Poultry Science

Pulsed electric field (PEF) processing of microalga *Chlorella vulgaris* and its digestibility in broiler feed

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ABSTRACT Microalgae have potentially beneficial effects on animal health and nutritional value when added to feed. Crucial hereby is that intracellular bioactive molecules are released in the intestinal tract. Digestibility of *Chlorella vulgaris* and its impact on total digestibility of broiler feed is a first step in assessing its characteristics as feed supplement. Different methods could be used to increase the digestibility of the algae. Among other, pulsed electric field (**PEF**) and freezing to disrupt autotrophic (**A**) and heterotrophic (**H**) *Chlorella vulgaris* cells was assessed to increase their availability followed by *in-vivo* trials. In these trials effect of algae type (A and H) and effect of PEF-processing was evaluated on the apparent nutrient digestibility. Pulsed electric field showed to have a disruption efficiency of 83.90% and 79.20% for heterotrophic and autotrophic *C. vulgaris* respectively. Freezing *C. vulgaris* only showed efficiencies ranging from 3.86 to 11.58%. In the *in-vivo* trials, microscopic counting of intact *C. vulgaris* cells showed an increase in digested intact *C. vulgaris* cells of PEF-processed *C. vulgaris* compared to nonprocessed cells ranging from 12.16% to 15.20%. Autotrophic *C. vulgaris* had a higher digestibility compared to heterotrophic *C. vulgaris*, with an increase of 7.29, 9.44, and 17.29% in digestibility of *C. vulgaris* in the 1, 2, and 5% feed respectively. Feeds with PEF-processed *C. vulgaris* showed no significant increase in digestibility compared to nonprocessed *C. vulgaris* supplemented feeds. The 5% *C. vulgaris* feeds showed lower fat digestibility than the 1 and 2% and control feeds. Protein digestibility was lower for all *C. vulgaris* feeds compared to the control feed. There was a significant linear decreasing effect ($P < 0.001$) for all digestibility parameters. Except for crude ash digestibility, which first lowered for the 1 and 2% feeds, but then increased at 5% inclusion. Considering this study, including low dosages of 1 and 2% of *C. vulgaris* in broiler feed does not compromise its digestibility.

Key words: broiler, cell disruption, *Chlorella vulgaris*, digestibility, poultry feed

2024 Poultry Science 103:103721
https://doi.org/10.1016/j.psj.2024.103721

INTRODUCTION

Broilers are the most produced and consumed terrestrial animals worldwide (OECD-FAO, 2021). They have been selected for their high production and protein conversion efficiency. However, the increased pressure, that is, increased average daily gain and shortened lifespan, on production has led to health problems in broilers. Since the ban of the prophylactic use of antibiotics by the European Commission (2003) (EC 1831/2003), there is a need for other sustainable additives to improve poultry health and production. Microalgae, as producers of bio-actives, could be used as additives in animal feed, both for aquaculture and for animal husbandry (Shah et al., 2018). Inclusion of small amounts of microalgae of for example, *Chorella, Scenedismus,* and *Arthrospira* can improve growth, health and product quality (Saadaoui et al., 2021). Microalgae can be grown heterotrophically and autotrophically. The former grow on organic carbon sources and nutrients, while the latter are phototrophic and use carbon dioxide for their (photosynthetic) growth. Mixotrophs, such as *Chlorella vulgaris*, are able to grow in both a heterotrophic (H) and an autotrophic (A) way. An advantage of microalgae for the use in food and feed is their production in (photo-)bioreactors and raceway ponds, hence no arable land has to be occupied. Moreover, microalgae are an emerging production system for high-quality end-products in

Received January 12, 2024.
Accepted March 31, 2024.

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chemical, pharmaceutical and other sectors. After extraction of those high value compounds, within the concept of bio-circularity and zero-waste, the residual biomass could be further used for the most valued purposes, for example animal feed. Considering the high growth in the microalgae sector, this resource is expected to be a significant feed supplement. *Chlorella vulgaris* is a unicellular, eukaryotic microalga with a high protein content up to 60%. Furthermore, it contains different bioactive molecules like pigments, carotenoids, minerals, vitamins and antioxidants (Abdelnour et al., 2019). Microalgae can as well have health-promoting effects on chickens or might even contribute to an augmented quality of the end-products. The reported microalgae in poultry feed trials have inclusion levels between 0.1 and 20%, with most dosages between 0.1 and 5% (Alfaia et al., 2021; Fries-Craft et al., 2023). Effects on both health and product quality are reported, improved effects on body weight, feed conversion ratio, histology of intestinal tissue, immunity, microbiome composition, antioxidant capacity, biochemical blood parameters, intestinal permeability, meat quality and egg quality (Janczyk et al., 2009; Kang et al., 2013a; Roques et al., 2022) (nonexhaustive list). These effects are both due to the nutritional value of microalgae as well as their health-promoting effects. Due to the wide variety in species and strains of *C. vulgaris*, they might give very different effects on the health of chickens. Differences between autotrophic and heterotrophic *C. vulgaris* are reported for the cellular content, however no studies have been done about their different effects on poultry. Autotrophic *C. vulgaris* might have more bioactive compounds, but on the other hand, Sajadian et al. (2018) found that heterotrophically grown *Chlorella* had a higher lipid content than autotrophically or mixotrophically grown *Chlorella*. Other studies showed the benefit of mixotrophic growth for improved cell composition, mainly for lipids and pigments, however, differences in protein content were not noted (Yun et al., 2021). A first step to assess the benefits of microalgae in poultry feed is determining whether algae need processing to break their rigid cell wall, which is specific for *Chlorella* (Safi et al., 2014b). Possible processing techniques are freezing, freeze-drying, (cold-)pasteurization, pulsed electric field (**PEF**), high pressure homogenization (**HPH**), bead milling, ultrasonication, microwave radiation and enzymatic disruption in order to increase the availability and digestibility of nutrients (Postma et al., 2016). Pulsed electric field is a nonthermal technique, mainly used in the food industry for preservation of food due to its microbial inactivation capacity (Jeyamkondan et al., 1999). This study examines different microalgae cell disruption techniques to improve microalgal nutrient availability for broilers. Freezing and PEF in both autotrophic and heterotrophic *C. vulgaris* were tested in this study because of their low-cost and high-throughput. Effects of different dosages and disruption methods of *C. vulgaris* on digestibility of broiler feed was tested using an *in-vivo* model, in which the feed additive effect rather than nutritional effect was evaluated.

MATERIALS AND METHODS

All experimental procedures in this study were in compliance with the European guidelines for the care and use of animals in research (Directive 2010/63/EU) and were approved by the Ethical Committee of the Research Institute for Agriculture, Fisheries and Food (**ILVO**), Merelbeke, Belgium under authorization number 2022/431.

Composition of Heterotrophic and Autotrophic C. vulgaris

Heterotrophic *C. vulgaris* was purchased from Aliga Microalgae, Hjørring, Denmark and autotrophic *C. vulgaris* from Algademy, Reggio Emilia, Italy. Table 1 shows the nutrient composition of both nonprocessed and PEF-processed algae. Following parameters were determined: gross energy (ISO, 1998), dry matter (**DM**) (103°C) (EC 1971, 1971), crude protein (**CP**) (N x 6.25) (ISO 5983-2, 2009), crude fat-B (**CF**) (ISO 6492, 1999), crude fiber (AOCS Ba 6a-05, 2017) and crude ash (ISO 5984, 2002).

Experiment 1: Pulsed Electric Field and Freezing to Disrupt C. vulgaris *Cells*

PEF was used for cell disruption of *C. vulgaris*. A watery 10% algae solution (m/v) was put in a 12.5 l chamber (25 × 20 × 25 cm) with an electrode gap of 20 cm and placed in a Pulsemaster (Pulsemaster B.V., Hapert, The Netherlands). The field strength was 1.5 kV/cm and a 30 kV voltage was used. The algae solution was exposed to 1,600 pulses with a total energy of 360 kJ, which gives 225 J/pulse. The amount of energy was given to 1 kg of biomass, which gives 360 J/g. After PEF treatment, the solution was spray dried in an Anhydro spray dryer (2 × 3.45 m) (SPX, NC). The solution was preheated to 52°C and dried with an airstream of 182°C.

The second technique assessed to disrupt the cells of the algae was freezing during 1 wk at -20°C, both as a powder and as a 10% (m/v) solution in water. A living culture (**LC**) (*C. vulgaris* 211-11b), grown in an Erlenmeyer flask at 22°C and 18L:6D, was used as a negative control, in which no disrupted cells are expected.

Evaluation of Cell Disruption With Fluorescence Microscopy

To validate the disruption of the cells, SYTOX Green (5 mM stock solution in DMSO, Thermo Fisher Scientific, Waltham, MA) was used to stain the DNA of disrupted cells. For each sample, 40 mg of algae powder was suspended in 50 mL of distilled water and vortexed.

Table 1. Analyzed composition of the heterotrophic and autotrophic (pulsed electric field (**PEF**)-processed) *C. vulgaris* on dry matter (**DM**) base.

Parameter	Heterotrophic	Autotrophic	Heterotrophic PEF	Autotrophic PEF	*C. vulgaris*	Soy
Gross energy (kcal/kg DM)	5126	4730	5044	4627	4586[c]	4698[d]
Rest fraction water (%)	4.92	5.01	5.55	5.46	5.83[a]	8.07[d]
Crude protein (% DM)	53.11	51.52	51.83	50.66	51.45[a]	37.69[d]
Crude fat (% DM)	11.27	8.25	11.05	7.35	12.18[a]	28.2[d]
Crude ash (% DM)	5.02	7.97	7.11	8.08	9.50[a]	4.29[d]
Crude fiber (% DM)	5.47	3.14	2.31	5.04	9.18[a]	5.44[d]
Amino acids (% DM)						
Alanine	3.87	3.33				5.00[b]
Arginine	12.33	7.54				7.40[b]
Asparagine	3.97	4.39				-
Cysteine	1.14	0.92				1.90[b]
Glutamine	5.15	5.18				-
Glycine	2.66	3.53				4.50[b]
Histidine	0.96	0.77				2.60[b]
Isoleucine	1.36	1.72				5.30[b]
Leucine	3.71	3.75				7.70[b]
Lysine	2.33	3.45				6.40[b]
Methionine	1.66	1.49				1.30[b]
Phenylalanine	2.01	2.15				5.00[b]
Proline	2.34	2.12				5.30[b]
Serine	1.72	1.97				5.80[b]
Threonine	1.85	2.25				4.00[b]
Tyrosine	7.73	7.57				3.20[b]
Valine	1.83	1.49				5.30[b]

The last 2 columns show values in *C. vulgaris* found in literature and the amino acid composition of soy, respectively, for comparison with the values of the algae used in this study.
[1]Yasin & Shalaby (2013).
[2]Safi et al. (2014b), reported in grams per 100 g of protein.
[3]Coelho et al. (2021).
[4]Etiosa et al. (2018).

From this suspension, 200 μL was transferred to a 24 well-plate and 2 drops of SYTOX Green were added. This was incubated for 15 min in a dark environment. The samples were subsequently visualized with a fluorescence microscope Axio Imager M2 (Zeiss, Oberkochen, Germany) using a green and red excitation filter. Multiple images (Figure 1) were taken from the same sample to calculate the mean cell disruption efficiency.

Evaluation of Cell Disruption Measuring Protein Release of C. vulgaris

The concentration of proteins released from suspended *C. vulgaris* cells in water, both for nonprocessed and PEF-processed algae was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). A watery 2% (m/v) solution of freeze-dried algae biomass was made, stirred for 1 h and subsequently centrifuged for 20 min at 10 000 *g* at room temperature, then protein concentration was determined on the supernatant, following manufacturer's instructions.

Two Digestibility Trials With Broilers (Experiment 2 and 3)

For each of the 2 trials, 180 one-day-old male broilers (Ross 308) were purchased from a commercial hatchery (Belgabroed, Merksplas, Belgium). The first days, they were group-housed on a solid floor covered with wood shavings. A 23L:1D light scheme and a room temperature of 32°C was used the first week, after which a 18L:6D scheme was used for the rest of the rearing period. The temperature of the room was gradually lowered by 4°C per week until the final temperature of 22°C was reached in wk 4. Chickens were vaccinated against Newcastle disease on d 17 with Nobilis (Intervet, Boxmeer, The Netherlands). On d 18, the broilers were relocated to digestibility units (L: 0.50 m, W: 0.40 m, H: 0.35 m). One unit with 3 birds was considered as one replication with a total of 6 units per treatment. A 4-d adaptation period was followed by 5 consecutive d of balance period according to the reference method (Bourdillon et al., 1990).

Total feed intake (**FI**) was determined and excreta were collected on d 3 and d 5 of the balance period. The feces were weighed and pooled per unit. Afterwards, homogenized subsamples from the feces were freeze-dried, ground and stored at -20°C. On the excreta, following analyses were performed: gross energy, dry matter (103°C), crude protein (N x 6.25), crude fat-B, crude fiber and crude ash, following the procedures as mentioned above. Apparent digestibility coefficients were calculated using the inert marker TiO_2 (0.4% in the feed). An example for crude fat (**CF**) is given in Equation 1. Apparent crude protein digestibility was corrected for the amount of uric acid found in the excreta (Marquardt, 1983).

$$\text{Fecal digestibility coefficient (\%)} = 1 - \left(\left(\frac{TiO_{2\ \text{feed}}}{TiO_{2\ \text{feces}}}\right) * \left(\frac{CF_{\ \text{feces}}}{CF_{\ \text{feed}}}\right)\right) * 100\% \quad (1)$$

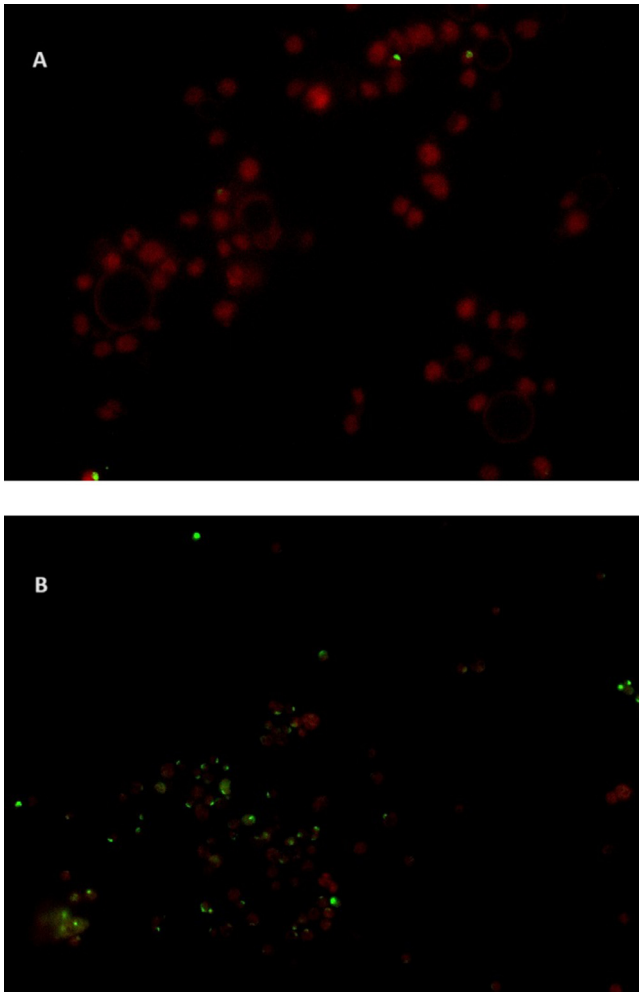

Figure 1. Fluorescence microscope image of nonprocessed *Chlorella vulgaris* stained with SYTOX Green (panel A) and Pulsed electric field (**PEF**)-processed *Chlorella vulgaris* stained with SYTOX Green (panel B). The green signal shows DNA coloration which indicates disrupted cell walls. Panel A shows intact cells (all red autofluorescence), panel B shows cells with disrupted walls, where the DNA is stained (green color).

Determination of Intact C. vulgaris *Cells in Feed and Feces*

Samples from feed and feces were diluted in water (1 g in 100 mL for the 1 and 2% feeds and 0.1 g in 100 mL for the 5% feeds) and vortexed for 30 s. Next, 10 μL of the solution was put in a Bürker counting chamber and the number of intact *C. vulgaris* cells was determined under a Laborlux D light microscope (Leica, Wetzlar, Germany). The digestibility coefficient of the intact *C. vulgaris* cells was calculated similar as for the other digestibility parameters described above, considering the inert ($\mathbf{TiO_2}$) marker.

Broiler Feed Composition and Nutrient Calculation

Broilers were fed a starter diet from d 1 to d 11, followed by a basic grower feed from d 12 to d 17. From d 18 to d 29, broilers were fed with the grower feed including *C. vulgaris* (Table 2). The analyzed feed composition is shown in Table 3.

Treatments in Experiment (E) 2 constituted of heterotrophic (H) *C. vulgaris* 1% (E2-H1%), H *C. vulgaris* 2% (E2-H2%), H *C. vulgaris* 5% (E2-H5%), autotrophic (A) *C. vulgaris* 1% (E2-A1%), A *C. vulgaris* 2% (E2-A2%), A *C. vulgaris* 5% (E2-A5%) and a control feed (E2-CON).

The treatments in Experiment (E) 3 were H-PEF *C. vulgaris* 5% (E3-HPEF5%), H *C. vulgaris* 5% (E3-H5%), A-PEF *C. vulgaris* 5% (E3-APEF5%), A *C. vulgaris* 5% (E3-A5%), A-PEF *C. vulgaris* 1% (E3-APEF1%), A *C. vulgaris* 1% (E3-A1%) and a control feed (E3-CON).

The 1% and 2% A and H algae treatments, both nonprocessed and PEF-processed (H/A 1% and 2%) were supplemented on-top of a control feed, since these inclusion levels had a small impact on the nutritional composition of the feed. The 5% A and H algae feeds, both nonprocessed and PEF-processed (H/A 5%) were reformulated, composition of the algae was taken into account for formulation of the feed (Table 1). Reference values were obtained from Barone et al. (2018) and Alfaia et al. (2021).

Statistical Analysis

Statistical analysis was performed with R version 4.1.2 for Windows (R Core Team, 2021). For cell disruption efficiencies and released protein concentration, least-square linear models were used with 'treatment' as independent variable. For microscopic data of the intact *C. vulgaris* cells, a least-square linear model was used with factors 'inclusion level' and 'algae type' in Experiment 2 and with 'treatment' as independent variable in Experiment 3. Linear model assumptions (normality and homoscedasticity) were verified by a visual check of the residuals plots. *P*-values of the variables were obtained with analysis of variance (ANOVA), a post hoc Tukey's range test (honest significant difference, **HSD**) was used to obtain adjusted *P*-values to account for multiple comparisons, with level of significance $\alpha = 0.05$. Digestibility data in Experiment 2 were analyzed using polynomial contrasts to study linear and quadratic effects of heterotrophic and autotrophic algae as treatments in this experiment were structured. A correction was done to adjust for the unequal spacing of the inclusion levels. Digestibility data in Experiment 3 were analyzed using ANOVA and Tukey's correction for multiple comparisons as this experiment set-up was not structured.

RESULTS

Feed Composition

Crude fat of H/A 5% was lower than that of H/A 1% and 2%, since formulation was done to obtain equal values of metabolizable energy and protein content. The difference is approximately 1.5% in crude fat content

Table 2. Feed composition and calculated nutrient composition for the starter, grower, and grower with algae for the digestibility trials.

	Starter	Grower basic (1 and 2%)[2]	Grower Heterotrophic (5%)	Grower Autotrophic (5%)
Ingredient (%)				
Wheat	48.37	51.61	55.92	55.60
Corn	15.00	15.00	15.00	15.00
Soybean	5.00	4.50	4.50	4.50
Soybean meal (48% CP)	23.34	22.08	14.65	14.93
Soy oil	1.00	-	-	-
Animal fat	2.05	2.95	0.71	0.77
Mineral and vitamin premix[1]	1.00	1.00	1.00	1.00
Feed chalk	0.44	0.97	0.95	0.95
Di-calcium phosphate	1.30	0.27	0.38	0.38
NaCl	0.10	0.16	0.11	0.11
Na-bicarbonate	0.40	0.29	0.36	0.36
L-lysine HCl	0.34	0.30	0.46	0.45
DL-methionine	0.31	0.26	0.29	0.29
L-threonine	0.20	0.12	0.17	0.17
Coccidiostat	0.05	-	-	-
NSP enzyme	0.01	0.01	0.01	0.01
Phytase	0.10	0.10	0.10	0.10
Titanium oxide	-	0.40	0.40	0.40
Heterotrophic *C. vulgaris*	-	-	5.00	-
Autotrophic *C. vulgaris*	-	-	-	5.00
Calculated nutrient composition				
Metabolizable energy (kcal/kg)	2806	2818	2818	2818
Moisture content (%)	10.96	13.36	13.34	13.27
Crude protein (%)	20.50	19.50	19.50	19.50
Crude fat (%)	8.03	6.55	4.45	4.77
Crude ash (%)	4.77	4.65	4.78	4.72
Crude fiber (%)	3.95	3.37	3.29	3.28
Dig. Lysine (%)	1.15	1.06	1.06	1.06
Dig. Methionine + cysteine (%)	0.86	0.80	0.80	0.80
Dig. Threonine (%)	0.81	0.69	0.69	0.69
Dig. Valine (%)	0.80	0.76	0.70	0.70
Ca (%)	0.85	0.80	0.80	0.80
Available P (%)	0.60	0.44	0.55	0.42
NaCl + KCl (mEq/kg)	250.57	238.37	204.00	200.39

For the 1 and 2% algae feed, the algae are mixed on top of the basic grower feed, the 5% algae feed was reformulated. (**NSP**: nonstarch polysaccharides, **CP**: crude protein).

[1]Vitamin and mineral premix composed of vitamin A/retinyl acetate 3a672a (10,00,000 IU/kg); vitamin D3 E671 (299,999.4 IU/kg); vitamin E 3a700 (all-rac-alpha-tocopheryl acetate) (5,000 IU/kg); vitamin K3 3a710 (250 mg/kg); vitamin B1/thiamine mononitrate 3a821 (200 mg/kg); vitamin B2/ribo-flavin (500 mg/kg); calcium D-pantothenate 3a841 (1,500 mg/kg); vitamin B6/pyridoxine hydrochloride 3a831 (400 mg/kg); vitamin B12/cyanocobala-min (2.5 mg/kg); niacinamide 3a315 (3,000 mg/kg); folic acid 3a316 (100 mg/kg); biotin/D-(+)-biotin 3a880 (15 mg/kg); choline chloride 3a890 (68,965.5 mg/kg); iron(II)sulphate (monohydrate) − iron E1 (4,920 mg/kg); copper(II)sulphate (pentahydrate) − copper E4 (2,000 mg/kg); zinc oxide 3b603 (6,000 mg/kg); manganese(II)oxide − manganese E5 (9,590.2 mg/kg); calcium iodate (anhydrous) − iodine 3b202 (120 mg/kg); sodium selenite -selenium E8 (36 mg/kg); sepiolite E562 (700 mg/kg); propyl gallate E310 (200 mg/kg); BHT E321 (300 mg/kg); citric acid E330.

[2]Algae in the 1 and 2% feeds were mixed on top.

Table 3. Analyzed nutrient composition for feeds for the digestibility trials with broilers.

Parameter	H 1%	H 2%	H 5%	A 1%	A 2%	A 5%	CON
Gross energy (kcal/kg)	3,606	3,620	3,504	3,571	3,631	3,481	3,577
Crude protein (%)	18.09	18.30	17.57	17.77	18.29	17.25	17.71
Crude fat (%)	5.41	5.20	3.59	5.24	5.13	3.53	4.90
Crude ash (%)	4.88	4.59	4.57	4.68	4.59	4.63	4.34
Crude fiber (%)	3.02	2.88	3.06	2.88	2.88	2.68	2.78
Parameter	**H 5%**	**H-PEF 5%**	**A 1%**	**A-PEF 1%**	**A 5%**	**A-PEF 5%**	**CON**
Gross energy (kcal/kg)	3,378	3,405	3,543	3,525	3,412	3,415	3,539
Crude protein (%)	17.04	17.20	17.68	17.21	16.96	16.93	17.25
Crude fat (%)	3.45	3.35	5.05	5.03	3.58	3.59	5.12
Crude ash (%)	5.47	4.80	4.53	4.46	4.83	4.63	4.63
Crude fiber (%)	2.41	2.44	2.43	2.35	2.31	2.42	2.44

A: autotrophic, H: Heterotrophic, PEF: Pulsed electric field, CON: control.

between the basic grower feeds with algae mixed on top and the reformulated feeds with 5% algae.

Experiment 1: Cell Disruption Efficiencies

PEF treatment resulted in the highest cell disruption efficiencies, both for autotrophic and heterotrophic *C. vulgaris*, which were 79.20 ± 5.60% and 83.90 ± 3.90% respectively (Table 4). The efficiency resulted from PEF was significantly higher than a freezing treatment ($P < 0.001$). Disruption efficiencies obtained with freezing were all lower than 10%. Freezing, both as a powder and as a solution, did not result in a significantly higher cell disruption as compared to the nonprocessed biomass. PEF nor freezing had a significantly different effect when applied on autotrophic or heterotrophic algae.

Protein Release of Nonprocessed and PEF-Processed C. Vulgaris PEF-processing of heterotrophic algae had no significant difference as compared to nonprocessed heterotrophic algae (4.16 ± 0.20% vs. 4.47± 0.53%; $P = 0.211$). PEF-processing of autotrophic algae had a lower protein release than nonprocessed autotrophic algae (3.68 ± 0.31% vs. 4.16 ± 0.22%; $P = 0.011$).

Experiment 2: Difference in Digestibility of Autotrophic and Heterotrophic C. vulgaris

Intact Chlorella Vulgaris Cells. Intact autotrophic *C. vulgaris* had a significantly higher digestibility compared to heterotrophic *C.* vulgaris, with an increase of 7.29, 9.44, and 17.29% in digestibility of intact *C. vulgaris* in the 1, 2, and 5% feed respectively ($P < 0.001$). E2-H5% and E2-A5% (63.02 ± 11.11% and 80.31 ± 3.16%) had a significantly lower digestibility compared to E2-H1% and E2-A1% (78.09 ± 1.21% and 85.38 ± 0.69%) ($P = 0.001$). The difference between algae included in E2-H/A (heterotrophic and autotrophic feed) 5% and E2-H/A 2% were not significant. Furthermore, algae included in E2-H/A 1% and E2-H/A 2% had no significant difference in their digestibility (Figure 2).

Table 4. Percentage of cell disruption (mean value ± 95% CI) for different disruption methods on autotrophic and heterotrophic *C. vulgaris*, evaluated with SYTOX Green staining.

Chlorella vulgaris	Disruption method	Disrupted cells (%)
Living culture	Nonprocessed	1.55 ± 2.31^{a}
Heterotrophic	Nonprocessed	11.58 ± 6.11^{b}
	Frozen (powder)	3.86 ± 2.15^{ab}
	Frozen (10% solution)	6.41 ± 1.99^{ab}
	Pulsed electric field	83.90 ± 3.90^{c}
Autotrophic	Nonprocessed	7.94 ± 2.03^{ab}
	Frozen (powder)	7.33 ± 2.38^{ab}
	Frozen (10% solution)	4.68 ± 1.12^{ab}
	Pulsed electric field	79.20 ± 5.60^{c}

The percentage is the number of disrupted cells, divided by the total number of cells.

CI: confidence interval (95%). Different letters show statistically significant differences between all groups (living culture, autotrophic, and heterotrophic with the different techniques). One-way ANOVA ($P < 0.001$), Tukey's Range Test, n = 5, $\alpha = 0.05$.

Digestibility Parameters of Broiler Feed. Heterotrophic and autotrophic algae had a significant linear effect ($P < 0.001$) on all digestibility parameters. Digestibility decreased with increasing algae inclusion. Except for crude ash digestibility, which first lowered for the 1 and 2% feeds, but then increased at 5% inclusion level. Furthermore, for heterotrophic algae, a significant quadratic response was found for crude fat ($P = 0.001$), crude protein ($P < 0.001$) and crude ash ($P < 0.001$). For autotrophic algae, a significant quadratic response was found for crude protein ($P = 0.001$) and crude ash ($P < 0.001$) (Table 5). Metabolizable energy of E2-H/A5% was lower than the control feed and E2-H/A1% and E2-H/A2%. E2-H/A5% feed had lower values compared to the control feed and to E2-H/A1% and E2-H/A2% for crude fat digestion. Crude protein digestibility of E2-H/A1%, E2-H/A2% and E2-H/A5% feed was lower than the control feed. The digestibility coefficients of crude fiber were all 0, except for E2-H5%, which means fiber is not digested.

Experiment 3: Effect of PEF-Processing on Digestibility of C. vulgaris

Intact Chlorella vulgaris Cells. PEF-processed *C. vulgaris* had a significantly higher digestibility as compared to nonprocessed *C. vulgaris*. E3-HPEF5%, E3-APEF5% and E3-APEF1% had respectively a 15.20, 12.16, and 12.66% higher digestibility than the E3-H5%, E3-A5% and E3-A1% feed ($P < 0.001$) (Figure 3).

Digestibility Parameters of Broiler Feed. Digestibility of crude ash of E3-HPEF5% was significantly higher than the nonprocessed E3-H5%, 42.9 ± 1.1% vs. 34.9 ± 1.7% (Table 6). Crude fiber was not digested. Crude protein digestibility of E3-A5% was lower than that of the control feed. Crude fat digestibility and metabolizable energy of E3-H/A5% was lower than E3-H/A1% and the control feed, as was observed in trial 1 ($P < 0.001$). There was no significant difference between PEF-processed and nonprocessed algae groups, neither for the autotrophic nor the heterotrophic algae.

DISCUSSION

The amounts of amino acids in autotrophic and heterotrophic *C. vulgaris* in this study were similar, except for arginine, which was more abundant in the heterotrophic algae. Miller et al. (1971) also showed that the amino acid and vitamin content of autotrophic and heterotrophic *Chlorella sorokiniana* does not differ remarkably. Protein and fat content in the algae samples in the current study were comparable to amounts mentioned by Safi et al. (2014b), 42 to 58% for proteins and 5 to 40% for lipids.

The first disruption method, freezing, was not able to disrupt *C. vulgaris* cells, the effectiveness might depend on the growth stage where the *C. vulgaris* was harvested. A study by Morris (1976) showed increased lysis of cells due to a temperature drop for cells harvested in the exponential phase, but not for those harvested in the

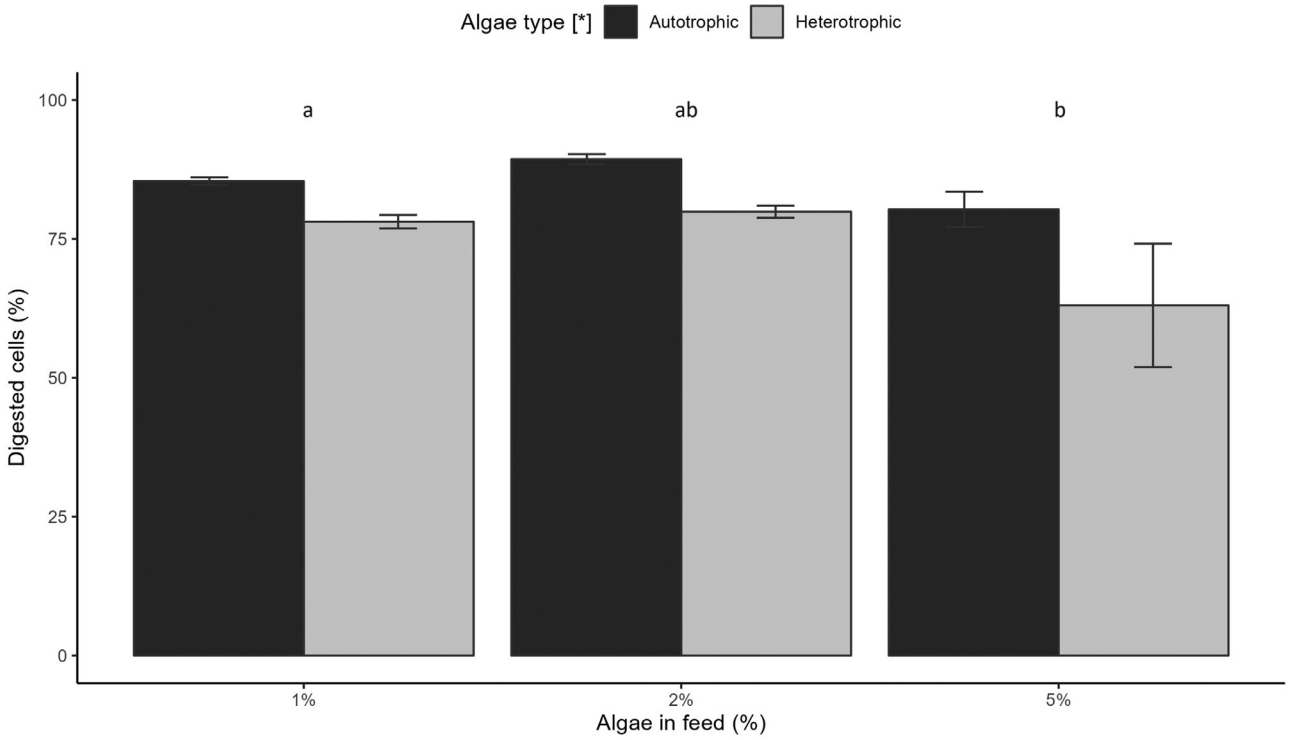

Figure 2. Digestibility of *Chlorella vulgaris* cells (mean value ± standard deviation) of the different feed treatments in trial 1. Error bars show the standard deviation. Different letters show statistically significant differences between inclusion levels, [*] shows a significant difference between algae types. ANOVA (2-way: factors "inclusion level" ($P = 0.001$) and "algae type" ($P < 0.001$), Tukey's Range Test, n = 3, $\alpha = 0.05$.

stationary phase. The biomass used in our study was harvested in the stationary phase. The second method to disrupt, PEF, was successful. After PEF-processing, the nutrient composition of the algae was not different, which suggests the treatment might not have any negative effects on the nutrient composition. Therefore, PEF could be an interesting technique because it perforates the cell wall, instead of completely breaking it so useful compounds like pigments, antioxidants and vitamins are not exposed to air and oxidized. Compared to freezing, PEF processing was a very effective technique to perforate *C. vulgaris* cells. The electric pulses cause a transmembrane potential. Since the cell membrane is only partly permeable for ions, charged groups accumulate in the cells, which causes perforation of the cells when these cannot longer sustain the increase in potential difference (Jeyamkondan et al., 1999). The protein release test gave no biologically relevant difference between PEF-processed and nonprocessed *C. vulgaris* cells, since differences were lower than 1%, on a total protein content of 55% proteins. It is possible that cells are perforated but proteins still are not able to leave the cell and dilute in water. Safi et al. (2014a) used the water-soluble protein from total protein fraction, released in the aqueous phase as an indicator for cell disruption. For *C. vulgaris*, this gave an increase from 9.7 ± 0.5 (nonprocessed sample) to 52.8 ± 0.6% with high-pressure homogenization treatment and to 9.0 ± 0.1% with manual grinding, to 18.1 ± 0.0% with ultrasonication and to 33.2 ± 0.0% with chemical treatment. It shows that these techniques do increase the protein release. PEF treatment in our study did not show an increase in protein release, which might be due to the fact that the cells were rather perforated. PEF-processing is very dependent on the parameters of the electric field that is used. Luengo et al. (2014) described the existence of reversible and irreversible disruption of *Chlorella* cells for pigment extraction. In the range between 20 and 25 kV/cm, irreversible electroporation occurs, even at short treatment times (5 pulses of 3 μs). At lower field strengths (10 kV/cm), reversible

Table 5. Digestibility parameters and metabolizable energy (mean values) of the different feed treatments in digestibility trial 1.

									P-value			
									H		A	
Parameter	CON	H 1%	H 2%	H 5%	A 1%	A 2%	A 5%	SEM	L	Q	L	Q
Metabolizable energy (n) (kcal)[a]	2,912.9	2,798.1	2,879.6	2,671.9	2,800.2	2,853.2	2,661.1	63.8	< 0.001	0.298	< 0.001	0.612
Gross energy (%)	76.25	72.69	74.68	71.48	73.15	73.90	71.28	1.65	< 0.001	0.565	< 0.001	0.273
Crude fat (%)	79.0	77.8	79.5	66.8	76.9	73.4	62.1	2.75	< 0.001	0.001	< 0.001	0.310
Crude protein (%)	83.9	79.5	80.6	79.3	80.2	80.3	77.3	0.85	< 0.001	< 0.001	< 0.001	0.001
Crude ash (%)	34.2	31.6	33.5	36.9	31.3	32.8	35.8	1.21	< 0.001	< 0.001	< 0.001	< 0.001
Crude fiber (%)	0.0	0.0	0.0	5.6	0.0	0.0	0.0	-	-	-	-	-

A, autotrophic, H: heterotrophic, CON: control. SEM: standard error of the means. Linear (L) and quadratic (Q) effects of H and A algae were determined by polynomial contrasts. [a]: apparent metabolizable energy (nitrogen corrected) of the feed.

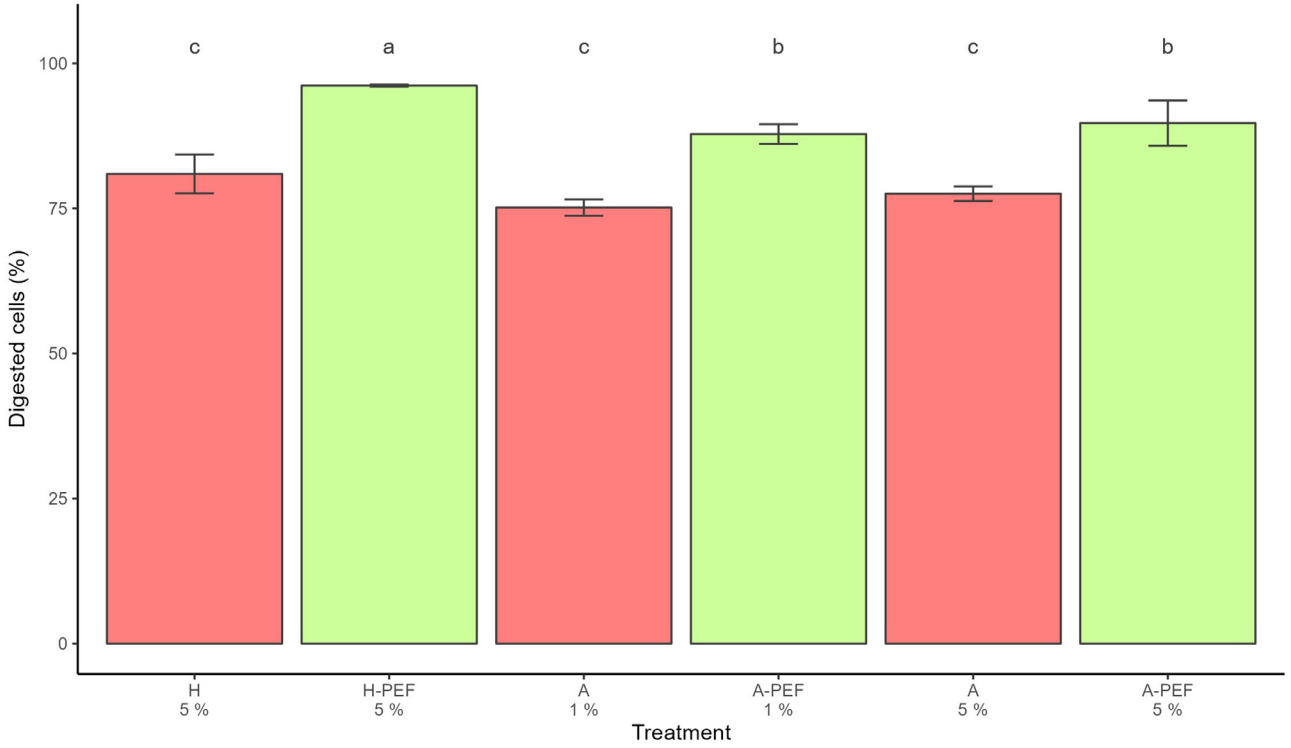

Figure 3. Digestibility of *Chlorella vulgaris* cells (mean value ± standard deviation) of the different feed treatments (red bars: nonprocessed *C. vulgaris*, green bars: PEF-processed C. vulgaris) in trial 2. A: Autotrophic, H: Heterotrophic, PEF: Pulsed electric field. Error bars show the standard deviation. Different letters show statistically significant differences between treatments. One-way ANOVA ($P < 0.001$), Tukey's Range Test, $n = 3$, $\alpha = 0.05$.

electroporation occurs, even at 50 pulses of 3 μs. Fields higher than 15 kV/cm and longer than 15 μs significantly increased the extraction yield of cellular components. In the present study, a field strength of 1.5 kV/cm was used, although 1,600 pulses were applied (total energy of 360 J per gram biomass). The study of Luengo et al. (2014) worked in a range from 0.009 to 0.059 J per gram biomass. Since in the current study, the applied energy was higher, the algae were disrupted irreversibly, which was confirmed with the Sytox Green staining and fluorescence microscopy evaluation.

Based on the counts of the intact *C. vulgaris* cells, the present study showed digestibility of autotrophic *C. vulgaris* to be significantly higher than the digestibility of heterotrophic cells. This might be due to the difference in crude fiber content, which is higher in heterotrophic *C. vulgaris* than in autotrophic *C. vulgaris* (5.75 vs. 3.31%). Crude fiber, which includes polysaccharides like (hemi-)cellulose are mainly present in the cell wall which might cause a lower digestibility of the cells. This might not be valid for all microalgae, and can depend on growth conditions, as Miller et al. (1971) showed that there was no difference in crude fiber content between auto- and heterotrophic *Chlorella sorokiniana*. Further research is needed to assess the digestibility of algae as nutrient rather than additives. In further studies, increasing the inclusion level of algae in the diets is needed to make conclusions about their digestibility.

Crude protein digestibility of the algae feeds was lower compared to the control feed, which might suggest that the proteins in the algae are less digestible than those from the other ingredients in the feed or the uptake of proteins in the feed is compromised due to the addition of microalgae. Other studies to the effects of additives on protein digestibility are inconclusive. A study by Mountzouris et al. (2011) showed

Table 6. Digestibility parameters and metabolizable energy (mean values) of the different feed treatments in digestibility trial 2.

Parameter	CON	H 5%	H-PEF 5%	A 1%	A-PEF 1%	A 5%	A-PEF 5%	SEM	*P*-value
Metabolizable energy (n) (kcal)[a]	2968.2^{a}	2828.9^{bc}	2886.9^{abc}	2910.3^{ab}	2890.7^{abc}	2825.6^{bc}	2809.2^{c}	50.27	< 0.001
Gross energy (%)	74.57^{a}	74.57^{a}	73.77^{a}	72.48^{a}	73.09^{a}	72.76^{a}	72.99^{a}	1.28	0.031
Crude fat (%)	77.1^{a}	65.1^{b}	66.7^{b}	75.3^{a}	75.0^{a}	65.9^{b}	65.7^{b}	2.06	< 0.001
Crude protein (%)	81.2^{ab}	80.4^{abc}	81.7^{a}	80.6^{abc}	79.6^{bc}	79.3^{c}	80.1^{abc}	0.96	0.001
Crude ash (%)	35.1^{b}	34.9^{b}	42.9^{a}	32.1^{c}	32.3^{c}	35.6^{b}	33.8^{bc}	1.17	< 0.001
Crude fiber (%)	0.00	0.0	0.0	0.0	0.0	0.0	0.0	-	-

A, autotrophic, H: heterotrophic, PEF: pulsed electric field, CON: control. SEM: standard error of the means. Different letters show statistically significant differences between treatments. ANOVA, Tukey's Range Test, $n = 6$, $\alpha = 0.05$. [a]: apparent metabolizable energy (nitrogen corrected) of the feed.

no effects of phytogenic feed additives on the ileal nutrient digestibility of crude proteins. A study by Hafeez et al. (2016) showed a significant increase in protein digestibility depending on the type of phytogenic additive. Since the crude ash digestibility increased with increasing inclusion level, *C. vulgaris* might enhance the uptake of minerals. This effect is also observed when the intestinal cells are in better conditions, for example due to the availability of antioxidants in the cells of the intestinal tract or when more digestive enzymes are available (Alagawany et al., 2018). Amad et al. (2011) found a linear increase in digestibility of crude fat, crude protein and crude ash with increasing inclusion levels of phytogenic feed additives. The crude fat digestibility and metabolizable energy of the 5% feeds, was lower than the other treatments groups and the control feed, although this might also be explained by the lower fat content of the 5% feeds, since these were reformulated while the algae were mixed on top for the 1 and 2% feeds. In this study, feeds were formulated for equal protein content and metabolizable energy, for this an equal fat content was not possible to formulate. Further studies should be done to investigate whether 5% inclusion can already have a negative impact on (fat) digestibility or the effects observed in this study were indeed due to the lower fat content of the feeds. This lower fat digestibility can potentially impact performance. Digestibility and availability of nutrients is related to broiler's body weight, average daily gain and feed conversion ratio. Only a few studies with inclusion levels higher than 2% can be found in literature. Even at lower inclusion levels, effects on performance already seem to be inconclusive. Kang et al. (2013b) found no significant effects of 1% inclusion of *C. vulgaris* on feed conversion ratio and feed intake, but a significant increase in body weight gain. These findings were also obtained by El-Bahr et al. (2020) and Roques et al. (2022), who conducted studies with inclusion levels of 0.1% and 0.8% respectively.

CONCLUSIONS

Pulsed electric field treatment of *C. vulgaris* had a high cell disruption efficiency, up to 80%, both for autotrophic and heterotrophic *C. vulgaris*. The digestibility trials showed that PEF-processed *C. vulgaris* cells were more digestible than nonprocessed *C. vulgaris* cells and autotrophic *C. vulgaris* cells had a higher digestibility compared to heterotrophic *C. vulgaris* cells. This indicates the beneficial effect of PEF treatment. Feeds with PEF-processed *C. vulgaris* had no significant difference in digestibility of crude protein, fat and ash compared to nonprocessed *C. vulgaris* supplemented feeds. Considering the above findings and the observation that including low dosages of *C. vulgaris* in broiler feed does not compromise its digestibility, further study to the use of *C. vulgaris* in broiler feed for their health-promoting effects is promising.

ACKNOWLEDGMENTS

This research was funded by the European Union (Horizon2020) under the project GeneBEcon, Grant Agreement No. 101061015. The authors would like to thank Jolien Vander Linden, Valentina Casciaro, Laid Douidah, Bart Ampe and the animal caretakers, technicians and researchers from the poultry research unit at ILVO (9090, Melle, Belgium) for help with the trials, sampling and analysis. We would also like to thank Katleen Coudijzer, Simon Van De Walle and Geert Van Royen from the Food Pilot of ILVO for the use of the Pulsemaster. Many thanks to Gregor Claus for proofreading the manuscript. This article was published with the support of the University Foundation of Belgium.

Authors Contributions: Sofie Van Nerom: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing—original draft, Visualization. Kobe Buyse: Conceptualization, Writing—review and editing. Filip Van Immerseel: Writing—review and editing, Supervision. Johan Robbens: Conceptualization, Methodology, Writing—review and editing, Supervision. Evelyne Delezie: Conceptualization, Methodology, Writing—review and editing, Supervision.

DISCLOSURES

The authors declare no conflicts of interest.

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