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Different microalgae species as a substitutive protein feed for soya bean meal in grass silage based dairy cow diets

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ABSTRACT

This experiment was conducted to evaluate different microalgae species as protein supplements in the nutrition of lactating dairy cows in comparison to soya bean meal. Four multiparous lactating Finnish Ayrshire cows (112 days in milk) were used in a balanced 4 × 4 Latin square study. Cows were fed separately fixed amount of cereal-sugar beet pulp based concentrate (12.5 kg/d) and grass silage ad libitum. Experimental treatments consisted of four isonitrogenous protein supplements: soya bean meal (SOY), *Spirulina platensis* (SPI), *Chlorella vulgaris* (CHL) and a mixture of *C. vulgaris* and *Nannochloropsis gaditana* (1:1 on dry matter (DM) basis; CHL-NAN). The substitution of soya bean meal by microalgae did not affect the quantity of total DM intake (DMI), but changed the composition of DMI by decreasing the concentrate:forage ratio of the diet ($P = 0.054$) owing to the poorer palatability of microalgae. Intake of methionine was increased ($P < 0.01$) and that of histidine decreased ($P < 0.01$) with microalgae diets compared to SOY, but no significant changes in arterial concentrations were observed. The digestibility of nutrients, milk or energy corrected milk (ECM) yield were not affected by dietary treatments. Though, owing to SPI, algae diets resulted in numerically +2.2 kg/d higher ECM yield than SOY. Microalgae diets tended to result in higher milk fat ($P = 0.073$), arterial acetic acid ($P = 0.055$) and non-esterified fatty acid ($P = 0.060$) concentrations than SOY. Milk fat ($P < 0.05$) and arterial acetic acid ($P = 0.010$) concentrations were increased and milk fat yield tended to increase ($P = 0.098$) on SPI compared to CHL and CHL-NAN. Urinary nitrogen excretion was also lower ($P < 0.05$) for microalgae diets than for SOY. Microalgae diets resulted in higher secretion of Δ16:2 ($P < 0.05$), *cis*-9, *cis*-12, *cis*-15 18:3 (α-linoleic acid; ALA) ($P < 0.05$), *cis*-6, *cis*-9, *cis*-12 18:3 ($P < 0.05$) and polyunsaturated fatty acids (PUFA) ($P < 0.05$) in milk than SOY. Secretion of *cis*-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 20:5 (eicosapentaenoic acid; EPA) in milk tended to be higher on CHL and CHL-NAN than on SPI ($P = 0.060$), and was higher on CHL-NAN than on CHL ($P < 0.05$). Also the omega-6:omega-3 ratio was lower ($P < 0.05$) for CHL-NAN than for CHL. The results suggest that microalgae are likely comparable protein feed to soya bean meal in dairy cow nutrition, especially if palatability of microalgae can be improved.

Abbreviations: ALA, α-linoleic acid; CHL, experimental treatment containing *Chlorella vulgaris*; CHL-NAN, experimental treatment containing *C. vulgaris* and *Nannochloropsis gaditana*; EPA, eicosapentaenoic acid; LA, linoleic acid; NUE, the efficiency of N utilisation for milk production; SOY, experimental treatment containing soya bean meal; SPI, experimental treatment containing *Spirulina platensis*

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1. Introduction

European Union (EU) is highly dependent on protein feed imports and especially the self-sufficiency of soya beans (*Glycine max*) is very low being only 5% (Bouxin, 2017). This makes livestock sector vulnerable to trade distortions, availability and price volatility of soya beans (Häusling, 2011; de Visser et al., 2014). The reduction of the protein deficit of EU is considered to have multiple ecological and socio-economic advantages (Häusling, 2011). This requires both the improvement of current protein feed production systems such as increased utilisation of pulses as well as the development of novel protein feed resources such as microalgae, that include both prokaryotic species such as cyanobacteria, and eukaryotic species such as chlorophytes belonging to green algae.

Due to their extremely rapid growth rate (Chisti, 2007), microalgae outyield conventional protein feed resources on area basis (van Krimpen et al., 2013). Because microalgae cultivation can take place in marginal or non-arable land, it has also been suggested that the feed use of microalgae has a potential to improve food security (Efroymson et al., 2017). The high production cost of microalgae (Pang et al., 2018) makes them currently an uncompetitive feed option, but the situation may change in the near future due to e.g. technical development and different policy interventions such as incentives and carbon taxation. In our previous experiment with dairy cows, the substitution of rapeseed meal by microalgae affected the composition but not total amount of DMI as the poor palatability of microalgae was compensated by increased silage intake and consequently the proportion of concentrate in the diet was decreased (Lamminen et al., 2017). Furthermore, feeding microalgae resulted in lower milk protein yield, efficiency of nitrogen utilisation (NUE), and milk production response to increased crude protein intake, which suggested lower protein value of microalgae than that of rapeseed meal. However, microalgae may perform more favourably in comparison to soya bean meal as rapeseed meal generally results in greater milk production response than soya bean meal on grass silage based diets (Huhtanen et al., 2011; Martineau et al., 2013).

The choice of microalgae species likely affects animal performance as the species differ greatly in chemical composition, including amino acid (AA) composition (Becker, 2013), but also in protein degradability (Costa et al., 2016) and cell wall construction. The effect of the latter has been demonstrated in biogas production where *Chlorella* sp. (chlorophyta) and *Nannochloropsis* sp. (ochrophyta) have resulted in lower fermentability than other strains due to resistant cell wall (Bohutskyi et al., 2014), and cyanobacteria showed higher fermentability than *C. vulgaris* (Mendez et al., 2015). Crude fat and EPA content is higher in *Nannochloropsis* sp. (Sukenik et al., 1993) than in *Spirulina platensis* (cyanobacterium) and *C. vulgaris* (Chacón-Lee and González-Mariño, 2010), which can have positive effect on milk EPA and PUFA concentration similarly to fish oil with high EPA content (Kairenius et al., 2015). However, as high dietary fat and especially PUFA concentration can decrease DMI (Onetti and Grummer, 2004; Weld and Armentano, 2017) e.g. via negative effects on ruminal fermentation (Allen, 2000), and oxidation of EPA can result in “fishy” odour compounds (Hammer and Schieberle, 2013), the effect of *N. gaditana* on DMI is likely more negative than that of *S. platensis* and *C. vulgaris*.

The objective of the current study was to compare the effects of different microalgae species (*S. platensis, C. vulgaris* and *N. gaditana*) and soya bean meal on dairy cow performance, milk fatty acid composition and N utilisation. We hypothesised that substitution of soya bean meal by microalgae would (1) decrease DMI owing to the poorer palatability of microalgae and especially the long chain PUFA in *N. gaditana*. In addition, (2) *S. platensis* would result in higher milk production than *C. vulgaris* and *N. gaditana* due to differences in cell wall composition and digestibility; and (3) inclusion of *N. gaditana* in the diet would increase the concentration of omega-3 fatty acids (FA) in milk.

2. Materials and methods

2.1. Animals, experimental design and diets

Study was conducted at the University of Helsinki research farm in Helsinki, Finland and approved by the National Animal Experiment Board in Finland according to the guidelines imposed by the European Union Directive 2010/63/EU and the current Finnish legislation on animal experimentation (Act on the Protection of Animals Used for Scientific or Educational Purposes 497/2013).

Four multiparous Finnish Ayrshire cows averaging 112 ± 21.6 d (mean ± SD) in milk were used in 4 × 4 Latin square study with four different protein feed rations and four 21 d periods, of which the latter 7 d formed a sampling period. At the beginning of the experiment, the cows had an average milk yield of 36.2 ± 3.77 kg/d, body weight (BW) of 652 ± 79.5 kg and body condition score of 2.7 ± 0.38 in a scale of 1–5 (Edmonson et al., 1989). The average weight change during the experiment was −0.263 ± 0.838 kg/d.

The cows were randomly assigned to four dietary treatments. Treatments consisted of pelleted cereal-sugar beet pulp (A-Rehu Ltd., Seinäjoki, Finland) supplemented with (1) pelleted soya bean supplement (**SOY**) (A-Rehu Ltd.), (2) *S. platensis* (**SPI**) (Duplaco B.V., Hengelo, the Netherlands), (3) *C. vulgaris* (**CHL**) (Duplaco B.V), or (4) mixture of *C. vulgaris* and *N. gaditana* (1:1 on DM basis; **CHL-NAN**) (Duplaco B.V). Terms of spirulina, chlorella and nannochloropsis are later used to describe *S. platensis*, *C. vulgaris* and *N. gaditana* used in current experiment, respectively, and collective term microalgae to describe both eukaryotic and prokaryotic microphytes. Soya bean supplement contained 833 g/kg DM of soya bean meal, the protein of which was isonitrogenously substituted totally by microalgae protein. Equal quantity of concentrate (12.5 kg/d on fresh matter basis) among diets was adjusted with cereal-sugar beet pulp. Small amount of molassess (Suomen Rehu Ltd., Hyvinkää, Finland) and molassed sugar beet pulp (Suomen Rehu Ltd.) were added to microalgae diets (SPI, CHL and CHL-NAN) to compensate for the contribution of these ingredients in soya bean supplement. Water was added to algae (around 130 ml/kg of concentrates) before mixing it daily with other concentrate components to bind algae powder on pellets. No water was added to concentrates on SOY. In addition to other concentrate components, cows

Table 1
Concentrate ingredient profiles of the experimental diets.

	Treatment[a]			
	SOY	SPI	CHL	CHL-NAN
Ingredients, kg DM/d				
Cereal-sugar beet pulp	9.09	9.64	9.44	9.18
Molassed sugar beet pulp		0.18	0.18	0.18
Molasses		0.06	0.06	0.06
Soya bean supplement	1.85			
Spirulina platensis		1.12		
Chlorella vulgaris			1.35	0.81
Nannochloropsis gaditana				0.82
Mineral-vitamin supplement	0.30	0.30	0.30	0.30
Total	11.2	11.3	11.3	11.4
N in concentrates, g/d	310	321	317	312
N in supplementary protein feed, g/d[b]	125	126	126	126

DM dry matter, N nitrogen.
[a] SOY = soya bean meal as a protein feed; SPI = *Spirulina platensis* as a protein feed; CHL = *Chlorella vulgaris* as a protein feed, CHL-NAN = mixture of *Chlorella vulgaris* and *Nannochloropsis gaditana* (1:1 on dry matter basis) as a protein feed.
[b] The protein feed of soya bean meal in soya bean supplement was isonitrogenously substituted by microalgae protein.

were offered mineral-vitamin supplement (Pihatto-Melli Plus, Raisioagro Ltd., Raisio, Finland). Cows had ad libitum access to water and grass silage, that was preserved from the secondary growth of timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) mixture, pre-wilted and ensiled with formic acid based additive applied at a rate of 6 l/1000 kg (AIV 2 Plus, Kemira Ltd., Helsinki, Finland). The complete concentrate profiles of the diets and the chemical composition of experimental feeds are described in Tables 1 and 2, respectively. Silage was offered to the animals three times (at 0900, 1400 and 1800 h) and concentrates five times (at 0600, 1100, 1430, 1700 and 1930) daily. Cows were housed in individual tie stalls equipped with Roughage Intake Control system (Insentec BV, Marknesse, the Netherlands) and separate concentrate troughs. Cows were milked twice daily at 0600 and 1700 h.

One animal was removed from the experiment due to feed intake related problems on microalgae diets. This resulted in two missing observations in the experiment, one on CHL (period 3) and the other on SPI (period 4) (n = 14).

2.2. Measurements, sampling and chemical analysis

Feed intake and milk yield of the cows were recorded daily throughout the experiment but only measurements on d 15 to 20 of each period were used for statistical analysis. The details of sampling and analysis of feed, blood, milk, urine and faeces have been described earlier (Lamminen et al., 2017). Briefly, blood samples from mammary vein and tail vessel were collected on d 21 at 0530, 0830 and 1130 h. Milk samples were collected over four consecutive milkings starting on d 18 at 1700 h, and analysed for fat, crude protein (CP), lactose and urea in commercial laboratory (Valio Ltd., Seinäjoki, Finland). For analysis of FA, unpreserved milk samples were composited according to yield and stored at −20 °C until analysed. Spot samples of faeces were obtained from the rectum of each cow at 0700 and 1600 h on d 17 to 20 of each period. Spot samples of urine were obtained by mild manual stimulation of the vulva on d 18 at 0530 and 1430 and on d 19 at 1000 and 1900 h. Cows were weighed on two consecutive days at the beginning and end of the experiment (CV 9600 Scale, Solotop Ltd., Helsinki, Finland).

The DM, organic matter (OM), Kjeldahl-nitrogen (N) or CP (Kjeldahl-N × 6.25), neutral detergent fibre (NDF), and ash insoluble ash (AIA) content of feed and faecal samples, crude fat content of concentrate components, and water soluble carbohydrate, NH_3-N, lactic acid, volatile fatty acid (VFA), indigestible NDF (iNDF), and in vitro digestible OM in DM (DOMD) content of silage, were determined as described in our previous paper (Lamminen et al., 2017). AIA was used as an internal marker to determine total tract apparent digestibility of the diets. Analysis of AA composition of feeds and plasma, as well as the plasma concentrations of acetic acid, β-hydroxybutyric acid (BHBA), glucose, insulin and non-esterified fatty acids (NEFA), and urine concentration of purine derivatives and urea, are also described earlier (Lamminen et al., 2017). For NDF analysis, crucibles with pore size of 40–100 µm were used for all samples and heat stable amylase for analysis of concentrate components. In addition, NDF concentration of microalgae was also analysed using crucibles with pore size 16–40 µm. Results of NDF are expressed exclusive of residual ash. Naming of AA were conducted according to International Union of Pure and Applied Chemistry (IUPAC). Terms Nπ (nitrogen atom closest to the side chain) and Nτ (nitrogen atom furthest from the side chain) are used later on to describe the position of methylated nitrogen atoms in the imidazole ring of histidine, according to the IUPAC recommendations. Thus, 3-methylhistidine, the product of muscle actin and myosin catabolism will be referred as Nτ-methylhistidine, and 1-methylhistidine, the product of anserine breakdown will be referred as Nπ-methylhistidine.

Feed samples destined for the analysis of medium and long chain FA were freeze-dried (Christ, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), ground to pass a 1 mm sieve and stored at −80 °C until analysis. Following the addition of tridecanoic acid as an internal standard (0.50 mg, T0502, Sigma-Aldrich, Helsinki, Finland), 1 mL of deionised water and adjusting the pH to 2.0 by 2 M hydrochloric acid, lipid in freeze-dried feeds (100 mg) was extracted twice using ultrasound and a 3:2 (4 ml, v:v) mixture of hexane and isopropanol as outlined previously (Shingfield et al., 2003a). Extracted lipids

Table 2
Chemical, amino acid (AA) and major fatty acid (FA) composition of experimental feeds.

	Silage[a]	Cereal-sugar beet pulp[b]	Molassed sugar beet pulp	Molasses	Mineral-vitamin supplement[c]	Soya bean supplement[d]	*Spirulina platensis*	*Chlorella vulgaris*	*Nanno-chloropsis gadiatana*
Dry matter, g/kg	272	900	879	703	992	878	947	948	962
Ash, g/kg DM	81.4	35.8	67.0	99.4	918	75.5	71.8	51.4	158
Crude protein, g/kg DM	135	124	111	68.8		439	693	586	385
Crude fat, g/kg DM		46.2	3.55			11.1	51.0	123	192
Neutral detergent fibre, g/kg DM[e]	506	361	341			158	0	0	90.0
Neutral detergent fibre, g/kg DM[f]							87.4	15.1	219
Starch, g/kg DM							66.2	54.0	26.1
Essential AA, g/kg crude protein									
Arginine	36.5	56.4	39.6	5.42		78.6	80.4	58.0	62.0
Histidine	16.4	22.0	27.6	3.32		27.4	17.7	18.3	17.6
Isoleucine	36.8	34.0	37.1	17.8		46.1	52.0	32.6	38.9
Leucine	68.3	66.2	57.7	17.8		79.9	83.4	78.9	79.1
Lysine	45.5	35.6	53.3	4.02		62.4	36.0	59.5	55.2
Methionine	11.8	9.72	10.0	13.9		9.76	22.9	20.8	19.4
Phenylalanine	44.4	45.7	34.0	6.56		55.3	50.8	45.3	44.8
Threonine	39.7	36.0	40.6	6.25		43.1	50.6	38.8	44.8
Tryptophan	13.9	18.6	14.7	7.81		20.5	12.5	15.6	14.3
Valine	48.5	48.2	50.1	14.9		47.0	57.8	48.1	50.4
Non-essential AA, g/kg crude protein									
Alanine	59.2	43.5	49.6	24.0		43.5	67.2	79.7	72.4
Aspartic acid	76.4	65.8	64.6	32.1		107	82.1	77.1	80.2
Cysteine	5.61	12.9	6.35	23.1		10.1	7.94	10.4	8.63
Glutamic acid	84.7	194	91.8	142		179	122	104	123
Glycine	45.3	45.2	40.9	15.9		45.8	52.4	51.4	51.3
Proline	46.8	77.9	42.4	12.6		53.0	35.5	47.4	54.8
Serine	39.8	44.6	44.1	9.13		56.0	50.2	36.1	40.0
Tyrosine	26.8	29.6	41.6	13.0		38.0	48.8	32.4	33.8
Σ Branched AA[g]	154	149	145	50.4		173	193	160	168
Σ Essential AA	362	372	365	97.7		470	464	416	427
Σ Non-essential AA[h]	385	513	381	272		532	466	439	464
Σ Total AA[i]	746	886	746	370		1003	930	855	890
Fatty acids, g/100 g FA									
16:0	16.6	19.8	22.2	18.8		14.7	45.6	15.8	24.3
cis-9 16:1	0.237	0.147	0.244			0.163	2.80	0.493	35.5
Δ16:2								26.4	
18:0	2.17	1.49	0.725	3.61		2.95	1.05	0.160	0.719
cis-9 18:1	5.47	20.4	13.6	31.2		20.5	2.73	2.47	5.38
cis-9, *cis*-12 18:2 (n-6)	18.7	50.1	50.1	34.5		50.5	23.4	48.5	1.401
cis-9, *cis*-12, *cis*-15 18:3 (n-3)	47.4	4.95	9.09	8.15		7.45	0.419	2.31	0.034
cis-6, *cis*-9, *cis*-12 18:3 (n-6)		0.027					19.9	0.033	0.224
cis-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 20:5 (n-3)								0.021	19.2
Σ Saturated FA	24.4	22.0	25.2	24.8		18.8	47.7	17.0	29.8
Σ Monounsaturated FA	8.73	22.8	15.6	32.6		23.2	8.11	5.51	43.0
Σ Polyunsaturated FA	66.9	55.2	59.2	42.6		57.9	44.2	51.0	24.4

DM dry matter.

[a] 32.2 g/kg DM of lactic acid, 7.19 g/kg DM of acetic acid, 1.23 g/kg DM of propionic acid, 0.223 g/kg DM of butyric acid, 130 g/kg DM of water soluble carbohydrates, 73.5 g/kg N of NH_3-N, 104 g/kg DM of indigestible neutral detergent fibre, 655 g/kg DM of in vitro digestible organic matter in DM, pH 4.20.

[b] Contained 360 g/kg of barley, 310 g/kg of barley feed, 200 g/kg of oat, 90 g/kg of molassed sugar beet pulp and 40 g/kg of molasses.

[c] Contained 207 g/kg of Ca, 105 g/kg of Na and 60.0 g/kg of Mg, 1400 mg/kg of Zn, 500 mg/kg of vitamin E, 465 mg/kg of Mn, 405 mg/kg of Cu, 53 mg/kg of I, 20 mg/kg of Se, 250,000 IU/kg of vitamin A and 35,000 IU/kg of vitamin D_3.

[d] Contained 833 g/kg DM of soya bean meal, 117 g/kg DM of molassed sugar beet pulp and 50.0 g/kg DM of molasses.

[e] Results of silage analysed without heat stable amylase and expressed exclusive of residual ash (NDFom), results of concentrate components analysed with heat stable amylase and expressed exclusive of residual ash (aNDFom). All feeds were analysed using crucibles with pore size of 40–100 µm.

[f] Results of microalgae analysed with heat stable amylase using crucibles with pore size of 16–40 µm, results are expressed exclusive of residual ash (aNDFom).

[g] Includes Ile, Leu and Val.

[h] Includes Ala, Asp, Cys, Glu, Gly, Pro, Ser and Tyr.

[i] Σ Essential AA + Σ non-essential AA.

were methylated using methanolic sodium methoxide at the laboratory room temperature (ca. 22 °C) for 5 min, followed by sulphuric acid in methanol (2:98, v:v) at 50 °C for 30 min (Shingfield et al., 2003a). Milk samples (1 ml) were extracted thrice using a mixture of ammonia, ethanol, diethylether and hexane (0.2:1.0:2.5:2.5, v:v). Lipid extracts were combined and evaporated to dryness at 40 °C under nitrogen. Samples were dissolved in hexane and methyl acetate and methylated by methanolic sodium methoxide at laboratory room temperature (ca. 22 °C) for 5 min (Shingfield et al., 2003a).

Fatty acid methyl esters (FAME) were analysed using a gas chromatograph (GC2010 Plus, Shimadzu, Kyoto, Japan) equipped with an autosampler (AOC-20s, Shimadzu), a split-splitless auto-injector (AOC-20i, Shimadzu), a flame-ionization detector and a 100-m fused silica capillary column (i.d. 0.25 mm) coated with a 0.2-μm film of cyanopropyl polysiloxane (CP-SIL 88, Agilent J&W, Santa Clara, CA, USA). Injector and detector temperatures were maintained at 240 °C. Total FAME profile in a 1 μl sample at a split ratio of 1:40 was determined using helium as a carrier gas at constant velocity of 16.5 cm/s and a temperature gradient program. Following sample injection, column temperature was maintained at 70 °C for 4 min, increased at a rate of 30 °C/min to 170 °C, held there for 30 min, increased at 15 °C/min to a final temperature of 220 °C and held there for 45 min. Peaks were identified by comparison of retention times with FAME standards (GLC463, Nu-Chek Prep, Elysian, MN, USA; 10-2100-9, 10-2600-12, 10-2800-7, 21-1412-7, 21-1413-7, 21-1600-8, 21-1614-7, 21-1615-7, Larodan, Malmö, Sweden) and by cross-referring to published isomeric profiles of milk fat (Shingfield et al., 2003a). The identification was further validated with a mass spectrometry (GCMS-QP2010 Ultra, Shimadzu). Milk and feed FA composition was expressed as a weight percentage of total FA using theoretical relative response factors (Wolff et al., 1995). To quantify FA in feeds, external standard curve of tridecanoic acid (0.50 mg, T0502, Sigma-Aldrich) and palmitic acid (0.07–3.00 mg, P0500, Sigma-Aldrich) methylated similarly to feeds was prepared.

2.3. Calculations and statistical analysis

Details of the calculation of DMI, ECM and metabolisable energy (ME) intake and balance have been described previously (Lamminen et al., 2017). The ME content and intake of silage was calculated based on DOMD according to Finnish nutrient requirements (Luke, 2018). The ME content of concentrate components (see the footnotes of Table 3 for details) was estimated based on the equation (MAFF, 1984):

ME (MJ/kg DM) = [15.2 × digestible CP (g/kg DM) + 34.2 × digestible crude fat (g/kg DM) + 12.8 × digestible crude fibre (g/kg DM) + 15.9 × digestible nitrogen free extract (NFE; g/kg DM)]/1000.

The crude fibre content of spirulina and chlorella was assumed to be zero based on zero NDF content in current experiments and that of nannochloropsis was based on Markovits et al. (1992), the NFE content of concentrates was calculated by difference of other macronutrients. In all microalgae species, the digestibility coefficients of CP (0.738), ether extract (0.625) and NFE (0.670) were based on Hintz et al. (1966), and digestibility coefficient of crude fibre (0.575 for nannochloropsis) was based on Sarker et al. (2016). For other concentrate components, the digestibility coefficients of Finnish feed tables (Luke, 2018) were used. The ME requirements for maintenance (MJ/d) and milk production (MJ/d and MJ/kg of ECM) were estimated according to Luke (2018).

The estimation of mammary plasma flow was based on the stoichiometric transfer of mammary phenylalanine and tyrosine uptake into milk according to Fick principle (Cant et al., 1993). Indirect estimation of microbial protein yield in the rumen and daily urine volume was based on urine concentration of purine derivatives assuming the creatinine excretion rate of 25 mg/kg of BW (Puhakka et al., 2016). The apparent transfer efficiency of FAs was estimated based on intake of a certain FA from feed (g/d) divided by the secretion of FA in milk fat (g/d). When estimating the apparent transfer efficiencies of FAs from literature without reported secretion FAs in milk, following equation was used:

Secretion of FA in milk (g/d) = [milk fat yield (g/d) × 0.94 × concentration of FA in milk (g/100 g FA)]/100,

where constant 0.94 refers to average proportion of FAs in milk lipids (Halmemies-Beauchet-Filleau et al., 2014).

All experimental data were subjected to analysis of variance using Mixed-procedure of SAS 9.3 version (Statistical Analysis Systems Institute Inc., Cary, NC, USA). The statistical model for analysis of variance was as follows:

$$Y_{ijklm} = \mu + A_i + P_j + D_k + + E_{ijklm},$$

where Y_{ijklm} is dependent variable, μ is overall mean, A is the effect of animal (random effect), P is the effect of period (fixed effect), D is the effect of diet (fixed effect) and E is the random residual error. The degrees of freedom were calculated according to the Satterthwaite method. Results are expressed as least squares mean for each treatment and standard error of mean (SEM). *P*-values ≤ 0.05 were regarded as significant, and $0.05 < P \leq 0.10$ were accepted as a tendency. Sums of squares of the treatment effects were further separated into single degree of freedom comparisons using orthogonal contrasts. The significance of substitution of soya bean meal by microalgae (SPI + CHL + CHL-NAN vs. SOY; abbreviated in tables as algae vs. SOY), spirulina by the two chlorella diets (CHL + CHL-NAN vs. SPI; abbreviated in tables as SPI vs. CHL), and chlorella by the diet containing both chlorella and nannochloropsis (CHL vs. CHL-NAN) were tested. Logarithmic or squared transformations were used to correct for deviations from normality and homoscedasticity of residuals. If transformations were needed, least squares means are reported from statistical analysis of untransformed and transformed values and SEM and *P*-values from analysis of transformed data.

Table 3
Effect of substitution of soya bean meal by different microalgae species on nutrient and energy intake.

	Treatment[a]					Significance[b]		
	SOY	SPI	CHL	CHL-NAN	SEM	Algae vs. SOY	SPI vs.CHL	CHL vs. CHL-NAN
Intake								
Silage dry matter, kg/d	10.6	12.9	10.9	12.8	0.51	0.034	0.16	0.045
Diet dry matter, kg/d	21.5	22.0	20.9	21.6	1.29	0.98	0.39	0.51
Organic matter, kg/d	19.9	20.4	19.4	19.9	1.21	0.95	0.38	0.58
Crude protein, kg/d[c]	11.1 (3.31)	11.4 (3.37)	10.6 (3.23)	10.6 (3.23)	1.33	0.80	0.36	0.96
Neutral detergent fibre, kg/d	8.82	9.32	8.55	9.09	0.450	0.56	0.20	0.20
ME intake, MJ/d[d]	527 (229)	526 (229)	494 (221)	509 (225)	56.1	0.59	0.50	0.71
ME balance, MJ/d	11.9	−11.9	−0.25	1.50	16.522	0.082	0.20	0.86
Concentrate proportion	0.511	0.408	0.477	0.397	0.0390	0.054	0.48	0.13
Crude protein concentration, g/kg DM	154	153	154	150	1.5	0.17	0.71	0.078
Amino acid intake, g/d								
Arginine	176	172	154	149	12.1	0.007	0.008	0.38
Histidine	69.2	61.6	60.0	59.0	4.27	0.004	0.31	0.66
Isoleucine	127	130	112	115	7.75	0.066	0.009	0.45
Leucine	234	236	223	225	15.7	0.44	0.22	0.84
Lysine	154	135	143	146	10.7	0.14	0.31	0.80
Methionine	35.3	45.1	42.1	41.5	2.96	0.005	0.100	0.77
Phenylalanine	157	155	145	145	9.7	0.035	0.047	0.96
Threonine	130	137	123	126	8.2	0.50	0.017	0.51
Tryptophan	56.2	50.7	51.1	49.8	3.64	0.038	0.92	0.62
Valine	160	168	154	157	10.7	1.00	0.076	0.73
Σ Branched AA[e]	520	534	489	497	34.1	0.40	0.070	0.71
Σ Essential AA	1299	1290	1207	1213	84.6	0.14	0.12	0.91
Σ Non-essential AA[f]	1536	1461	1397	1395	103.5	0.060	0.30	0.98
Σ Total AA[g]	2835	2751	2604	2607	187.9	0.083	0.20	0.98
Fatty acid intake, g/d								
16:0[h]	58.3 (76.0)	76.1 (86.8)	64.5 (78.8)	63.5 (78.8)	8.88	0.052	0.046	0.86
cis-9 16:1	0.759	1.12	1.16	7.36	0.7646	0.024	0.020	0.003
Δ 16:2	< 0.01	< 0.01	10.2	5.42	1.077	0.006	0.003	0.020
cis-9 18:1	58.7	52.8	54.2	49.9	5.33	0.077	0.83	0.30
cis-9, *cis*-12 18:2 (n-6)	154	145	159	141	14.3	0.51	0.64	0.14
cis-9, *cis*-12, *cis*-15 18:3 (n-3)	95.8	111	97.0	110	4.93	0.099	0.28	0.11
cis-6, *cis*-9, *cis*-12 18:3 (n-6)	0.060	5.40	0.161	0.099	0.4509	0.015	< 0.001	0.92
cis-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17 20:5 (n-3)	< 0.01	< 0.01	0.109	3.46	0.3304	0.025	0.008	0.002
Σ Saturated FA[h]	91.5 (95.2)	118 (108)	100 (98.1)	103 (100)	12.75	0.049	0.067	0.71
Σ Monounsaturated FA[i]	1.84 (69.8)	1.81 (65.2)	1.82 (66.4)	1.82 (68.5)	0.049	0.34	0.77	0.95
Σ Polyunsaturated FA	251	263	268	262	17.4	0.20	0.86	0.64
Σ Total FA[h]	1744 (416)	1920 (437)	1932 (432)	1901 (431)	252.0	0.22	0.99	0.86

SEM standard error of the mean, AA amino acid, FA fatty acid, ME metabolisable energy. For treatments SOY and CHL-NAN SEM must be multiplied by 0.8306.

[a] SOY = soya bean meal as a protein feed; SPI = *Spirulina platensis* as a protein feed; CHL = *Chlorella vulgaris* as a protein feed, CHL-NAN = mixture of *Chlorella vulgaris* and *Nannochloropsis gaditana* (1:1 on dry matter basis) as a protein feed.

[b] Significance of substitution of soya bean meal by microalgae (algae vs. SOY), *Spirulina platensis* by the two diets containing *Chlorella vulgaris* (SPI vs. CHL), and *Chlorella vulgaris* by the mixture of *Chlorella vulgaris* and *Nannochloropsis gaditana* (CHL vs. CHL-NAN).

[c] Squared transformation of crude protein intake, original values are presented below the squared values.

[d] Squared transformation of ME intake divided by 100. Original values are presented in parenthesis below the squared values. Silage on average 10.5 MJ/kg DM, cereal-sugar beet pulp 12.4 MJ/kg DM, molassed sugar beet pulp 12.0 MJ/kg DM, molasses 12.6 MJ/kg DM, soya bean meal 12.8 MJ/kg DM, *Spirulina platensis* 10.8 MJ/kg DM, *Chlorella vulgaris* 11.7 MJ/kg DM, *Nannochloropsis gaditana* 11.1 MJ/kg DM.

[e] Includes Ile, Leu and Val.

[f] Includes Ala, Asp, Cys, Glu, Gly, Pro, Ser and Tyr.

[g] Σ Essential AA + Σ non-essential AA.

[h] Squared transformation of 16:0, saturated and total FA intake divided by 100, original values are presented in parenthesis below the squared values.

[i] Logarithmic transformation of monounsaturated FA intake, original values are presented in parenthesis below the logarithmic values.

3. Results

3.1. Diet composition

The chemical composition of feeds is presented in Table 2. The CP content and in vitro DOMD of grass silage was relatively low, and fermentation quality was good based on low concentration of fermentation acids and the proportion of NH_3-N from total N (see the footnotes of Table 2 for details). The chemical composition of spirulina and chlorella differed from other experimental feeds with markedly higher CP content. The NDF concentration of microalgae was dependent on the pore size of the crucibles used. For pore size 40–100 µm, NDF concentration was 90 g/kg DM for nannochloropsis but zero for spirulina and chlorella, whereas with pore size 16–40 µm, NDF concentration was 15–219 g/kg DM in all microalgae. In comparison to soya bean supplement, the concentrations of histidine and tryptophan were lower and that of methionine higher in microalgae. The concentration of lysine was markedly lower in spirulina than in soya bean supplement, chlorella and nannochloropsis. Of protein feeds (soya bean supplement and microalgae), the concentration of crude fat was lowest in soya bean supplement and the FA profiles of protein feeds clearly differed from each other. The most abundant FAs in soya bean supplement were (in sequence of decreasing concentration) *cis*-9, *cis*-12 18:2 (linoleic acid; LA), *cis*-9 18:1 and 16:0, in spirulina 16:0, LA and *cis*-6, *cis*-9, *cis*-12 18:3, in chlorella LA, Δ16:2 (location and configuration of double bonds undetermined) and 16:0, and in nannochloropsis *cis*-9 16:1, 16:0 and EPA. The concentration of minor FA in microalgae is presented in Supplementary Table 1.

3.2. Feed and nutrient intake, digestibility and milk production

The substitution of soya bean meal by microalgae increased ($P = 0.034$) silage intake and tended to decrease ($P = 0.054$) the proportion of concentrate in the diet, but did not affect ($P = 0.98$) total DMI (Table 3). However, the feed intake response on microalgae inclusion in the diet varied greatly between individual animals (Supplementary Fig. 1). The substitution of soya bean meal by microalgae tended ($P = 0.082$) to result lower ME balances. Intake of arginine ($P = 0.007$), histidine ($P = 0.004$), phenylalanine ($P = 0.035$) and tryptophan ($P = 0.038$) were decreased and that of isoleucine ($P = 0.066$), non-essential AA (NEAA; $P = 0.060$) and total AA (TAA; $P = 0.083$) tended to decrease when microalgae substituted soya bean supplement in the diet. On the other hand, intake of methionine was increased ($P = 0.005$) on microalgae diets compared to soya bean meal. Substitution of soya bean meal by microalgae increased the intake of *cis*-9 16:1 ($P = 0.024$), Δ16:2 ($P = 0.006$), *cis*-6, *cis*-9, *cis*-12 18:3 ($P = 0.015$), EPA ($P = 0.025$) and saturated FA (SFA; $P = 0.049$), and tended to increase those of 16:0 ($P = 0.052$) and ALA ($P = 0.099$). Microalgae diets tended to ($P = 0.077$) result in lower intake of *cis*-9 18:1 than soya bean meal diet.

Spirulina resulted in higher intakes of Arg ($P = 0.008$), Ile ($P = 0.009$), Phe ($P = 0.047$) and Thr ($P = 0.017$) than chlorella diets. In addition, intake of Met ($P = 0.100$), Val ($P = 0.076$) and branched chain AA (BCAA; $P = 0.070$) tended to be higher on spirulina than on chlorella diets. Intake of 16:0 ($P = 0.046$) and *cis*-6, *cis*-9, *cis*-12 18:3 ($P < 0.001$) were higher and that of *cis*-9 16:1 ($P = 0.020$), Δ16:2 ($P = 0.003$) and EPA ($P = 0.008$) were lower on spirulina than chlorella diets. Silage intake was increased ($P = 0.045$) when nannochloropsis substituted half of the chlorella in the diet. Compared to pure chlorella, mixture of chlorella and nannochloropsis resulted in higher intakes of *cis*-9 16:1 ($P = 0.003$) and EPA ($P = 0.002$) and lower intake of Δ16:2 ($P = 0.020$).

Apparent digestibility of nutrients, milk and ECM yield, or milk protein and lactose concentration and yield or urea (MUN) concentration were not affected ($P \geq 0.165$) by dietary treatments (Table 4). Milk fat concentration tended to be higher ($P = 0.073$) on microalgae treatments compared to soya bean meal, and was higher ($P = 0.028$) on spirulina compared to chlorella treatments. In addition, there was a tendency ($P = 0.098$) for increased milk fat yield on spirulina compared to chlorella treatments.

3.3. Energy, nitrogen and amino acid metabolism

The substitution of soya bean meal by microalgae decreased excretion of urinary N ($P = 0.039$) and urinary urea N ($P = 0.047$), and tended ($P = 0.096$) to decrease the proportion of dietary N excreted in urine ($P = 0.096$) (Table 5). Ruminal microbial N outflow tended ($P = 0.053$) to be higher on spirulina compared to chlorella diets.

Arterial concentrations of plasma metabolites are presented in Table 6 and mammary uptakes in Supplementary Table 2. Microalgae diets resulted in lower ($P = 0.018$) arterial insulin concentration and higher mammary uptake of acetic acid ($P = 0.021$) than soya bean meal. In addition, arterial concentration of acetic acid ($P = 0.055$) and NEFA ($P = 0.060$) tended to be higher on microalgae diets than on soya bean meal. When comparing spirulina to chlorella diets, SPI resulted in higher arterial concentration ($P = 0.010$) and mammary uptake ($P = 0.007$) of acetic acid. Compared to pure chlorella, mixture of chlorella and nannochloropsis tended to result in lower arterial concentration of insulin ($P = 0.062$).

Arterial concentrations of AA and carnosine are presented in Table 6 and mammary uptakes in Supplementary Table 2. Only few differences in plasma essential AA (EAA) were observed when comparing different protein sources. On microalgae diets, arterial concentration of tryptophan tended to be lower ($P = 0.062$) than on soya bean meal, whereas mammary uptake ($P = 0.014$) of tryptophan was higher than on soya bean meal. Mammary uptake of arginine was decreased when soya bean meal was substituted by microalgae ($P = 0.035$) and when spirulina was substituted by chlorella ($P = 0.049$).

3.4. Milk fatty acids

The concentration of major and some biologically relevant FAs with emphasis on 18-carbon isomers is presented in Table 7. The

Table 4
Effect of substitution of soya bean meal by different microalgae species on apparent total tract digestibility of nutrients, milk yield and milk composition.

	Treatment[a]					Significance[b]		
	SOY	SPI	CHL	CHL-NAN	SEM	Algae vs. SOY	SPI vs. CHL	CHL vs. CHL-NAN
Apparent total tract digestibility, g/kg								
Dry matter	651	641	650	651	12.7	0.78	0.51	0.92
Organic matter	659	650	661	661	12.7	0.86	0.44	0.98
Neutral detergent fibre	474	504	491	516	28.1	0.25	1.00	0.43
Crude protein	617	602	609	606	16.1	0.50	0.78	0.89
Yield								
Milk, kg/d	29.7	32.1	29.9	30.8	1.86	0.52	0.46	0.72
Energy corrected milk, kg/d	29.3	33.9	30.0	30.5	2.02	0.29	0.17	0.83
Fat, g/d	1215	1484	1261	1287	98.2	0.19	0.098	0.82
Protein, g/d	952	1043	957	969	61.5	0.47	0.23	0.85
Lactose, g/d	1320	1427	1324	1360	95.5	0.62	0.49	0.78
Milk composition								
Fat, g/kg	41.0	45.0	41.4	42.2	1.57	0.073	0.028	0.48
Protein, g/kg	32.2	32.2	31.6	31.5	1.34	0.62	0.54	0.95
Lactose, g/kg	44.5	44.3	44.4	44.2	0.49	0.76	0.92	0.81
Urea N, mg/dL	11.2	9.34	11.3	10.3	1.354	0.49	0.36	0.55
Energy corrected milk (kg/d):dry matter intake (kg/d)	1.37	1.55	1.48	1.43	0.130	0.19	0.32	0.63

SEM standard error of the mean, N nitrogen. For treatments SOY and CHL-NAN SEM must be multiplied by 0.8306.

[a] SOY = soya bean meal as a protein feed; SPI = *Spirulina platensis* as a protein feed; CHL = *Chlorella vulgaris* as a protein feed, CHL-NAN = mixture of *Chlorella vulgaris* and *Nannochloropsis gaditana* (1:1 on dry matter basis) as a protein feed.

[b] Significance of substitution of soya bean meal by microalgae (algae vs. SOY), *Spirulina platensis* by the two diets containing *Chlorella vulgaris* (SPI vs. CHL), and *Chlorella vulgaris* by the mixture of *Chlorella vulgaris* and *Nannochloropsis gaditana* (CHL vs. CHL-NAN).

Table 5
Effect of substitution of soya bean meal by different microalgae species on nitrogen (N) metabolism in lactating dairy cows.

	Treatment[a]					Significance[b]		
	SOY	SPI	CHL	CHL-NAN	SEM	Algae vs. SOY	SPI vs. CHL	CHL vs. CHL-NAN
N intake, g/d[c]	283 (530)	292 (539)	273 (516)	271 (517)	34.0	0.80	0.36	0.96
Ruminal microbial N flow, g/d[d]	330	337	290	300	34.9	0.18	0.053	0.62
Excretion in milk								
Milk N, g/d	149	163	150	152	9.6	0.47	0.23	0.85
Milk N:N intake	0.282	0.308	0.295	0.291	0.0183	0.19	0.37	1.00
Excretion in urine								
Urine, L/d[e]	24.6	20.9	21.5	23.7	2.09	0.30	0.54	0.46
Urinary urea N, g/d	84.5	66.0	72.5	58.1	9.53	0.047	0.94	0.18
Urinary N, g/d	147	127	133	121	12.1	0.039	0.97	0.25
Urinary urea N:urinary N	0.575	0.513	0.535	0.470	0.0347	0.12	0.83	0.22
Urinary N:N intake	0.276	0.237	0.256	0.237	0.0221	0.096	0.62	0.39
Excretion in faeces								
Faecal N, g/d[f]	202	217	204	207	18.0	0.53	0.43	0.83
Faecal N:N intake	0.383	0.398	0.391	0.394	0.0161	0.50	0.78	0.89
N balance, g/d[g]	32.6	32.4	29.9	36.9	20.12	0.97	0.95	0.70

SEM standard error of the mean. For treatments SOY and CHL-NAN SEM must be multiplied by 0.8306.

[a] SOY = soya bean meal as a protein feed; SPI = *Spirulina platensis* as a protein feed; CHL = *Chlorella vulgaris* as a protein feed, CHL-NAN = mixture of *Chlorella vulgaris* and *Nannochloropsis gaditana* (1:1 on dry matter basis) as a protein feed.

[b] Significance of substitution of soya bean meal by microalgae (algae vs. SOY), *Spirulina platensis* by the two diets containing *Chlorella vulgaris* (SPI vs. CHL), and *Chlorella vulgaris* by the mixture of *Chlorella vulgaris* and *Nannochloropsis gaditana* (CHL vs. CHL-NAN).

[c] Squared transformation of nitrogen intake divided by 1000, original values are presented in parenthesis below the squared values.

[d] Estimated microbial N outflow from the rumen based on urinary purine derivative excretion (Puhakka et al., 2016).

[e] Estimated from urinary excretion of creatinine according to Puhakka et al. (2016).

[f] Calculated as [1 – (apparent digestibility of N (g/kg)/1000)] × N intake (g/d).

[g] Calculated as N intake (g/d) – [N in milk (g/d) + N in feces (g/d) + N in urine (g/d)].

concentration of other FAs and secretion of all FAs is presented in Supplementary Tables 3 and 4, respectively. In general, concentration and secretion of FAs in milk were affected by different protein supplements, however, in many cases the changes were very small although statistically significant. The concentration of Δ16:2 ($P = 0.024$) and omega-3 FAs ($P = 0.029$) in milk fat were higher on microalgae diets than on soya bean meal, whereas the opposite was true for 16:0 ($P = 0.029$) and SFA ($P = 0.038$). In addition,

Table 6
Effect of substitution of soya bean meal by different microalgae species on arterial concentration of plasma metabolites, amino acids (AA) and carnosine in lactating cows.

	Treatment[a]					Significance[b]		
	SOY	SPI	CHL	CHL-NAN	SEM	Algae vs. SOY	SPI vs. CHL	CHL vs. CHL-NAN
Plasma metabolites								
Acetic acid, mmol/L	1.25	2.62	1.47	1.36	0.212	0.055	0.010	0.71
BHBA, mmol/L[c]	0.768	2.07	1.03	0.871	0.5447	0.35	0.16	0.83
Glucose, mmol/L	3.63	3.40	3.56	3.62	0.136	0.44	0.30	0.74
Insulin, µIU/ml	13.5	13.4	11.1	9.11	1.131	0.018	0.009	0.062
NEFA, mmol/L[d]	0.113	0.254	0.194	0.197	0.0775	0.060	0.28	0.97
Essential AA, µmol/L								
Arginine	87.5	83.7	75.9	83.8	9.30	0.30	0.59	0.33
Histidine	63.4	52.3	56.9	45.5	7.12	0.14	0.91	0.25
Isoleucine[e]	219 (147)	491 (207)	287 (167)	302 (173)	118.2	0.25	0.19	0.92
Leucine	156	188	180	158	21.9	0.42	0.51	0.49
Lysine	107	116	109	102	9.1	0.89	0.40	0.59
Methionine	24.5	25.9	20.9	21.7	2.76	0.58	0.24	0.83
Phenylalanine	56.8	58.8	54.0	53.5	3.28	0.71	0.28	0.91
Threonine	129	123	109	117	9.5	0.25	0.42	0.55
Tryptophan	41.5	40.5	36.4	39.1	1.14	0.062	0.12	0.15
Valine	318	352	347	319	29.7	0.47	0.59	0.48
Non-essential AA, µmol/L								
Alanine	277	262	263	295	32.5	0.91	0.70	0.50
β-alanine	3.65	3.75	2.51	3.53	0.763	0.53	0.33	0.24
Asparagine	64.3	63.8	53.3	57.0	5.72	0.34	0.29	0.65
Aspartic acid	6.14	7.50	5.11	4.02	1.244	0.66	0.13	0.54
Citrulline	65.3	70.0	79.7	76.7	6.89	0.13	0.27	0.69
Cysteine	26.0	24.2	22.4	24.0	1.72	0.16	0.57	0.44
Glutamic acid	66.7	80.2	59.4	74.4	4.67	0.38	0.078	0.069
Glutamine	232	220	211	238	16.5	0.58	0.82	0.26
Glycine	337	332	340	348	36.7	0.92	0.71	0.81
Nτ-Methylhistidine[f]	5.62	4.84	5.64	5.58	0.905	0.78	0.53	0.96
Nπ-Methylhistidine[f]	2.81	2.13	3.25	2.51	0.385	0.67	0.19	0.21
Ornithine	49.7	46.9	45.6	45.3	4.88	0.43	0.80	0.97
Proline	98.7	90.6	87.8	92.5	9.17	0.37	0.97	0.69
Serine	103	92.9	84.3	67.7	15.00	0.23	0.41	0.45
Taurine	37.9	37.2	32.0	36.5	5.08	0.31	0.34	0.21
Tyrosine	49.9	50.6	41.0	42.9	3.57	0.20	0.091	0.67
Σ Branched AA[g]	621	748	695	651	75.1	0.33	0.42	0.65
Σ Essential AA	1131	1251	1160	1113	90.9	0.64	0.32	0.69
Σ Non-essential AA[h]	1262	1245	1190	1270	95.7	0.80	0.90	0.56
Σ Total AA[i]	2393	2506	2360	2383	120.4	0.86	0.41	0.89
Carnosine, µmol/L	23.2	24.3	22.8	22.4	5.31	0.98	0.54	0.89

SEM standard error of the mean. For treatments SOY and CHL-NAN SEM must be multiplied by 0.8306.

[a] SOY = soya bean meal as a protein feed; SPI = *Spirulina platensis* as a protein feed; CHL = *Chlorella vulgaris* as a protein feed, CHL-NAN = mixture of *Chlorella vulgaris* and *Nannochloropsis gaditana* (1:1 on dry matter basis) as a protein feed.

[b] Significance of substitution of soya bean meal by microalgae (algae vs. SOY), *Spirulina platensis* by the two diets containing *Chlorella vulgaris* (SPI vs. CHL), and *Chlorella vulgaris* by the mixture of *Chlorella vulgaris* and *Nannochloropsis gaditana* (CHL vs. CHL-NAN).

[c] β-hydroxybutyrate.

[d] Non-esterified fatty acids.

[e] Squared transformation of isoleucine divided by 100, original values are presented in parenthesis below the squared values.

[f] IUPAC nomenclature. Nτ-methylhistidine = the product of muscle actin and myosin catabolism; Nπ-methylhistidine = the product of anserine breakdown.

[g] Isoleucine, leucine and valine.

[h] Includes Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser and Tyr.

[i] Σ Essential AA + Σ non-essential AA.

the concentration of ALA ($P = 0.077$) and PUFA ($P = 0.055$) tended to be higher on microalgae diets than on soya bean meal. Compared to soya bean meal, microalgae diets increased the secretion of Δ16:2 ($P = 0.014$), ALA ($P = 0.037$), *cis*-6, *cis*-9, *cis*-12 18:3 ($P = 0.027$) and polyunsaturated FA (PUFA; $P = 0.037$).

The concentration of *cis*-6, *cis*-9, *cis*-12 18:3 ($P = 0.003$) in milk fat was higher on spirulina than chlorella diets. The opposite was true for Δ16:2 ($P = 0.010$), LA ($P = 0.022$), EPA ($P = 0.045$), PUFA ($P = 0.015$), omega-3 FAs ($P = 0.022$) and omega-6 FAs ($P = 0.021$). Spirulina resulted in higher secretion of 14:0 ($P = 0.028$), 16:0 ($P = 0.024$), *cis*-6, *cis*-9, *cis*-12 18:3 ($P = 0.002$), Σ < 16-carbon FAs ($P = 0.043$) and SFA ($P = 0.045$) in milk than chlorella diets. The opposite was true for Δ16:2 ($P = 0.006$). Also the

Table 7
Effect of substitution of soya bean meal by different microalgae species on the concentration of major and some biologically relevant fatty acids (FA) in milk and the apparent transfer efficiency some FAs from feed to milk in lactating cows.

	Treatment[a]					Significance[b]		
	SOY	SPI	CHL	CHL-NAN	SEM	Algae vs. SOY	SPI vs. CHL	CHL vs. CHL-NAN
FA composition, g/100 g of total FA								
4:0	3.60	3.74	3.70	3.69	0.050	0.040	0.40	0.81
6:0	2.28	2.33	2.29	2.34	0.088	0.002	0.032	0.002
8:0	1.38	1.40	1.38	1.42	0.077	0.30	0.88	0.14
10:0	3.07	3.10	2.97	3.06	0.265	0.78	0.40	0.42
12:0	3.53	3.53	3.32	3.43	0.309	0.32	0.20	0.41
14:0	11.6	11.5	10.7	11.1	0.660	0.021	0.026	0.096
cis-9 14:1	0.847	0.905	0.751	0.871	0.0490	0.94	0.19	0.14
16:0	30.6	29.7	27.9	28.7	0.867	0.029	0.099	0.30
cis-9 16:1	1.04	1.16	1.12	1.64	0.206	0.078	0.19	0.028
Δ16:2	0.012	0.002	0.431	0.191	0.0608	0.024	0.010	0.031
18:0	12.0	10.9	11.9	10.9	0.579	0.22	0.40	0.21
cis-9 18:1[c]	17.2	19.1	19.0	18.6	1.89	0.092	0.83	0.74
cis-11 18:1	0.498	0.496	0.561	0.552	0.1178	0.16	0.084	0.77
trans-10 18:1	0.173	0.160	0.175	0.160	0.0253	0.65	0.73	0.53
trans-11 18:1	0.998	1.00	0.879	1.06	0.0889	0.81	0.69	0.093
cis-9,*cis*-12 18:2 (n-6)	2.14	1.92	3.41	2.41	0.310	0.13	0.022	0.030
cis-9,*trans*-11 18:2[d]	0.404	0.441	0.393	0.448	0.0398	0.48	0.61	0.23
trans-11,*cis*-15 18:2	0.100	0.135	0.105	0.150	0.0149	0.039	0.57	0.024
cis-9,*cis*-12,*cis*-15 18:3 (n-3)	0.427	0.460	0.565	0.530	0.0516	0.077	0.14	0.54
cis-6,*cis*-9,*cis*-12 18:3 (n-6)	0.030	0.064	0.032	0.028	0.0057	0.061	0.003	0.50
20:0	0.202	0.163	0.195	0.290	0.0174	0.46	0.020	0.014
cis-5,*cis*-8,*cis*-11,*cis*-14, *cis*-17 20:5 (n-3)	0.048	0.040	0.066	0.206	0.0286	0.12	0.045	0.018
Σ < 16-carbon FA	28.7	28.7	27.2	28.0	1.48	0.12	0.060	0.15
Σ Unidentified FA	0.948	0.959	1.01	1.06	0.0755	0.26	0.25	0.44
Σ Saturated FA	72.2	70.3	68.2	68.7	2.09	0.038	0.22	0.73
Σ Monounsaturated FA[7]	23.4	25.5	25.5	25.9	2.25	0.089	0.86	0.79
Σ Polyunsaturated FA	3.44	3.30	4.85	4.13	0.372	0.055	0.015	0.090
Σ n-3 FA[e]	0.537	0.559	0.705	0.833	0.0657	0.029	0.022	0.12
Σ n-6 FA[e]	2.40	2.20	3.68	2.70	0.318	0.11	0.021	0.030
Ratio n-6/n-3 FA	4.51	4.06	5.43	3.22	0.338	0.47	0.55	0.008
Apparent transfer efficiency of FAs								
cis-9,*cis*-12 18:2 (n-6)	0.161	0.195	0.265	0.211	0.0212	0.023	0.11	0.075
cis-9,*cis*-12,*cis*-15 18:3 (n-3)	0.054	0.062	0.074	0.061	0.0045	0.068	0.36	0.095
Σ n-3 FA[e]	0.069	0.073	0.091	0.092	0.0058	0.045	0.058	0.83
Σ n-6 FA[e]	0.179	0.214	0.285	0.233	0.0227	0.025	0.11	0.101

SEM standard error of the mean. For treatments SOY and CHL-NAN SEM must be multiplied by 0.8306.

[a] SOY = soya bean meal as a protein feed; SPI = *Spirulina platensis* as a protein feed; CHL = *Chlorella vulgaris* as a protein feed, CHL-NAN = mixture of *Chlorella vulgaris* and *Nannochloropsis gaditana* (1:1 on dry matter basis) as a protein feed.

[b] Significance of substitution of soya bean meal by microalgae (algae vs. SOY), *Spirulina platensis* by the two diets containing *Chlorella vulgaris* (SPI vs. CHL), and *Chlorella vulgaris* by the mixture of *Chlorella vulgaris* and *Nannochloropsis gaditana* (CHL vs. CHL-NAN).

[c] Contains *trans*-15 18:1 as a minor component.

[d] Contains *trans*-7,*cis*-9 18:2 and *trans*-8,*cis*-10 18:2 as minor components.

[e] Includes omega-3 (n-3) or omega-6 (n-6) fatty acids listed on this table and Supplementary Table 3.

secretion of EPA tended to ($P = 0.060$) be lower on spirulina than on chlorella diets. The concentration of EPA ($P = 0.018$) in milk fat was higher on mixture of chlorella and nannochloropsis than on pure chlorella. The opposite was true for LA ($P = 0.030$). Compared to pure chlorella, mixture of chlorella and nannochloropsis resulted in higher secretion of EPA ($P = 0.019$) but lower secretion of Δ16:2 ($P = 0.018$) and LA ($P = 0.048$). Also the ratio of omega-6 to omega-3 FAs was lower for mixture of chlorella and nannochloropsis than pure chlorella ($P = 0.023$).

Compared to soya bean meal, microalgae diets resulted in higher apparent transfer efficiencies of LA ($P = 0.023$), omega-3 FAs ($P = 0.045$) and omega-6 FAs ($P = 0.025$) from feed to milk (Table 7). Also the apparent transfer of ALA tended to be higher for microalgae than for soya bean meal ($P = 0.068$). The apparent transfer efficiency of EPA was 0.707 (SE 8.2177) for mixture of chlorella and nannochloropsis but it is not calculated for other diets due to negligible EPA in feeds. There was tendency for lower apparent transfer efficiency of omega-3 FAs ($P = 0.058$) on spirulina than chlorella diets, and that of LA ($P = 0.075$) in mixture of chlorella and nannochloropsis than in pure chlorella.

4. Discussion

This study compared the protein value of different microalgae species to that of soya bean meal in grass silage and cereal based

diets of lactating dairy cows. The results of current experiment were influenced by feed intake problems of some but not all individual animals on microalgae diets leading to one missing observation on SPI and other on CHL and large standard errors.

4.1. Microalgae composition

The chemical composition of spirulina and chlorella with high CP concentration fit in the range summarised by Becker (2013), and was similar to those in our previous experiments (Lamminen et al., 2017). The chemical composition of nannochloropsis with moderate CP and higher crude fat concentration than in spirulina and chlorella is in agreement with Robelloso-Fuentes et al. (2001). The fatty acid profile with high concentration of EPA in current experiment is typical for nannochloropsis (Sukenik et al., 1993; Robelloso-Fuentes et al., 2001). The zero NDF concentration in spirulina and chlorella when analysed with crucibles having pore size 40–100 μm is in agreement with our previous experiments (Lamminen et al., 2017). However, when analysed with crucibles having smaller pore size (16–40 μm), NDF results were obtained for all microalgae, indicating need to further develop the NDF determination for analysis of feed materials with very small particle size e.g. with addition of a microfiber filtering aid (Raffrenato and Van Amburgh, 2011). Indeed, the cell size of microalgae being 10 × 60–500 μm for spirulina (filamentous cell shape), 2.5 μm for chlorella (spherical cell shape) and 3.0 μm for *Nannochloropsis* sp. (spherical cell shape) (Griffiths et al., 2012) suggests that reliable NDF determination in microalgae using crucibles with pore size > 40 μm as recommended in the standard of Mertens (2002) is very challenging. The relatively high NDF concentration (219 g/kg DM) in nannochloropsis obtained with smaller pore size crucibles is not surprising given the cell wall composition of this species. The bilayered cell wall of nannochloropsis consists of cellulosic inner wall and outer hydrophobic algaenan layer closely resembling the cutan of vascular plants (Scholz et al., 2014). The NDF concentration of 87.4 g/kg DM of spirulina obtained with smaller pore size crucibles is in line with Costa et al. (2016) who used crucibles with pore size of 40–60 μm (P. Isherwood, personal communication). These authors also report higher NDF concentrations for spirulina than for *Chlorella pyrenoidosa*, which is in agreement with our results of chlorella having NDF concentration of only 15 g/kg DM. However, this is surprising because the cell wall of chlorella is commonly reported to be more rigid than that of evolutionary older cyanobacteria (e.g. Mendez et al., 2015). The main component of spirulina cell walls is murein (peptidoglycan) (Lee, 2008), but some cyanobacteria species may have cellulose as minor extracellular components in slime tubes, sheaths and extracellular slime (Nobles et al., 2001). There is no general agreement with the cell wall construction of *Chlorella* sp. probably due to high variability of the species (Bernaerts et al., 2018), however, proteinaceous polymers instead of carbohydrates seem to be the main determinant of cell wall digestibility of the species (Mahdy et al., 2015).

4.2. Feed intake and digestibility

The substitution of soya bean meal by microalgae decreased concentrate intake as indicated by the decreasing proportion of concentrate in the diet. However, as silage intake was simultaneously increased, the composition but not total amount of DMI was changed by microalgae inclusion in the diet. On the other hand, there seems to be individual differences in preference of microalgae, as no shift in the composition of DMI were seen on half of the animals, whereas the proportion of concentrate in the diet was dramatically dropped on microalgae diets on the rest of the animals. Palatability problems with large quantities of microalgae in ruminant diets have also been reported earlier (Hintz et al., 1966; Van Emon et al., 2015; Lamminen et al., 2017). The shift in feeding pattern in current experiment was most pronounced on CHL-NAN with 2.2 kg/d higher silage intake and 2.2 kg/d lower concentrate intake compared to SOY. Due to the differences in CP concentration, the daily microalgae dosage was higher on CHL-NAN than on SPI and CHL (+0.51 and +0.30 kg DM/d, respectively), which might have further emphasised the palatability problems on this diet.

High dietary fat content has a potential to decrease DMI (e.g. Onetti and Grummer, 2004) especially if there is a high content of unsaturated FA (Weld and Armentano, 2017). However, the crude fat concentration of the concentrates (39.2, 44.5, 53.1 and 60.0 g/kg DM for SOY, SPI, CHL and CHL-NAN, respectively) was moderate, which on forage based diets likely has quantitatively very small effect on DMI (Huhtanen et al., 2008). Despite of the slight differences in concentrate crude fat concentration among microalgae diets, total FA intake was similar (on average 433 g/d) as the proportion of other petroleum benzine soluble compounds than FA (e.g. pigments and waxes) was higher in nannochloropsis and chlorella (0.849 and 0.730 of crude fat, respectively) than in spirulina (0.442 of crude fat).

Odour and taste properties of the feed are important factors in feed selection of ruminants (Cannas et al., 2009). As conservative eaters ruminants tend to prefer familiarity over novelty (Rapisarda et al., 2012), which might to some extent explain the DMI responses to microalgae in current and earlier experiments. When comparing different plant based concentrates, sheep favour feeds rich in aldehydes (green, fruity and rancid aromas), and avoid feeds rich in sulphuric (garlic, meaty and fishy aromas) and terpenic (solvent, spice and wood aromas) compounds (Rapisarda et al., 2012). In the current experiment, clear fishy odour was observed in nannochloropsis whereas the odour of spirulina and chlorella was more neutral. With regard to nannochloropsis, this contradicts the results of Van Durme et al. (2013) reporting that the aroma of *N. oculata* paste was dominated by 'grassy, vegetable, cucumber' flavours for human perception. In addition, they detected no sulphuric aroma compounds in these microalgae, and even the content of terpenes was relative low. However, the aroma profile of microalgae can change during processing and storing as several different aroma compounds can arise from the oxidation of unsaturated FAs (Sérot et al., 2002) including fishy odour compounds from EPA (Hammer and Schieberle, 2013), and microbial degradation of sulphuric AAs produces sulphuric aroma compounds (Seefeldt and Weimer, 2000).

Despite of the dramatic changes in composition of DMI, intake or apparent total tract digestibility of OM, CP and NDF were not affected by the algae treatments in current experiment. Differences in nutrient digestibility were expected between microalgae

species because they vary in composition and rigidity of cell walls, as already discussed. The results on biogas production suggest that the fermentability of cyanobacteria (such as spirulina) is higher than that of chlorella (Mendez et al., 2015), and the more rigid cell wall structure of *Chlorella* sp. and *Nannochloropsis* sp. can hinder their digestion (Bohutskyi et al., 2014). On the other hand, it is very likely that the method of apparent total tract digestibility measurement was not sensitive enough to pinpoint the effects of single diet ingredient on digestibility especially when also the concentrate to forage ratio varied between diets.

4.3. Milk production and energy balance

Despite of palatability problems on microalgae diets, milk and ECM yields were not significantly affected by substitution of soya bean meal by microalgae or the species of microalgae. However, the milk and ECM yields on microalgae diets were on average 1.2 kg/d and 2.2 kg/d, respectively, higher than on SOY, but the differences did not reach statistical significance. The increased milk fat production on microalgae diets and especially SPI are likely related to differences in plasma metabolites. Milk fat can originate either from more acetate or butyrate intensive ruminal fermentation, or body lipid mobilisation, both leading to increased amounts of milk fat precursors in plasma, namely acetic acid, BHBA and NEFA (Chilliard et al., 2000). Indeed, arterial acetic acid concentrations tended to increase and mammary uptake of acetic acid was increased in current experiment when soya bean meal was substituted by microalgae, the concentration and mammary uptake being twofold higher on SPI than on SOY. Increased proportion of silage on microalgae diets may have favoured the growth of acetate producing rumen bacteria (France and Dijkstra, 2005). Also decreasing energy balance, which was observed as a tendency on microalgae diets compared to SOY, is known to affect milk fat to protein ratio by increasing milk fat concentration and decreasing that of milk protein (e.g. Duffield et al., 1997). However, no significant effects on arterial or mammary uptake of BHBA were seen, but the concentration and mammary uptake on SPI were two- to threefold higher than on other treatments. Arterial NEFA concentrations tended to increase by microalgae inclusion in the diet, but treatments had no effect on mammary uptake of NEFA.

Regardless of the concentrate related feed intake problems and lower energy balance on microalgae diets, milk lactose and arterial concentrations of glucose were not affected by any of the treatments, and glucose concentrations were relatively high (on average 3.55 mmol/l) in comparison to reference values of 2.6–3.8 mmol/l measured on clinically normal lactating dairy cows (Cozzi et al., 2011).

4.4. Nitrogen utilisation and AA metabolism

The supply of N and energy was likely sub-optimally balanced on all treatments as indicated by the MUN concentrations being slightly below the suggested threshold of 11.7 mg/dl for optimal degradable N:ME ratio of the diets (Nousiainen et al., 2004). This also explains the somewhat higher NUE in current experiment in comparison to average value of 0.28 on North European experimental diets (Huhtanen and Hristov, 2009). Environmental-wise, microalgae diets performed better than soya bean meal as indicated by lower urinary N and urinary urea N secretion, the most susceptible forms of N for environmental losses (Bussink and Oenema, 1998). Moreover, in line with the milk production responses, SPI resulted in numerically highest NUE and numerically lowest MUN concentrations, suggesting more efficient nutrient utilisation on SPI than on other diets. These indicate potential to reduce environmental N load of dairy production by substituting soya bean meal with microalgae and especially spirulina. Similar beneficial results regarding higher NUE (Shingfield et al., 2003b; Gidlund et al., 2015; Rinne et al., 2015), and lower MUN (Shingfield et al., 2003b; Gidlund et al., 2015; Paula et al., 2018) and urinary N losses (Shingfield et al., 2003b; Rinne et al., 2015; Paula et al., 2018) have been achieved with rapeseed meal in comparison to soya bean meal. However, the value of microalgae as protein feed is likely between that of soya bean meal and rapeseed meal as in our previous experiments (Lamminen et al., 2017) milk protein yield and NUE was decreased in medium but not in low-yielding dairy cows when rapeseed meal was substituted by microalgae.

The positive N balance (on average 33 g/d) on all experimental treatments was obviously an overestimation resulting most likely from underestimation of excreted urine volume. However, the spot sampling technique of urine and faeces still allows the determination of treatment differences within experiment. Positive N balances have been measured even when total collection of urine has been used (Hassanat et al., 2013; Reynolds et al., 2014). Indeed, overestimation of N balance is not unique for the current experiment, but common in experiments with lactating cows (Spanghero and Kowalski, 1997; Reynolds and Kristensen, 2008; Spek et al., 2013). This is likely caused by minor cumulative losses of N through routes that are not taken into account (Reynolds and Kristensen, 2008). No muscle protein reserves were likely mobilised as no changes were observed during the experiment on arterial concentration of Nτ-methylhistidine, a product of muscle actin and myosin catabolism (Ratchmacher, 2000). This supports positive or zero N balance in the current experiment.

The microalgae studied in current experiment were rich in methionine, but poor sources of histidine compared to soya bean meal, which is notable as methionine is typically the first AA limiting milk production on soya based diets (Casper and Schingoethe, 1988; Pisulewski et al., 1996), and histidine on grass silage and cereal based diets (Kim et al., 1999; Vanhatalo et al., 1999). The intake of methionine was increased as microalgae substituted soya bean meal in the diet, and was numerically highest on spirulina. This may explain the changes in N excretion patterns, as the balancing of AA supply in metabolisable protein has decreased plasma urea concentration and increased NUE (Haque et al., 2012). No significant effects were seen on arterial concentrations or mammary uptakes of methionine, but spirulina resulted in +18.7 mmol/d higher uptake than other diets. In spite of decreased histidine intake by microalgae inclusion in the diet, histidine unlikely limited milk production in current experiment as indicated by high arterial plasma concentrations compared to that of 45.3–49.4 μmol/l in meta-analysis of Patton et al. (2015), and lack of responses on plasmatic carnosine, an endogenic histidine reserve.

4.5. Milk FA composition

Only minor changes in milk FA composition were expected because the crude fat concentration of the diets remained relatively low, as already discussed. The changes induced by the substitution of soya bean meal by microalgae mirrored the FA composition of these microalgae and changes in silage DM consumption, but also lower energy balance, acetate supply or both.

Higher silage intake on microalgae diets resulted in higher intake of ALA leading also to higher secretion of ALA in milk fat. Inclusion of EPA-rich nannochloropsis in the diet resulted in four-fold increase in milk EPA concentration in comparison to other diets without no signs of milk fat depression. Previously, slightly lower milk EPA concentrations (0.17 vs. 0.21 g/100 g FA) and much lower apparent transfer efficiency from diet to milk (0.02 vs. 0.71) were achieved with 300 g/d fish oil supplementation (Kairenius et al., 2015) compared to CHL-NAN, respectively. The omega-6:omega-3 FA ratio in milk was on CHL-NAN closest to recommended ratio of 1–2 for human nutrition (Simopoulos, 2002), whereas the ratio on CHL was most unfavourable mainly due to higher milk LA secretion than other diets (+17.0, +14.0 and +12.2 g/d in comparison to SOY, SPI and CHL-NAN, respectively). The numerical increase on LA intake was quantitatively too small on CHL in comparison to SOY (+5 g/d) to explain the difference in milk secretion between these diets. In addition, the elongation of 16-carbon acyl chain in animal is considered negligible (Palmquist, 2006), thus it unlikely explains the differences in milk LA secretion. The apparent efficiency of transfer of LA, but also omega-3 and omega-6 FAs from diet to milk was higher on microalgae diets compared to SOY. Same was true also in Franklin et al. (1999) for LA and ALA with rumen unprotected *Schizochytrium* sp. microalgae in comparison to soya bean meal and *Schizochytrium* sp. protected from ruminal biohydrogenation with xylose coating. Reason for this remains unclear, but these observations suggest that the unsaturated FA metabolism might differ between lipids in microalgae and soya bean meal.

Increased milk fat production on SPI originated either from changes in ruminal fermentation or increased mobilisation of body fat caused by lower energy balance, as already discussed. Of the milk fat precursors, acetate and BHBA are used in synthesis of de novo FA (< 16-carbon FAs, and to some extent 16:0) whereas > 16-carbon FA taken up from the blood by the mammary gland originate from NEFA and triglyceride-rich lipoproteins (Chilliard et al., 2000). Consistent with increased arterial acetic acid concentrations, spirulina resulted in higher secretion and concentration of < 16-carbon FA, and higher secretion of 16:0 in milk than chlorella diets. Bovine adipose tissue is mainly compromised of *cis*-9 18:1, 18:0 and 16:0 FAs (Enser et al., 1996) and increased concentrations of *cis*-9 18:1 (Jorjong et al., 2014) are associated with elevated plasma NEFA concentrations. Of these indicators, only the concentration of *cis*-9 18:1 in milk fat tended to increase on microalgae diets, and coincided with tendency to increasing plasma NEFA concentrations on these diets.

5. Conclusions

Current study demonstrated the suitability of non-defatted and protein-rich microalgae for the nutrition of lactating dairy cows. Microalgae inclusion in the diet did not affect total amount of DMI but changed the composition of DMI by increasing silage intake, and thus lead to decreasing proportion of concentrate in the diet. Despite of poorer palatability of microalgae concentrates, microalgae diets resulted in milk and ECM yields similar to soya bean meal, with spirulina having numerically the highest yields. This may be attributed to increased methionine intake on microalgae diets. No differences in apparent digestibility of nutrients were found between dietary treatments. Owing to spirulina, microalgae diets increased milk fat concentration either via more acetate intensive rumen fermentation or increased body lipid mobilisation. Nannochloropsis inclusion in the diet resulted in most favourable omega-6:omega-3 ratio for human nutrition and fourfold increase in milk EPA concentration without adverse effects on milk fat production. Milk FA results imply that the unsaturated FA metabolism of cows fed unicellular microalgae might differ from soya bean meal. The results of current experiment suggest that microalgae are at least as good protein feed as soya bean meal in the nutrition of lactating dairy cows especially if palatability of microalgae diets can be improved.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

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