolysed to glucose and quantified using an enzymatic glucose UV-test (Boehringer Mannheim, 1994) via the spectrophotometric determination (Type Lambda 25, PerkinElmer, Waltham, MA, USA) of NADPH at 340 nm.

The organic residue was defined as the difference between 1,000 and the sum of the concentrations of CA, CP, EE and α-linked glucose expressed as g/kg dry matter and was a measure of the sum of all constituents that could not be identified by any of the applied methods.

Concentrations of AA were determined as described by Rodehutscord, Kapocius, Timmler, and Dieckmann (2004) with minor modifications, using the non-cell-disrupted MAP for analysis. Briefly, samples were oxidized using a mixture of performic acid, hydrogen peroxide and phenol, and then hydrolysed with hydrochloric acid (6 M) containing 1 g/L phenol for 24 hr at 110°C. Norleucine was used as the internal standard. Amino acids were separated and detected by ion-exchange chromatography using an AA analyser (Hitachi, L-8900, Tokyo, Japan) with post-column ninhydrin derivatization. Methionine and cysteine were determined as methionine sulphone and cysteic acid respectively. Asparagine and aspartic acid, as well as glutamine and glutamic acid, could not be distinguished by analysis and are therefore labelled as Asx and Glx. Tryptophan was determined separately after alkaline hydrolysis with barium hydroxide (Scheuermann & Eckstein, 1986) by reverse-phase chromatography and fluorescence detection (Agilent 1100 HPLC, Agilent, Waldbronn, Germany) at an excitation wavelength of 283 nm and an emission wavelength of 355 nm. Amino acids are expressed as g/16 g N.

The IVPD was determined according to Boisen and Fernández (1995) as an estimation of protein digestibility in pigs, including some modifications to ensure suitability for microalgae. Analyses were performed for the non-cell-disrupted and the cell-disrupted sample of each microalgae product in fourfold replication. The samples were suspended in a phosphate buffer solution and 0.2 M hydrochloric acid and pH was adjusted to 2.0 using 5 M hydrochloric acid and 4 M NaOH. Porcine pepsin (107190, Merck KGaA, Darmstadt, Germany) and thymol, to avoid microbial contamination, were added and the samples were incubated under constant stirring in a water bath for 6 hr at 40°C. NaOH (0.6 M) and a phosphate buffer solution were added and pH was adjusted to 6.8 as described previously. Porcine pancreatin (P1750, Sigma-Aldrich, Saint Louis, MO, USA) was added and the samples were incubated in a water bath under constant stirring at 40°C for 18 hr. Subsequently, the samples were cooled to 20°C and insoluble protein was precipitated using 20% sulfosalicylic acid. Two aliquots of the sample solution were centrifuged and soluble nitrogen was analysed in the supernatant using Kjeldahl steam distillation. IVPD was calculated as the amount of soluble nitrogen relative to the amount of initially incubated nitrogen.

Fatty acid methyl esters (FAME) were prepared as suggested by Kramer et al. (1997) with a combination of sodium methylate solution and boron trifluoride. FAME standards included a mixture of 52 components purchased from Nu-Chek Prep (GLC 674, Elysian, USA) and completed by the addition of C18:4 n-3 (Sigma-Aldrich, Schnelldorf, Germany). The FAME were quantified using a gas chromatograph (Agilent 6890, Agilent, Waldbronn, Germany) equipped with a flame ionization detector, an automatic split/splitless injector and an SP2380 column (30 m, 250 µm, 0.2 µm; Supelco, Taufkirchen, Germany). Helium was the carrier gas with a column head pressure of 95 kPa. The temperature programme was as follows: 2 min at 40°C, 4°C/min to 130°C, 2°C/min to 160°C, 0.8°C/min to 175°C, 30°C/min to 250 and a hold time of 15 min. Other gas chromatography conditions were as follows: injector temperature 250°C, split mode, split flow 12.6 ml/min, split 10:1, total flow 16.4 ml/min, injection volume 0.5 µl, detector temperature 260°C, H_2 flow 33 ml/min, air flow 400 ml/min and combined flow 30 ml/min. The FAME C18:1 t6 and C18:1 t9, C18:2 n-6 and C19:1 t7, C20:3 n-6 and C20:4 n-6 as well as C22:4 n-6 and C24:1 could not be distinguished by analysis; therefore, the sum of the respective FAME is presented.

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis [dihydrogen phosphate] or $InsP_6$) and other inositol phosphate isomers ($InsP_X$) were determined as described by Zeller, Schollenberger, Kühn, and Rodehutscord (2015). In brief, samples were extracted using 0.2 M ethylenediaminetetraacetic acid and 0.1 M sodium fluoride at pH 10. Subsequently, high-performance ion-exchange chromatography with post-column derivatization (Dionex ICS-3000, Idstein, Germany, CarboPac® PA 200 column) was used for analysis.

2.3 | Calculations and statistical methods

Means and ranges were determined for each genus, and the coefficient of variation (CV) was calculated for the genera *Arthrospira* and *Chlorella* using PROC MEANS of SAS (Version 9.3, SAS Institute, Cary, NC, USA). In case the analysed values were between the limits of detection and quantification of the respective compound, the average value between the limits of detection and quantification was used for subsequent statistical analyses. If the analysed values were below the limit of detection, 0 was used for statistical analyses. The range and CV were only determined when more than 50% of the analysed values for the respective genera were above the limit of quantification. In cases where more than 50% of the analysed values were below the limit of quantification, this is indicated in the respective tables with "o."

3 | RESULTS AND DISCUSSION

3.1 | Proximate nutrient composition, α-linked glucose and gross energy

Crude protein was the main component of almost all analysed microalgae samples, and the mean CP concentration ranged from 431 g/kg dry DM in *Nannochloropsis* to 690 g/kg DM in *Arthrospira* (Table 2). The mean CP concentration in *Chlorella* was 502 g/kg DM, but ranged from 81 to 623 g/kg DM and showed a high CV (34%). This considerable intra-genus variability in the CP concentration also applied to the other genera. Different studies have shown that protein concentration can be affected by factors such as temperature (Morris, Glover, & Yentsch, 1974), light intensity (Morris et al., 1974), salinity (Khatoon et al., 2014) and N (Michelon et al., 2016; Piorreck, Baasch, & Pohl, 1984) or phosphorus supply (Healey & Hendzel, 1979; Michelon et al., 2016). Furthermore, the time of harvest may have led to differences in the CP concentration as CP levels change during algae growth phases (Fernández-Reiriz et al., 1989; Piorreck & Pohl, 1984; Whyte, 1987). These factors have presumably led to considerable intra-genera variability in CP concentration. Nevertheless, the values reported herein are in general accordance with previously published findings (Becker, 2007; Tibbetts, Milley, & Lall, 2015).

Mean CA concentration ranged from 71 g/kg DM in *Arthrospira* and *Chlorella* to 169 g/kg DM in *Phaeodactylum*. The mean CA concentration in *Chlorella* was high compared with reported values (Tibbetts, Whitney, et al., 2015). In contrast, other studies (Kent, Welladsen, Mangott, & LI, 2015; Matos et al., 2016; Tibbetts, Milley, & Lall, 2015) have reported values that are consistent with the values reported in the present study. In the marine and brackish genera *Phaeodactylum* and *Nannochloropsis* CA concentrations were notably high (>10%). High ash concentrations may limit the application quantities, as ash dilutes the nutrient and energy concentration of food and feed products. Furthermore, considerable intra-genera variation was observed in *Chlorella* and *Nannochloropsis*. The CV was 36% and 50% for these two genera respectively. This variation was also apparent in the mineral composition, and may result from differences in the mineral composition of the cultivation medium (Barsanti & Gualtieri, 2014; Grobbelaar, 2013) or may represent variable amounts of residues of the cultivation medium, which has not been removed completely during downstream processing. Therefore, standardization of culture medium and downstream processing techniques appears necessary to avoid high and unpredictable CA and mineral concentrations.

The mean EE concentration varied from 63 g/kg DM in *Arthrospira* to 188 g/kg DM in *Nannochloropsis*. Similar to the findings for CP and CA, considerable intra-genera variability was observed for EE. This is likely related to the variable cultivation conditions, as the EE concentration can be affected by nutrient supply (Reitan, Rainuzzo, & Olsen, 1994; Rodolfi et al., 2009), irradiance (Rodolfi et al., 2009) or temperature (Converti, Casazza, Ortiz, Perego, & Del Borghi, 2009). Determination of lipid concentration with gravimetric solvent extraction methods like EE determination may overestimate true lipid concentration of microalgae as it may extract non-lipid compounds like lipid soluble proteins, soluble carbohydrates, chlorophyll or other pigments (Laurens et al., 2012). Therefore, variability in EE concentrations in the investigated microalgae might also result from

TABLE 2 Proximate nutrient composition, α-linked glucose and gross energy concentration of 16 microalgae products [g/kg dry matter (DM) unless otherwise stated]

	Arthrospira (*n* = 2)		*Chlorella* (*n* = 8)			*Nannochloropsis* (*n* = 4)			*Phaeodactylum* (*n* = 2)	
	Mean	Range	Mean	Range	CV [%]	Mean	Range	CV [%]	Mean	Range
Crude ash	71	64–77	71	22–102	36	144	62–237	50	169	161–177
Ether extract	63	51–74	157	75–549	101	188	162–252	23	113	99–127
Crude protein	690	651–729	502	81–623	34	431	350–500	17	446	429–462
α-linked glucose	50	28–72	84	31–143	48	4.0	1.0–7.0	74	0.5	n.d.–1.0
Organic residue	127	92–162	187	85–253	31	233	184–272	19	272	250–294
Gross energy [MJ/kg DM]	22.6	22.5–22.6	23.1	19.7–30.4	14	23.5	22.0–24.7	5	19.5	19.2–19.7

Note. Organic residue: 1,000 − Crude ash − Crude protein − Ether extract − α-linked glucose; CV: Coefficient of variation; n.d.: not detected.

varying amounts of compounds which were extracted in addition to true lipids.

Mean gross energy content varied from 19.5 MJ/kg DM in *Phaeodactylum* to 23.5 MJ/kg DM in *Nannochloropsis*. Mean concentrations of α-linked glucose were generally low, and varied from 0.5 g/kg DM in *Phaeodactylum* to 84 g/kg DM in *Chlorella*. We prefer to not term this fraction as "starch" because the applied assay included acid hydrolysis followed by enzymatic hydrolysis using the enzyme amyloglucosidase, cleaving α-1,4 and α-1,6-glucan bonds of polysaccharides. Microalgae have been described to contain glucose as monosaccharides besides starch (de Jesus Raposo, de Morais, & de Morais, 2013, 2014; Sui, Gizaw, & BeMiller, 2012) and with α-1,4 or α-1,6-glucan bonds (e.g., chrysolaminarin in *Phaeodactylum*) (Goo et al., 2013; Gügi et al., 2015). Such polysaccharides might have been degraded to glucose by acid hydrolysis or amyloglucosidase or both and hence might have mimicked starch. Consequently, it cannot be distinguished between α-linked glucose and true starch. The official methods (VDLUFA, 1976) for the determination of neutral (method 6.5.1) and acid (method 6.5.2) detergent fibre were used to characterize the non-starch polysaccharide fraction of the MAP. Nevertheless, neither these original methods, nor modifications (e.g., filter materials, inclusion of centrifugation) yielded reliable and repeatable results. Therefore, the use of these methods is not appropriate for the characterization of the non-starch polysaccharide fraction of microalgae, and cannot be recommended for microalgae. Mass balancing of the analysed fractions to 1,000 g/kg DM revealed an organic residue, which could not be assigned to the analysed fractions. As the fraction of non-starch polysaccharide could not be considered in this calculation, the organic residue is assumed to consist mainly of this fraction. For *Chlorella* and *Nannochloropsis* species the occurrence of aliphatic, non-hydrolysable bio-macromolecules, termed algaenans, was described (Gelin et al., 1999) and might have contributed to the organic residue in these genera. Additionally, the monosaccharides of nucleic acids might have considerably contributed to this fraction as nucleic acid concentration of microalgae can make up 4%–6% of dry matter (Becker, 2013). Furthermore, the organic residue may consist of various soluble and insoluble carbohydrate-like compounds.

3.2 | Protein and amino acid composition

The TP concentration varied from 336 g/kg DM in *Phaeodactylum* to 620 g/kg DM in *Arthrospira*, and the sum of AA ranged from 375 to 628 g/kg DM respectively (Table 3). As found for the CP concentration, considerable intra- and inter-genera variations were observed in the TP concentration and in the sum of AA. The concentration of TP and the sum of AA were generally lower than the CP concentration, which presumably resulted from the existence of NPN compounds. Furthermore, there was high correspondence between the TP concentration and the sum of AA, which indicates that TP determination is a good measure of protein in microalgae when data on AA concentrations are not available.

Mean NPN concentrations were 70, 86, 47 and 110 g/kg DM, which equals 10%, 16%, 11% and 25% of CP for *Arthrospira*, *Chlorella*, *Nannochloropsis* and *Phaeodactylum* respectively. Among others, NPN compounds in microalgae can be free AA (Al-Amoudi & Flynn, 1989; Dortch, Clayton, Thoresen, & Ahmed, 1984; Flynn, Garrido, Zapata, Öpik, & Hipkin, 1992), nucleic acids (Dortch et al., 1984; Lourenço, Barbarino, Marquez, & Aidar, 1998; Lourenço et al., 2004) and nitrogenous pigments, such as chlorophylls (Lourenço et al., 1998, 2004). Furthermore, NPN compounds can originate from intracellular inorganic nitrogenous compounds, such as nitrate, nitrite and ammonium (Dortch, 1982; Dortch et al., 1984; Lourenço et al., 1998, 2004). In several MAP, the sum of analysed AA was greater

TABLE 3 Nitrogenous compounds and in vitro crude protein digestibility (IVPD) of 16 microalgae products [g/kg dry matter unless otherwise stated]

	Arthrospira (*n* = 2)		*Chlorella* (*n* = 8)			*Nannochloropsis* (*n* = 4)			*Phaeodactylum* (*n* = 2)	
	Mean	Range	Mean	Range	CV [%]	Mean	Range	CV [%]	Mean	Range
N × 6.25	690	651–729	502	81–623	34	431	350–500	17	446	429–462
N × 4.78	529	497–561	387	61–481	35	331	270–382	17	345	331–360
N × k[a]	658	619–698	421	66–523	35	342	280–396	17	352	337–366
True protein	620	614–626	415	74–518	36	384	319–435	14	336	326–346
Sum of AA	628	582–675	416	70–491	34	380	295–453	20	375	369–381
NPN	70	37–103	86	7.0–298	106	47	31–65	40	110	103–116
IVDP [%][b]										
Non-cell-disrupted	74	71–78	79	72–86	5	54	48–59	9	77	76–77
Cell disrupted	78	73–83	84	80–94	5	79	78–80	1	83	82–84

Notes. NPN: Non-protein-nitrogen = Crude protein (N × 6.25)—True protein; Sum of AA: Sum of the 20 analysed amino acids; CV: Coefficient of variation.

[a]Species-specific nitrogen-to-protein conversion factor (Lopez et al., 2010; Lourenço et al., 2004; Tibbetts, Whitney, et al., 2015). [b]IVPD according to the method of Boisen and Fernández (1995) measured in non-cell-disrupted and cell-disrupted microalgae biomass.

than the true protein concentration, which indicates the existence of free AA in the samples of the present study. NPN concentrations were within the range previously described in the literature (Becker, 2007; Templeton & Laurens, 2015). Nevertheless, variability in the proportion of NPN in the total N was high within genera. This variation may result from a varying N supply and harvest regime, as assimilation of nitrogenous compounds in microalgae cells can be affected by the amount (Al-Amoudi & Flynn, 1989; Dortch, 1982; Dortch et al., 1984; Flynn et al., 1992; Lourenço et al., 2004) and source of N supplied (Dortch, 1982; Dortch et al., 1984; Flynn et al., 1992) and changes during the life cycle (Lourenço et al., 1998, 2004). The high amounts of NPN compounds may lead to an overestimation of protein concentration when applying the general nitrogen-to-protein conversion factor of 6.25 to microalgae (Lee & Picard, 1982). Lourenço et al. (2004) reported a generally accepted nitrogen-to-protein conversion factor for microalgae of 4.78. Application of this factor to the microalgae biomass used in the present study appeared to underestimate the protein concentration when compared with the sum of the AA or TP concentration. The application of species-specific nitrogen-to-protein conversion factors (Lopez et al., 2010; Lourenço et al., 2004; Tibbetts, Whitney, et al., 2015) led to a closer agreement between calculated and analysed protein concentrations. Nevertheless, unpredictable variability in the NPN concentration, even within microalgae species, restricts the applicability of such factors. Thus, the determination of TP concentration appears more reasonable than the use of species-specific conversion factors as it considers NPN compounds. Therefore, when data on the AA composition are not available, analysis of TP concentration should be preferred.

In the non-cell-disrupted MAP, mean IVPD was highest in *Chlorella* (79%) and lowest in *Nannochloropsis* (54%). Cell disruption increased IVPD in all genera, but the extent of the increase was variable. In *Nannochloropsis*, cell disruption led to a 49% increase relative to the non-cell-disrupted samples. The increase was considerably lower in *Arthrospira* (5%), *Chlorella* (6%) and *Phaeodactylum* (8%). Variability within genera was highest in *Chlorella* products and lowest in *Phaeodactylum*, regardless of cell disruption. In *Nannochloropsis*, the cell-disrupted samples showed a considerably lower variability (78%–80%) than the non-cell-disrupted ones (48%–59%). In vitro digestibility values for non-cell-disrupted *Arthrospira* and *Chlorella* biomass of 75% and 70% reported by Batista et al., (2017) are in accordance with the results of the present study, whereas the in vitro digestibility of 50% for *Phaeodactylum* was considerable lower than the results of the present study. (Tibbetts, Milley & Lall, 2015) reported IVPD values of 88%, 87%, 85% and 89% for non-cell-disrupted *Arthrospira*, *Chlorella*, *Nannochloropsis* and *Phaeodactylum* respectively. Compared to the IVPD values of the present study, these values are higher for all microalgae genera even when the cell-disrupted samples are considered and especially for non-cell-disrupted *Nannochloropsis*. Furthermore, the authors compiled IVPD values of five studies for *Chlorella* and of two studies for *Arthrospira*. The values ranged from 27% to 97% for Chlorella and from 70% to 85% for *Arthrospira*. They primarily attributed this wide range to methodical differences, such as differences in the enzyme mixtures, assay conditions, sample processing and cell disruption. In a further study, Tibbetts and co-workers used three approaches to determine protein digestibility for non-ruminant animals of different microalgae species. Values for protein solubility, dilute pepsin digestibility and two phase gastric/pancreatic digestibility of protein for the same *Chlorella vulgaris* sample were 84%, 80% and 70% respectively (Tibbetts, MacPherson, McGinn, & Fredeen, 2016). Therefore, methodical differences might also explain discrepancy between the results of the present study and previous results. The differences in the present study between microalgae genera in the extent of the effect of the cell disruption may be related to different cell wall structure and composition. For example, *Arthrospira* has a thin cell wall made of four layers of fibrils and peptidoglycan (van Eykelenburg, 1977) which can be expected to be easily digested by pepsin and pancreatin. Therefore, the disruption of cells does only release a small amount of additionally soluble nitrogen. On the other hand, cell walls of *Nannochloropsis* consist of a cellulosic inner cell wall which is surrounded by an algaenan layer (Scholz et al., 2014). Both are expected to be hardly digested by the applied enzymes. Therefore, the amount of additionally released nitrogen by cell disruption was high in the genera. Low IVPD of non-cell-disrupted *Nannochloropsis* is in accordance with the results of Cavonius, Albers, and Undeland, (2016) who reported a low degree of protein hydrolysis of *Nannochloropsis* in a multistage in vitro system. Furthermore, the authors observed an increase of the degree of protein hydrolysis by cell disruption. For *Chlorella*, it has been shown that ultrasound treatment or high-pressure homogenization can significantly increase CP and amino acid digestibility in rats (Janczyk, Franke, & Souffrant, 2007; Janczyk, Wolf, & Souffrant, 2005) and Atlantic salmon (Tibbetts et al., 2017). Furthermore, an increase of in vitro digestibility in *Scenedesmus* algae was observed after cell disruption treatment with a ball mill (Hedenskog, Enebo, Vendlová, & Prokes, 1969). Because of methodical and animal species differences immediate comparisons between studies do not appear to be reasonable. Nevertheless, the general increasing effect induced by cell disruption might be explained by higher accessibility of nutrients after destruction of the microalgae cell walls which appears to be independent from microalgae and animal species. However, high-pressure homogenization and electroporation decreased CP and amino acid digestibility in rats (Janczyk et al., 2005, 2007; Komaki et al., 1998), indicating that there are cell disruption methods which do not lead to an enhanced nutrient accessibility with all microalgae species.

The mean Lys concentration varied from 4.0 g/16 g N in *Arthrospira* protein to 5.6 g/16 g N in *Chlorella* and *Nannochloropsis* protein (Table 4). All MAP had low concentrations of Met and Cys, whereby the range for mean Met concentration was 1.7 in *Chlorella* to 2.1 g/16 g N in *Arthrospira* and mean Cys concentration ranged from 0.6 g/16 g N in *Phaeodactylum* to 0.8 in *Chlorella* protein. Mean concentration of Trp ranged from 1.2 g/16 g N in *Arthrospira* to 1.6 g/16 g N in *Nannochloropsis*. All MAP possessed a high concentration of Glx and Asx. The mean Glx concentration varied from

TABLE 4 Amino acid composition of protein of 16 microalgae products [g/16 g of nitrogen]

	Arthrospira (*n* = 2)		*Chlorella* (*n* = 8)			*Nannochloropsis* (*n* = 4)			*Phaeodactylum* (*n* = 2)	
	Mean	Range	Mean	Range	CV [%]	Mean	Range	CV [%]	Mean	Range
Ala	7.2	6.8–7.6	7.0	3.9–8.3	20	6.6	6.2–6.9	5	6.8	6.4–7.2
Arg	6.6	6.3–7.0	7.4	5.2–20	68	5.2	5.1–5.4	3	5.0	4.8–5.2
Asx	9.6	9.4–9.8	7.6	3.3–9.0	24	8.4	8.2–8.6	2	9.6	9.5–9.6
Cys	0.7	0.7–0.7	0.8	0.6–0.9	15	0.7	0.6–0.8	9	0.6	0.6–0.6
Glx	14	13–16	12	9.9–23	39	11	9.9–12	9	11	11–11
Gly	4.4	4.1–4.7	4.6	2.1–5.2	23	5.2	5.1–5.4	2	4.9	4.6–5.3
His	1.7	1.6–1.9	2.0	1.0–2.2	21	2.0	2.0–2.1	2	1.7	1.7–1.7
Ile	4.8	4.6–5.0	2.9	1.3–3.4	24	3.8	3.6–4.2	8	3.9	3.7–4.0
Leu	8.1	7.8–8.5	7.1	3.1–8.6	24	8.1	7.9–8.4	3	7.0	6.8–7.1
Lys	4.0	3.6–4.4	5.6	2.2–7.0	29	5.6	5.2–6.0	6	4.9	4.7–5.1
Met	2.1	1.9–2.3	1.7	0.7–1.9	25	1.9	1.7–2.1	8	2.0	2.0–2.1
Phe	4.0	3.7–4.3	3.9	1.5–4.5	25	4.6	4.4–4.8	3	4.7	4.6–4.8
Pro	3.3	2.9–3.7	3.8	1.5–4.6	26	5.9	4.3–7.8	27	3.4	3.1–3.6
Ser	4.9	4.7–5.0	3.8	1.9–5.4	26	4.3	4.2–4.3	1	4.3	4.1–4.4
Thr	4.6	4.3–4.8	4.1	1.8–5.3	25	4.7	4.5–4.9	3	4.7	4.5–5.0
Trp	1.2	1.1–1.2	1.5	0.6–1.8	24	1.6	1.5–1.8	9	1.3	1.3–1.3
Tyr	3.6	3.3–3.8	2.7	1.1–3.1	24	2.9	2.8–3.2	6	2.7	2.7–2.8
Val	5.6	5.5–5.6	4.5	2.1–5.4	23	5.0	4.8–5.4	5	4.8	4.5–5.2

Note. CV: Coefficient of variation.

11 g/16 g N in *Nannochloropsis* and *Phaeodactylum* to 14 g/16 g N in *Arthrospira*, and the mean Asx concentration ranged from 7.6 g/16 g N in *Chlorella* to 9.6 g/16 g N in *Arthrospira* and *Phaeodactylum*. When compared with rapeseed meal, the concentrations of Ala, Arg, Asp, Gly, Leu, Met, Phe, Ser, Thr, Trp, Tyr and Val were higher or similar, whereas the concentrations of Cys, Glu and His were lower in all investigated microalgae genera. The Lys concentration was similar in *Chlorella* and *Nannochloropsis* products, but lower in *Arthrospira* and *Phaeodactylum* products, when compared with rapeseed meal. The AA profile of almost all investigated MAP revealed lower concentrations of Arg, Asp, Cys, Glu, His, Lys, Phe, Pro and Tyr, but similar or higher concentrations of Ala, Gly, Ile, Leu, Met, Ser, Thr, Trp and Val than soybean meal (Agroscope, c2011-2016; DLG, c2006–2010). Thus, considering their high protein concentration, microalgae are promising alternative protein sources for animal nutrition. Nevertheless, for a conclusive evaluation of the nutritional value of microalgae protein, data on the digestibility of AA is needed in addition to CP composition and IVPD. Currently, data on AA digestibility of microalgae is very scarce with only two studies containing data on the AA digestibility of *Chlorella vulgaris* in rats (Janczyk et al., 2005) and Atlantic salmon (Tibbetts et al., 2017). In rats, apparent AA digestibility of diets containing 21% of untreated, electroporated or ultrasonic-treated was low: In the untreated *Chlorella vulgaris* biomass apparent AA digestibility of the diets ranged from 35% (Tyr) to 78% (Trp), from 37% (Ile, Cys) to 74% (Arg) in the electroporated and from 48% (Cys) to 77% (Arg) in the ultrasonic-treated biomass with a high uncertainty expressed by a high standard deviation between experimental replicates (3%–38%) (Janczyk et al., 2005).

A high CV (>15) for all AA in *Chlorella* resulted from one MAP with a very high NPN concentration (48% of CP) from heterotrophic cultivation. When this sample was excluded from the calculation, the CV for *Chlorella* was less than 10% for almost all AA. Furthermore, except for the MAP rich in NPN compounds, the AA composition of the proteins was highly similar amongst the investigated MAP. When CV was calculated across all MAP, excluding the NPN-rich MAP from the calculation, the CV was smaller than 15% for almost all AA. The AA with higher variability between species were Ile, Lys and Pro, with a CV of 16.3%, 18.2% and 29.1% respectively. These few exceptions may have nutritional importance; for example, Lys is one of the first limiting amino acids in farm animals. Nevertheless, Volkman and Brown (2006) compiled data from 5 studies and 37 microalgae species and also found a high similarity in the AA composition, with mean values consistent with the findings of the present study. Furthermore, studies have shown that the AA composition of the protein is not affected by irradiance (Brown, Dunstan, et al., 1993), growth phase, harvest regime (Brown, Garland, Jeffrey, Jameson, & Leroy, 1993) or nutrient supply (Daume, Long, & Crouch, 2003; von Alvensleben, Magnusson, & Heimann, 2016). This indicates that the protein composition is quite stable in microalgae, even between different genera which was already observed previously (Brown et al., 1997).

TABLE 5 Fatty acid composition of 16 microalgae products [g/100 g of total fatty acids]

	Arthrospira (*n* = 2)		*Chlorella* (*n* = 8)			*Nannochloropsis* (*n* = 4)			*Phaeodactylum* (*n* = 2)	
	Mean	Range	Mean	Range	CV [%]	Mean	Range	CV [%]	Mean	Range
C8:0	<LQo		<LQo			0.2	0.1–0.4	41	<LQo	
C10:0	7.7	4.3–11	0.2o			0.2	0.1–0.2	31	<LQo	
C12:0	<LQo		0.1	n.d.–0.4	118	0.3	0.3–0.4	11	<LQo	
C14:0	0.6	0.2–0.9	1.1	0.5–2.3	56	4.3	3.7–4.8	12	9.6	9.1–10
C16:0	45	45–45	22	17–26	14	20	15–31	35	18	16–21
C16:1 n-7	4.1	3.5–4.8	5.3	0.6–19	117	21	13–28	28	23	16–29
C18:0	0.8	0.6–0.9	1.9	0.3–4.0	68	0.3	0.1–0.7	78	0.5	0.4–0.6
C18:1 n-9	3.5	2.6–4.4	15	3.2–66	141	5.8	2.4–11	61	2.4	1.2–3.7
C18:1 n-7	0.5	0.5–0.6	2.7	n.d.–11	130	0.6	0.3–1	63	3.2	2.3–4.0
C18:1 t6+ C18:1 t9	n.d.o		<LQo			n.d.o			0.1o	
C18:2 n-6+ C19:1 t7	18	13–23	30	10–68	60	5.4	2.9–11	75	1.9	1.2–2.6
C18:3 n-6	18	17–19	<LQo			0.4	0.2–0.8	60	0.5	0.3–0.6
C18:3 n-3	0.1	0.1–0.1	20	1.6–37	64	3.1	0.2–12	186	0.6	0.6–0.7
C18:4 n-3	0.1	0.1–0.1	0.1	n.d.–0.5	130	0.1o			0.6	0.4–0.9
C20:0	0.1	0.1–0.1	0.3	0.1–0.5	59	<LQo			<LQo	
C20:3 n-3	0.4	0.4–0.4	n.d.o			0.3	0.2–0.4	31	0.1	0.1–0.1
C20:3 n-6+ C20:4 n-6	0.1o		0.1o			6.8	5.4–8.2	20	2.3	0.9–3.6
C20:5 n-3	<LQo		0.1o			31	16–44	40	33	24–42
C22:0	<LQo		0.1	<LQ–0.1	42	n.d.o			0.1	0.1–0.1
C22:2 n-6	<LQo		<LQo			<LQo			n.d.o	
C22:4 n-6+ C24:1 n-9	0.8	0.3–1.3	<LQo			<LQo			0.1	0.1–0.2
C22:5 n-3	n.d.o		n.d.o			<LQo			0.6	0.1–1.0
C22:5 n-6	<LQo		<LQo			<LQo			0.1o	
C22:6 n-3	<LQo		<LQo			<LQo			1.9	1.6–2.3
C24:0	0.2	0.1–0.4	0.2	0.1–0.2	35	<LQo			1.5	1.2–1.8
∑SFA	54	51–58	26	22–31	11	26	20–36	28	30	28–32
∑MUFA	8.1	6.6–9.7	23	4.7–67	86	27	21–39	29	28	20–37
∑PUFA	37	32–43	51	12–71	37	47	25–57	33	42	31–52
n3:n6-ratio	0.02	0.01–0.02	0.9	0.05–1.8	79	2.7	1.9–3.9	33	8.1	6.6–9.6

Note. SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acids; LQ: Limit of quantification (0.05); <LQ: Value was below the limit of quantification; n.d.: Value was below the limit of detection; o: More than 50% of analysed values of the respective genera were below the limit of quantification; thus other statistical values were not determined.

3.3 | Fatty acid composition

Arthrospira products were rich in saturated fatty acids, especially in C16:0, which made up 45% of the total fatty acids (Table 5). Other fatty acids present in *Arthrospira* in appreciable amounts were C18:2 n-6+ C19:1 t7 and C18:3 n-6. The fraction of polyunsaturated fatty acids (PUFA) in *Arthrospira* oil almost completely consisted of n-6 fatty acids. Interestingly, C18:3 n-6, a fatty acid not prevalent in larger quantities in oils of plant and fats of animal origin, makes up almost half of the total PUFA. The only other known sources of C18:3 n-6 are seeds of evening primrose, borage or black currant and fungi (*Mortierella*, *Aspergillus*) (Certik & Shimizu, 1999; Gill & Valivety, 1997). When metabolized by the animal, the fatty acid C18:3 n-6 is elongated to C20:3 cis n-6, which acts as a precursor of prostaglandin E_1 and 15-hydroxyeicosatrienoic acid. These metabolites attenuate inflammatory and proliferative processes (Ziboh, 2008). In *Arthrospira*, C18:3 n-6 appears to be associated with photosynthesis, as it is located in the galactosyl diglycerides of the chloroplasts

(Nichols & Wood, 1968) and its concentration is affected by the light regime (Ronda & Lele, 2008).

Chlorella products contained an oil rich in PUFA (51% of total fatty acids), mainly C18:2 n-6+ C19:1 t7 and C18:3 n-3, with mean concentrations of 30% and 20% of the total fatty acids respectively. Additionally, C16:0 and C18:1 n-9 were present in higher concentrations. Thus, with the exception of a higher C18:3 n-3 concentration in *Chlorella*, the fatty acid composition of many plant seed oils, such as rapeseed or sunflower (Sakhno, 2010), is quite similar to that of *Chlorella* oil.

Nannochloropsis and *Phaeodactylum* had considerable amounts of the omega-3 fatty acid C20:5 n-3. This can be metabolized to 3-series prostaglandins, thromboxane A_3, and 5-series leukotrienes, which have vasodilatory, platelet anti-aggregatory and neutrophil aggregatory activities (Lee & Hwang, 2008). Therefore, C20:5 n-3 is suggested to have health benefits, for instance, by reducing the risk for cardiovascular disease or cancer (Lunn & Theobald, 2006). Furthermore, *Nannochloropsis* and *Phaeodactylum* products had a mean n3:n6-ratio of 2.7 and 8.1 respectively. Accordingly, both genera are rich sources of omega-3 fatty acids. As long-chain PUFA from microalgae are enriched in milk (Glover et al., 2012; Vahmani, Fredeen, & Glover, 2013) or eggs (Bruneel et al., 2013; Fredriksson, Elwinger, & Pickova, 2006; Lemahieu et al., 2013), *Phaeodactylum* and *Nannochloropsis* may be direct or indirect sources for omeg-3 fatty acids for human nutrition, when fed to cows and laying hens. C16:0 and C16:1 n-7 were present in major concentrations (about 20% of the total fatty acids) in *Nannochloropsis* and *Phaeodactylum* products. The latter contained higher amounts of C14:0 than the other investigated microalgae genera (9.6% of the total fatty acids). In *Nannochloropsis* products, the FAME C20:3 n-6+ C20:4 n-6, C18:2 n-6+ C19:1 t7 and C18:1 n-9 were present at higher concentrations than in *Phaeodactylum* products.

Despite the occurrence of predominant fatty acids in all investigated algae genera, the relative amounts varied appreciably within genera, and ranged considerably, as described by Volkman, Brown, Dunstan, and Jeffrey (1993) for marine algae from the class *Eustigmatophyceae*. Fatty acid composition is affected by factors such as nutrient limitation (Gong, Guo, Wan, Liang, & Jiang, 2013; Reitan et al., 1994; Yongmanitchai & Ward, 1991), temperature (Renaud, Thinh, Lambrinidis, & Parry, 2002) and CO_2 supply (Tsuzuki, Ohnuma, Sato, Takaku, & Kawaguchi, 1990). Furthermore, fatty acid composition changes during the growth phase (Ben-Amotz, Tornabene, & Thomas, 1985; Costard, Machado, Barbarino, Martino, & Lourenço, 2012; Dunstan, Volkman, Barrett, & Garland, 1993). Therefore, differences in fatty acid profiles within genera presumably result from different cultivation conditions.

TABLE 6 Mineral composition of 16 microalgae products

	Arthrospira (*n* = 2)		*Chlorella* (*n* = 8)			*Nannochloropsis* (*n* = 4)			*Phaeodactylum* (*n* = 2)		
	Mean	Range	Mean	Range	CV [%]	Mean	Range	CV [%]	Mean	Range	LQ
Major elements [g/kg dry matter]											
Calcium	1.4	1.0–1.8	3.0	0.2–7.8	105	5.9	2.6–11	69	16	8.2–24	0.01
Magnesium	2.8	2.5–3.2	2.1	0.4–3.0	42	5.3	2.4–11	69	4.3	2.2–6.5	0.005
Phosphorus	8.3	7.5–9.2	11	3.2–16	40	13	11–17	18	25	23–28	0.005
Potassium	11	9.5–13	17	3.3–24	44	15	10–22	30	16	15–18	0.02
Sodium	5.6	4.9–6.2	1.4	0.1–5.3	140	25	3.9–52	79	17	12–21	0.02
Sulphur	6.7	6.0–7.5	4.7	0.6–7.6	47	6.1	4.8–7.1	18	10	8.4–12	0.02
Trace elements [mg/kg dry matter]											
Copper	1.0	0.7–1.4	11	0.3–23	81	15	1.5–24	70	3.5	2.2–4.8	0.05
Iron	423	260–586	638	5.8–1,773	97	627	254–1,101	56	1,553	973–2,134	2.0
Iodine	0.3	0.1–0.6	0.3	0.1–1.1	132	1.8	0.5–3.7	76	1.3	1.0–1.7	0.05
Manganese	26	20–32	34	9.7–57	60	69	36–124	57	174	165–184	2.0
Selenium	0.3	0.1–0.4	0.1○			0.1	<LQ–0.3	86	0.1	0.1–0.1	0.05
Zinc	7.7	6.5–8.8	49	29–96	52	55	33–78	39	38	38–39	2.0
Heavy metals [mg/kg dry matter]											
Arsenic	1.3	0.2–2.4	0.1○			0.6	<LQ–1.5	108	<LQ○		0.05
Cadmium	0.1	0.1–0.1	<LQ○			0.1○			<LQ○		0.05
Lead	0.4	0.4–0.4	0.1○			2.2	0.3–7.4	155	0.2	0.1–0.3	0.05
Mercury	<LQ○		<LQ○			<LQ○			<LQ○		0.05

Note. LQ: Limit of quantification; <LQ: Value was below limit of quantification of the respective analysis; ○: More than 50% of analysed values of the respective genera were below the limit of quantification; thus, other statistical values were not determined; CV: Coefficient of variation.

3.4 | Minerals

The highest mean Ca concentrations were present in *Phaeodactylum* (16 g/kg DM) products and lowest in *Arthrospira* (1.4 g/kg DM) products. In all genera, except for *Arthrospira*, high variation was observed (Table 6). Tibbetts, Milley & Lall (2015) reported lower Ca concentrations of 0.9 and 2.6 g/kg DM for *Nannochloropsis granulata* and *Phaeodactylum tricornutum* respectively. The range of 3.6–8.0 g Ca/kg DM described in the literature for *Chlorella* (Batista, Gouveia, Bandarra, Franco, & Raymundo, 2013; Tibbetts, Milley & Lall, 2015; Tokuşoglu & Ünal, 2003) is generally consistent with the values of the present study. The Ca levels reported for *Arthrospira* vary among studies. Kyntäjä, Partanen, Siljander-Rasi, and Jalava (2014) reported Ca concentrations that were in line with the present values, whereas other authors reported higher values (Batista et al., 2013; Tokuşoglu & Ünal, 2003).

Mean Mg concentrations varied from 2.1 g/kg DM in *Chlorella* to 5.3 g/kg DM in *Nannochloropsis*, and mean K values varied from 11 to 17 g/kg DM in *Arthrospira* and *Chlorella* respectively. Variation in Mg and K concentrations was lower than variation in Ca. However, CV exceeded 30% for *Chlorella* and *Nannochloropsis*, which is still high. Mean Na concentrations were highest in *Nannochloropsis* (25 g Na/kg DM) and lowest in *Chlorella* (1.4 g Na/kg DM). Compared with common feedstuffs (Agroscope, c2011-2016), these values are notably high, especially in the marine or brackish genera *Phaeodactylum* and *Nannochloropsis*. The mean S concentration varied from 4.7 g/kg DM in *Chlorella* to 10 g/kg DM in *Phaeodactylum*, which is line with values reported in the literature (Rebolloso-Fuentes, Navarro-Pérez, García-Camacho, Ramos-Miras, & Guil-Guerrero, 2001; Tibbetts, Mlley & Lall, 2015) but higher than that in common feedstuffs, such as cereal grains or soybean meal (Agroscope, c2011-2016).

The P concentrations varied from 8.3 g/kg DM in *Arthrospira* to 25 g/kg DM in *Phaeodactylum*, which is higher than in cereal grains (Rodehutscord et al., 2016) or common protein rich feedstuffs, such as soybean meal (Agroscope, c2011-2016). Concentrations of $InsP_6$, $InsP_5$, $InsP_4$ and $InsP_3$ were below the detection limit (<1 μmol/g DM) in all analysed samples (data not shown). This is in contrast to common plant feedstuffs, where phytate represents the primary storage form of P (Eeckhout & de Paepe, 1994; Rodehutscord et al., 2016). Microalgae primarily store inorganic P as polyphosphate in vacuoles or granules (Cembella, Antia, & Harrison, 1984; Eixler, Karsten, & Selig, 2006), which can serve as both a reservoir of energy and phosphate (Kornberg, 1995). Since the utilization of phytate-P is limited in non-ruminant animals, the P present in microalgae may be utilized to a higher extent than in common plant materials. Still, further investigations are necessary to evaluate the P utilization of microalgae in farm animals.

Mean Cu concentration varied from 1.0 to 15 mg/kg DM in *Arthrospira* and *Nannochloropsis*, respectively, and variability was high in *Chlorella* and *Nannochloropsis* (CV 81% and 70% respectively). Mean Fe concentrations were lowest in *Arthrospira* (423 mg/kg DM) and highest in *Phaeodactylum* (1,553 mg/kg DM), and additionally, considerable variation in the Fe concentration was observed in all investigated genera. Variability in Mn and Zn was lower than in Fe, although it was still considerably high. The mean concentration of Mn varied from 26 to 174 mg/kg DM in *Arthrospira* and *Phaeodactylum*, and the mean Zn concentration ranged from 7.7 to 55 mg/kg DM in *Arthrospira* and *Nannochloropsis* respectively. The I concentration was highest in *Nannochloropsis* (1.8 mg/kg DM) and lowest in *Arthrospira* and *Chlorella* (0.3 mg/kg DM). Selenium was detected only in traces in all genera. Available data on trace elements in microalgae are in some cases consistent, and in other cases different from the results of the present study (Batista et al., 2013; Fabregas & Herrero, 1986; Tibbetts, Whitney, et al., 2015; Tibbetts, Milley, & Lall, 2015). Concentrations of the heavy metals As, Cd, Pb, and Hg were below the respective limits of quantification (Table 6) in almost all MAP. This indicates that presence of these heavy metals in MAP do not need to be of concern, which might not be applicable if industrial waste streams (e.g., industrial flue gas, agro-industrial waste water) are used as substrates for microalgae cultivation.

As optimization of media composition for microalgae cultivation has been an objective of research for decades, many recipes for media exist in the literature, which are applied in different facilities (Barsanti & Gualtieri, 2014; Grobbelaar, 2013). Mineral uptake is related to the composition of the cultivation media (Lee & Picard, 1982), including nutrient availability and nutrient ratios, as well as physical factors that affect microalgal growth (e.g., light, temperature, turbulence) (Grobbelaar, 2013). Therefore, intra-genera variation, which was found for most minerals and investigated microalgae genera, and inconsistencies with previously published data, were likely caused by differences in the composition of the applied cultivation media and cultivation process parameters. Furthermore, it is possible that the investigated MAP contained residues of the cultivation media, which were not completely removed during downstream processing. Thus, as explained for CA, variability in mineral composition may also result from differences in the downstream processing of the investigated MAP.

4 | CONCLUSIONS

The investigated commercially available MAP were characterized by an overall high protein concentration and a favourable AA composition. Furthermore, the MAP showed high concentrations of minerals and some of them different essential fatty acids, which are proposed to have health benefits. Nevertheless, the chemical composition of the MAP varied considerably, especially in terms of proximate nutrients and mineral composition, even within genera. This variability probably resulted from varying cultivation conditions used in different cultivation facilities. Thus, the use of general means may not be appropriate for the characterization of microalgae for utilization in food and feed production. Consequently, there is a need for analytical characterization to be applied to each produced microalgae batch or product when they are intended to be used for the production of food and feed. To the extent possible, standardization of cultivation conditions should be strived for, in order to allow better predictions

of the nutrient composition. In addition, their processing properties (e.g., behaviour during pelleting process) and the bioavailability of individual constituents are almost unknown, and should therefore be investigated in both human and animal nutrition. Furthermore, it will be necessary to evaluate relationships between the nutritive value and cultivation conditions to achieve an application-oriented and effective microalgae biomass production.

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5.2. Manuscript 2

VARIABILITY OF *IN VITRO* RUMINAL FERMENTATION AND NUTRITIONAL VALUE OF CELL-DISRUPTED AND NONDISRUPTED MICROALGAE FOR RUMINANTS

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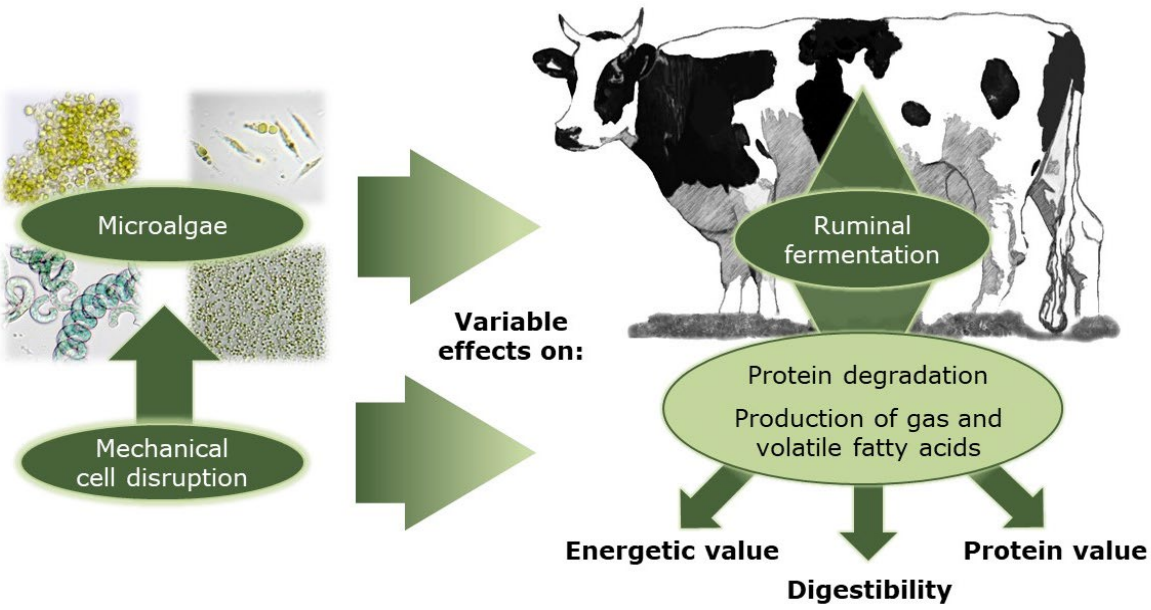
GRAPHICAL ABSTRACT:



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ORIGINAL RESEARCH

WILEY GLOBAL CHANGE BIOLOGY BIOENERGY

Variability of *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 (Ryckebosch, 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 (DELTA 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 CO_2 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

into each of the pre-warmed syringes, which were immediately placed into a rotating disk placed in an oven and incubated for 72 hr at 39°C. Six subsequent runs were conducted and each run contained one replicate of the cell-disrupted and the nondisrupted material of each MAP. In addition, three standard concentrate and three standard hay samples with known gas production (GP) were included in each run, as well as four syringes containing only the buffered rumen fluid that were termed as blanks. All syringes were allocated randomly to the rotating disk. The GP of the MAP, blanks, and standard samples was recorded after 2, 4, 6, 8, 12, 24, 32, 48, and 72 hr. The GP at each incubation time was corrected for the GP of the blanks and standard samples and an exponential equation was fitted to the data for the cell-disrupted and the nondisrupted material of each MAP using PROC NLMIXED of SAS (version 9.3 for Windows, SAS Institute, Cary, NC, USA):

$$GP = b \times (1 - e^{-c \times t})$$

where GP (ml 200 mg^{-1} DM) is the GP after t hours of incubation, b is the potential GP (pGP, ml 200 mg^{-1} DM), c (% hr^{-1}) is the rate constant of GP, and t is the time of incubation (hr) (Westreicher-Kristen, Steingass, & Rodehutscord, 2012). The GP after 24 hr of incubation (ml 200 mg^{-1} DM) was used to estimate digestibility of organic matter (dOM) and metabolizable energy (ME) by inserting GP data and data on chemical analyses into the following equations described by Menke and Steingass (1988):

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

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

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.

2.4 | Experiment 2: Production of methane and volatile fatty acids

Experiment 2 was carried out similar to Experiment 1 with the following modifications: Incubation lasted 24 hr and approximately 180 mg DM of each MAP was weighed into the syringes. Each run contained six blanks, three hay, and three concentrate standard samples, and one replicate of the cell-disrupted and the nondisrupted material of each MAP. Total GP and methane concentration were recorded after 24 hr of incubation. The methane concentration was measured using an infrared-methane analyzer (Pronova Analysentechnik GmbH Co. KG, Berlin, Germany), which was calibrated with a reference gas (13.0 vol % CH_4; Westfalen AG, Münster, Germany). The CH_4-volume (ml) was calculated by multiplying the methane concentration (%) by the total GP (ml) and standardized to 180 mg DM per syringe. Gas and methane production were corrected for gas and methane production of the blanks. The CH_4-concentration of the produced gas was calculated as the CH_4-volume (ml 180 mg^{-1} DM) relative to the total GP (ml 180 mg^{-1} DM). For the analysis of VFA, the liquid incubation residue of each syringe was collected and incubation was stopped immediately by cooling in an ice bath. Two aliquots of the incubation residue were taken under continuous stirring and stored at about −30°C. The preparation of samples and the analysis of VFA was performed as described by Wischer et al. (2013). In brief, samples were thawed, stirred and then centrifuged at 4,000 *g* for 5 min. One milliliter of the supernatant was transferred into an Erlenmeyer flask and 0.1 ml of an internal standard solution (80 mM 2-methylvaleric acid in 50% formic acid) was added. Afterward, samples were frozen in an alcohol bath at −20°C and were vacuum distilled. The distillates were analyzed in duplicate using a gas chromatograph (Hewlett-Packard 6890; Agilent, Waldbronn, Germany) equipped with a flame-ionization detector and a HP-FFAP fused silica capillary column (25 m × 0.32 mm, film thickness 0.5 µm, HP 7683; Agilent). The amounts of individual VFA were corrected for VFA contained in the blanks. Total VFA (mmol/L) was calculated as the sum of acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate (mmol/L).

2.5 | Experiment 3: Utilizable crude protein at the duodenum and ruminally undegradable crude protein

The Extended Hohenheim Gas Test method (Raab, Cafantaris, 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%

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 $Na_2HPO_4 \cdot 12 H_2O\ L^{-1}$, 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:

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

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 (DELTA 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 pancreatin solution (68.05 g/L KH_2PO_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, μ is the overall mean, MAG_x is the fixed effect of the microalgae

genera (*Arthrospira*, *Chlorella*, *Nannochloropsis*, *Phaeodactylum*), CD_y is the fixed effect of the cell disruption (cell-disrupted, nondisrupted), $MAG_x \times CD_y$ 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 x 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)

	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)	
Cell disruption (CD)	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**
Microalgae genera (MAG)										
Arthrospira ($n = 2$)	24.5^{a}	22.2^{b}	26.4^{a}	24.8^{b}*	14.2^{c}	12.3^{d}*	72^{a}	70^{a}*	11.3^{a}	11.0^{b}*
	22–28	19–26	23–30	22–28	14–15	11–14	72–73	70–71	11–11	11–11
Chlorella ($n = 8$)	17.2^{b}	28.6^{a}*	24.0^{b}	32.8^{a}*	8.9^{d}	13.5^{c}*	58^{b}	67^{b}*	10.8^{ab}	12.7^{a}*
	9–24	23–37	12–34	23–40	3–21	9–19	39–65	41–80	9.0–17	11–17
Nannochloropsis ($n = 4$)	14.9^{c}	18.6^{c}*	16.6^{c}	18.7^{c}*	15.3^{b}	27.5^{a}*	57^{b}	60^{c}*	10.6^{b}	11.1^{b}
	13–19	16–23	14–20	15–24	8–23	23–30	52–60	57–62	9.3–11	10–12
Phaeodactylum ($n = 2$)	12.1^{d}	16.9^{c}*	12.6^{d}	17.3^{d}*	35.3^{a}	23.6^{b}*	57^{b}	61^{c}*	8.7^{c}	9.2^{c}*
	9–16	15–19	9–16	14–20	27–44	21–26	54–60	59–63	8.6–8.7	9.2–9.5
Pooled *SEM*	1.01		0.38		0.38		0.98		0.21	
p-Values (ANOVA)										
MAG	<0.001		<0.001		<0.001		<0.001		<0.001	
CD	<0.001		<0.001		<0.001		<0.001		<0.001	
MAG × CD	<0.001		<0.001		<0.001		<0.001		<0.001	

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 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)

	GP after 24 hr (ml 180 mg^{-1} DM)		CH_4-volume (ml 180 mg^{-1} DM)		CH_4-concentration in GP (%)	
Cell disruption (CD)	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**
Microalgae genera (MAG)						
Arthrospira ($n = 2$)	22.4^{a}	19.4^{b}*	5.6^{a}	5.4^{a}	25.2^{a}	29.0^{a}
	21–24	17–22	5.4–5.8	5.1–5.7	23–28	24–34
Chlorella ($n = 8$)	16.2^{b}	23.3^{a}*	3.3^{b}	4.8^{b}*	21.3^{bc}	20.4^{b}
	8–23	18–27	1.5–4.4	2.5–6.0	9–32	12–22
Nannochloropsis ($n = 4$)	12.7^{c}	18.3^{b}*	2.8^{c}	3.3^{c}*	22.8^{ab}	18.0^{c}*
	10–15	15–22	2.0–3.3	2.8–4.1	19–29	16–19
Phaeodactylum ($n = 2$)	11.5^{c}	15.0^{c}*	2.4^{d}	3.2^{c}*	20.5^{c}	21.1^{b}
	9–14	15–15	1.9–2.8	2.9–3.3	20–21	20–22
Pooled *SEM*	0.67		0.15		0.94	
p-Values (ANOVA)						
MAG	<0.001		<0.001		<0.001	
CD	<0.001		<0.001		0.604	
MAG × CD	<0.001		<0.001		<0.001	

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.

Nannochloropsis, but did not affect 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 MAG × 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 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 *Phaeodactylum* and did not have a significant effect in *Arthrospira* and *Nannochloropsis*. 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 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)

	Acetate		Propionate		Isobutyrate		Butyrate		Isovalerate		Valerate		Total VFA [mmol/L]	
Cell disruption (CD)	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**
Microalgae genera (MAG)														
Arthrospira ($n = 2$)	51.9^{b}	51.6^{c}	19.2^{b}	19.2^{c}	4.7	4.9	10.2^{b}	10.1^{b}	9.5	9.5	4.5	4.6	22.4^{a}	23.6^{a}*
	50–53	51–52	18–20	18–21	4.3–5.3	4.7–5.1	10–11	10–10	8.3–11	8.6–10	3.9–5.2	4.1–5.1	21–24	23–24
Chlorella ($n = 8$)	57.2^{a}	56.0^{a}	21.1^{ab}	22.4^{b}	2.2	2.6	12.3^{a}	10.7^{b}*	3.4	4.3	3.8	4.0	15.6^{b}	21.5^{b}*
	49–64	50–59	13–41	16–38	0.6–3.4	0.5–3.8	8.3–22	8.7–16	0.7–5.3	0.7–6.4	2.0–4.9	2.1–4.8	10–23	11–31
Nannochloropsis ($n = 4$)	52.2^{b}	48.0^{d}*	20.8^{ab}	25.1^{a}*	2.6	2.6	12.3^{a}	13.2^{a}	5.1	5.2	7.0	5.9	12.3^{c}	17.3^{c}*
	47–54	44–51	19–22	21–33	1.8–3.7	1.5–3.6	11–15	12–15	3.4–7.4	3.1–7.2	3.6–8.3	3.5–7.5	10–14	16–20
Phaeodactylum ($n = 2$)	53.2^{b}	54.1^{b}	22.2^{a}	19.1^{c}*	3.1	3.7	10.3^{b}	11.1^{b}	6.2	7.1	4.9	4.9	11.4^{c}	14.5^{d}*
	52–54	54–55	20–24	19–19	2.8–3.5	3.4–3.9	7.6–13	10–12	5.4–6.9	6.2–7.8	4.2–5.5	4.5–5.2	9.6–13	14–15
Pooled *SEM*	0.68		0.88		0.15		0.47		0.32		0.24		0.50	
p-Values (ANOVA)														
MAG	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
CD	0.006		0.232		0.007		0.900		0.033		0.245		<0.001	
MAG × CD	0.004		<0.001		0.140		0.027		0.280		0.156		<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 ($p \leq 0.05$) in case of a significant interaction effect. Different superscripts within one column indicate significant mean differences ($p \leq 0.05$) in case of a significant interaction effect.

TABLE 4 Crude protein (CP), utilizable CP (uCP) and ruminally undegradable CP (RUP) after 8 and 48 hr of incubation and intestinal digestibility of RUP (IDP) of four microalgae genera. Mean, range, pooled standard error (*SEM*) and results of two-factorial analysis of variance (ANOVA)

			uCP [g kg^{-1} DM]				RUP [% of CP]					
	CP[1] [g kg^{-1} DM]		8 hr		48 hr		8 hr		48 hr		IDP [% of RUP]	
Cell disruption (CD)	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**	**n-cd**	**cd**
Microalgae genera (MAG)												
Arthrospira ($n = 2$)	692	690	477^{a}	505^{a}*	300^{a}	287^{a}	60^{b}	64^{b}	40^{d}	38^{c}	27^{b}	26^{c}
	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	470^{a}	457^{b}*	296^{a}	216^{c}*	76^{a}	71^{a}*	52^{c}	35^{c}*	43^{a}	44^{b}
	516–629	517–623	413–498	421–489	182–415	160–279	69–84	62–79	25–73	23–45	26–53	25–52
Nannochloropsis ($n = 4$)	432	431	357^{b}	344^{c}	272^{b}	248^{b}*	76^{a}	69^{a}*	61^{a}	57^{a}	43^{a}	41^{b}
	353–500	350–500	302–415	282–391	214–321	230–268	69–82	69–71	54–69	52–64	28–57	35–49
Phaeodactylum ($n = 2$)	451	446	298^{c}	289^{d}	260^{b}	205^{c}*	58^{b}	54^{c}*	56^{b}	43^{b}*	40^{a}	49^{a}*
	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	
p-Values (ANOVA)												
MAG	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
CD	0.869		0.681		<0.001		<0.001		<0.001		0.306	
MAG × CD	0.999		<0.001		<0.001		<0.001		<0.001		0.011	

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.

[1]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 genera) within one trait ($p \leq 0.05$) in case of a significant interaction effect.

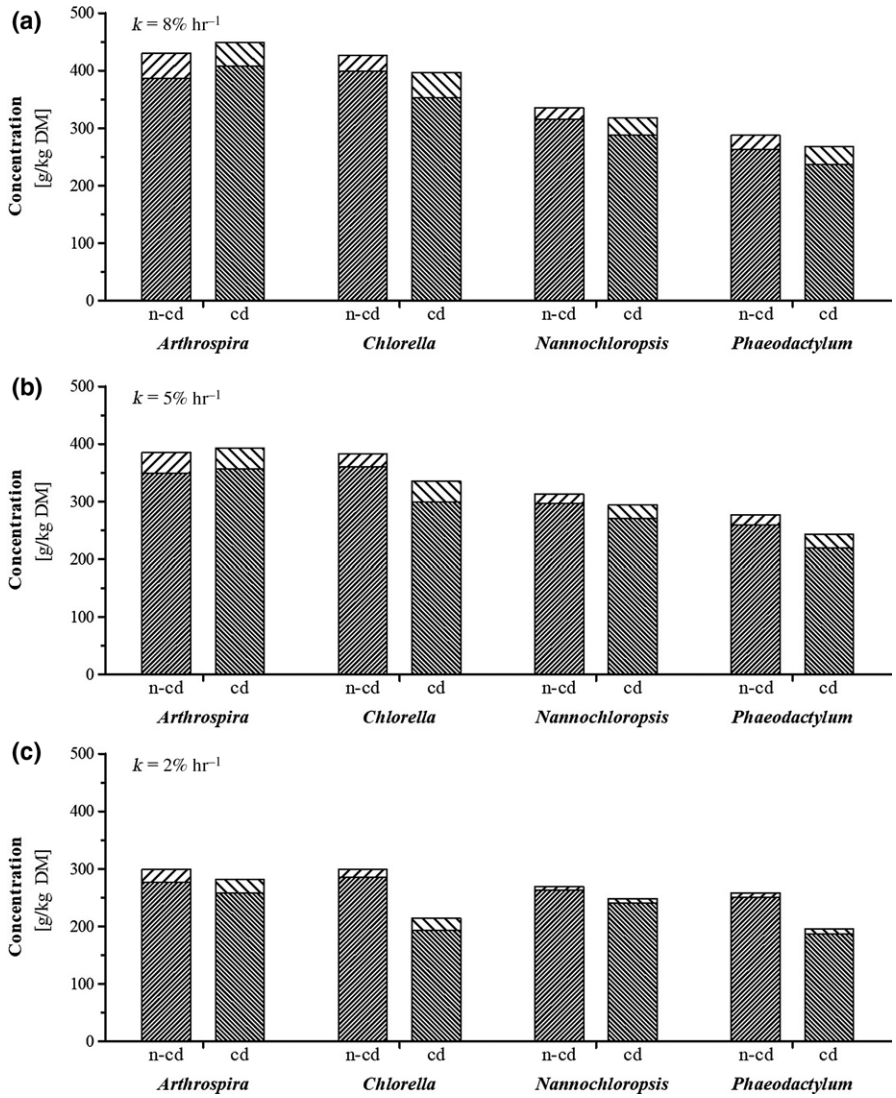

FIGURE 1 **Differentiation of effective uCP in effective RUP and microbial protein at different passage rates (k) (▨ ▨ Effective RUP; ▨ ▨ Microbial protein; a: 8% hr^{-1}, b: 5% hr^{-1}, c: 2% hr^{-1}). cd: cell-disrupted microalgae biomass (stirred ball mill); n-cd: nondisrupted microalgae biomass**

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 *Chlorella* or *Nannochloropsis* products in a continuous rumen fermentation system. Lipid-extracted *Nannochloropsis* products consistently decreased microbial efficiency compared to soybean meal, while microbial efficiency was increased with some lipid-extracted *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 *Chlorella pyrenoidosa*, *Nannochloropsis*, and *Schizochytrium* sp. was lower than or on a similar level to that of soybean meal, while only *Dunaliella salina* and *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 microalgae 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 microalgae 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 microalgae 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 microalgae 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 biomasses and lipid-extracted microalgae 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 CH_4-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 CH_4-volume. Amino acids are converted to ammonia and α-keto acids during deamination and subsequently produce H^+ ions, which contribute to the CH_4-pool in the rumen (Hossain, Sherasia, Phondba, 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 CH_4-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 CH_4-volume ($r = -0.75$ for RUP after 48 hr of incubation).

4.5 | Intra- and inter-genera variability

The nutrient composition of microalgae has been shown to be highly variable between and within microalgae genera (Wild et al., 2018), hence raising the issue whether the nutritional value is also variable. Data on this issue in ruminants are scarce with only one study investigating the ruminal fermentation of several *Nannochloropsis* and *Chlorella* samples differing in cultivation and harvesting methodologies (Lodge-Ivey et al., 2014). As in the current study, ruminal fermentation traits differed between samples of the same microalgae species as well as between microalgae species. Ruminal fermentation is affected by, among other factors, the supplied nutrients and their availability for microbial fermentation. Therefore, variation in the ruminal fermentation of microalgae genera may be explained by intrinsic differences in the nutrient composition of the microalgae. Variability in the nutrient availability between microalgae genera might be explained by different structures of the whole cell, differences in allocation of ruminal fermentable compounds in the microalgae cell, and by differences in the structure and composition of microalgae cell walls. Reasons for a variable nutrient composition within microalgae genera are likely to be related to differences in the cultivation conditions and have been discussed before (Wild et al., 2018). Variability in the nutrient availability within microalgae genera might also be related to differences in the cultivation process or harvest regime that affect the development of whole cell and cell wall structure, as well as the synthesis and storage of ruminally undegradable compounds. To the best of our knowledge, there are no studies investigating the relationship between cultivation conditions and the nutrient availability for farm animals and therefore studies are necessary to further investigate this issue.

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 Eykelenburg, 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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5.3. Manuscript 3

CHEMICAL COMPOSITION AND NUTRITIONAL CHARACTERISTICS FOR RUMINANTS OF THE MICROALGAE *CHLORELLA VULGARIS* OBTAINED USING DIFFERENT CULTIVATION CONDITIONS

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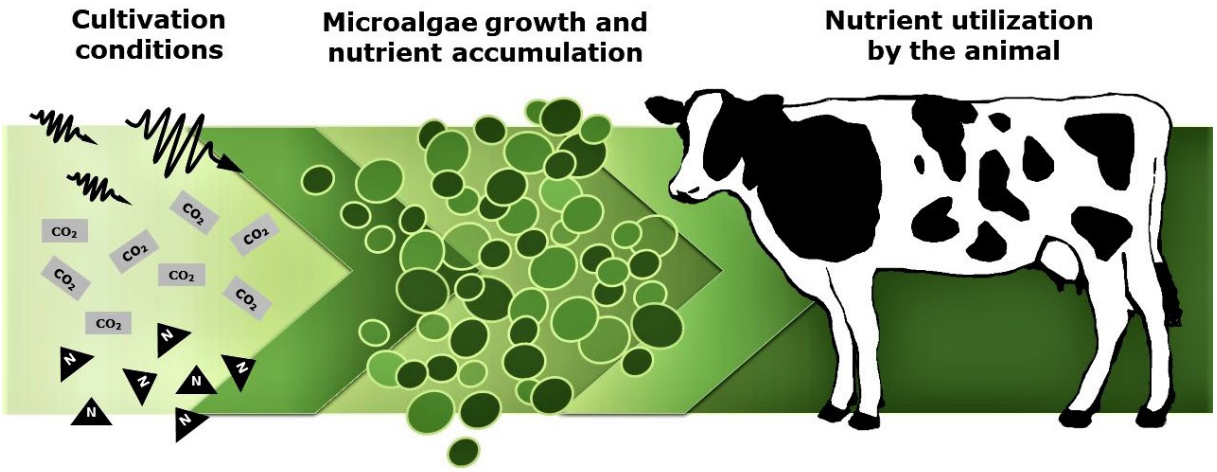
GRAPHICAL ABSTRACT:



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Chemical composition and nutritional characteristics for ruminants of the microalgae *Chlorella vulgaris* obtained using different cultivation conditions

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ABSTRACT

The objective of the present study was to investigate the nutritional value of microalgae for ruminants. *Chlorella vulgaris* was cultivated on a laboratory scale under nitrogen depleted, CO_2 depleted, or saturated conditions. Furthermore, an outdoor cultivation under saturated conditions was carried out to demonstrate the transferability of the results to a pilot scale. In vitro methods were used to determine characteristics of the nutritional value (e.g., gas production, digestibility, and related energy and protein characteristics) of *C. vulgaris* and were complemented by a comprehensive analysis of the nutrient composition (e.g., proximate nutrients, amino acids, and fatty acid composition).

Nutrient composition of *C. vulgaris* was affected by cultivation conditions in terms of proximate composition and fatty acid composition, wherein CO_2 deficiency had the most pronounced effects. Characteristics of the nutritional value were also significantly affected. Changes in ruminal fermentation characteristics (gas production) were rather small and nitrogen and CO_2 deficiency had adverse effects on the protein value of microalgae for ruminants. Laboratory and outdoor cultivation yielded similar results, so that transferability of laboratory results to a pilot scale appears feasible. Nevertheless, it will be necessary to investigate further cultivation strategies that not only achieve a high productivity, but also have a positive impact on nutrient utilization by the animal and hence on the nutritional value of microalgae.

1. Introduction

Microalgae are a heterogeneous group of unicellular or simple multicellular photosynthetic organisms. Some species have garnered interest for potential application as alternative sources of protein and lipids or high value compounds such as omega-3 fatty acids, carotenoids, or vitamins in human and animal nutrition. In addition, they can be cultivated without the use of arable land and, therefore, provide an opportunity to produce feed products on currently idle land without further stressing competition with food production.

Microalgae growth and pattern of nutrient accumulation is highly dependent on the cultivation process, including environmental factors (e.g., light, temperature, and salinity) and nutrient supply (e.g., carbon, nitrogen, and phosphorus) [1]. The variable factors have multiple effects on chemical composition and productivity and responses can be specific to the microalgae species or strain [2]. Consequently, nutrient composition can be highly variable between and within microalgae species [3], which may be detrimental when microalgae are intended for use in industrial food and feed production, where standardised raw materials are needed. Nevertheless, this issue also provides an opportunity to shift microalgae composition to a desired pattern [2] and hence produce biomass fitted to specific requirements of the food and feed industry. The effects of specific cultivation conditions on chemical composition have been the subject of numerous studies [4–7]. Nevertheless, the nutritional value of feedstuffs is not only determined by the chemical composition, but is also dependent on the utilization of nutrients by the animal. Nutrient utilization in ruminants is principally determined by ruminal microbial fermentation. However, there are no

Abbreviations: AA, amino acids; CA, crude ash; CDM, cell dry mass; CP, crude protein; CV, coefficient of variation; DM, dry matter; dOM, digestibility of organic matter; EE, ether extract; GP, gas production; IDP, intestinal digestibility of ruminally undegradable crude protein; ME, metabolisable energy; MP, microbial crude protein; NPN, non-protein nitrogen; pGP, potential gas production; RUP, ruminally undegradable crude protein; TP, true protein; uCP, utilizable crude protein at the duodenum

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studies investigating the effects of varying cultivation conditions on ruminal fermentation and the consequences on the nutritional value of microalgae for ruminants yet.

Accordingly, the objective of the present study was to investigate the nutritional value of microalgae for ruminants when cultivated using different conditions (nitrogen and CO_2 supply and environmental factors). The microalgae *Chlorella vulgaris* (*C. vulgaris*), which is approved for the application as a feedstuff in the EU [8], was cultivated on a laboratory scale under saturated, nitrogen-depleted, and CO_2-depleted conditions. Furthermore, an outdoor cultivation under saturated conditions was carried out to demonstrate transferability on pilot scale. Standardised in vitro methods were used to determine characteristics of the nutritional value of the microalgae *C. vulgaris* and were complemented by a comprehensive analysis of the nutrient composition.

2. Materials and methods

2.1. Cultivation of microalgae, harvest, and sample preparation

Liquid pre-cultures of the strain *Chlorella vulgaris* SAG 211–12 were cultivated in 500 mL shaking flasks on an orbital shaker (KS 501 digital, Ika-Werke GmbH & Co. KG, Staufen, Germany). Pre-cultures were aerated with 5% CO_2 and incubated at 25 °C, 100 rpm, pH 7.5, and a photon flux density of 160 µmol $m^{-2} s^{-1}$ (photosynthetic active radiation) supplied by warm-white LED illumination (MS6L083AT, Nichia, Tokushima, Japan). The pre-culture medium was 1-fold TAP medium without acetate. Experimental cultures were cultivated in a 37 L flat-panel-photobioreactor (constructed exactly as published by San Pedro et al. [9]) in batch cultures under continuous light provided by nine halogen spotlights (mean photon flux density: 377 µmol photons $m^{-2} s^{-1}$). Cultures were grown on a 2.5-fold concentrated and modified TAP medium [10,11], without tris(hydroxymethyl)-aminomethane or acetate, at pH 7.5 and 25 °C (Table 1). A cooling coil installed at the reactors head plate ensured temperature regulation. Aeration was provided by a gassing tube at the bottom of the photobioreactor connected to mass flow controllers (MKS Instruments, Andover, MA, USA). For the control culture, 2.5% NH_4OH was used for pH control and as a nitrogen source; 2% CO_2 enriched air was used for gas supply. For the nitrogen limited culture (N-), CO_2 supply was maintained at 2%, but 1 M NaOH was used for pH control. A third culture was carried out with four phases of variable CO_2 supply (CO_2-): CO_2 concentration of supplied air was set to 0.5% for ten days followed by a four-day period with a CO_2 concentration of 0.04% and this sequence of phases with variable CO_2 supply was repeated twice. A fourth cultivation was carried out to validate transferability of laboratory cultures to a pilot scale with real environmental conditions (Outdoor). An identically constructed 230 L photobioreactor was used for the cultivation at the outdoor facility of KIT, Campus Nord. The mean total photon flux density was 475 µmol photons $m^{-2} s^{-1}$ and the mean diffuse light component was 212 µmol photons $m^{-2} s^{-1}$. Mean pH value was 7.4 and 2.5% NH_4OH was used for pH control during the night phase. CO_2 concentration in the supply air was controlled using a PI controller with minimum CO_2 concentrations of 1% during the night phase and 5% during the day phase. Mean temperature was 21.4 °C, with minimum and maximum temperatures of 11 and 25 °C, respectively. The four cultivation variants were produced in four $n = 1$ batches, hereafter referred to as cultivation batches. Since the four cultivation batches were operated under varying growth conditions, different cultivation periods were necessary to reach a biomass concentration sufficient for harvest, e.g. 2–3 g L^{-1}. The exact biomass dry weight values at the end of cultivation were 2.8, 2.1, 2.0 and 3.1 g L^{-1} for the batch Control, N-, CO_2– and Outdoor, respectively.

Cell dry mass (CDM) of experimental cultures was monitored daily in duplicate. For this, duplicate 20 mL samples were taken from the culture broth, centrifuged (15 min, 4850 × g, 4 °C, Rotina 420R, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany), washed, and dried (48 h, 80 °C). The cell pellets were cooled to room temperature and weighed. Biomass productivity was calculated by a linear fit of CDM concentration (g L^{-1}) against the cultivation time (t):

$$\text{Biomass productivity (g CDM } L^{-1}d^{-1}) = \frac{CDM_t}{t}.$$

The specific growth rate μ in the linear growth phase was calculated by the linear correlation:

$$\text{Specific growth rate } (d^{-1}) = \mu_{linear} \times CDM_t \approx \text{const.} \approx \left(\frac{CDM_t - CDM_0}{t - t_0}\right)$$

where *CDM* represents the cell dry mass (g L^{-1}) and *t* represents the respective cultivation time (d). Additionally, the nutrient productivity was calculated for selected nutrients by multiplying the biomass productivity with the concentration of the respective nutrient concentration at harvesting.

Cultures were harvested by two subsequent centrifugation steps (7 min at 3300 × g, 4 °C, Rotina 420R, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany), yielding a highly concentrated microalgae paste. The microalgae paste was lyophilized for 96 h (Alpha 1–2 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The lyophilized microalgae biomass was pulverised with a ball mill (MM 400, Retsch GmbH, Haan, Germany) four times for 20 s at a grinding frequency of 30 Hz. Ground samples were stored at approximately −20 °C until analyses.

2.2. Analysis of nutrient composition

All analyses of nutrient composition were performed in duplicate. The official methods of the Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten [12] were used to determine concentrations of crude ash (CA, method 8.1), crude protein (CP, N × 6.25, method 4.1.1), true protein (TP, method 4.4.1), and ether extract (EE, method 5.1.1 B). Concentration of α-linked glucose was assayed enzymatically as described by Wild et al. [3]. The difference between 1000 g kg^{-1} and the sum of the concentrations of CA, CP, EE, and α-linked glucose was defined as the organic residue, and was a measure of the sum of all constituents that could not be identified by any of the applied methods. Gross energy concentration was determined using a bomb calorimeter (C 200; Ika-Werke GmbH & Co. KG, Staufen, Germany) and benzoic acid as standard.

The difference between CP and TP relative to CP concentration was

Table 1
Cultivation conditions of *Chlorella vulgaris* samples.

	Control	N-	CO_2-	Outdoor
Photobioreactor	Flat-panel-reactor, 37 L	Flat-panel-reactor, 37 L	Flat-panel-reactor, 37 L	Flat-panel-reactor, 230 L
Temperature	25 °C	25 °C	25 °C	11–25 °C
pH	7.5	7.5	7.5	7.4
Nitrogen supply	N +	N-	N +	N +
CO_2 supply	+ CO_2 (2%)	+ CO_2 (2%)	- CO_2 (≤ 0.5%)	+ CO_2 (1–5%)
Light	Continuous	Continuous	Continuous	Day/Night-rhythm
Cultivation period, d	13	9	23	15

the non-protein nitrogen (NPN). The generally accepted nitrogen-to-protein conversion factor for microalgae of 4.78 [13] and a species-specific conversion factor for *C. vulgaris* of 5.14 [14] were used to calculate protein concentration in addition to CP concentration by multiplying nitrogen concentration by the respective factor.

Determination of amino acid content (AA) was carried out as described by Rodehutscord et al. [15] with minor modifications and concentrations were expressed as g AA per 16 g N. In brief, microalgae samples were oxidised using a mixture of performic acid, hydrogen peroxide, and phenol and then hydrolysed using hydrochloric acid (6 M) containing 1 g L^{-1} phenol for 24 h at 110 °C. Separation and detection of AA was done by ion-exchange chromatography using an AA analyser (Hitachi, L-8900, Tokyo, Japan) and post column derivatisation was carried out using ninhydrin. Asparagine and aspartic acid, as well as glutamine and glutamic acid, could not be distinguished from each other by analysis and were therefore labelled as Asx and Glx, respectively. Tryptophan was determined by reverse-phase chromatography and fluorescence detection (Agilent 1100 HPLC, Agilent, Waldbronn, Germany) at an excitation wavelength of 283 nm and an emission wavelength of 355 nm after alkaline hydrolysis with barium hydroxide [16].

The fatty acid composition was determined using the method of Lepage and Roy [17], including minor modifications described by Meiser et al. [18]. Briefly, samples were diluted with methanol/acetyl chloride (20:1 v/v) for transesterification. Fatty acid methyl esters were analysed using a gas chromatograph (Agilent 7890A, Agilent, Santa Clara, CA, USA) equipped with a flame-ionisation detector and a fused silica SPB-PUFA capillary column (30 m × 0.32 mm, film thickness 0.2 µm, 24,323 Supelco, Sigma–Aldrich, St. Louis, MO, USA). A certified C_4 – C_{24} fatty acid methyl ester mixture (18919-1AMP Supelco, Sigma-Aldrich, St. Louis, MO, USA) was used as standard.

2.3. Determination of characteristics of the nutritional value for ruminants

2.3.1. Animals and diet

For Experiment 1 and Experiment 2, two ruminally fistulated Jersey cows served as donor animals for the rumen fluid. The investigations were conducted over a period of four weeks and the cows were offered a total mixed ration composed of 28% maize silage, 25% grass silage, 21% hay, 20% concentrate mixture, 3% barley straw, 2% mineral mixture, and 1% rapeseed meal (by dry matter; DM). Cows had ad libitum access to feed and water and were housed in groups. Mean DM intake was 12 kg day^{-1}.

2.3.2. Experiment 1: Gas production kinetics, metabolisable energy, and digestibility of organic matter

In vitro gas production, metabolisable energy, and digestibility of organic matter were determined using the Hohenheim Gas Test method [19]. In brief, 200 mg of DM of the microalgae samples was weighed into 100 mL syringes. Syringes were closed and rendered airtight with greased plungers and pre-warmed. A buffered mineral solution was kept at 39 °C under continuous stirring and flushing with CO_2. Rumen fluid of two cows was collected prior to the morning feeding, mixed, and filtered through two layers of cheesecloth. The rumen fluid was mixed with the reduced buffer solution and 30 mL of the rumen fluid-buffer solution was dispensed into the syringes which were immediately incubated for 72 h at 39 °C. Four experimental runs with two replicates per sample in each run were performed. In addition to the microalgae samples, each run contained four syringes that only contained the rumen fluid-buffer solution (blanks), three replicates of a standard concentrate sample and three replicates of a standard hay sample with known gas production (GP). The order of syringes was fully randomised for each run. After 2, 4, 6, 8, 12, 24, 32, 48, and 72 h of incubation, GP was recorded. The GP of microalgae samples was corrected for GP of standard samples and blanks at each incubation time. An exponential equation was fitted to the GP data for each run separately, using PROC NLMIXED of SAS (version 9.3 for Windows, SAS Institute, Cary, NC, USA):

$$GP = pGP \times (1 - e^{-c \times t}),$$

where *GP* (mL 200 mg^{-1} DM) is the GP after *t* hours of incubation, *pGP* is the potential GP (mL 200 mg^{-1} DM), *c* (% h^{-1}) is the rate constant of GP, and *t* is the time of incubation (h) [20].

Metabolisable energy (ME) and digestibility of organic matter (dOM) were estimated by inserting GP after 24 h of incubation (mL 200 mg^{-1} DM) and data on the nutrient composition into the following equations of Menke and Steingaß [19]:

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

$$ME\ (MJ\ kg^{-1}DM) = 1.68 + 0.1418\ GP_{24} + 0.0073\ CP + 0.0217\ EE - 0.0028\ CA$$

where GP_{24} is the gas production after 24 h of incubation (mL 200 mg^{-1} DM), and *CP*, *EE*, and *CA* are the concentrations of crude protein, ether extract, and crude ash in g kg^{-1} DM, respectively. Results for each sample were averaged per run.

2.3.3. Experiment 2: Utilizable crude protein at the duodenum, ruminally undegradable crude protein, and microbial protein

Utilizable crude protein at the duodenum (uCP), ruminally undegradable crude protein (RUP), and microbial crude protein (MP) were determined using the Extended Hohenheim Gas Test method [21] with the additions of Steingaß et al. [22]. Incubations were carried out similar to Experiment 1 with the following modifications: 130 mg DM of the microalgae samples with and without the addition of 130 mg of a carbohydrate mixture (50% corn starch, 30% cellulose, and 20% sucrose) was weighed. Six subsequent runs were performed and each run comprised an incubation over 8 h and an incubation over 48 h. Each incubation contained two replicates of each microalgae sample, four blanks, and three replicates of a standard concentrate sample with known GP and uCP concentrations (cf. Section 2.3.2). The GP was recorded after 8 and 48 h of incubation and syringes were then immediately put on ice to stop further microbial fermentation. Steam distillation with subsequent titration (Vapodest 50, C. Gerhardt GmbH & Co. KG, Königswinter, Germany) was used to determine NH_3-N in incubation residues. For this, 15 mL of phosphate buffer (90 g $Na_2HPO_4 \cdot 12\ H_2O$ L^{-1}, adjusted to pH 11.0 using sodium hydroxide) was added to the incubation residue, distilled NH_3 was trapped in 3% boric acid, and titration was carried out with 0.05 M HCl. Concentration of uCP was calculated for the syringes without carbohydrate addition after 8 and 48 h of incubation and was averaged per run:

$$uCP\ (g\ kg^{-1}DM) = ((N_{sample} - (NH3 - N_{sample} - NH3 - N_{blank}))/\text{initial weight}) \times 6.25 \times 1000$$

where N_{sample} is the amount of N added by the microalgae sample (mg), NH_3-N_{sample} and NH_3-N_{blank} are the NH_3-N concentrations of microalgae and blank incubation residues (mg), respectively, and *initial weight* is the amount of microalgae initially incubated (mg DM).

For the calculation of RUP, a linear regression was fitted to the GP and NH_3-N values of microalgae samples with and without carbohydrate addition within one run [21]. The difference between the y-intercept and the NH_3-N concentration of blanks was the rumen degradable nitrogen. Ruminally undegradable nitrogen was calculated as the difference between the amount of N added by the microalgae sample and the rumen degradable nitrogen. The amount of ruminally undegradable nitrogen relative to the amount of N added by the microalgae sample was the proportion of ruminally undegradable nitrogen. The concentration of RUP (g kg^{-1} DM) was calculated by multiplying the CP concentration of microalgae samples with the proportion of ruminally undegradable nitrogen. The MP was the difference between the uCP and RUP relative to uCP.

2.3.4. Experiment 3: Intestinal digestibility of ruminally undegradable crude protein

Intestinal digestibility of RUP (IDP) was determined using a three-step enzymatic in vitro method [23], including the modifications of Wild et al. [24] to ensure applicability for microalgae. Three subsequent experimental runs were performed. Ruminal protein degradation was simulated by the application of a *Streptomyces griseus* protease [25] in this assay. For this, a protease solution (borate-phosphate buffer and 1.0 U mL^{-1} *Streptomyces griseus* protease; Type XIV, ≥ 3.5 units mg^{-1} solid, P5147, Sigma–Aldrich, St. Louis, MO, USA) was added to the samples in an amount corresponding to 41 U g^{-1} TP and samples were incubated at 39 °C with continuous stirring for 18 h. Intestinal digestion was simulated by subsequent treatment of the incubation residues with pepsin (0.1 M HCl at pH 1.9 containing 1 g L^{-1} of pepsin; P7012, Sigma-Aldrich, St. Louis, MO, USA) and pancreatin (68.05 g L^{-1} KH_2PO_4, 50 mg L^{-1} thymol and 3 g L^{-1} pancreatin; P7545, Sigma-Aldrich, St. Louis, MO, USA; adjusted to pH 7.8) at 38 °C for 1 and 24 h, respectively. Incubation was stopped by the addition of trichloroacetic acid. Samples were centrifuged and the supernatant was used for analysis of the soluble N by the Kjeldahl method [12]. The IDP was calculated as the amount of soluble N relative to the amount of N incubated with pepsin and pancreatin.

2.4. Statistical analysis

Data on the nutrient composition is shown as the mean of the laboratory replicates and the coefficient of variation (CV) was calculated as the variation of the four cultivation batches in order to illustrate variation between cultivation conditions. If values were below the limit of detection, 0 was used for calculations and it was indicated as "not detected" in the tables.

Data on the characteristics of the nutritional value for ruminants were subjected to a one-factorial analysis of variance using PROC MIXED of SAS. The replicates derived from the in vitro incubations of Experiment 1, 2 and 3 were averaged per experimental run for each of the microalgae samples before subjecting data to analysis of variance and the averaged values were considered as experimental replicates in the statistical model. Cultivation was set as a fixed effect and the experimental run was a random effect. Model assumptions were checked on the residuals. Differences between least square means were tested using *t*-test and significance was declared at $p < 0.05$.

3. Results

3.1. Growth and productivity characteristics of microalgae cultivation

Growth rate and biomass productivity of the Control were 0.11 d^{-1} and 0.22 g CDM L^{-1} d^{-1}, respectively (Table 2). Growth rate and biomass productivity of the nitrogen deficient culture were on a similar level to those of the Control, but were reduced in the CO_2– condition. The outdoor cultivated culture had similar biomass productivity (0.21 g CDM L^{-1} d^{-1}) as the Control, but growth rate was considerably lower (0.05 d^{-1}).

Table 2
Growth rate, biomass productivity and nutrient productivity of *Chlorella vulgaris* depending on variable cultivation conditions[†].

	Control	N-	CO_2-	Outdoor
Growth rate, d^{-1}	0.11	0.10	0.04	0.05
Biomass productivity, g CDM L^{-1} d^{-1}	0.22	0.23	0.09	0.21
Nutrient productivity[‡]	mg^{-L-d}			
Crude protein	116	105	30	122
Ether extract	35	44	31	33
Digestible organic matter	127	116	43	119
Utilizable crude protein[1]	84	78	19	94

[†] Control: Positive control, saturation; N-: Nitrogen deficiency; CO_2-: CO_2 deficiency; Outdoor: Outdoor cultivation.
[‡] Nutrient productivity was calculated by multiplying the biomass productivity with the concentration of respective nutrient at harvesting.
[1] Utilizable crude protein after 8 h of incubation.

Table 3
Proximate nutrients, α-linked glucose, organic residue and gross energy of *Chlorella vulgaris* depending on variable cultivation conditions[†].

	Control	N-	CO_2-	Outdoor	CV
Proximate nutrients	g kg^{-1} DM				%
Crude ash (CA)	37	35	31	45	16
Crude protein (CP)	526	455	328	579	23
Ether extract (EE)	157	192	341	158	41
α-linked glucose	4.0	6.0	7.0	2.0	47
Organic residue	276	312	293	216	15
Gross energy, MJ kg^{-1} DM	24.2	24.4	26.3	24.3	4

Data shown as mean of two laboratory replicates and coefficient of variation (CV).
Organic residue: 1000 – CA – CL – CP − α-linked glucose.
[†] Control: Positive control, saturation; N-: Nitrogen deficiency; CO_2-: CO_2 deficiency; Outdoor: Outdoor cultivation.

3.2. Nutrient composition

In the Control, N-, and Outdoor conditions, CP was the main component with concentrations of 526, 455, and 579 g kg^{-1} DM, respectively, while in the CO_2– condition, EE was the main component (341 g kg^{-1} DM) (Table 3). Concentration of CA varied between 31 and 45 g kg^{-1} DM in the CO_2 deficient and the outdoor cultivated sample, respectively. Concentrations of α-linked glucose were low overall. The organic residue was highest in the nitrogen deficient sample (312 g kg^{-1} DM) and lowest in the outdoor cultivated sample (216 g kg^{-1} DM). Gross energy concentration was very similar in Control, N-, and Outdoor conditions, but considerably higher in CO_2-.

The proportion of NPN varied between 8.7 and 13.2% of CP in Control and CO_2-, respectively (Table 4). The protein concentration calculated either with a general nitrogen-to-protein conversion factor for microalgae or with a species specific factor for *C. vulgaris* showed the same ranking as CP, TP, and sum of AA. Variability expressed by the CV was high (≥15%) for proximate nutrient and nitrogenous compounds. Concentration of Lys varied between 4.87 and 5.06 g per 16 g N and concentration of Met ranged from 1.89 to 2.02 g per 16 g N. All samples had low concentrations of Cys (0.85 to 1.08 g Cys per 16 g N) and high concentrations of Glx (9.38 g to 11.71 g Glx per 16 g N). Overall, the variation in AA composition was low, expressed by a CV lower than 15% for all AA.

The predominant fatty acids were C18:3 n-3, C18:2 n-6 cis, and C16:0 in all samples and the CO_2– condition additionally had high proportions of C18:1 n-9 cis (Table 5). Nevertheless, proportions of individual fatty acids were highly variable between the samples. Compared to the Control, the proportion of C18:2 n-6 cis decreased and proportion of C18:1 n-9 increased in the samples cultivated under nitrogen and CO_2 deficiency, while in the sample cultivated outdoor, the proportion of C18:3 n-3 increased at the expense of C18:2 n-6 cis when compared to the Control.

3.3. Characteristics of the nutritional value for ruminants

Cultivation significantly affected digestibility of organic matter, metabolisable energy, and all traits related to GP ($p < 0.001$, Table 6). After 8 h of incubation, GP was decreased in the N- and Outdoor conditions compared to the Control and increased in the CO_2- condition. After 24 h of incubation, GP was highest in the Control (21.5 mL 200 mg^{-1} DM) and significantly lower in N- and Outdoor conditions. The pGP was lowest in N- (17.5 mL 200 mg^{-1} DM), intermediate in CO_2- and Outdoor, and highest in the Control (24.5 mL 200 mg^{-1} DM). Compared to the Control (10.8%

Table 4
Nitrogenous compounds and amino acid composition of the protein of *Chlorella vulgaris* depending on variable cultivation conditions[†].

	Control	N-	CO_2-	Outdoor	CV
Nitrogenous compounds	g kg^{-1} DM, unless otherwise stated				%
Nitrogen × 6.25 (CP)	526	455	328	579	23
Nitrogen × 5.14	432	375	267	478	23
Nitrogen × 4.78	402	349	249	445	23
True protein (TP)	480	403	285	521	25
Non-protein nitrogen, % of CP	8.7	11.5	13.2	10.0	18
Sum of analysed AA	465	394	277	498	24
Amino acids	g amino acids per 16 g nitrogen				%
Ala	7.98	7.89	7.84	7.93	0.8
Arg	5.59	6.70	5.58	5.37	10
Asx	8.75	8.51	8.48	8.51	1.5
Cys	0.95	1.08	1.07	0.85	11
Glx	11.71	11.03	10.70	9.38	9.2
Gly	5.40	5.21	5.12	5.49	3.2
His	2.41	2.70	2.32	2.14	9.8
Ile	2.79	2.44	2.77	3.16	11
Leu	8.25	7.74	7.62	8.36	4.6
Lys	5.06	4.92	4.88	4.87	1.8
Met	1.98	1.89	1.89	2.02	3.4
Phe	4.87	4.55	4.30	4.91	6.1
Pro	4.26	4.31	4.39	4.46	2.0
Ser	4.45	4.42	4.27	4.30	2.0
Thr	4.22	4.13	4.24	4.25	1.3
Trp	2.03	1.89	1.52	1.87	12
Tyr	3.17	3.12	3.05	3.25	2.7
Val	4.56	4.04	4.30	4.94	8.6

Data shown as mean of two laboratory replicates and coefficient of variation (CV). Non-protein nitrogen: CP (N × 6.25) – TP; Sum of analysed AA: Sum of 20 analysed amino acids.
[†] Control: Positive control, saturation; N-: nitrogen deficiency; CO_2-: CO_2 deficiency; Outdoor: outdoor cultivation.

Table 5
Fatty acid composition of *Chlorella vulgaris* depending on variable cultivation conditions[†].

Fatty acids	Control	N-	CO_2-	Outdoor	CV
	g per 100 g of total analysed fatty acids				%
C14:0	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C14:1 n-5	2.98	1.43	0.46	3.78	69
C15:1	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C16:0	25.0	20.8	16.9	23.3	16
C16:1 n-7	0.72	1.39	0.93	0.89	29
C17:0	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C17:1	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C18:0	*n.d.*	*n.d.*	0.46	*n.d.*	200
C18:1 n-9 cis	4.36	12.3	32.2	3.27	103
C18:1 n-9 trans	1.48	3.47	3.45	4.14	37
C18:2 n-6 cis	32.3	21.6	16.7	19.1	31
C18:2 n-6 trans	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C18:3 n-6	*n.d.*	*n.d.*	*n.d.*	2.07	200
C18:3 n-3	33.1	38.9	28.9	43.4	18
C20:0	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C20:1 n-9	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C20:2 n-6	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C20:3 n-6	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C20:3 n-3	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C21:0	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C20:5 n-3	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C20:4 n-6	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C22:0	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C22:1 n-9	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C22:2 n-6	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C23:0	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C22:6 n-3	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C24:0	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
C24:1 n-9	*n.d.*	*n.d.*	*n.d.*	*n.d.*	
ΣSFA	25.0	20.8	17.4	23.3	15
ΣMUFA	9.54	18.6	37.0	12.1	64
ΣPUFA	65.5	60.6	45.6	64.6	16
Total analysed fatty acids, g kg^{-1} DM	60.0	89.1	204	60.7	66

Data shown as mean of two laboratory replicates and coefficient of variation (CV).
SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acid, PUFA: Polyunsaturated fatty acids.
n.d.: not detected.
[†] Control: Positive control, saturation; N-: Nitrogen deficiency; CO_2-: CO_2 deficiency; Outdoor: Outdoor cultivation.

h^{-1}), the rate constant of GP was increased by nitrogen (13.9% h^{-1}) and CO_2 deficiency (21.3% h^{-1}) and unaffected when cultivated outdoors (9.35% h^{-1}). The dOM values of the Control and Outdoor treatments were not significantly different. Compared to the Control, the dOM was significantly decreased by nitrogen and CO_2 deficiency. Outdoor cultivation did also not affect ME concentration compared to the Control, while nitrogen deficiency decreased and CO_2 deficiency increased concentrations of ME compared to the Control (11.9 MJ ME kg^{-1} DM).

All investigated traits related to the protein value of *C. vulgaris* were significantly affected by cultivation ($p < 0.01$), with the exception of IDP (Table 7). Compared to the Control (382 g kg^{-1} DM), concentrations of uCP after 8 h of incubation were decreased by nitrogen and CO_2 deficiency and increased by outdoor cultivation. The effect of cultivation on uCP after 48 h of incubation was the same. However, with values between 195 (CO_2-) and 299 (Outdoor) g uCP kg^{-1} DM, the level was lower than that after 8 h of incubation for all treatments.

After 8 h of incubation, RUP was highest in the Outdoor (73% of CP) and lowest in the CO_2– (51% of CP) condition, while after 48 h of incubation, RUP was highest in the CO_2– (54% of CP) and lowest in the Outdoor (47% of CP) condition. Compared to the Control, the MP after 8 h of incubation was unaffected by nitrogen deficiency, increased by CO_2 deficiency, and decreased when cultivation was operated outdoors. After 48 h of incubation, MP concentrations of Control, CO_2-, and Outdoor conditions were not significantly different, but were significantly higher in the N- treatment. The IDP ranged between 42 and 48% of RUP in the Control and N- conditions, respectively, but was not significantly affected by cultivation ($p > 0.05$).

4. Discussion

The investigated biomasses were derived from four single cultivation batches. Although replicated cultivation could not be performed, the observed differences in nutrient composition can be very likely associated with the applied cultivation conditions. Many preliminary experiments in small scale reactors (1–2 *l*) prior to the cultivations done herein have shown similar effects of cultivation conditions on biomass composition [26]. Furthermore, the observed shifts in nutrient composition are in good agreement with previously published results [7,27–31].

The proximate nutrient composition of the *C. vulgaris* biomass investigated in the current study was in the range of previously reported nutrient profiles of *C. vulgaris* [3,32]. Nitrogen and CO_2 deficiency appears to have led to an increase in EE concentration as well as to a shift in fatty acid composition towards monounsaturated fatty acids. According to Hu et al. [33], nitrogen is the most critical single nutrient affecting lipid accumulation in microalgae. They compiled studies with microalgae species of various taxa, generally observing a trend towards the accumulation of lipids, particularly triacylglycerol, in response to nitrogen deficiency. Nevertheless, in the present study, the clearer effects on lipid accumulation were observed in the batch cultivated under CO_2 deficiency than in the sample cultivated under nitrogen deficiency, likely because nitrogen reduction was not complete and residual nitrogen was available at the end of cultivation in the nitrogen deficient culture. Accumulation of lipids may be a mechanism for carbon and

Table 6
Gas production, digestibility of organic matter and metabolisable energy of *Chlorella vulgaris* depending on variable cultivation conditions[†]. Mean, pooled standard error (SEM) and results of one-factorial analysis of variance (ANOVA); $n = 4$ experimental replicates derived from subsequent in vitro incubations.

	Control	N-	CO_2-	Outdoor	Pooled SEM	*p*-Values (ANOVA)
Gas production (GP)						
GP after 8 h, mL 200 mg^{-1} DM	13.9^{b}	11.6^{c}	17.0^{a}	11.7^{c}	0.71	< 0.001
GP after 24 h, mL 200 mg^{-1} DM	21.5^{a}	16.2^{b}	20.6^{a}	17.9^{b}	0.66	< 0.001
Potential GP, mL 200 mg^{-1} DM	24.5^{a}	17.5^{c}	20.8^{b}	21.5^{b}	0.56	< 0.001
Rate constant of GP, % h^{-1}	10.8^{c}	13.9^{b}	21.3^{a}	9.35^{c}	0.99	< 0.001
Digestibility of organic matter, %	59.8^{a}	52.3^{b}	49.8^{c}	59.5^{a}	0.56	< 0.001
Metabolisable energy, MJ kg^{-1} DM	11.9^{b}	11.4^{c}	14.3^{a}	11.8^{b}	0.10	< 0.001

[a-c]: Different superscripts within one row indicate significant mean differences ($p \leq 0.05$).
[†] Control: Positive control, saturation; N-: Nitrogen deficiency; CO_2-: CO_2 deficiency; Outdoor: Outdoor cultivation.

Table 7
Crude protein, utilizable CP (uCP), ruminally undegradable CP, microbial CP (MP) after 8 and 48 h of incubation and intestinal digestibility of RUP (IDP) of *Chlorella vulgaris* depending on variable cultivation conditions[†]. Mean, pooled standard error (SEM), and results of one-factorial analysis of variance (ANOVA); $n = 6$ experimental replicates derived from subsequent in vitro incubations, unless otherwise stated.

	Control	N-	CO_2-	Outdoor	Pooled SEM	*p*-Values (ANOVA)
Crude protein (CP), g kg^{-1} DM	526	455	328	579		
After 8 h of incubation						
uCP, g kg^{-1} DM	382^{b}	338^{c}	214^{d}	448^{a}	12.8	< 0.001
RUP, % of CP	65^{b}	66^{b}	51^{c}	73^{a}	3.93	< 0.001
MP, % of uCP	11^{b}	11^{b}	23^{a}	6^{c}	2.45	< 0.001
After 48 h of incubation						
uCP, g kg^{-1} DM	271^{b}	247^{c}	195^{d}	299^{a}	6.40	< 0.001
RUP, % of CP	48^{b}	49^{b}	54^{a}	47^{b}	1.32	< 0.001
MP, % of uCP	8^{b}	11^{a}	9^{b}	9^{b}	0.91	0.035
IDP[‡], % of RUP ($n = 3$)	42	48	45	47	3.04	0.413

[a-d]: Different superscripts within one row indicate significant mean differences ($p \leq 0.05$).
[†] Control: Positive control, saturation; N-: Nitrogen deficiency; CO_2-: CO_2 deficiency; Outdoor: Outdoor cultivation.
[‡] The IDP was determined with an enzymatic three-step in vitro system.

energy storage under nitrogen and CO_2 stress in this *Chlorella* strain. The higher proportion of NPN (Table 4) in both deficient cultures may have been caused by a reduction in protein synthesis by the microalgae cells. Despite similar nutrient supply, the Outdoor sample achieved a higher CP concentration than the Control. When storage compounds are accumulated during the day, microalgae can convert these compounds to proteins during the night, if nitrogen is available. This can lead to an increase of protein concentration of up to 70% [34]. Since ATP dependent steps are involved in the metabolic pathways, the storage compounds are partly degraded in the respiratory chain, leading to biomass losses of up to 10%, which can be even higher under unfavourable growth conditions (e.g., low temperature and photon flux density) [35–37]. Therefore, it is possible that dissimilation of storage compounds during night phases increased the relative proportions of CP in the Outdoor sample.

Detected fatty acids were the same for all samples, with the exception of minor concentrations of C18:0 in CO_2- and C18:3 n-6 in Outdoor sample. The presence of C18:3 n-6 is rather surprising, since it is usually not present in *C. vulgaris* [3], while that of palmitic acid, oleic acid, linoleic acid, and linolenic acid as major fatty acids is typical for *C. vulgaris* [38]. Although no indication of contamination was observed during microscopically examination of the sample, it cannot be ruled out that the presence of C18:3 n-6 in the outdoor cultivated sample was caused by contamination with other microalgae or microorganisms.

In contrast to the fatty acid composition, the amino acid composition of the protein was not observably different between the investigated microalgae biomasses. This is in accordance with findings of previous studies, in which nutrient supply, irradiance, growth phase, and harvest regime did not affect AA composition of microalgae proteins [39–42]. Furthermore, several authors observed high similarity of AA profiles even across different microalgae genera [3,43]. It is not surprising that AA composition is relatively constant even under stress conditions, as it may be expected to be genetically fixed. Changes might be possible when ratios of protein fractions with distinct AA compositions are shifted, as has been shown in cereal grains [44]. Furthermore, genetic engineering methods could be applied to extensively modify the AA profiles of microalgae, but this might have adverse effects on consumer acceptance of microalgae products.

Compared to the sum of AA, CP determination appeared to overestimate, and the application of the general nitrogen to protein conversion factor of 4.78 [13] for microalgae appeared to underestimate the protein concentration of *C. vulgaris*. True protein determination with the Barnstein method and application of a species specific nitrogen to protein conversion factor for *C. vulgaris* [14] were in good agreement with the sum of AA and might therefore be applied for protein analysis when data on AA composition are not available. Nevertheless, it is worth noting that even a species specific factor may be inaccurate because of high variability of NPN concentrations of microalgae [3] and therefore analysis of TP in addition to CP is recommended.

Utilization of nutrients in ruminants is primarily determined by microbial fermentation in the rumen. The production of gas is highly related to the degradation of nutrients in the rumen and thus the digestibility of a feedstuff. Consequently, GP is an important measure for the extent of degradation in the rumen and GP kinetics (pGP and rate constant of GP) provide insights into the dynamics of the fermentation processes. Potential GP ranged from 17.5 to 24.5 mL 200 mg^{-1} DM and was at a consistent low level, which is in accordance with a previous study with commercially available microalgae products [24]. The rate constant of GP was highest under CO_2 deficiency. Consequently, GP of CO_2- sample approached the asymptote (pGP) first, after approximately 30 h of incubation, while the Control, N-, and Outdoor samples approached the asymptote of GP later, after approximately 60, 40, and 65 h of incubation, respectively. Accordingly, ruminal fermentation appeared to cease with longer retention time in the CO_2 deficient sample. This is in accordance with the slight change of uCP and RUP over incubation time (cf. Table 7) in this sample. It might be that the high EE concentration (341 g kg^{-1} DM) of CO_2- has hampered microbial fermentation, because high lipid concentrations are known to have negative impact on ruminal fermentation [45]. Since microalgae cells were not disrupted in this study, and CO_2– formed lipid bodies (observed by microscopically examination of the samples), it is possible that the inhibitory effect of the lipids on microbial fermentation was delayed until cell wall and membranes were degraded and lipids were

released. This assumption might also explain the high GP of the CO_2– sample after 8 h of incubation (17.0 mL 200 mg^{-1} DM), which was only slightly increased during further incubation. Furthermore, the lowest dOM and highest GE and ME levels of CO_2– sample were likely related to the high EE concentration of this sample. Nevertheless, since tolerance of EE is rather limited in ruminants [45], it should be used in small quantities, if at all, ensuring a maximum EE content of 4% in total diet DM. Overall low GP and dOM of the other samples might also be related to the relatively high EE concentrations (157–192 g^{-1} kg DM) compared to common feedstuffs. The amount of ruminally fermentable polysaccharides is assumed to be low in microalgae [24]. Additionally, Han and McCormick [46] suggested that microalgae carbohydrates are less fermentable in the rumen than carbohydrates of soybean meal, explaining the overall low level of ruminal fermentation characteristics. On the other hand, low ruminal fermentation indicates that the enzyme activity in the rumen is not well adjusted to microalgae ingredients. Taxonomic composition of rumen microbiota [47,48], as well as enzymes synthesized by the microbiota [49], shifts and adapts to the provided diet. Therefore, it is possible that rumen microbiota can adapt to microalgae when they are provided long-term and hence increase ruminal fermentation of microalgae, but this requires further investigation.

Feedstuffs high in RUP are regarded as valuable protein sources for ruminants, provided that the intestinal digestibility of RUP is high. In the current study, ruminal CP degradation appeared to be particularly affected by CO_2 deficiency and outdoor cultivation, when compared to the control culture. Since AA composition of CP did not appear to be different among the investigated biomasses, it appears unlikely that differences in the type of protein led to the variation in ruminal CP degradation. Nevertheless, it is possible that differences in the structure of microalgae cells affected ruminal CP degradation. For example, some *Chlorella* species tend to form larger cells under favourable conditions, while under nutrient depletion more small cells are formed because of the increased surface area to volume ratio and the resulting faster nutrient uptake [50,51]. Increased surface area to volume ratio could also lead to higher accessibility of microalgae cells to enzymes of the rumen microbiota. On the other hand, the proportion of poorly degradable cell wall compounds may be expected to be higher with a larger number of small cells. Consequently, variability in the cell size distribution caused by cultivation effects might explain the variation in CP degradation, but it will be necessary to further investigate the importance of this issue.

High uCP and RUP and the low level of MP in the present study are in general accordance with the findings of a previous study with commercially available microalgae products [24]. Nevertheless, after 8 h of incubation, uCP and RUP values determined in the current study were slightly lower, particularly for N- and CO_2– samples, when compared to the mean values for non-disrupted *Chlorella* of 470 g uCP kg^{-1} DM and 76% RUP of CP previously published. After 48 h of incubation, accordance of uCP and RUP values with previous data [24] was higher and variation between the samples of the present study was lower. Since CP degradation was incomplete even with longer incubation time, and the proportion of RUP in CP was quite similar for all samples after 48 h of incubation, it appears that there is some kind of core protein in *Chlorella*, which is undegradable in the rumen. This assumption is strengthened by the fact that IDP was not significantly different between the investigated microalgae samples, indicating that the microalgae protein passing the rumen undegraded has very similar characteristics, regardless of its fermentation characteristics in the rumen. As observed previously [24,52], synthesis of MP from ruminally degraded CP was relatively low for *C. vulgaris* in the current study, with more pronounced differences between the samples after 8 h of incubation. The low level of MP is likely related to an energy deficiency for microbial growth, due to the apparently low amounts of ruminally fermentable polysaccharides [24]. The characterisation of the non-starch polysaccharide fraction was not part of the current study, therefore it was not possible to clarify whether accumulation of ruminally fermentable polysaccharides was affected in the investigated *Chlorella* samples. Differences in the amounts and type of polysaccharides might have led to variation in the energy supply for MP synthesis, but this requires further investigation. Interestingly, MP synthesis appeared to be highest with the CO_2 deficient culture and lowest with the outdoor cultivated sample. As mentioned before, high EE concentrations, as present in the CO_2- sample, usually hamper ruminal fermentation and thereby synthesis of MP. Therefore, it is rather surprising that the CO_2- sample had the highest MP values in the current study. Nevertheless, since the effects of oils on rumen microbiota vary for different microbial species and fatty acids [53], it is possible that only specific microbial groups (e.g., protozoa) were affected by the high EE concentrations, but overall MP synthesis was not reduced.

Because of the high level of uCP and RUP, especially in the Control and Outdoor samples, where *C. vulgaris* was cultivated under favourable conditions, microalgae biomass appears to have a high potential as protein source for ruminants. The similarity of the Control and outdoor cultures in terms of protein value suggests that the laboratory data is transferable to a pilot scale. However, nutrient deficient conditions led to somewhat adverse effects on the protein value by the reduction of uCP and RUP, especially in the CO_2- culture. The effects of nitrogen deficiency were less marked because of the lower impact on CP, but were also adverse in terms of the protein value. Consequently, adequate supply of CO_2 and nitrogen must be ensured during cultivation, when *C. vulgaris* is intended for use as a protein source for ruminants. In addition, the relatively low level of IDP in all samples restricts the potential of *C. vulgaris* as protein source. Since the applied cultivation conditions did not have a significant effect on IDP, further strategies (e.g., processing techniques, further cultivation factors, and specific strain selection) are needed to increase IDP and thereby the protein value of *C. vulgaris*.

Most of the investigated nutritional characteristics were significantly affected by cultivation conditions, but the overall change was rather small or in some cases even adverse (e.g., dOM, pGP, RUP, and uCP). Consequently, the applied cultivation conditions appear not to be suitable to considerably shift ruminal fermentation to increase the nutritional value for ruminants. Nevertheless, since a previous study by our working group with commercially available microalgae products [24] revealed higher variability in ruminal fermentation characteristics and the nutritional value of *C. vulgaris*, it appears that there might be influencing factors that could enhance the nutritional value of microalgae for ruminants. It is possible that the supply of other nutrients (e.g., phosphorus, sulphur, iron, or magnesium), environmental factors (e.g., temperature, pH, or salinity), a thorough cell disruption, or more extreme applications of the applied factors would have more pronounced effects on the nutrient utilization of the animal and thus the feeding value. Additionally, targeted selection of microalgae species or specific strains that accumulate polysaccharides instead of lipids under stress conditions could enable the production of microalgae biomasses with higher ruminal degradability, although this requires further investigation.

The biomass productivity of the control cultivation (0.22 g CDM L^{-1} d^{-1}) was in the same order of magnitude as previously published results [54]. The N- and the outdoor cultivations reached similar values of 0.23 and 0.21 g CDM L^{-1} d^{-1}, respectively. The lowest productivity was observed in the CO_2 deficient culture (0.09 g CDM L^{-1} d^{-1}). It is likely that the cause of the low productivity and growth rate in the CO_2– sample was a lack of carbon. Interestingly, productivity appeared not to be reduced in the N- cultivation, despite the deficient supply of nitrogen. In the outdoor cultivation, the slightly reduced productivity could be explained by a deficiency of energy, i.e., photons. Although the mean total photon flux density of the outdoor cultivation (475 μmol photons m^{-2} s^{-1}) was higher than the applied photon flux density indoors (377 μmol photons m^{-2} s^{-1}), the effects of day/night cycles and the reflection of photons from the photobioreactor

surface due to steep incidence angles led to a reduction in productivity. Corresponding to the above-mentioned phenomena, nutrient productivity (Table 2) was considerably reduced in the CO_2– cultivation. In the case of N deficiency, production of CP, uCP, and digestible organic matter was reduced despite the slightly higher biomass productivity. In contrast, the high concentrations of CP and uCP in the outdoor culture were able to compensate for its slightly lower biomass productivity and the Outdoor cultivation yielded the highest CP and uCP productivities. These observations show that an optimized productivity may not necessarily mean an optimized product yield, when concentrations of utilizable nutrients are reduced and vice versa. Therefore, optimization of microalgae production for application as a feedstuff should not only consider the productivity of the process, but must also evaluate the accumulation of nutrients and in particular their utilization by the animal. Additionally, production of different nutrients may be contradictory. For example, the N- treatment yielded the highest EE productivity, but the productivity of CP, uCP, and dOM was impaired. Therefore, optimization strategies may be variable, depending on the intended application.

5. Conclusions

Nutrient composition, as well as characteristics of the nutritional value were affected by the cultivation process of *C. vulgaris*. Nutrient deficient conditions led to adverse effects in terms of digestibility, protein value, and nutrient productivity. Therefore, it will be necessary to investigate further strategies that not only achieve a high productivity of cultivation but also considerably enhance nutrient utilization and thus the nutritional value for the animal. Outdoor cultivation yielded similar or even higher values than the laboratory control culture for most of the investigated nutritional characteristics. Therefore, it appears reasonable to transfer data on nutritional characteristics of laboratory *C. vulgaris* cultivations to a pilot scale, but it will be necessary to confirm these findings for further cultivation conditions and microalgae species utilizing a large number of cultivations.

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Declaration of authors contributions

KW, AT, HS, CP, and MR designed the study, regularly discussed its progress and interpreted the results. AT, MK, and CP planned and conducted the algae cultivation. KW performed chemical and statistical analyses and conducted the in vitro experiments. KW and AT drafted the article; HS and MR critically revised the article. MR obtained the funding of the work.

Conflict of interest statement

The authors declare no conflict of interests.

Statement of animal rights

All experimental procedures concerning the handling and treatment of the animals were conducted in accordance with the German animal welfare legislation and were approved by the Regierungspräsidium Stuttgart, Germany.

Declaration of authors agreement to authorship and submission

All authors declare agreement to authorship and to submission of this article.

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CHAPTER 6

SUMMARY

6. SUMMARY

The predicted increase of the world population will lead to a continuous increase in the demand for meat and dairy products. The concurrent decline of arable land per capita additionally strengthens the need for improved utilization of feed resources, as well as 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 heterogonous group of unicellular photosynthetic organisms that have raised interest for application in the feed sector because of their high potential of production of high value compounds. They can be cultivated without the use of arable and, thus, provide the opportunity of feed production on currently idle land without competing with food production. Nevertheless, systematic data on nutritional characteristics of microalgae are rare, particularly for ruminants. The high diversity of species and the high capacity of microalgae to adapt to environmental conditions is a challenge to deal with, because findings on nutritional properties might not be easily generalised for different microalgae species or possibly not even for the same species. Many microalgae species develop robust cell walls that might restrict their nutritional value. Literature results indicate that cell disruption might enhance nutrient availability. Nevertheless, investigations on the effects of cell disruption methods on the nutritional value of microalgae for farm animals are scarce and have not been made in ruminant animals.

The main objective of the present thesis was to systematically determine nutritional characteristics of microalgae and evaluate the suitability of microalgae as feedstuffs, particularly for ruminants. The experiments comprised a comprehensive characterization of microalgae nutrient profiles with chemical-analytical methods and the determination of extent and dynamics of nutrient utilization using *in vitro* methods.

In order to generate a comprehensive database on nutrient composition of microalgae, 16 commercial microalgae biomasses of four genera (*Arthrospira*, *Chlorella*, *Nannochloropsis*, and *Phaeodactylum*) were analysed utilizing established methods for food and feed evaluation (Manuscript 1). These investigations included determination of the *in vitro* crude protein (CP) digestibility for pigs. Nutrient analyses showed a considerable variation particularly in concentrations of proximate nutrients, minerals, and fatty acids, both among and within genera. This variation presumably resulted from varying cultivation conditions and it was concluded that general mean values are not appropriate to characterize microalgae in terms nutrient composition.

Manuscript 2 aimed to determine characteristics of the nutritional value of microalgae for ruminants utilizing different *in vitro* methods. The commercial biomasses included in Manuscript 1 were investigated using the Hohenheim Gas Test method. The investigations comprised the determination of several ruminal fermentation characteristics, of the energy value, and of the protein value. A three-step enzymatic *in vitro* system was used to estimate intestinal digestibility of ruminally undegraded CP (IDP). Ruminal fermentation of the investigated microalgae biomasses was overall low, which was indicated by an overall low level of production of gas and volatile fatty acids, and a low ruminal CP degradation. As a result of low ruminal fermentation, microalgae biomasses were characterized by high concentrations of ruminally undegradable CP (RUP; 386, 399, 315, and 263 g RUP/kg DM at passage rate of 8 %/h for *Arthrospira*, *Chlorella*, *Nannochloropsis*, and *Phaeodactylum*, respectively). Thus, microalgae appear to be potential alternative protein sources for high performing animals. However, this was contradicted by low IDP, which was determined for microalgae in the present thesis for the first time (mean values for *Arthrospira*, *Chlorella*, *Nannochloropsis*, and *Phaeodactylum* were 27, 43, 43, and 40 % of RUP respectively). The variation observed in nutrient profiles was reflected in several nutritional characteristics.

A common objective of Manuscript 1 and Manuscript 2 was to investigate whether cell disruption affects nutritional characteristics of microalgae. In Manuscript 1, effects of cell disruption on *in vitro* CP digestibility for pigs were investigated and in Manuscript 2 several *in vitro* methods were utilized to investigate effects of cell disruption on the nutritional value of microalgae for ruminants. Mechanical cell disruption with a ball mill enhanced *in vitro* CP digestibility and ruminal fermentation in most of the samples, presumably by the destruction of cells and hence an increase in nutrient accessibility. Nevertheless, concerning the protein value of microalgae in ruminants, application of mechanical cell disruption cannot not be recommended because it decreased RUP but did not increase IDP so that intestinal digestible RUP was overall decreased by cell disruption.

The aim of the third manuscript was to investigate the effects of variable cultivation conditions on nutrient composition and nutritional characteristics for ruminants of the microalgae *Chlorella vulgaris*. *Chlorella vulgaris* was cultivated under varying conditions (saturation, nitrogen and CO_2 depletion, outdoor cultivation). The obtained biomasses were analysed for their nutrient composition and their nutritional value for ruminants using different *in vitro* methods. Both, nutrient composition and characteristics of the nutritional value for

ruminants were affected by the cultivation process. Nutrient deficient conditions had rather adverse effects in terms of digestibility, protein value, and nutrient productivity.

It can be concluded that microalgae have potential as alternative protein source for ruminants. Nevertheless, this potential is contradicted by low IDP, but the findings obtained herein have to be verified in future studies. Furthermore, the results of the present thesis show that nutrient composition and ruminal fermentation characteristics of microalgae vary considerably between and in many cases even within microalgae genera. Thus, to the extent possible, it should be strived for a standardisation of cultivation conditions, in order allow better predictions of nutritional characteristics of microalgae.

CHAPTER 7

ZUSAMMENFASSUNG

7. ZUSAMMENFASSUNG

Der prognostizierte Anstieg der Weltbevölkerung wird zu einem kontinuierlich steigenden Bedarf an Fleisch- und Milchprodukten führen. Der gleichzeitige Rückgang an landwirtschaftlichen Nutzflächen erhöht zusätzlich die Notwendigkeit einer verbesserten Verwertung von Futterressourcen sowie die Etablierung von alternativen Futterquellen, die nicht mit der Produktion von Lebensmitteln konkurrieren oder unabhängig von landwirtschaftlichen Nutzflächen erzeugt werden können. Mikroalgen sind eine heterogene Gruppe von einzelligen Mikroorganismen, die zur Photosynthese fähig sind. Aufgrund ihres hohen Potentials hochwertige Inhaltstoffen zu synthetisieren, haben sie Interesse für die Anwendung im Futtermittelsektor erweckt. Sie können unabhängig von landwirtschaftlichen Nutzflächen erzeugt werden, sodass sie die Produktion von Futtermitteln auf derzeitigen Brachflächen ermöglichen, ohne mit der Lebensmittelproduktion zu konkurrieren. Allerdings gibt es derzeit nur sehr wenige systematische Daten zu ihren Futterwerteigenschaften, insbesondere für Wiederkäuer. Die große Diversität an Mikroalgenspezies und ihr hohes Potential, sich an Umweltbedingungen anzupassen, stellen eine große Herausforderung dar, da Ergebnisse zum Nährwert einer Mikroalgenspezies wahrscheinlich nicht auf andere Spezies übertragen werden können. Dies gilt möglicherweise sogar innerhalb einer Mikroalgenspezies. Darüber hinaus entwickeln viele Mikroalgen stabile Zellwände, die ihren Futterwert möglicherweise stark einschränken. Literaturergebnisse weisen darauf hin, dass ein Zellwandaufschluss die Nährstoffverfügbarkeit erhöhen kann. Allerdings ist dies bei landwirtschaftlichen Nutztieren noch weitgehend unerforscht.

Das Hauptziel der vorliegenden Arbeit war es, systematisch Futterwerteigenschaften von Mikroalgen zu untersuchen und ihre Eignung als Futtermittel, insbesondere für Wiederkäuer, zu bewerten. Die Untersuchungen beinhalteten eine umfassende Charakterisierung der Nährstoffprofile von Mikroalgen mit Labormethoden, sowie die Bestimmung von Ausmaß und Dynamik der Nährstoffverwertung mit *in vitro* Methoden.

Um eine umfassende Datengrundlage zu den Nährstoffprofilen von Mikroalgen zu schaffen, wurden 16 kommerzielle Mikroalgenbiomassen von vier Gattungen (*Arthrospira, Chlorella, Nannochloropsis* und *Phaeodactylum*) mit etablierten Methoden der Lebens- und Futtermittelbewertung untersucht (Manuskript 1). Diese Untersuchungen beinhalteten die Bestimmung der Verdaulichkeit des Rohproteins (XP) von Mikroalgen für Schweine. Die Nährstoffanalytik zeigte sowohl zwischen als auch innerhalb der Gattungen eine erhebliche Variation, insbesondere in den Konzentrationen von Rohnährstoffen, Mineralstoffen und

Fettsäuren. Diese Variation resultierte vermutlich aus unterschiedlichen Kultivierungsbedingungen und es wurde geschlussfolgert, dass allgemeingültige Mittelwerte ungeeignet sind, um Mikroalgen hinsichtlich ihrer Nährstoffzusammensetzung zu charakterisieren.

Das Ziel der Untersuchungen für Manuskript 2 war es, Futterwerteigenschaften von Mikroalgen für Wiederkäuer mit verschiedenen *in vitro* Methoden zu bestimmen. Die kommerziellen Biomassen aus Manuskript 1 wurden mit der Methode des Hohenheimer Futterwerttests untersucht. Die Untersuchungen umfassten die Bestimmung verschiedener Charakteristika der ruminalen Fermentation, des Energiewertes und des Proteinwertes. Ein dreistufiges, enzymatisches *in vitro* System wurde verwendetet, um die intestinale Verdaulichkeit des ruminal nicht abbaubaren XP (IDP) zu ermitteln. Die ruminale Fermentation der untersuchten Mikroalgen war gering, was anhand des generell niedrigen Niveaus der Produktion von Gas und flüchtigen Fettsäuren, sowie dem niedrigen ruminalen XP-Abbau deutlich wurde. Als Folge des niedrigen ruminalen XP-Abbaus wurden die Mikroalgenbiomassen durch hohe Konzentrationen an ruminal nicht abbaubaren XP (UDP) geprägt (386, 399, 315 und 263 g UDP/kg Trockenmasse bei einer Passagerate von 8 %/h für *Arthrospira, Chlorella, Nannochloropsis* und *Phaeodactylum*). Demnach scheinen Mikroalgen als alternative Proteinquelle Potential zu haben, insbesondere für Hochleistungstiere. Allerdings ist dieses Potential durch die geringe IDP, die im Rahmen der vorliegenden Arbeit das erste Mal für Mikroalgen ermittelt wurde, möglicherweise stark eingeschränkt (Mittelwerte für *Arthrospira, Chlorella, Nannochloropsis* und *Phaeodactylum*: 27, 43, 43 und 40 % des UDP). Die Variation, die bei den Nährstoffprofilen beobachtet wurde, spiegelte sich auch in verschiedenen Futterwerteigenschaften für Wiederkäuer wider.

Ein gemeinsames Ziel von Manuskript 1 und Manuskript 2 war es, zu untersuchen ob ein Zellwandaufschluss die Futterwerteigenschaften von Mikroalgen beeinflusst. In Manuskript 1 wurde der Effekt eines Zellwandschlusses auf die *in vitro* XP-Verdaulichkeit von Mikroalgen beim Schwein untersucht. In Manuskript 2 wurden verschiedene *in vitro* Methoden genutzt um die Effekte eines Zellwandaufschlusses auf Futterwerteigenschaften für Wiederkäuer zu untersuchen. Ein mechanischer Zellwandaufschluss mit einer Rührwerkskugelmühle erhöhte bei den meisten Proben die *in vitro* XP-Verdaulichkeit und die ruminale Fermentation, was wahrscheinlich auf die Zerstörung der Zellwände und die daraus resultierende Erhöhung der Nährstoffverfügbarkeit zurückzuführen war. Allerdings führte dies zu einer geringeren Konzentration an UDP, wobei die IDP nicht zunahm, sodass die Menge an intestinal

verdaulichem UDP generell sank. Daher ist die Anwendung eines mechanischen Zellwandaufschlusses von Mikroalgen hinsichtlich ihres Proteinwertes für Wiederkäuer als ungünstig zu bewerten und kann nicht empfohlen werden.

Das Ziel des dritten Manuskripts war es, die Effekte von verschiedenen Kultivierungsbedingungen auf die Nährstoffzusammensetzung und die Futterwerteigenschaften für Wiederkäuer bei der Mikroalge *Chlorella vulgaris* zu untersuchen. *Chlorella vulgaris* wurde bei variierenden Bedingungen (Nährstoffsättigung, Stickstoff- und CO_2-Limitierung, Freiland) kultiviert. Die gewonnenen Biomassen wurden hinsichtlich ihrer Nährstoffzusammensetzung und ihrer Futterwerteigenschaften für Wiederkäuer mit *in vitro* Methoden untersucht. Sowohl die Nährstoffzusammensetzung als auch Futterwerteigenschaften für Wiederkäuer wurden durch den Kultivierungsprozess beeinflusst. Hinsichtlich der Verdaulichkeit und des Proteinwertes, sowie hinsichtlich der Nährstoffproduktivität hatte eine Nährstofflimitierung eher ungünstige Effekte.

Zusammenfassend lässt sich sagen, dass Mikroalgen Potential als alternative Proteinquelle für Wiederkäuer haben. Allerdings ist dieses Potential durch die niedrige IDP möglicherweise stark einschränkt. Daher sollten die Ergebnisse der vorliegenden Arbeit in zukünftigen Studien verifiziert werden. Außerdem zeigen die Ergebnisse dieser Arbeit, dass sowohl die Nährstoffzusammensetzung, als auch die Futterwerteigenschaften von Mikroalgen für Wiederkäuer einer großen Variation unterliegen. Die gilt nicht nur zwischen den untersuchten Mikroalgengattungen, sondern in vielen Fällen auch innerhalb einer Gattung. Daher sollte, soweit möglich, eine Standardisierung der Kultivierungsbedingungen angestrebt werden, um eine bessere Vorhersagbarkeit von Futterwerteigenschaften für Mikroalgen zu ermöglichen.

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CURRICULUM VITAE

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Stuttgart-Hohenheim, 14.02.2019

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DECLARATION IN LIEU OF AN OATH ON INDEPENDENT WORK

according to Section 18 (3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

"Investigations on nutritional characteristics of microalgae with emphasis on ruminants"

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

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I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Stuttgart-Hohenheim, 14.02.2019

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