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Apparent digestibility of nutrients, energy, essential amino acids and fatty acids of juvenile Atlantic salmon (*Salmo salar* L.) diets containing whole-cell or cell-ruptured *Chlorella vulgaris* meals at five dietary inclusion levels

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ABSTRACT

Chlorella vulgaris, one of the most studied microalga for industrial applications, has never before been assessed as a potential 'low-trophic' ingredient for Atlantic salmon (*Salmo salar* L.). The effects on apparent nutrient digestibility coefficients (ADCs) by dietary inclusion of whole-cell or cell-ruptured *C. vulgaris* meals at five levels were determined. Integrity of nutrients, energy, essential amino acids (EAAs) and fatty acids were well-preserved after cell-rupture processing. Based on microscopy and protein solubility, two Microfluidizer® passes were sufficient for complete cell-rupture as no improvement in solubility ($P = 0.998$) was achieved with a third pass. Whole-cell *C. vulgaris* meal reduced ADCs for dry matter, protein, lipid and energy at inclusion as low as 6–12% ($P \leq 0.035$), whereas carbohydrate ADC was not affected up to 24% ($P \geq 0.980$) and was significantly improved at 30% ($P = 0.028$). Similarly, starch ADC was not affected by inclusion of whole-cell *C. vulgaris* meal at any level ($P = 0.256$). Inclusion of cell-ruptured *C. vulgaris* meal did not affect ADCs for dry matter when included up to 30% ($P \geq 0.900$), protein up to 24% ($P \geq 0.092$) or lipid up to 18% ($P \geq 0.124$). Energy ADC was not affected up to 12% ($P \geq 0.530$) but reduced at higher levels ($P \leq 0.009$). Inclusion of cell-ruptured *C. vulgaris* meal at all levels improved carbohydrate ADC ($P \leq 0.002$), due to increased starch digestibility. In fact, starch ADCs of diets containing 18–30% was significantly higher than that of the algae-free control diet ($P \leq 0.009$). Similarly, phosphorous ADC was higher in diets containing 18–30% *C. vulgaris* meals than the algae-free control diet. Dietary ADCs were not affected by moderate inclusion (up to 18%) of whole-cell *C. vulgaris* meal for most EAAs ($P \geq 0.116$), while ADCs for leucine and phenylalanine were reduced when over 12%. Dietary ADCs were not affected by high inclusion (24–30%) of cell-ruptured *C. vulgaris* meal for any EAA ($P \geq 0.076$) and tryptophan and lysine were confirmed as the most limiting EAAs in *C. vulgaris* meals for Atlantic salmon. Reduced energy digestibility of diets containing *C. vulgaris* meals was associated with significantly lower ADCs for palmitic, oleic, linoleic and linolenic acids with whole-cell *C. vulgaris* meal at > 6–12% ($P \leq 0.022$) and palmitic and oleic acids with cell-ruptured *C. vulgaris* meal at > 12–18%. Nevertheless, inclusion of either *C. vulgaris* meal up to 30% did not affect dietary ADCs for palmitoleic acid, eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) ($P \geq 0.126$). Single ingredient ADCs of nutrients, energy, EAAs and major fatty acids are reported for the first time for juvenile Atlantic salmon fed diets containing whole-cell and cell-ruptured *C. vulgaris* meals.

1. Introduction

Global demand for compound aquafeeds is around 32 million metric tonnes (MMT), is growing at ~12% annually and will soon reach almost 50 MMT as world aquaculture production is projected to double within the next 10–15 years (Kobayashi et al., 2015; Tacon and Metian, 2008, 2015). Conventional sources of fish meal and oil obtained from the reduction of pelagic forage fish have reached or exceeded their maximum sustainable limits, are becoming increasingly cost-prohibitive and wild populations may become even more pressured by global climate change (Nasopoulou and Zabetakis, 2012; Lazard, 2017) and cannot be depended upon to keep pace with growing aquafeed demand. Second-generation feed ingredients derived from terrestrial crops are now widely used in salmon feeds globally, but they are not without

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limitations. Most are lacking in certain functional properties and nutritional profiles, they may alter final product quality and, like marine resources; their growing demand is increasing their price and their agricultural production is becoming increasingly ecologically unsustainable (Draganovic et al., 2013; Hixson, 2014; Li et al., 2009; Turchini et al., 2009). The increased use of these ingredients has forced global salmon production to shift its alignment to terrestrial agriculture, which occupies large aerial footprints and is heavily dependent on fossil fuel-based fertilizers, chemical pesticides and freshwater irrigation (Fry et al., 2016; Pahlow et al., 2015). In an effort to develop more sustainable salmonid feeds based on 'lower-trophic' ingredients, microalgae have been proposed as promising candidates (Hemaiswarya et al., 2011; Roy and Pal, 2014). Despite this encouraging trend, the required species-specific digestibility data is either inadequate (or non-existent) and the effect of inclusion of these novel ingredients on the physical properties of compound aquafeeds is unknown. In particular, *Chlorella* species have been consumed by humans for thousands of years and in commercially cultivation since 1961. They are generally regarded by dieticians, animal nutritionists and the health-conscious public to be packed with essential nutrients, bioactive molecules, antioxidants and other health promoting compounds often loosely termed as Chlorella Growth Factor (CGF) and they are generally free of known allergens and do not produce bio-toxins (Liu and Hu, 2013). While this makes them attractive organisms for food supplements and sustainable animal feeds (Grigorova, 2005; Geetha et al., 2010; Draganovic et al., 2013; Liu and Hu, 2013; Vecina et al., 2014; Yaakob et al., 2014; Kotrbáček et al., 2015; Maisashvili et al., 2015; Yang et al., 2015; Chen et al., 2016; Kim et al., 2016; Alavi and Golmakani, 2017; Xie et al., 2017), there has never been an adequate strategic assessment of its nutritional quality as a feed ingredient for Atlantic salmon (*Salmo salar* L.).

Annual production of *Chlorella* remains small (~2000 t) and this is related to high production costs and technological challenges associated with current cultivation systems (Brennan and Owende, 2010; Liu and Hu, 2013; Priyadarshani and Rath, 2012; Pulz and Gross, 2004; Walker, 2009). In order for *Chlorella* to be seriously considered as a routine input for aquaculture feeds, it will require a massive scale-up of industrial production. And, even if this is technically and economically possible, the aquafeed sector will demand a consistent product and reliable supply at a price that is competitive with fish meal and other high-protein plant-based feedstuffs in order to penetrate this market space. This has not been the case over the past half century where *Chlorella* has been produced and marketed to the relatively niche human dietary supplement market as a poorly regulated nutraceutical (Bagchi, 2006). Görs et al. (2010) reported that of the numerous *Chlorella*-based products on the market, quality control was poor and most were contaminated with bacteria, cyanobacteria and other unlisted algal species, contained highly variable levels of chlorophyll and its breakdown products and were highly heterogeneous in their nutrient composition. This lack of product uniformity and quality control should not be tolerated in aquafeeds and quality assurance must be made a priority.

The microalga under investigation in this study is a freshwater chlorophytic (green) spherical species (generally 2–10 μm in diameter) that has been proposed for industrial mass algaculture as a suitable platform for bioremediation and as a feedstock for renewable energies (Lee et al., 2013; Xu et al., 2006; Yang et al., 2015). Surprisingly, despite the fact that *Chlorella* spp. are some of the most biotechnologically relevant microalgae for industrial applications, including commodity protein production (Barka and Blecker, 2016; Chen et al., 2016; Görs et al., 2010; Liu and Hu, 2013; Morris et al., 2008; Safi et al., 2014a; Waghmare et al., 2016; Xie et al., 2017), there has never been a suitable assessment of their nutritional quality in diets for most fish and specifically for farmed Atlantic salmon. In fact, while *C. vulgaris* has been assigned an International Feed Number (IFN 5-20-658), standard fish nutrition references contain no data on its general composition, amino acid profile and nutrient digestibility (Halver and Hardy, 2002; NRC, 2011). Most photoautotrophic microalgae-based ingredients with potential for aquafeeds are presently lacking the required compositional data and digestibility coefficients required for feed formulation (Shields and Lupatsch, 2012). This is particularly relevant here given the fact that it is well-known that chlorophytic microalgae in the *Chlorella* genus possess rigid, recalcitrant cell walls, although species differences do exist in their actual compositional make-up (Domozych et al., 2012; Liu and Hu, 2013; He et al., 2016). Since reduced digestibility of diets containing *Chlorella* microalgae with their rigid cells walls is likely to occur, it is our belief that jumping too far ahead in its nutritional evaluation in feeding trials without adequate examination of the first bottleneck for nutrient assimilation (*e.g.*, digestibility) is premature. While cognizant of the fact that nutrient digestibility data does not always directly predict fish performance (de Carvalho et al., 2016), it was felt that the optimal level of dietary inclusion of *Chlorella* meals should be determined based on the 'target' animal's ability to digest it in different processed forms (*e.g.*, intact cells and ruptured cells) and over a realistic range of dietary inclusion levels prior to growth performance and animal health studies. Previous studies with our own proprietary strain of *C. vulgaris* have demonstrated its good potential for rapid cell division and lipid accumulation; high in energy-rich oleic acid and health-promoting 18-series PUFAs linoleic acid and linolenic acid (MacDougall et al., 2011; Tibbetts et al., 2015). Additionally, the biomass proved to be a rich source of key essential amino acids, minerals and trace elements and carotenoids (Tibbetts et al., 2015). Subsequent *in vitro* studies using monogastric (porcine) derived digestive enzymes identified it as having the highest protein solubility, dilute pepsin digestibility and 2-phase gastric/pancreatic digestibility compared to other algal species assayed (Tibbetts et al., 2016). However, *in vitro* digestibility data is often preliminary, comparative in nature and rarely 'species-specific' so these results required further *in vivo* validation with the target species (*e.g.*, Atlantic salmon). It is for these reasons that *C. vulgaris* was chosen for investigation as a novel sustainable ingredient for salmon feeds in the present study. However, it is well-known that green chlorophytic microalgae like *Chlorella* generally possess a recalcitrant cell wall so it was investigated in different forms (*e.g.*, intact cells and ruptured cells) and over a range of dietary inclusion levels (0–30%). The objectives of this study were to comprehensively characterize the biochemical composition of whole-cell and cell-ruptured *C. vulgaris* meals and to determine the effect of dietary inclusion of these *C. vulgaris* meals on *in vivo* ADCs of a reference Atlantic salmon diet when provided at different dietary inclusion levels (0, 6, 12, 18, 24 and 30%).

2. Materials and methods

2.1. Test ingredients

The *C. vulgaris* microalgae used in this study is a proprietary green chlorophyte isolated from freshwater lakes in Alberta, Canada (East-Central region between N52 to 56° and W110 to 113°). Details of DNA sequence identification, maintenance of immobilized unicellular stocks, mobilization of starter cultures and mass cultivation in 1000 L 'Brite-Box' photobioreactors (PBRs) are described in Tibbetts et al. (2015). Cultures from 22 production campaigns (22,000 L total volume) were pooled for these studies. The cultures were harvested between 5 and 6 days into stationary phase (~39×10^6 cells/mL) using a process centrifuge (model Z101, CEPA Carl Padberg Zentrifugenbau GmbH., Lahr, Germany) equipped with a 10 L collection chamber at 15,760 × *g* and immediately frozen at −20 °C. Frozen paste (~25% solids) was lyophilized for 72 h at a low shelf temperature (< 5 °C) in a large capacity freeze-dryer (model 35EL, The Virtis Company, Gardiner, NY) to a final moisture content of < 4%. Freeze-dried *C. vulgaris* biomass was pulverized (to pass through a 0.5 mm screen) at 10,000 rpm using a laboratory ultra-centrifugal mill (model ZM200, Retsch GmbH., Haan,

Germany) equipped with a Retsch pneumatic auto-feeder (model DR100) and then stabilized with 500 mg/kg (0.05%) ethoxyquin (≥ 75% pure, Sigma-Aldrich, Cat. E8260, International Feed Number 8-01-841). This material was stored at − 20 °C and half of it was used directly as the whole-cell *C. vulgaris* meal.

The remaining half was reconstituted with demineralized water at an initial ratio of 1-to-5 (*w/v*), which was increased to 1-to-6 (*w/v*) after the second processing pass due to increasing material viscosity. Reconstituted *C. vulgaris* biomass was cell-ruptured by high pressure homogenization using a bench-top laboratory Microfluidizer® (model M-110P, Microfluidics International Corporation, Westwood, MA). The operating parameters were 25,000 psi (1724 bar) at a flow rate of approximately 75 mL/min through two in-series 'Z' configuration interaction chambers including a 200 μm module (model H30Z diamond) and an 87 μm module (model G10Z diamond) and the material was passed through the instrument thrice. To minimize possible thermal damage related to high shear forces, the product passed through a chilling coil surrounded by crushed ice immediately upon exit of the 87 μm module. Additionally, microfluidized product was collected directly into a container immersed in crushed ice and stored at 4 °C between passes. Samples (15 mL) were collected and immediately frozen at − 20 °C prior to processing and after single, double and triple passes through the Microfluidizer®. These samples were later used to examine the extent of cell-rupture throughout the processing using microscopy and a protein solubility assay. Final microfluidized product (after triple pass) was partially dewatered using a Rotavapor (model R152, Büchi Labortechnik AG, Flawil, Switzerland) connected to an ethylene glycol recirculating chiller (model UKT3000, Lauda-Brinkmann, Delran, New Jersey) set to − 15 °C. Several 10 L batches were processed in a 20 L capacity evaporation bulb at 50 °C and 85 rpm. Microfluidized and partially dewatered material (~20% solids) was lyophilized for 112 h at a low shelf temperature (< 5 °C) to a final moisture content of < 4%. This cell-ruptured *C. vulgaris* meal was stored at − 20 °C until use.

2.2. Test diets

A practical-ingredient basal diet was formulated to meet the known nutritional requirements of juvenile Atlantic salmon (Table 1). Aliquots from this common lot were blended (% *w*/w basis) with either whole-cell or cell-ruptured *C. vulgaris* meals at ratios of 100:0 (reference diet), 94:6, 88:12, 82:18, 76:24 and 70:30 and all test diets were supplemented with chromic oxide (Cr_2O_3, 0.5% *w*/w basis) as the inert digestion indicator. Dry ingredients of the basal diet were finely ground (< 500 μm) using a laboratory ultra-centrifugal mill (model ZM200, Retsch GmbH., Haan, Germany). Micronutrients (vitamin, mineral and amino acid supplements) were pre-mixed with wheat flour using a twin-shell blender (Paterson-Kelly, East Stroudsburg, PA) prior to addition to the main ingredient mixture. All ingredients were thoroughly blended in a Hobart mixer (Model H600T, Rapids Machinery Co., Troy, OH) and steam pelleted into 2.5 mm pellets (California Pellet Mill Co., San Francisco, CA). The pellets were dried in a forced-air drier at 80 °C for 120 min to form dry, sinking pellets and stored in air-tight containers at − 20 °C until use. Diets were screened to remove fines prior to feeding.

Table 1
Formulation of the basal diet used to measure *in vivo* apparent digestibility of diets containing whole-cell and cell-ruptured *Chlorella vulgaris* meals at different inclusion levels (as-fed basis).

Ingredient (%)[a]	
Fish meal (68% CP)	28.00
Poultry by-product meal (64% CP)	9.00
Wheat gluten meal (86% CP)	8.50
Soy protein concentrate (62% CP)	7.80
Corn protein concentrate (79% CP)	7.80
Blood meal (93% CP)	5.00
Fish oil	14.80
Wheat flour	14.09
Calcium phosphate, monobasic	3.50
Vitamin mixture[b]	0.40
Mineral mixture[b]	0.40
Choline chloride	0.40
Salt, NaCl	0.25
Vitamin C, ascorbic acid 'Stay-C 35'	0.03
Vitamin E, α-tocopherol	0.03

[a] All dietary ingredients were supplied by EWOS/Cargill Canada (Surrey, BC, Canada).
[b] EWOS/Cargill Canada freshwater salmonid mixture.

2.3. In vivo digestibility

In vivo ADCs of nutrients, energy, essential amino acids and fatty acids of test diets and single-ingredients whole-cell and cell-ruptured *C. vulgaris* meals were measured using the indirect digestibility determination method (NRC, 2011). Specially-designed tanks as described in Tibbetts et al. (2006) were used for passive collection of naturally egested faecal material from fish voluntarily consuming the various test diets. Digestibility measurements were made using 600 pre-smolt Atlantic salmon (average weight; 40.4 ± 2.7 g/fish) obtained from a local hatchery (Northern Harvest Seafarms Fish Hatchery, Cardigan, PE, Canada). Upon arrival, the fish were acclimated in two identical 900 L tanks at 7 °C (12 L/min) for 1 month. During this period, the fish were hand-fed 3.0 mm extruded salmonid feed (EWOS/Cargill Canada, Surrey, BC, Canada) to apparent voluntary satiety twice daily during the week (08:30 and 15:30 h) and once daily on weekends (10:00 h). The analyzed composition (as-fed basis) of this diet was: moisture 6.4%, crude protein 49.7%, lipid 18.7%, ash 11.3% and gross energy 22.7 MJ/kg. Prior to their transfer to digestibility tanks, the fish were gradually weaned onto the experimental reference diet over a 7-day period. They were then acclimated to their new tanks and gradually weaned onto their assigned test diet over a 12-day period before commencing faecal sample collections. The collection period lasted until a minimum of 70 g of wet faecal material was collected from each tank (11–13 days) and each of 11 test diets was fed to duplicate tanks (initial stocking density, 10.1 ± 0.2 kg/m^3). De-gassed and oxygenated freshwater from a well was supplied to each tank at a flow rate of 5 L/min in a flow-through system and water temperatures and dissolved oxygen levels were recorded daily (12.8 ± 0.2 °C and 10.7 ± 0.9 mg/L, respectively). During the experimental period, fish were hand-fed to apparent voluntary satiety twice daily during the week (08:30 and 14:30 h) and once daily on weekends (10:00 h). The tanks were checked daily for dead or moribund fish and none were found throughout the study. Each day, after the final feeding, the tanks and faecal collection columns were thoroughly cleaned with a brush to remove residual particulate matter (faeces and uneaten feed) and rinsed thoroughly with freshwater. Faecal samples were collected each morning (08:00 h) into 50 mL plastic conical bottom tubes, centrifuged (4000 rpm [2560 × *g*] for 20 min at 4 °C) and the supernatant carefully decanted and discarded and each sample stored in a sealed container at − 20 °C for the duration of the collection period. Faecal samples (~21% solids) were lyophilized for 72 h at a low shelf temperature (< 5 °C) to a final moisture content of < 1%. The study was conducted in compliance with guidelines set out by the Canadian Council on Animal Care (2005).

2.4. Analytical techniques

C. vulgaris meals, test diets and lyophilized faecal samples were analyzed using similar procedures. Moisture and ash contents were determined gravimetrically by drying in an oven at 105 °C and by incineration in a muffle furnace at 550 °C for 18 h. Nitrogen (N) contents were determined by elemental analysis (950 °C furnace) using a Leco N analyzer (model FP-528, Leco Corporation, St. Joseph, MI) with ultra-

high purity oxygen as the combustion gas and ultra-high purity helium as the carrier gas. Crude protein contents of *C. vulgaris* meals were calculated using the conventional nitrogen-to-protein (N-to-P) conversion factor (N × 6.25) and a *C. vulgaris*-specific N-to-P conversion factor (N × 5.14; Tibbetts et al., 2015) while N × 6.25 was used for the test diets and lyophilized faecal samples. Protein solubility of *C. vulgaris* prior to processing and after single, double and triple passes through the Microfluidizer® was estimated by incubation of 250 mg of freeze-dried sample in 0.2% potassium hydroxide (0.036 N KOH, pH 13) for 20 min at 22 °C with head-over-heals agitation (Tibbetts et al., 2016). Lipids were extracted by methanolic HCl *in-situ* transesterification (McGinn et al., 2012) and the corresponding fatty acid methyl esters (FAMEs) were separated and quantified by GC-FID (Omegawax 250 column, Agilent 7890). Individual FAs, along with an internal standard (C19:0; methyl nonadecanoate, Fluka), were identified by comparing retention times to two FA reference mixtures (Supelco 37 and PUFA No. 3, Sigma-Aldrich). Carbohydrate contents were determined by colorimetry using phenol and sulfuric acid following acid hydrolysis (2.5 M HCl at 95 °C for 3 h) (Dubois et al., 1956; Sukenik et al., 1993). Final results were determined against a dextrose standard curve (0–100 μg/mL; D-glucose, solid, > 99% pure, Sigma-Aldrich, Cat. G5400). Starch contents were determined by the α-amylase and amyloglucosidase method (Fernandes et al., 2012) using a Total Starch Assay Kit (K-TSTA, Megazyme International Ireland Ltd., Wicklow, Ireland) accepted by AOAC (Official Method 996.11) and AACC (Method 76.13). Crude fibre contents of the *C. vulgaris* meals and test diets were estimated using the ANKOM filter bag technique according to AOCS (2005) (Gross energy (MJ/kg) contents were measured using an isoperibol oxygen bomb calorimeter (model 6200, Parr Instrument Company, Moline, IL) equipped with a Parr 6510 water handling system for closed-loop operation. Chromic oxide concentrations of test diets and lyophilized faecal samples were determined by flame atomic absorption spectrophotometry (model iCE 3000 Series AA, Thermo Fisher Scientific, Waltham, MA) following phosphoric acid and potassium bromide digestion (Williams et al., 1962). Amino acid concentrations were determined using the Waters Pico-Tag RP-HPLC method (Heinriksen and Meredith, 1984; White et al., 1986). Protein digestibility-corrected amino acid score (PDCAAS) was calculated according to Schaafsma (2000) relative to the NRC (2011) essential amino acid requirements of juvenile Atlantic salmon reared in freshwater and the essential amino acid index (EAAI) was calculated according to Oser (1951) relative to an ideal protein pattern (egg albumin). For determination of carotenoid concentrations, 10 mg of sample was extracted (× 3) at room temperature with 5.0 mL of $CHCl_3$:MeOH (1-to-1 *v*/v) for 15 min followed by sonication (15 min) and the combined extracts were dried under N_2 gas. Extracts were then dissolved in 1.0 mL MeOH and stored at − 20 °C prior to HPLC analysis. All carotenoid extractions were conducted under low light. Carotenoids analysis was performed using an Agilent 1200 series HPLC with an YMC carotenoid column (5 μm, 2 × 250 mm, YMC Co. Ltd., Japan) eluted with 50 mM NH_4OAc in MeOH/TBME linear gradients at 0.2 mL/min flow rate for 60 min. Standard curves of astaxanthin, α- and β-carotene, canthaxanthin, fucoxanthin, lutein, lycopene and zeaxanthin at 450 nm were used for calculation of specific carotenoid levels. Elemental compositions were measured by ICP-AES according to SW-846 Method 6010C and mercury was measured following reference method 7471B (EPA, 2007). Concentrations of minerals, trace elements and heavy metals were determined using element-specific wavelengths on an IRIS Intrepid II spectrometer (Thermo Fisher Scientific, Waltham, MA). All analytical work was conducted in triplicate.

2.5. Calculations and statistical methods

Protein solubility was calculated on a dry-weight basis as:

$$\text{Protein solubility (\%)} = 100 \times \frac{\text{Protein in initial sample} - \text{Protein in dry residue}}{\text{Protein in initial sample}}$$

In vivo ADCs of nutrients, energy, essential amino acids and fatty acids (all referred to as 'Nutrient') of the diets were calculated on a dry-weight basis according to NRC (2011):

$$\text{Dry matter ADC (\%)} = 100 - \frac{\text{Chromic oxide in diet}}{\text{Chromic oxide in faeces}}$$

$$\text{Nutrient ADC (\%)} = 100 - \frac{\text{Chromic oxide in diet}}{\text{Chromic oxide in faeces}} \times \frac{\text{Nutrient in faeces}}{\text{Nutrient in diet}}$$

Using these data, *in vivo* ADCs of nutrients, energy, essential amino acids and fatty acids for the single *C. vulgaris* meals were calculated on a dry-weight basis according to NRC (2011):

$$\text{Nutrient ADC (\%)} = \text{ADC of test diet} + (\text{ADC of test diet} - \text{ADC of reference diet}) \times \frac{\rho \text{ reference diet} \times \text{D reference diet}}{\rho \text{ test ingredient} \times \text{D test ingredient}}$$

Where 'ρ' represents the proportion of the reference diet or test ingredient in the combined test diet and 'D' represents the dry-weight nutrient (or energy) content of the reference diet or test ingredient.

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

3. Results

3.1. Composition of test ingredients

Microscopic images and protein solubility of *C. vulgaris* prior to processing and after single, double and triple Microfluidizer® passes are shown in Fig. 1. The images demonstrate a progression of product uniformity with each consecutive pass, indicating successful cell-rupture and homogeneity of the algal slurry. Consistent with progressive cell-rupture, protein solubility also significantly increased ($P < 0.001$) from the initial un-processed suspension to the slurry after single, double and triple passes. No significant difference in protein solubility was found between double and triple passes ($P = 0.997$). Biochemical composition of whole-cell and cell-ruptured *C. vulgaris* meals is shown in Table 2. Whole-cell and cell-ruptured *C. vulgaris* meals contained statistically similar levels of ash ($P = 0.138$), lipid ($P = 0.976$), carbohydrate ($P = 0.502$) and starch ($P = 0.572$). Although statistical differences were observed for their levels of dry matter ($P < 0.001$), N × 6.25 crude protein ($P = 0.002$), N × 5.14 crude protein ($P = 0.002$) and gross energy ($P < 0.001$), these are due to the high repeatability (*e.g.*, low variability) between analytical replicates and the very narrow ranges (< 1% and 0.3 MJ/kg) would have little biological or practical importance. A significant difference ($P < 0.001$) in crude fibre levels between whole-cell and cell-ruptured *C. vulgaris* meals was observed, which may have practical importance. Essential amino acid compositions were statistically similar ($P \geq 0.058$) for histidine, isoleucine, leucine, lysine, phenylalanine, tryptophan and valine. Although likely of little practical importance, small differences ($P \leq 0.040$) were observed for arginine, methionine, methionine + cysteine, phenylalanine + tyrosine and threonine resulting in a minor (< 0.02) difference in their EAAI. The PDCAAS values were high (0.87–1.29) for leucine, phenylalanine, phenylalanine + tyrosine, threonine and valine and moderately high (0.47–0.72) for arginine, histidine, isoleucine, methionine and methionine + cysteine. Tryptophan and lysine had the lowest PDCAAS values (0.04–0.48) which confirms them as the most limiting EAAs in *C. vulgaris* meals for Atlantic

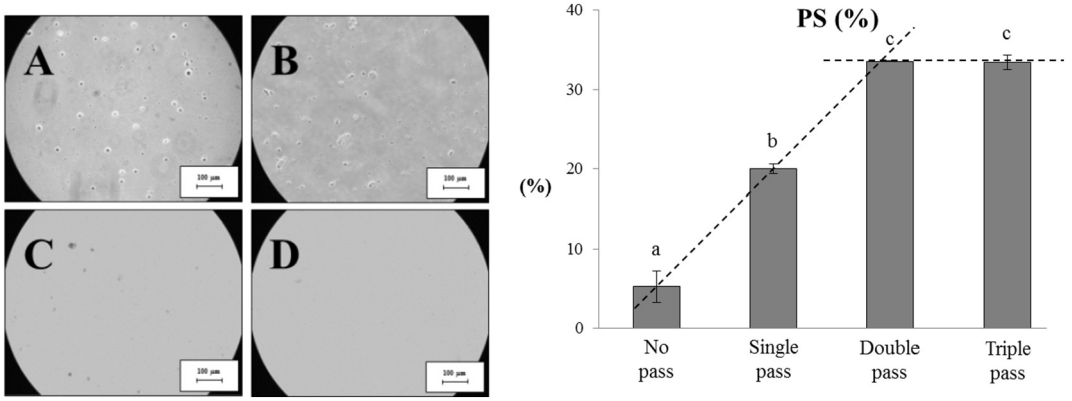

Fig. 1. Microscopic images (1:100 dilution; 20 × magnification) and protein solubility (in 0.2% KOH) of *C. vulgaris* biomass prior to processing (**A**) and after single (**B**), double (**C**) and triple (**D**) Microfluidizer® passes. Values having different superscript letters are significantly different ($P < 0.05$).

salmon, which is in generally agreement with other alternative salmon feed ingredients (Halver and Hardy, 2002). The majority (> 85%) of lipid in the *C. vulgaris* meals was composed of oleic acid, linoleic acid, palmitic acid, linolenic acid, ALA and hexadecadienoic acid. Major fatty acid groups were statistically similar ($P \geq 0.185$) for monounsaturates and n-6 polyunsaturates while small (< 2%) differences ($P \leq 0.019$) were observed for saturates, polyunsaturates and n-3 polyunsaturates. These findings resulted in a small (< 0.05), but significant ($P = 0.003$) modification in the n-3:n-6 ratio. The compositions of major minerals were statistically similar ($P \geq 0.102$) for calcium, magnesium, phosphorous, potassium, sodium and the Ca:P ratio. Trace element compositions were statistically similar ($P \geq 0.415$) for copper, iron, manganese and zinc, while the low level of selenium found in whole-cell *C. vulgaris* meal was not detected in cell-ruptured *C. vulgaris* meal. Heavy metal concentrations were statistically similar ($P \geq 0.239$) for arsenic, lead and mercury while cadmium levels were significantly different ($P < 0.001$), although very low. Measured heavy metals were present at concentrations several magnitudes lower than the maximum allowable levels for animals feed ingredients (EU, 2002). Given the notable color difference between whole-cell *C. vulgaris* meal (green) and cell-ruptured *C. vulgaris* meal (brown), it is not surprising that significant differences were observed for all carotenoids measured. Significantly higher ($P \leq 0.002$) concentrations were observed in whole-cell than cell-ruptured *C. vulgaris* meals for astaxanthin, α-carotene, β-carotene, fucoxanthin and lutein. Alternatively, significantly higher ($P \leq 0.001$) levels were found in cell-ruptured than whole-cell *C. vulgaris* meals for canthaxanthin and zeaxanthin. The majority (> 85%) of carotenoids in *C. vulgaris* meals were composed of lutein, β-carotene and zeaxanthin. In the case of cell-ruptured *C. vulgaris* meal, pheophorbide *a* was observed at far higher ($P < 0.001$) concentrations than its whole-cell counterpart. This is not surprising as pheophorbide is a known primary product of chlorophyll breakdown (Head et al., 1994) with suspected anti-cancer bioactivity (Mimouni et al., 2012; Tang et al., 2010) but has also been suspected to cause skin irritations and photosensibilization in some humans when consumed at high levels (Görs et al., 2010; van der Spiegel et al., 2013).

3.2. Composition of test diets

Nutrients, energy, essential amino acid and fatty acid composition of the test diets are shown in Table 3. The test diets had similar levels of moisture, ash, lipid, starch, crude fibre, phosphorous and gross energy. Dietary crude protein levels varied inversely with the *C. vulgaris* meal substitution level and, in the opposite manner, dietary carbohydrate levels increased with increasing *C. vulgaris* meal substitution level. It is important to note that the dietary carbohydrate range of diets used in this study (15–23%) is within the range recommended by NRC (2011) for salmonids and marine fish (15–25%). Additionally, all test diets used in this study meet or exceed the minimum recommended dietary levels of digestible protein (DP), digestible energy (DE) and DP/DE ratio of 36%, 18 MJ/kg and 20 g DP/MJ DE, respectively for Atlantic salmon reared in freshwater (NRC, 2011). As a result of the comparatively 'complete' amino acid profile of the *C. vulgaris* meals, the essential amino acid compositions of the test diets were highly similar. The majority of lipid (~80%) in the test diets was composed of palmitic acid, palmitoleic acid, oleic acid, linoleic acid, linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Increasing dietary inclusion of *C. vulgaris* meals did not cause any appreciable change in total saturates, monounsaturates or polyunsaturates. However, n-3 polyunsaturates decreased moderately and n-6 polyunsaturates doubled, resulting in a 3-fold decrease in the dietary n-3:n-6 ratio.

3.3. Feed intake

While not a major focus for the purposes of this digestibility study, the fish were carefully fed twice daily to apparent satiation and feed consumption was monitored. Excessive or inadequate feeding levels can alter gut transit times in fish which may influence nutrient digestion rates, and hence, ADC values. As such, observing feed palatability is important in a good digestibility study (Jobling, 2016). Monitoring feed consumption was particularly relevant in this case given the documented negative effects of dietary inclusion of green algae on feed palatability for other farmed monogastric species like poultry and swine (Gatrell et al., 2014) and Atlantic cod, *Gadus morhua* (Walker and Berlinsky, 2011). The duplicate *in vivo* digestibility trials were conducted consecutively with new fish of statistically the same initial starting weight (1st replicate, 39.5 ± 3.2 g/fish; 2nd replicate, 41.2 ± 1.9 g/fish; $P = 0.133$) and no significant differences in feed intake were found between duplicate trials for each diet; reference diet ($P = 0.396$), whole-cell *C. vulgaris* diets ($P \geq 0.117$) and cell-ruptured *C. vulgaris* diets ($P \geq 0.079$). As such, feed intake data for each replicate could be pooled by diet and no significant differences were found for diets containing whole-cell or cell-ruptured *C. vulgaris* meal at each inclusion levels of 0% 'reference' ($P = 0.396$), 6% ($P = 0.817$), 12% ($P = 0.929$), 18% ($P = 0.275$), 24% ($P = 0.652$) or 30% ($P = 0.954$). In comparison with the reference diet, all *C. vulgaris*-supplemented test diets were accepted equally well by the fish throughout the trial; having consumed statistically similar ($P = 0.974$) amounts of feed (0.6 ± 0.1 g feed/fish/day; equivalent to 1.5 ± 0.1% of BW/day).

3.4. In vivo digestibility of test diets

In vivo ADCs of nutrients, energy, essential amino acids and major fatty acids in the test diets are shown in Tables 4, 5, 6 and 7 and Fig. 2. The reference diet (algae-free control) was digested at expected levels;

Table 2
Biochemical composition of whole-cell and cell-ruptured *Chlorella vulgaris* meals (dry weight basis)[a].

	Chlorella vulgaris meals	
	Whole-cell	Cell-ruptured
Proximate composition (%)		
Dry matter	96.4 ± 0.1[a]	97.3 ± 0.1[b]
Ash	3.3 ± 0.1[ns]	3.2 ± 0.1
Crude protein (N × 6.25)	30.4 ± 0.1[a]	29.8 ± 0.1[b]
Crude protein (N × 5.14)	25.0 ± 0.1[a]	24.5 ± 0.1[b]
Lipid	26.0 ± 0.7[ns]	26.1 ± 0.2
Carbohydrate	24.5 ± 2.9[ns]	25.6 ± 0.6
Starch	13.0 ± 0.4[ns]	13.2 ± 0.1
Crude fibre	9.9 ± 0.4[a]	2.1 ± 0.1[b]
Gross energy (MJ/kg)	25.0 ± 0.0[a]	24.7 ± 0.0[b]
Essential amino acids (%)[b]		
Arginine	1.67 ± 0.01[a] {0.68}	1.50 ± 0.03[b] {0.71}
Histidine	0.52 ± 0.02[ns] {0.48}	0.49 ± 0.00 {0.52}
Isoleucine	0.94 ± 0.01[ns] {0.63}	0.93 ± 0.01 {0.72}
Leucine	2.05 ± 0.03[ns] {1.01}	2.08 ± 0.06 {1.17}
Lysine	1.41 ± 0.02[ns] {0.43}	1.37 ± 0.02 {0.48}
Methionine	0.45 ± 0.00[b] {0.47}	0.47 ± 0.01[a] {0.57}
Methionine + Cysteine	0.74 ± 0.00[a] {0.49}	0.70 ± 0.01[b] {0.54}
Phenylalanine	1.38 ± 0.00[ns] {1.13}	1.37 ± 0.01 {1.29}
Phenylalanine + Tyrosine	2.41 ± 0.00[a] {0.99}	2.30 ± 0.02[b] {1.08}
Threonine	1.29 ± 0.07[b] {0.87}	1.53 ± 0.01[a] {1.17}
Tryptophan	0.02 ± 0.00[ns] {0.05}	0.02 ± 0.00 {0.04}
Valine	1.51 ± 0.01[ns] {0.93}	1.50 ± 0.02 {1.06}
EAAI	0.87 ± 0.01[b]	0.89 ± 0.00[a]
Fatty acids (% of total FAs)		
14:0	0.74 ± 0.01[b]	0.82 ± 0.04[a]
14:1n-5	0.40 ± 0.00[ns]	0.38 ± 0.04
15:0	0.12 ± 0.00[b]	0.12 ± 0.00[a]
16:0	17.12 ± 0.15[b]	17.60 ± 0.04[a]
16:1n-5	0.75 ± 0.01[b]	0.77 ± 0.00[a]
16:1n-7	1.61 ± 0.02[b]	1.65 ± 0.00[a]
16:2n-6	4.78 ± 0.01[a]	4.74 ± 0.01[b]
16:4n-1	0.52 ± 0.00[a]	0.07 ± 0.00[b]
17:0	0.17 ± 0.00[b]	0.18 ± 0.00[a]
17:1	6.74 ± 0.06[a]	6.40 ± 0.05[b]
18:0	1.91 ± 0.03[b]	1.96 ± 0.01[a]
18:1n-7	0.52 ± 0.00[ns]	0.52 ± 0.00
18:1n-9	30.60 ± 0.14[b]	31.28 ± 0.08[a]
18:2n-6	19.72 ± 0.01[ns]	19.80 ± 0.10
18:3n-3 (ALA)	13.64 ± 0.16[a]	12.79 ± 0.13[b]
18:4n-3	0.12 ± 0.03[ns]	0.11 ± 0.02
20:0	0.15 ± 0.00[b]	0.17 ± 0.00[a]
20:2n-9	0.14 ± 0.00[b]	0.15 ± 0.00[a]
20:4n-3	0.11 ± 0.01[ns]	0.12 ± 0.01
22:0	0.14 ± 0.03[ns]	0.11 ± 0.02
22:4n-6	< DL[c]	0.12 ± 0.00
22:5n-6	0.14 ± 0.01[ns]	0.17 ± 0.06
Σ SFA	21.1 ± 0.2[b]	21.5 ± 0.0[a]
Σ MUFA	42.1 ± 0.1[ns]	42.1 ± 0.1
Σ PUFA	40.8 ± 0.3[a]	39.2 ± 0.1[b]
Σ n-3 PUFA	14.5 ± 0.3[a]	13.5 ± 0.1[b]
Σ n-6 PUFA	25.6 ± 0.0[ns]	25.5 ± 0.1
n-3:n-6	0.57 ± 0.01[a]	0.53 ± 0.00[b]
Minerals (%)		
Calcium	0.33 ± 0.01[ns]	0.34 ± 0.01
Magnesium	0.16 ± 0.00[ns]	0.16 ± 0.00
Phosphorous	0.53 ± 0.02[ns]	0.54 ± 0.01
Potassium	0.62 ± 0.01[ns]	0.64 ± 0.01
Sodium	0.16 ± 0.00[ns]	0.17 ± 0.00
Ca:P ratio	0.62 ± 0.01[ns]	0.62 ± 0.01
Trace elements (mg/kg)		
Copper	23.4 ± 1.0[ns]	23.9 ± 0.5
Iron	345.1 ± 1.6[ns]	346.4 ± 2.1
Manganese	50.0 ± 1.3[ns]	50.9 ± 1.0
Selenium	0.7 ± 0.3	< DL
Zinc	21.1 ± 0.5[a]	22.2 ± 0.4[b]
Heavy metals (ppm)[d]		
Arsenic	0.6 ± 0.1[ns]	0.6 ± 0.0
Cadmium	0.2 ± 0.0[a]	0.1 ± 0.0[b]
Lead	< DL	< DL
Mercury	< DL	< DL
Carotenoids (mg/100 g)		
Astaxanthin	14.2 ± 0.9	< DL
Canthaxanthin	4.2 ± 0.1[b]	5.9 ± 0.2[a]
α-carotene	22.7 ± 1.3[a]	18.3 ± 1.1[b]
β-carotene	29.4 ± 1.2[a]	23.3 ± 1.5[b]
Fucoxanthin	7.8 ± 0.7	< DL
Lutein	222.9 ± 12.9[a]	169.7 ± 2.8[b]
Lycopene	< DL	< DL
Pheophorbide *a*	5.9 ± 1.7[b]	252.0 ± 33.1[a]
Zeaxanthin	23.1 ± 0.6[b]	28.4 ± 0.9[a]
Other	139.6 ± 9.2[ns]	144.5 ± 2.4

[a] Values within the same row having different superscript letters are significantly different ($P < 0.05$).
[b] Values in {parentheses} indicate the PDCAAS relative to the NRC (2011) essential amino acid requirements of juvenile Atlantic salmon reared in freshwater.
[c] Below detection limit.
[d] Maximum allowable concentration (ppm) in animal feed ingredients = arsenic (2–4), cadmium (0.5–1.0), lead (10–40) and mercury (0.1–0.4).

confirming its high nutritional quality. Although nutrient digestibility of all the test diets remained relatively high, dietary inclusion of *C. vulgaris* meals significantly ($P < 0.001$) affected nutrient ADCs to varying degrees. Inclusion of whole-cell *C. vulgaris* meal at all dietary inclusion levels significantly ($P \leq 0.035$) reduced ADCs for dry matter, lipid and energy while protein ADC was only significantly reduced ($P \leq 0.040$) at inclusion levels higher than of 6%. Carbohydrate ADC was unaffected ($P \geq 0.980$) by inclusion levels of 6–24% while at the 30% inclusion level, it was significantly improved ($P = 0.028$). Similarly, starch ADC was not significantly affected ($P = 0.256$) by dietary inclusion of whole-cell *C. vulgaris* meal at any level (Fig. 2). Alternatively, cell-ruptured *C. vulgaris* meal did not significantly affect dry matter ADC ($P \geq 0.900$) at any dietary inclusion level. Protein ADC was not significantly affected at inclusion levels of 6–24% ($P \geq 0.092$) but was reduced at 30% ($P = 0.006$). Lipid ADC was not significantly affected at inclusion levels of 6–18% ($P \geq 0.124$) but was reduced at 24–30% ($P \leq 0.003$). Carbohydrate ADC was significantly improved at all inclusion levels ($P \leq 0.002$) and this was in part due to improvements in the digestibility of the starch in cell-ruptured *C. vulgaris* meal which, in fact, resulted in significantly higher ($P \leq 0.009$) starch ADCs when included at high levels (18–30%) (Fig. 2). In a similar manner, digestibility of dietary phosphorous was higher in diets containing *C. vulgaris* meals than the algae-free control diet, particularly those containing high (18–30%) inclusion levels which were significantly higher ($P \leq 0.019$) than the reference diet. Energy ADC was not significantly affected at inclusion levels of 6–12% ($P \geq 0.112$) but was reduced at 18–30% ($P \leq 0.009$).

The digestibility of essential amino acids in the reference diet was high. Compared to the reference diet, the digestibility of essential amino acids were not affected for diets containing whole-cell *C. vulgaris* meal at relatively high inclusion levels of 18–30% for arginine, histidine, isoleucine, lysine, methionine, threonine, tryptophan and valine, while ADCs for leucine and phenylalanine were reduced at levels higher than 12%. Dietary inclusion of cell-ruptured *C. vulgaris* meal up to the highest level of 30% had no effect on essential amino acid ADC for any of the 10 essential amino acids. The digestibility of major fatty acids in the reference diet was high. Reduced lipid digestibility shown previously was related to significantly lower dietary ADCs for palmitic acid, oleic acid, linoleic acid and linolenic acid (ALA) with inclusion of whole-cell *C. vulgaris* meal at levels as low as 6% or cell-ruptured *C. vulgaris* meal at levels higher than 12% of diet. Alternatively, digestibility was not significantly affected by any inclusion level (up to 30% of diet) of either whole-cell or cell-ruptured *C. vulgaris* meals for palmitoleic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid

Table 3
Biochemical composition of diets containing whole-cell and cell-ruptured *Chlorella vulgaris* meals at different inclusion levels (as-fed basis).

		Whole-cell *C. vulgaris* meal					Cell-ruptured *C. vulgaris* meal				
	Reference	6%	12%	18%	24%	30%	6%	12%	18%	24%	30%
Proximate composition (% of diet)											
Moisture	7.1	6.9	6.6	6.7	6.7	6.6	7.1	7.4	7.5	7.5	7.4
Ash	10.1	9.3	9.3	8.7	8.4	8.1	9.5	9.3	9.0	8.4	8.0
Crude protein	50.2	49.3	48.1	46.6	45.6	43.5	48.8	46.9	46.3	44.2	43.6
Lipid	17.8	17.7	18.0	18.6	18.5	18.3	17.3	17.9	19.0	17.6	17.5
Carbohydrate	14.8	16.9	18.0	19.4	20.8	23.5	17.3	18.4	18.2	22.3	23.5
Starch	7.5	7.8	8.3	8.6	9.0	9.4	7.9	8.2	8.6	8.8	9.0
Crude fibre	0.8	1.0	1.9	2.1	2.0	2.3	0.9	1.2	0.8	1.4	1.2
Phosphorous	1.9	1.8	1.7	1.6	1.5	1.5	1.8	1.7	1.7	1.6	1.5
Energy (MJ/kg)	22.0	22.1	22.2	22.3	22.4	22.6	22.0	22.2	22.3	22.5	22.6
DP	46.9	45.7	44.3	42.8	41.4	39.3	45.5	43.6	42.8	40.8	40.0
DE (MJ/kg)	19.2	18.7	18.6	18.2	17.8	17.8	19.0	19.1	19.0	19.1	19.0
DP/DE ratio (g DP/MJ DE)	24.4	24.4	23.8	23.5	23.3	22.1	23.9	22.8	22.5	21.4	21.1
Essential amino acids (% of diet)											
Arginine	2.1	2.1	2.3	2.2	2.0	1.9	2.2	2.1	1.9	2.0	1.8
Histidine	1.1	1.0	1.1	1.1	1.0	0.9	1.1	1.0	0.9	1.0	0.9
Isoleucine	1.5	1.5	1.5	1.5	1.3	1.3	1.5	1.4	1.4	1.4	1.3
Leucine	3.5	3.3	3.2	3.3	3.0	2.9	3.4	3.2	3.2	3.0	2.9
Lysine	2.0	2.0	1.7	1.7	1.5	1.8	1.9	1.6	1.6	1.5	1.4
Methionine	0.9	0.8	0.8	0.8	0.7	0.7	0.8	0.7	0.7	0.7	0.7
Phenylalanine	2.0	1.9	1.8	1.8	1.6	1.7	1.9	1.8	1.8	1.7	1.7
Threonine	1.3	1.3	1.5	1.4	1.3	1.2	1.4	1.3	1.2	1.4	1.3
Tryptophan	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.1	0.1	0.1	0.1
Valine	2.1	2.1	2.2	2.1	1.9	1.8	2.2	2.0	1.9	2.0	1.9
Fatty acids (% of diet)											
14:0	0.9	0.8	0.8	0.7	0.6	0.6	0.7	0.7	0.8	0.7	0.6
16:0	2.8	2.9	2.9	3.1	3.0	3.0	2.7	2.9	3.2	2.9	2.9
16:1n-7	1.1	1.0	0.9	0.8	0.7	0.7	0.9	0.9	0.9	0.7	0.6
16:2n-6	nd	nd	nd	0.2	0.3	0.3	nd	0.1	0.2	0.2	0.3
17:1	nd	nd	0.2	0.3	0.4	0.4	nd	0.2	0.2	0.3	0.4
18:0	0.6	0.6	0.6	0.6	0.5	0.5	0.6	0.6	0.6	0.5	0.5
18:1n-7	0.7	0.7	0.6	0.6	0.5	0.5	0.6	0.6	0.6	0.5	0.5
18:1n-9	2.8	3.0	3.3	3.6	3.8	3.9	2.9	3.2	3.7	3.6	3.8
18:2n-6	1.2	1.5	1.7	1.9	2.1	2.2	1.4	1.6	1.9	2.0	2.1
18:3n-3 (ALA)	0.2	0.4	0.6	0.8	1.0	1.1	0.4	0.5	0.7	0.8	0.9
18:4n-3	0.5	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.3	0.3
20:0	1.5	1.2	1.3	1.1	1.1	1.0	1.3	1.3	1.3	1.0	0.9
20:1n-9	0.6	0.7	0.5	0.6	0.5	0.4	0.6	0.5	0.6	0.4	0.4
20:5n-3 (EPA)	1.8	1.7	1.6	1.5	1.4	1.3	1.7	1.6	1.7	1.3	1.2
22:1n-9	1.7	1.6	1.5	1.4	1.2	1.1	1.5	1.5	1.5	1.2	1.1
22:6n-3 (DHA)	1.4	1.3	1.2	1.1	1.0	1.0	1.3	1.2	1.2	1.0	0.9
Σ SFA	5.8	3.7	5.5	5.4	5.2	5.1	5.3	5.4	5.9	5.1	5.0
Σ MUFA	6.9	6.9	7.0	7.3	7.1	7.1	6.5	6.9	7.4	6.8	6.8
Σ PUFA	5.1	5.3	5.5	5.9	6.1	6.1	5.4	5.5	6.1	5.6	5.7
Σ n-3 PUFA	3.9	3.8	3.8	3.8	3.8	3.6	3.9	3.8	4.0	3.4	3.3
Σ n-6 PUFA	1.2	1.5	1.7	2.1	2.4	2.5	1.4	1.7	2.1	2.2	2.4
n-3:n-6	3.1	2.5	2.2	1.8	1.6	1.4	2.7	2.2	1.9	1.6	1.4

Table 4
Apparent digestibility coefficients (% ADCs) of nutrients and energy in diets containing whole-cell and cell-ruptured *Chlorella vulgaris* meals at different inclusion levels[a].

	Dry matter	Protein	Lipid	Carbohydrate	Phosphorous	Energy
Diet						
Reference	78.9 ± 0.3^{a}	93.4 ± 0.1^{a}	95.8 ± 1.1^{a}	35.4 ± 1.4^{e}	42.9 ± 2.5^{d}	87.3 ± 0.2^{a}
Whole-cell *C. vulgaris* meal						
6%	76.9 ± 0.1^{b}	92.6 ± 0.1^{a,b}	92.9 ± 0.3^{bcde}	35.5 ± 0.4^{e}	44.0 ± 2.3^{cd}	84.7 ± 0.3^{bc}
12%	76.4 ± 0.3^{bc}	92.1 ± 0.2^{b}	91.9 ± 0.2^{cde}	36.5 ± 1.2^{de}	46.0 ± 1.2^{bcd}	83.6 ± 0.0^{c}
18%	75.1 ± 0.3^{c}	91.8 ± 0.4^{bc}	90.3 ± 0.5^{e}	35.2 ± 0.2^{e}	47.5 ± 0.7^{a,bcd}	81.8 ± 0.5^{d}
24%	73.2 ± 0.3^{d}	90.7 ± 0.8^{cd}	87.0 ± 1.3^{f}	35.4 ± 1.7^{e}	45.2 ± 0.8^{cd}	79.3 ± 0.4^{e}
30%	72.9 ± 0.6^{d}	90.4 ± 0.3^{d}	86.4 ± 0.1^{f}	39.8 ± 1.3^{cd}	49.2 ± 1.5^{a,bc}	78.8 ± 0.4^{e}
Cell-ruptured *C. vulgaris* meal						
6%	78.7 ± 0.5^{a}	93.3 ± 0.3^{a,b}	95.1 ± 1.3^{a,b}	41.5 ± 1.0^{bc}	47.4 ± 0.6^{a,bcd}	86.5 ± 0.7^{a,b}
12%	78.7 ± 0.5^{a}	93.0 ± 0.3^{a,b}	94.6 ± 0.3^{a,bc}	45.3 ± 0.8^{b}	47.3 ± 0.6^{a,bcd}	86.0 ± 0.4^{a,b}
18%	78.8 ± 0.0^{a}	92.5 ± 0.1^{a,b}	93.5 ± 0.3^{a,bcd}	44.9 ± 0.2^{b}	51.3 ± 1.0^{a,b}	85.4 ± 0.0^{bc}
24%	78.9 ± 0.4^{a}	92.3 ± 0.1^{a,b}	91.7 ± 0.3^{de}	55.4 ± 1.2^{a}	51.8 ± 0.6^{a}	85.1 ± 0.2^{bc}
30%	78.4 ± 0.1^{a}	91.7 ± 0.0^{bc}	90.7 ± 0.0^{e}	56.4 ± 0.1^{a}	50.2 ± 1.4^{a,bc}	84.1 ± 0.2^{c}

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

Table 5
Apparent digestibility coefficients (% ADCs) of essential amino acids in diets containing whole-cell and cell-ruptured *Chlorella vulgaris* meals at different inclusion levels[a].

Diet	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Tryptophan	Valine
Reference	96.1 ± 0.2^{a,b}	95.9 ± 0.2^{a,b}	93.7 ± 0.0^{a,b}	94.9 ± 0.0^{a,b}	96.8 ± 0.0^{a,b}	94.7 ± 0.1^{a,bcd}	94.4 ± 0.0^{a}	93.4 ± 0.2^{a}	97.4 ± 0.2^{a}	94.5 ± 0.0^{a,b}
Whole-cell *C. vulgaris* meal										
6%	96.0 ± 0.1^{a,bc}	95.6 ± 0.1^{a,bc}	93.1 ± 0.2^{a,b}	93.9 ± 0.1^{bc}	96.7 ± 0.0^{a,b}	93.9 ± 0.1^{de}	93.5 ± 0.1^{a,b}	92.5 ± 0.1^{a,bc}	97.3 ± 0.2^{a,b}	93.8 ± 0.2^{a,b}
12%	96.7 ± 0.5^{a}	96.2 ± 0.4^{a}	93.4 ± 0.3^{a,b}	94.2 ± 0.3^{a,bc}	96.5 ± 0.5^{a,b}	94.5 ± 0.0^{bcde}	93.2 ± 0.3^{a,bc}	93.9 ± 0.5^{a}	97.3 ± 0.2^{a,b}	94.4 ± 0.3^{a,b}
18%	95.8 ± 0.2^{a,bc}	95.4 ± 0.0^{a,bcd}	92.7 ± 0.1^{b}	93.4 ± 0.2^{c}	95.8 ± 0.0^{a,b}	93.9 ± 0.2^{cde}	92.2 ± 0.2^{bcd}	92.5 ± 0.0^{a,bc}	97.3 ± 0.2^{a,b}	93.6 ± 0.0^{b}
24%	94.6 ± 0.1^{c}	94.1 ± 0.0^{d}	90.7 ± 0.9^{c}	91.8 ± 0.8^{d}	95.1 ± 0.8^{b}	92.6 ± 0.7^{f}	90.4 ± 1.4^{d}	90.7 ± 0.6^{c}	96.3 ± 0.3^{a,bc}	91.8 ± 0.6^{c}
30%	92.7 ± 0.3^{d}	92.4 ± 0.3^{e}	90.6 ± 0.3^{c}	91.4 ± 0.3^{d}	96.9 ± 0.4^{a}	92.3 ± 0.2^{f}	91.3 ± 0.3^{cd}	87.4 ± 0.1^{d}	97.1 ± 0.1^{a,bc}	90.4 ± 0.2^{d}
Cell-ruptured *C. vulgaris* meal										
6%	96.3 ± 0.3^{a}	95.7 ± 0.3^{a,bc}	94.3 ± 0.0^{a}	95.2 ± 0.1^{a}	97.3 ± 0.2^{a}	95.4 ± 0.0^{a,b}	94.9 ± 0.1^{a}	93.5 ± 0.6^{a}	97.9 ± 0.1^{a}	94.8 ± 0.2^{a}
12%	95.6 ± 0.1^{a,bc}	94.6 ± 0.1^{bcd}	93.6 ± 0.3^{a,b}	94.8 ± 0.3^{a,b}	96.9 ± 0.3^{a}	94.8 ± 0.2^{a,bc}	94.6 ± 0.4^{a}	92.2 ± 0.0^{a,bc}	97.4 ± 0.3^{a,b}	94.1 ± 0.2^{a,b}
18%	95.0 ± 0.9^{bc}	94.4 ± 1.0^{cd}	93.3 ± 0.1^{a,b}	94.5 ± 0.0^{a,bc}	97.2 ± 0.3^{a}	94.6 ± 0.1^{bcde}	94.5 ± 0.3^{a}	91.1 ± 1.2^{bc}	97.2 ± 0.2^{a,bc}	93.5 ± 0.4^{b}
24%	95.7 ± 0.1^{a,bc}	95.2 ± 0.1^{a,bcd}	93.1 ± 0.3^{a,b}	94.3 ± 0.1^{a,bc}	95.8 ± 0.7^{a,b}	95.6 ± 0.1^{a}	93.3 ± 0.8^{a,bc}	92.7 ± 0.2^{a,b}	96.9 ± 0.0^{a,bc}	93.8 ± 0.1^{a,b}
30%	95.7 ± 0.0^{a,bc}	95.4 ± 0.0^{a,bcd}	93.0 ± 0.1^{a,b}	94.3 ± 0.1^{a,bc}	95.6 ± 0.6^{a,b}	93.6 ± 0.2^{e}	93.1 ± 0.0^{a,bc}	92.8 ± 0.1^{a,b}	96.1 ± 0.7^{c}	93.9 ± 0.1^{a-}

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

(DHA). The digestibility of major fatty acid groups in the reference diet was high for all groups. Digestibility of all fatty acid groups in diets containing whole-cell *C. vulgaris* meal were significantly reduced even at the lowest inclusion levels of 6–12% for saturates, monounsaturates, polyunsaturates, n-3 polyunsaturates and n-6 polyunsaturates. As for cell-ruptured *C. vulgaris* meal, digestibility of saturates was significantly reduced at inclusion levels above 6% while high inclusion levels (≥ 24%) had no significant effects on ADCs for monounsaturates, polyunsaturates, n-3 polyunsaturates and n-6 polyunsaturates.

3.5. *In vivo* digestibility of single test ingredients

The *in vivo* ADCs of nutrients, energy, essential amino acids and major fatty acids of the single ingredients are shown in Tables 8 and 9. Cell-rupture significantly improved ($P < 0.001$) digestibility of protein, lipid, energy, carbohydrate and starch. Essential amino acid ADCs were significantly higher ($P \leq 0.045$) for cell-ruptured *C. vulgaris* meal than its whole-cell counterpart for isoleucine, leucine, methionine, phenylalanine, valine and threonine. No significant differences ($P \geq 0.286$) were observed between the two *C. vulgaris* meals for arginine, histidine, lysine and tryptophan. Cell-rupture significantly improved digestibility ($P < 0.001$) of oleic acid, linoleic acid and linolenic acid (ALA) while no significant difference was found for palmitic acid ($P = 0.687$).

4. Discussion

The total amount of a particular nutrient consumed by fish (intake) rarely reflects the amount that is accessible from the digestive tract; which ultimately determines the amount bioavailable for anabolic purposes (*e.g.*, growth, maintenance, tissue repair and reproduction). This reality is precisely the impetus for the importance of reliable nutrient digestibility data for novel feed ingredients such as those studied here. While there are several general factors that affect *in vivo* digestibility between various fish studies such species, culture conditions, composition of test ingredients, diet formulation, faecal sampling, calculations, *etc*. (de Carvalho et al., 2016), the predominant factor within this study is surely *C. vulgaris* meal cell wall rupture (or lack there-of). While the aquaculture nutrition data remains scarce, *Chlorella* meals have been incorporated into test diets for some farmed fish such as crucian carp, *Carassius auratus*, Nile tilapia, *Oreochromis niloticus*, channel catfish, *Ictalurus punctatus*, olive flounder, *Paralichthys olivaceus*, brown trout, *Salmo trutta* and Atlantic salmon, *Salmo salar* as a replacement for other dietary protein sources (Grammes et al., 2013; Kupchinsky et al., 2015; Lupatsch and Blake, 2013; Rahimnejad et al., 2016; Shi et al., 2017a, 2017b; Saberi et al., 2017). The *Chlorella* meals used in these studies varied widely in their production conditions, nutrient composition and were included in test diets at highly variable levels (*e.g.*, from < 5 to > 75%) and (with the exception of Nile tilapia) none of the studies measured nutrient digestibility of the test diets or the single ingredient *Chlorella* meals. Reported *in vivo* protein ADC for *C. vulgaris* and *C. pyrenoidosa* measured with rats is highly variable at 45–89% (Janczyk et al., 2005; Komaki et al., 1998; Lubitz, 1963;) as were ADCs for other dietary components (*e.g.*, and dry matter, protein, lipid, organic matter, carbohydrate, crude fibre and energy) for tilapia and rats at 15–93% (Lubitz, 1963; Lupatsch and Blake, 2013). These studies have led to upper dietary inclusion recommendation for *Chlorella* meals of 10–15% for flounder and rats and 26–71% for warmwater herbivorous/omnivorous fish like carp, tilapia and catfish based on varying performance parameters (*e.g.*, feed intake, growth rate, nutrient utilization, blood histochemistry, organ weights, carcass yields, sensory evaluation, digestive enzyme activities and intestinal histology). While not primarily focused on growth performance and nutrient utilization, the study by Grammes et al. (2013), which involved feeding test diets containing *C. vulgaris* meal to Atlantic salmon, is worth discussing. They reported that adding 'cracked' *C. vulgaris* meal (supplied by Synergy

Table 6
Apparent digestibility coefficients (% ADCs) of major fatty acids in diets containing whole-cell and cell-ruptured *Chlorella vulgaris* meals at different inclusion levels[a].

	Palmitic Acid (16:0)	Palmitoleic Acid (16:1n-7)	Oleic Acid (18:1n-9)	Linoleic Acid (18:2n-6)	Linolenic Acid (ALA) (18:3n-3)	Eicosapentaenoic Acid (EPA) (20:5n-3)	Docosahexaenoic Acid (DHA) (22:6n-3)
Diet							
Reference	90.2 ± 1.8[a]	98.8 ± 1.6[ns]	97.2 ± 0.8[a]	96.2 ± 0.1[a]	100.0 ± 0.0[a]	100.0 ± 0.0[ns]	97.5 ± 0.0[a,b]
Whole-cell *C. vulgaris* meal							
6%	86.9 ± 0.4[a,b]	97.6 ± 0.4	91.9 ± 0.4[d]	91.1 ± 0.0[c]	87.3 ± 0.1[c]	100.0 ± 0.0	96.7 ± 0.4[a,b]
12%	85.9 ± 0.8[b]	99.4 ± 0.9	89.0 ± 0.0[e]	89.1 ± 0.9[d]	85.6 ± 2.3[c]	100.0 ± 0.0	96.7 ± 0.0[a,b]
18%	84.3 ± 1.1[bc]	100.0 ± 0.0	84.6 ± 1.0[f]	85.0 ± 0.3[e]	81.7 ± 0.1[d]	100.0 ± 0.0	97.7 ± 0.9[a,b]
24%	80.6 ± 0.2[cde]	98.3 ± 2.3	79.8 ± 1.0[g]	80.5 ± 1.1[f]	78.4 ± 1.3[de]	99.5 ± 0.7	98.0 ± 2.8[a,b]
30%	80.5 ± 0.3[de]	100.0 ± 0.0	78.6 ± 0.0[g]	79.3 ± 0.3[f]	77.1 ± 0.7[e]	100.0 ± 0.0	100.0 ± 0.0[a]
Cell-ruptured *C. vulgaris* meal							
6%	87.7 ± 2.1[a,b]	98.5 ± 2.1	96.1 ± 0.9[a,b]	95.9 ± 0.4[a]	100.0 ± 0.0[a]	100.0 ± 0.0	97.5 ± 0.5[a,b]
12%	86.5 ± 0.0[a,b]	98.7 ± 1.8	95.5 ± 0.3[a,b]	95.7 ± 0.3[a]	100.0 ± 0.0[a]	100.0 ± 0.0	97.0 ± 0.1[a,b]
18%	84.2 ± 0.6[bcd]	97.3 ± 0.3	94.6 ± 0.2[bc]	95.4 ± 0.2[a,b]	96.7 ± 0.3[a,b]	100.0 ± 0.0	96.8 ± 0.3[a,b]
24%	80.0 ± 0.3[e]	96.6 ± 0.4	93.0 ± 0.4[cd]	94.4 ± 0.3[a,b]	95.7 ± 0.3[b]	100.0 ± 0.0	95.3 ± 0.0[b]
30%	77.3 ± 0.1[e]	96.3 ± 0.2	91.7 ± 0.2[d]	93.7 ± 0.2[b]	95.1 ± 0.3[b]	100.0 ± 0.0	95.3 ± 0.3[b]

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

Table 7
Apparent digestibility coefficients (% ADCs) of fatty acid groups in diets containing whole-cell and cell-ruptured *Chlorella vulgaris* meals at different inclusion levels[a].

	Saturates	Monounsaturates	Polyunsaturates	n-3 polyunsaturates	n-6 polyunsaturates
Diet					
Reference	93.0 ± 1.3[a]	96.3 ± 1.6[a]	98.4 ± 0.0[a]	99.1 ± 0.0[a]	96.2 ± 0.1[a]
Whole-cell *C. vulgaris* meal					
6%	89.9 ± 1.1[a,b]	93.4 ± 0.0[a,b]	95.5 ± 0.1[c]	97.5 ± 0.2[a,b]	90.4 ± 0.9[b]
12%	89.3 ± 0.8[b]	92.6 ± 0.4[a,b]	93.7 ± 0.6[d]	96.7 ± 0.4[bc]	87.3 ± 1.2[c]
18%	88.6 ± 0.8[bcd]	90.7 ± 0.7[b]	91.3 ± 0.0[e]	95.5 ± 0.3[c]	83.8 ± 0.3[d]
24%	85.8 ± 0.2[cde]	86.6 ± 2.1[c]	88.0 ± 1.2[f]	93.5 ± 1.4[d]	79.2 ± 1.0[e]
30%	85.7 ± 0.2[de]	86.5 ± 0.1[c]	86.8 ± 0.3[f]	93.0 ± 0.2[d]	78.0 ± 0.4[e]
Cell-ruptured *C. vulgaris* meal					
6%	90.1 ± 1.9[a,b]	96.2 ± 1.8[a]	98.3 ± 0.2[a]	99.1 ± 0.2[a]	96.0 ± 0.2[a]
12%	89.2 ± 0.0[bc]	95.9 ± 0.6[a]	98.1 ± 0.1[a]	99.0 ± 0.0[a]	95.9 ± 0.4[a]
18%	87.4 ± 0.5[bcd]	94.9 ± 0.3[a]	97.5 ± 0.2[a,b]	98.4 ± 0.1[a,b]	95.8 ± 0.2[a]
24%	83.8 ± 0.3[e]	93.6 ± 0.4[a,b]	96.6 ± 0.1[a,bc]	97.7 ± 0.1[a,b]	95.0 ± 0.3[a]
30%	81.5 ± 0.1[f]	92.7 ± 0.0[a,b]	96.2 ± 0.1[bc]	97.4 ± 0.0[a,b]	94.5 ± 0.2[a]

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

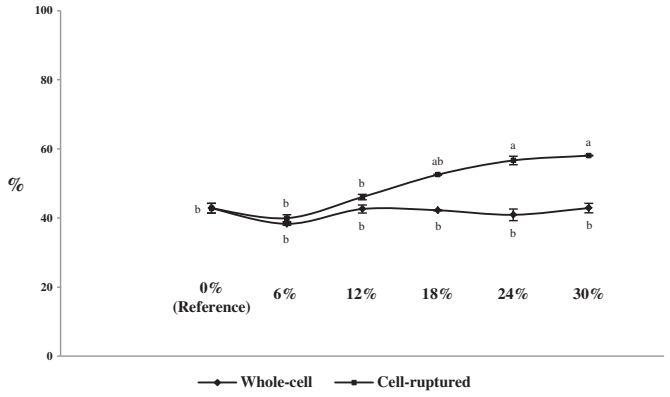

Fig. 2. Starch digestibility (%) of diets containing whole-cell (diamonds) and cell-ruptured (squares) *Chlorella vulgaris* meals at different inclusion levels.

Natural Products Pty Ltd.) at 20% of the diet to a feed containing a relatively high level of soybean meal (also 20%) counteracted the negative effects of soybean meal induced enteropathy (SBMIE) in the fish. Intestinal health parameters such as organ weights, intestinal histopathology and morphometrics, gene expression, amino acid metabolism and intestinal microbiota were restored to near normal levels with a 50:50 mix of soybean meal and *C. vulgaris* meal relative to a fish meal-based control diet containing no soybean meal. However, inclusion of 20% dietary *C. vulgaris* meal reduced growth rates over the relatively short feeding period (4 weeks) and this may be related to *C. vulgaris* meal digestibility, although it was unfortunately not measured in their study. Our results may support this notion as we have demonstrated that nutrient digestibility of diets containing > 6–12% whole-cell *C. vulgaris* meal reduces nutrient digestibility for Atlantic salmon and the same case was generally true for diets containing 12–24% cell-ruptured *C. vulgaris* meal. However, it is unknown as to what extent the 'cracked' *C. vulgaris* meal used in their study was cell-ruptured. As a result, it is difficult to predict if the 20% inclusion levels used exceeds its permissible inclusion rate for acceptable nutrient digestibility in relation to the actual extent of its cell wall disruption. Taken together, their study combined with the digestibility data in this study using a completely intact cell-wall meal and a fully ruptured meal, it is possible that similar beneficial intestinal health effects might be observed at more appropriate dietary inclusion levels that do not negatively impact dietary nutrient digestibility or growth performance of farmed Atlantic salmon and this area warrants further investigation.

Many different methods have been used for disruption of *C. vulgaris* cell walls with varying degrees of success and realistic potential for industrial scale-up (Safi et al., 2014a; Günerken et al., 2015). The chosen method for cell-rupture used in this study (microfluidics) was selected because of its high shear and pressure consistency, which is claimed to result in a high degree of product particle size uniformity,

Table 8
Apparent digestibility coefficients[a] (% ADCs) of nutrients and energy of the single ingredient whole-cell and cell-ruptured *Chlorella vulgaris* meals[b].

	Chlorella vulgaris meals	
	Whole-cell	Cell-ruptured
Protein		
6%	70.2 ± 3.2[ns]	89.7 ± 8.2[ns]
12%	75.8 ± 2.1	87.8 ± 4.8
18%	79.8 ± 3.4	85.5 ± 1.1
24%	77.0 ± 4.7	86.5 ± 0.8
30%	79.5 ± 1.2	85.4 ± 0.2
Pooled ADC	76.5 ± 4.4	87.0 ± 3.6*
Lipid		
6%	59.7 ± 3.7[ns]	86.8 ± 13.2[ns]
12%	70.9 ± 1.4	87.8 ± 1.7
18%	70.9 ± 2.3	85.2 ± 1.2
24%	65.6 ± 4.5	82.2 ± 1.0
30%	69.9 ± 0.2	82.1 ± 0.0
Pooled ADC	67.4 ± 5.0	84.8 ± 5.1*
Energy		
6%	44.8 ± 5.6[ns]	73.6 ± 12.1[ns]
12%	57.3 ± 0.2	76.7 ± 3.0
18%	57.6 ± 2.5	76.6 ± 0.0
24%	55.0 ± 1.8	77.9 ± 1.0
30%	59.6 ± 1.4	76.5 ± 0.6
Pooled ADC	54.9 ± 6.0	76.3 ± 4.4*
Carbohydrate		
6%	36.2 ± 2.8[ns]	79.2 ± 7.2[a]
12%	39.8 ± 4.5	75.7 ± 3.2[a,b]
18%	34.9 ± 0.5	62.8 ± 0.5[b,c]
24%	35.4 ± 4.0	87.5 ± 3.2[a]
30%	45.0 ± 2.9	82.7 ± 0.2[a]
Pooled ADC	38.3 ± 4.7	81.3 ± 5.7*
Starch		
6%	−7.3 ± 0.9[b,c]	11.0 ± 9.2[b,c]
12%	41.5 ± 14.1[a]	62.4 ± 20.8[a]
18%	40.3 ± 4.3[a]	83.8 ± 5.6[a]
24%	36.5 ± 6.4[a]	88.3 ± 10.3[a]
30%	43.0 ± 1.6[a]	84.2 ± 0.8[a]
Pooled ADC	40.3 ± 6.6	79.6 ± 14.2*

[a] Average values across dietary inclusion levels and a significant difference between whole-cell and cell-ruptured *C. vulgaris* meals is denoted by an asterix (*).
[b] Values within the same column within each nutrient having different superscript letters are significantly different ($P < 0.05$).
[c] Value removed.

and for the fact that it is a solely mechanical process (*e.g.*, free of chemicals, enzymes and solvents) which results in a meal that does not require post-processing clean-up. Additionally, it has been suggested that protein yield is higher and protein quality and amino acid profile is better maintained using high-pressure homogenization (mechanical) processing than by chemical (alkali) methods (Safi et al., 2014b). Perhaps of most importance for an applied application such as this, however, is that unlike many other cell-rupture methods (*e.g.*, pH or temperature shock, ultrasonication, electroporation, chemical/enzymatic degradation, *etc.*) microfluidics technology is highly scalable to industrial levels (Samarasinghe et al., 2012; Günerken et al., 2015). To our knowledge, microfluidics technology has only been previously investigated with *Chlorella* sp. to evaluate its effect on *in vitro* bioaccessibility of its carotenoids (Cha et al., 2011, 2012). This is the first study to evaluate its effects on biochemical composition and *in vivo* digestibility of nutrients, energy, essential amino acids and fatty acids for Atlantic salmon. As demonstrated by others, the increased progression of cell wall disruption throughout processing of microalgae is generally correlated with increasing solubilization of cell wall and intracellular proteins (Safi et al., 2014b) and possible de-activation of heat-labile anti-nutritional compounds (Drew et al., 2007); however, excessive processing has the potential to damage protein quality and is unnecessarily costly. This study showed that two Microfluidizer® passes were sufficient for complete cell wall rupture based on microscopy and protein solubility. As no significant improvement in protein solubility was achieved beyond the second pass, this indicates the opportunity to reduce production costs and energy consumption associated with this type of processing. Previous authors have indicated that even just a single pass may be sufficient to achieve > 90% cell-rupture for some *Chlorella* species under a processing regime optimized for variables such as process feeding rate and initial slurry cell density (Doucha and Lívanský, 2008). However, our finding that a single pass was not sufficient for adequate cell-rupture of *C. vulgaris* is consistent with Halim et al. (2016) for *Nannochloropsis* sp. where they reported only a 50% cell-rupture success after a single pass. The fully cell-ruptured, highly homogenous slurry achieved in the present study after 2 passes at 25000 psi is also consistent with a report for a related species (*C. ellipsoidea*) where the authors found that a processing pressure of 20,000 psi resulted in greater slurry particle homogeneity than those processed at lower pressures of 5000 to 10,000 psi (Cha et al., 2012). A double pass with a minimum of 20,000 psi was also reported to be the optimal high pressure homogenization treatment for extraction of proteins, lipids and sugars from other *Chlorella* sp. and *N. oculata* (Lee et al., 2013; Safi et al., 2014b; Samarasinghe et al., 2012; Shene et al., 2016).

While the nutrient content of *C. vulgaris* is highly variable in the literature, which reflects the wide diversity of its cultivation and harvesting strategies, the protein content is generally one of the most important factors when it is being evaluated for potential inclusion in aquafeeds. Although the total amount of protein in *C. vulgaris* varies widely in the literature (often by multiple magnitudes), the amino acid profile of that protein (and microalgal protein in general) generally remains quite conserved (Brown et al., 1997; Safi et al., 2014b). The amino acid profiles of the *C. vulgaris* meals used in this study are remarkably similar to those in a recent multivariate analysis of amino acid composition in several *Chlorella* strains (Wei et al., 2011). The protein contents of the *C. vulgaris* meals used in this study were estimated by conversion of elemental nitrogen content data to protein values using an appropriate 'species-specific' nitrogen-to-protein (N-to-P) conversion factor (N × 5.14; Tibbetts et al., 2015), which has been the recommended method by other experts working in this emerging field (González López et al., 2010; Laurens et al., 2012; Lourenço et al., 2004; Templeton and Laurens, 2015). It is encouraging that the N-to-P conversion factor we used (N × 5.14 based on 22 amino acids) is remarkably close to the average value reported recently for *C. vulgaris* as food, feed and fuel (N × 5.00 based on 18 amino acids) by Templeton and Laurens (2015). Differences in the biochemical composition of whole-cell and cell-ruptured *C. vulgaris* meals were very minimal in this study (with the exception of carotenoids) and this finding is consistent with Komaki et al. (1998) who concluded that the chemical composition of *C. vulgaris* was scarcely altered after a similar processing method (high pressure homogenization). With regard to the digestibility of energy from whole-cell *C. vulgaris* as a potential feed ingredient, the consensus is encouraging. *In vitro* two-phase porcine gastric/pancreatic DE content has been reported at 15 MJ/kg (Tibbetts et al., 2016), which corroborates the *in vivo* DE content measured with laboratory rats at 15 MJ/kg (Komaki et al., 1998). Here we report a highly similar *in vivo* DE (14 MJ/kg) measured with Atlantic salmon, which can be significantly increased to 19 MJ/kg with cell-rupture. Komaki et al. (1998) further reported that *in vivo* ADC of crude fibre in *C. vulgaris* biomass is low in rats (37–41%) and this is surely causative for lower energy ADC for Atlantic salmon, especially given the large difference in crude fibre levels between whole-cell (10%) and cell-ruptured *C. vulgaris* meals (2%) used in this study. On the other hand, these authors reported that cell-rupture processing only caused small improvements in protein digestibility and DE content for rats (< 1.5% and < 0.5 MJ/kg, respectively), while we observed far larger improvements for salmon (> 10% and > 5 MJ/kg, respectively).

With regard to whole-cell *C. vulgaris* meal, since dietary dry matter and energy ADCs dropped off in a relatively dose-dependent manner

Table 9
Apparent digestibility coefficients[a] (% ADCs) of essential amino acids and major fatty acids of the single ingredient whole-cell and cell-ruptured *Chlorella vulgaris* meals[b].

	Chlorella vulgaris meals			*Chlorella vulgaris* meals	
	Whole-cell	Cell-ruptured		Whole-cell	Cell-ruptured
Arginine			Methionine		
6%	93.9 ± 3.3^{a,b}	103.2 ± 8.0^{ns}	6%	66.9 ± 2.7$^{c,\#}$	112.7 ± 0.5$^{a,\#}$
12%	103.2 ± 5.8$^{b,\#}$	90.8 ± 1.6	12%	92.2 ± 0.4^{a}	96.8 ± 3.3^{b,c}
18%	94.3 ± 1.3^{a,b}	88.8 ± 6.6	18%	87.3 ± 1.6^{a,b}	93.6 ± 1.3^{b,c}
24%	88.4 ± 0.5^{a,b}	93.9 ± 0.8	24%	80.3 ± 4.6^{b}	100.5 ± 0.5^{b}
30%	83.3 ± 1.3$^{a,\#}$	94.6 ± 0.0	30%	83.4 ± 0.8^{b}	89.6 ± 0.9^{c}
Pooled ADC	92.2 ± 3.3	94.3 ± 6.3	Pooled ADC	85.8 ± 5.1	95.1 ± 4.5*
Histidine			Phenylalanine		
6%	87.2 ± 2.3^{a,b}	88.5 ± 12.6^{ns}	6%	73.7 ± 3.5^{ns}	108.7 ± 3.3$^{a,\#}$
12%	102.7 ± 7.6$^{b,\#}$	73.1 ± 1.9	12%	81.5 ± 3.4	96.7 ± 4.5^{a,b}
18%	90.7 ± 0.1^{a,b}	80.2 ± 10.1	18%	78.7 ± 1.2	95.3 ± 2.2^{b}
24%	83.1 ± 0.3^{a,b}	90.6 ± 0.5	24%	76.8 ± 3.6	88.8 ± 4.2^{b}
30%	77.9 ± 1.3$^{a,\#}$	93.1 ± 0.2	30%	81.8 ± 1.1	89.2 ± 0.0^{b}
Pooled ADC	87.0 ± 3.6	85.1 ± 9.5	Pooled ADC	78.5 ± 3.8	92.5 ± 4.5*
Isoleucine			Threonine		
6%	76.3 ± 5.0^{b}	109.6 ± 0.1$^{b,\#}$	6%	77.4 ± 2.5^{c}	91.9 ± 4.5^{ns}
12%	89.9 ± 4.3$^{a,\#}$	92.4 ± 4.1^{a}	12%	96.5 ± 2.4$^{a,\#}$	83.9 ± 0.1
18%	84.8 ± 1.0^{a,b}	90.4 ± 1.2^{a}	18%	87.6 ± 0.3$^{b,\#}$	83.9 ± 3.0
24%	76.5 ± 5.1^{b}	89.8 ± 1.7^{a}	24%	81.5 ± 2.7^{b,c}	90.6 ± 0.9
30%	79.4 ± 1.3^{a,b}	90.5 ± 0.5^{a}	30%	73.3 ± 0.3^{c}	91.5 ± 0.2
Pooled ADC	79.2 ± 4.6	90.8 ± 2.0*	Pooled ADC	77.4 ± 4.0	88.4 ± 4.3*
Leucine			Tryptophan		
6%	68.1 ± 4.0$^{a,\#}$	104.8 ± 2.3$^{a,\#}$	6%	80.3 ± 21.9^{ns}	194.2 ± 14.7$^{a,\#}$
12%	85.7 ± 4.5^{b}	93.5 ± 3.9^{a,b}	12%	86.0 ± 13.2	94.4 ± 20.6^{b}
18%	81.7 ± 1.6^{b}	91.7 ± 0.2^{b}	18%	90.5 ± 6.5	86.8 ± 10.3^{b}
24%	76.8 ± 5.0^{a,b}	91.2 ± 0.8^{b}	24%	71.8 ± 7.0	80.4 ± 0.3^{b}
30%	79.2 ± 1.3^{a,b}	92.4 ± 0.5^{b}	30%	90.0 ± 1.6	68.8 ± 14.9^{b}
Pooled ADC	80.8 ± 4.4	92.2 ± 1.8*	Pooled ADC	83.7 ± 11.7	82.6 ± 14.4
Lysine			Valine		
6%	94.5 ± 0.9^{ns}	109.5 ± 4.2$^{a,\#}$	6%	78.4 ± 3.8^{b}	102.9 ± 4.4$^{a,*,\#}$
12%	93.4 ± 5.8	97.5 ± 2.7^{a,b}	12%	92.7 ± 1.9$^{a,\#}$	89.8 ± 2.8^{b}
18%	89.8 ± 0.3	99.5 ± 1.8^{a,b}	18%	87.5 ± 0.4^{a,b}	87.1 ± 3.3^{b}
24%	89.0 ± 3.7	92.3 ± 3.4^{b}	24%	80.3 ± 3.0^{b}	90.8 ± 0.5^{b}
30%	97.3 ± 1.8	92.7 ± 2.0^{b}	30%	78.0 ± 0.9^{b}	92.2 ± 0.5^{b}
Pooled ADC	92.8 ± 4.0	95.5 ± 3.8	Pooled ADC	81.0 ± 4.5	90.0 ± 2.6*
Palmitic acid (16:0)			Oleic acid (18:1n-9)		
6%	51.7 ± 5.2^{ns}	63.5 ± 22.4^{ns}	6%	59.1 ± 3.0^{a,b}	89.8 ± 6.2^{ns}
12%	64.1 ± 5.1	68.6 ± 0.1	12%	63.6 ± 0.1^{a}	90.6 ± 1.1
18%	65.0 ± 4.6	64.5 ± 2.4	18%	58.1 ± 3.0^{a,b}	89.1 ± 0.6
24%	59.5 ± 0.7	58.3 ± 1.0	24%	52.8 ± 2.6$^{b,\#}$	86.8 ± 0.9
30%	64.6 ± 0.7	57.1 ± 0.2	30%	56.7 ± 0.0^{a,b}	85.4 ± 0.4
Pooled ADC	61.0 ± 6.0	62.4 ± 8.7	Pooled ADC	59.4 ± 3.2	88.3 ± 2.9*
Linoleic acid (18:2n-6)			Linolenic acid, ALA (18:3n-3)		
6%	67.0 ± 0.3^{a,b}	94.4 ± 2.0^{ns}	6%	63.2 ± 0.3^{ns}	100.0 ± 0.0$^{a,\#}$
12%	71.1 ± 3.3$^{a,\#}$	94.4 ± 0.9	12%	67.2 ± 5.2	100.0 ± 0.0$^{a,\#}$
18%	65.3 ± 0.7^{a,b}	94.1 ± 0.6	18%	62.8 ± 0.2	93.6 ± 0.5^{b}
24%	59.5 ± 2.5^{b}	92.2 ± 0.7	24%	58.7 ± 2.5	92.3 ± 0.5^{b},c
30%	61.7 ± 0.7^{b}	91.3 ± 0.4	30%	60.1 ± 1.2	91.7 ± 0.5^{c}
Pooled ADC	63.4 ± 3.3	93.3 ± 1.6*	Pooled ADC	62.4 ± 3.7	92.5 ± 1.0*

[#]Value removed.
[a] Average values across dietary inclusion levels and a significant difference between whole-cell and cell-ruptured *C. vulgaris* meals is denoted by an asterix (*).
[b] Values within the same column within each nutrient having different superscript letters are significantly different ($P < 0.05$).

with increasing inclusion level from 0 to 30%, this clearly indicates that the digestibility of the major macronutrients (*e.g.*, protein, lipid, carbohydrate) were affected. However, dry matter and energy ADC data do not indicate which of these constituent nutrients might be the causative agent(s). Our results showed that the carbohydrate, starch and protein fractions of whole-cell *C. vulgaris* meal had little to do with this reduction in overall diet digestibility, while it is clear that this effect was most highly related to the lipid fraction. With regards to cell-ruptured *C. vulgaris* meal, since dry matter ADCs were unaffected, it might appear at first glance that ADC of the major nutrients was not affected by dietary inclusion of cell-ruptured *C. vulgaris* meal even up to the highest (30%) inclusion level. However, since energy ADC drops slightly at the highest inclusion levels (24–30%) this indicates that, indeed, ADCs of some 'energy-yielding' nutrients were impacted; despite cell wall rupture. Again, the data clearly showed that the carbohydrate, starch and protein fractions had little to do with this reduction in overall diet digestibility and it was predominantly related to the lipid fraction. At least for the protein fraction, this has been confirmed through the essential amino acid *in vivo* ADC data where protein quality of *C. vulgaris* meals used in this study proved to be high for juvenile Atlantic salmon. These are encouraging findings since the mass industrial production of *C. vulgaris* is likely to be associated with a bioenergy strategy. Under this scenario, the lipid fraction would be essentially removed (or at least greatly reduced) from the product and used elsewhere for biodiesel production, leaving behind the high-value protein- and starch and carbohydrate-rich fraction for feed applications. The finding that dietary carbohydrate and starch digestibilities actually increased with rising dietary inclusion level is also highly encouraging. Dietary sources of carbohydrate presently used in aquafeeds typically come from cereal grains such as wheat, corn and/or rice because they are abundantly available at low cost and provide both dietary energy (calories) and functionality for pellet production. However, while they are highly

digestible sources of energy for farmed 'warmwater' fish species such as carp, tilapia and catfish, their utilization by 'coldwater' farmed fish species such as salmonids is less efficient (Kamalam et al., 2017; NRC, 2011). This can be clearly seen in the digestibility data for the reference diet (algae-free control) used in this study, where the ADCs for carbohydrate (35%) and starch (43%) from these ingredients is relatively low and is representative of the scenario with commercially pelleted salmon feeds (NRC, 2011). Feed extrusion technology, which more effectively gelatinizes starch, may increase these values somewhat and it appears from our data that the inclusion of cell-ruptured *C. vulgaris* meal in farmed salmon feeds has the potential to improve this even further (Fig. 2). Additionally, since cellulose is likely the predominant component of the crude fibre fraction of *C. vulgaris* meals, rather than hemicellulose, pectin or lignin (Li et al., 2015; Tibbetts et al., 2015) and crude fibre levels in the test diets increased concomitantly with increasing inclusion of *C. vulgaris* meals, pellet quality of salmonid feeds may also be improved (Hansen and Storebakken, 2007). This effect was observed anecdotally, however pellet hardness and durability tests were beyond the scope of this project. The effect of inclusion of algal meals on the quality of finished animals feed pellets has received almost no attention (Boney and Moritz, 2017) and the impact of dietary inclusion of *C. vulgaris* meals in extruded salmon feeds on nutritional value, rheological properties and finished pellet quality are warranted.

Of course it is important to try to understand why the lipid fraction of *C. vulgaris* meals is predominantly responsible for reductions in dietary energy digestibility for Atlantic salmon and there are several possible explanations. First, the recalcitrant cell wall of *C. vulgaris* is the most obvious cause of lower digestibility of algae and this was clearly demonstrated in the present study between whole-cell and cell-ruptured of *C. vulgaris* meals. However, as we also demonstrated, while this may be somewhat ameliorated by cell-rupture processing, it likely won't fully eliminate it and it will incur additional processing costs which industry will have to consider. Secondly, further examination of the digestibility of individual FAs revealed that reduced digestibility of the lipid fraction of *C. vulgaris* meals was due to significantly lower ADCs of four fatty acids (palmitic, oleic, linoleic and linolenic) with inclusion of whole-cell *C. vulgaris* meal at levels as low as 6% and two fatty acids (palmitic and oleic) with cell-ruptured *C. vulgaris* meal at inclusion levels higher than 12%. Alternatively, lipid digestibility was not significantly affected by any inclusion level (up to 30% of diet) of either whole-cell or cell-ruptured *C. vulgaris* meals for palmitoleic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Thirdly, the reductions in lipid digestibility were also in accordance with progressively decreasing n-3:n-6 PUFA ratios from 3.1 to 1.4. It is well documented that water temperature in the salmon culture environment, fatty acid characteristics of the test ingredients (*e.g.*, source, chain length, degree of saturation, melting point, *etc.*) and dietary n-3:n-6 ratios all impact lipid digestion in farmed salmonids (Hua and Bureau, 2009; Huguet et al., 2015). Of course temperature can be ruled out as a factor in this case because the entire study was conducted at constant water temperature (12.8 ± 0.2 °C). However, as mentioned, with rising inclusion level of *C. vulgaris* meals in the test diets, the dietary n-3:n-6 fatty acid ratios were reduced almost 3-fold from 3.1 for the algae-free control diet to 1.4 for diets containing the highest inclusion level. This alteration was caused by decreasing total levels of dietary eicosapentaenoic acid, EPA (20:5n-3; from 1.8 to 1.2% of diet) and docosahexaenoic acid, DHA (22:6n-3, from 1.4 to 0.9% of diet) and concomitant increasing levels of linoleic acid (18:2n-6) from 1.2 to 2.2% of diet. Recent studies have demonstrated that all dietary PUFA is generally highly digested by several coldwater farmed fish, but that n-3 PUFA is more highly digestible than its n-6 counterpart and, as such, alternations in the n-3:n-6 ratio can directly affect lipid digestibility (Francis et al., 2007; Bandarra et al., 2011; Eroldogan et al., 2013). Finally, a recent *in vitro* study has suggested that a similar *Chlorella* strain (*C. sorokiniana*) cultivated in the same manner contained monogalactosyldiacylglycerols (MGDGs) that effectively inhibited pancreatic lipase activity (Banskota et al., 2016). Further studies are required, but if confirmed *in vivo*, the presence of these MGDGs in *Chlorella* meals may offer a possible explanation for the observed reductions in dietary lipid digestibility in this study.

While the *in vivo* digestibility of essential amino acids from diets containing *C. vulgaris* meals has been reported for laboratory rats (Janczyk et al., 2005), it has never been reported for any farmed livestock or aquaculture species and confirmation of the most limiting EAAs in *C. vulgaris* meals for Atlantic salmon has not been known until now. In addition, the aforementioned work with rats did not report essential amino acid ADCs for the single test ingredients; only the complete test diets. Diet digestibility of essential amino acids for rats was variable depending upon the type of meal processing for arginine (72–77%), histidine (45–62%), isoleucine (37–54%), leucine (46–63%), lysine (47–63%), methionine (55–72%), phenylalanine (40–58%), threonine (43–58%), tryptophan (67–82%) and valine (42–59%) while those of the algae-free control diet were much higher (83–95%). The highest values were consistently for the *C. vulgaris* meal processed using sonication (54–82%), lowest when processed by electroporation (37–74%) and intermediate for spray-dried meal (45–78%). Regardless, these values are all low and indicative that the processing methods used (*e.g.*, spray drying, electroporation and sonication) were either ineffective at cell wall rupture and/or were detrimental to protein quality, or both. In fact, one of the most commonly-used laboratory methods for microalgal cell wall rupture (*e.g.*, sonication) was recently shown to be ineffective for protein extraction from *Chlorella* sp. (Al-Zuhair et al., 2017). On the other hand, the processing used in the current study (*e.g.*, freeze-drying followed by microfluidization) was effective at maintaining high protein quality based *in vivo* EAA digestibility for both the test diets and single ingredients for arginine (94–97%), histidine (85–96%), isoleucine (91–94%), leucine (92–95%), lysine (95–97%), methionine (92–96%), phenylalanine (90–95%), threonine (87–94%), tryptophan (83–98%) and valine (90–95%).

For nutrient ADC values of single test ingredients to be useful for future diet formulation, it must be demonstrated that the assumption that the ADCs of the single ingredient and those of the reference diet portion of the combined test diet are digested independently of one another is proven correct (Cho et al., 1982). This is most adequately assessed by using several tests feeds with a range of reference diet and test ingredient levels in the combined diet (Jobling, 2016). When this assumption is proven correct, the 'predicted' and 'measured' ADCs of the combined test diets regressed against each another should result in a highly significant linear relationship. To test and confirm this assumption, we correlated predicted and measured values for both *C. vulgaris* meals (whole-cell and cell-ruptured) included in the test diets over a wide range (0–30%) and confirmed this relationship ($r \geq 0.975$, $R^2 \geq 0.951$, $P < 0.001$). This is not a new concept and the method used to calculate predicted values has been presented by several authors, however, while the assumption has been validated for other commercially-important farmed species like rainbow trout, *Oncorhynchus mykiss* (Cho et al., 1982; Cho and Kaushik, 1990), channel catfish, *Ictalurus punctatus* (Wilson and Poe, 1985), tilapia, *Oreochromis niloticus*, ayu, *Plecoglossus altivelis*, and carp, *Cyprinus carpio* (Watanabe et al., 1996a, 1996b), seabass, *Dicentrarchus labrax* (da Silva and Oliva-Teles, 1998), silver perch, *Bidyanus bidyanus* (Allan et al., 1999), Australian short-finned eel, *Anguilla australis* (Engin and Carter, 2002) and Atlantic cod, *Gadus morhua* (Tibbetts et al., 2006) fed diets containing a variety of alternative feed ingredients, this is the first for Atlantic salmon fed diets containing *C. vulgaris* meals.

In conclusion, this study showed that of the major energy-yielding nutrients (*e.g.*, carbohydrate, protein and lipid), protein quality is high for both whole-cell and cell-ruptured *C. vulgaris* meals (EAAI, 0.87–0.89) and that tryptophan and lysine are the most limiting EAAs in *C. vulgaris* meals for juvenile Atlantic salmon reared in freshwater, based on their low PDCAAS values. Digestibility of EAAs is high (83–95%) for cell-ruptured *C. vulgaris* meal and inclusion of cell-

ruptured *C. vulgaris* meal increased dietary carbohydrate and starch digestibility by up to 20%. Alternatively, lipid digestibility appears to be the limiting factor responsible for reductions in energy digestibility. This study demonstrated that cell-rupture processing greatly improved nutrient digestibility, but due to the fatty acid profile of *C. vulgaris*, it may not be enough and the additional processing costs of lipid-extraction (*e.g.*, defatting) must be considered. However, this finding is encouraging since the mass industrial production of *C. vulgaris* is likely to be associated with a bioenergy strategy. Under this scenario, the lipid fraction would be essentially removed (or at least greatly reduced) from the product and used elsewhere for other biotechnological applications (*e.g.*, biodiesel), leaving behind the high-value concentrated protein, starch and carbohydrate-rich fractions for salmon feed applications. Admittedly, the relatively low crude protein contents (< 30%) of the present *C. vulgaris* meals would likely preclude their use and further work to develop cost-effective methods to enhance protein levels is highly encouraged. Such measures should include harvesting of *C. vulgaris* biomass during the exponential growth phase and defatting and/or fractionation to produce algal protein concentrates (APCs). Based on the digestibility data presented here, optimum dietary inclusion levels must be determined based on animal health and performance, nutrient utilization efficiency, waste output and final product quality. In addition, the fish health and consumer safety aspect of using these products in aquaculture feeds must be established. In this regard, work is currently underway using a zebrafish (*Danio rerio*) model to assess the toxicity (or lack there-of) of whole-cell and cell-ruptured *C. vulgaris* meals using a Fish Embryo Toxicity (FET) assay and a General and Behavioural Toxicity (GBT) assay (Lammer et al., 2009; Ellis et al., 2014). Finally, in order for *Chlorella* meals to be seriously considered as a routine input for aquaculture feeds, it will require a massive scale-up of industrial production. And, even if this is technically and economically possible, the aquafeed sector will demand a consistent product and reliable supply at a price that is competitive with fish meal and other high-protein plant-based feedstuffs in order to penetrate this market space. It is expected that the comprehensive biochemical composition and Atlantic salmon specific *in vivo* digestibility data generated in the present study will provide a solid first step towards a comprehensive strategic assessment of the nutritional quality of *C. vulgaris* meals as 'low-trophic' feed ingredients for Atlantic salmon that takes into account both cell-rupture (or lack thereof) and dietary inclusion level.

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