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RESEARCH

Mechanical cell disruption of the green microalga *Chloroidium saccharophilum*: Effects on protein digestibility and aroma profile

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Abstract

The efficiency and outcomes of mechanically disrupting microalgal cell walls depend on various factors and are case-specific. In general, bead milling is reproducible and scalable, and it can successfully increase the digestibility of microalgal biomass. However, its effects on the aroma of microalgal biomass have not yet been assessed. This study is the first to report the use of the NanoWitt-100 bead mill to break down microalgal biomass. Optimization of processing time and cell concentration revealed that the optimal conditions were a milling time of 15 min, and a cell concentration of 50 g L^{-1}. Under these conditions, we then compared the in vitro protein and amino acid digestibility as well as volatile organic compound (VOC) profiles of undisrupted and disrupted samples. Bead milling substantially increased total protein digestibility from 62.2 ± 2.28 to $99.7 \pm 0.06\%$. The digestibilities of all amino acids and the in vitro digestible indispensable amino acid scores (DIAAS) also increased. Although the release of VOCs increased in the disrupted biomass, the VOC profiles indicated an improvement in the final aroma of the biomass. This outcome was due to a reduction in acid concentration and an increase in ester concentration as a proportion of the total VOCs. Overall, these findings support further scalability tests, sensory analysis, and in vivo trials using microalgae disrupted with the bead mill NanoWitt-100.

Keywords Microalgal biotechnology · Bead milling · NanoWitt-100 · Infogest · In vitro DIAAS · Volatile organic compounds (VOCs)

Introduction

Microalgae are a highly diverse group of microscopic organisms characterized by the ability to perform oxygenic photosynthesis (Raven and Giordano 2014). Over the last few decades, microalgae have attracted significant interest from industry and academia because they (i) grow faster and fix CO_2 more efficiently than conventional crops; (ii) require less water than conventional agriculture; (iii) do not require arable land for cultivation; (iv) produce and accumulate valuable biomolecules; and (v) can replace industrial bioprocesses in a more sustainable way (Chisti 2007; Rodolfi et al. 2009). For these and other reasons, several microalgal strains have been used in the food, feed, pharmaceutical, and energy industries (Borowitzka et al. 2013).

Harvesting and downstream processing in microalgal biotechnology are costly and energy intensive, and they still represent the sector's main technological bottlenecks (Acién et al. 2012). Cell disruption, for example, is an essential downstream process for implementing a biorefinery approach, which aims to exploit all fractions of the biomass (Anastas and Zimmerman 2003). This process is also important for microalgal applications where intracellular compounds must be released or the microalgal biomass must be digested (Postma et al. 2018). From lab to commercial scale, several techniques are commonly used to disrupt microalgal cells. Conventionally, they can be classified into mechanical and non mechanical methods. Mechanical methods break down the cell walls in a non specific manner using impact forces and solid shear forces (e.g., bead milling), liquid shear forces (e.g., high-pressure homogenization), wave-based shear forces

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(e.g., ultrasonication), energy transfer through currents (e.g., pulsed electric field), or heat (e.g., thermolysis). Non mechanical methods involve cell lysis with chemical agents, enzymes, or osmotic shock. These methods are considered milder and more selective because cell walls are permeabilized rather than shredded (Günerken et al. 2015). Overall, the available literature reports inconsistent information, partly due to the wide variation in the composition and thickness of microalgal cell walls, making predictions and extrapolations highly challenging (Günerken et al. 2015). It is also attributed to the limited scalability of the equipment used.

Cell disruption plays an important role in the food and feed applications of microalgae because it releases intracellular compounds, making them more accessible to digestive enzymes; it also alters their organoleptic properties, such as aroma (Prabakaran and Ravindran 2011). Studies have shown that cell disruption through bead milling and high-pressure homogenization can increase the in vitro and in vivo digestibility of microalgal cells (Tibbetts et al. 2017; Wild et al. 2018). However, the effects of cell disruption on digestibility are not directly proportional because antinutritional compounds may also be released, which can inhibit the activity of digestive enzymes (Janczyk et al. 2007). Therefore, a thorough investigation of the digestibility of disrupted microalgal cells is necessary. Microalgae are often proposed as alternative sources of protein, but these claims are usually based solely on protein quantity. A more accurate assessment should include protein quality, defined by the content, digestibility, and bioavailability of essential amino acids (Williamson et al. 2024). The Food and Agriculture Organization (FAO) (2013) proposed the use of the digestible indispensable amino acid score (DIAAS) to evaluate protein quality. However, to date, very few DIAAS values have been reported for microalgae (Williamson et al. 2024).

The odor of microalgal biomass is closely related to the profile of its volatile organic compounds (VOCs), which are released when cell walls break down (Hosoglu 2018). The overall diversity of aroma compounds is widely varied (www.thegoodscentcompany.com). The release of these molecules can significantly affect the final aroma of microalgal biomass and, consequently, consumer acceptance. For example, a recent study showed that a trained panel of evaluators detected intense grassy and fishy odors after *Nannochloropsis* sp. cells were disrupted (Coleman et al. 2023). These odors correlated with the release of unsaturated aldehydes, ketones, amines, and alcohols. Studies of this kind are scarce in the literature. The morphophysiological diversity and metabolic plasticity of microalgae suggest that these characteristics may be species specific or even strain specific.

This study investigated the impact of cell disruption through bead milling on the protein digestibility and VOC profiles of the green microalga *Chloroidium saccharophilum*. For the first time, the Nanowitt-100 (Frewitt, Switzerland), a robust, automated, and readily scalable milling machine, was used to break down microalgal cells. The chlorophyte *C. saccharophilum* was selected as the focus of this study because it has been previously characterized and proposed as a good candidate for use in animal feed based on its growth and biochemical composition (Kurpan et al. 2025; Wahl et al. 2025). The processing time and cell concentration for bead milling were first optimized. Next, the optimal conditions were used to compare the in vitro protein and amino acid digestibility as well as the VOC profiles of undisrupted and disrupted biomass. To the best of the authors' knowledge, this is the first report of the DIAAS values for microalgae. Similarly, very little information on the impact of cell disruption on the VOC profiles of microalgae has been reported.

Materials and methods

Organism and cultivation conditions

Chloroidium saccharophilum (FAM 27962) was isolated from the Swiss plateau and is now part of AlgoScope, the microalgae culture collection of Agroscope (Switzerland; Kurpan et al. 2025). The biomass proximate composition is shown in Table 1. Cells were batch-cultured photoautotrophically in 5-L glass bottles containing 4 L of Doucha's growth medium (0.55 g $CO(NH_2)_2$, 0.12 g KH_2PO_4, 0.10 g $MgSO_4.7H_2O$, 0.02 g Na_2EDTA Fe, 0.09 g $CaCl_2.2H_2O$, and 2 mL microelement solution; Doucha and Lívanský 2006). The bottles were inoculated with 0.2 g L^{-1} of dry biomass

Table 1 Proximate composition of *Chloroidium saccharophilum* FAM 27962 photoautotrophically grown in Doucha's medium at 22.5°C, 150 µmol photons m^{-2} s^{-1}, and 4% CO_2

Strain	Parameters (g kg^{-1})				
	Moisture	Proteins	Carbohydrates	Lipids	Ash
C. saccharophilum FAM 27962	29.7 ± 1.13	403 ± 0.68	197 ± 13.2	137 ± 4.24	53.5 ± 0.35

from cultures in the exponential growth phase and maintained in an incubator (Multitron, Infors-HT) at 22.5°C with 115 rpm orbital agitation, continuous illumination of 150 µmol photons m^{-2} s^{-1}, and 4% CO_2 atmosphere.

Bead milling

The optimization of two parameters was investigated for cell disruption through bead milling: cell concentration and milling time. For this reason, at the end of the exponential growth phase, cultures were diluted or concentrated with distilled water to achieve final concentrations of 1, 10, and 50 g L^{-1} of dry biomass. Samples were concentrated by resuspending the pellets after centrifugation at 1500 × *g* (Bio Stratos, Thermo Scientific). Aliquots of 250 mL of cell suspensions with known concentrations were then bead milled using the NanoWitt-100 (Frewitt, Switzerland) in recirculation mode, 500 mL min^{-1} flow rate, and rotor speed of 4.7 m s^{-1} at room temperature (23 ± 2 °C). The bead chamber was filled at 60% capacity with 0.1–0.2 mm zirconium beads. This technology is protected by an international patent (EP2307145). Samples were taken after 0, 5, 15, 60, and 120 min of milling. The "0 min" sample was taken immediately after the beads were pumped into the milling chamber, which was done at a lower rotor speed and took 2–3 min. The samples were frozen immediately after bead milling until further analysis.

Size classes and microscopy

Immediately after sampling, the suspensions were evaluated for particle size classes using laser diffraction and checked through optical microscopy. The particle size distribution of the samples was measured using a Mastersizer 3000 laser diffraction analyzer (Malvern Instruments, UK) via the wet dispersion method, with distilled water used as the dispersant. An appropriate amount of the sample was first dispersed in distilled water and then transferred into the Hydro EV wet dispersion unit at a stirring speed of 2,000 rpm. The refractive index and the absorption index were set at 1.50 and 0.01, respectively. Measurement was initiated once the obscuration level reached the range 0.1% – 5%. Each sample was measured thrice, and the average values were recorded. The instrument automatically provided particle size distribution data, including characteristic values, such as D10, D50, and D90. The system was thoroughly rinsed with distilled water before and after each measurement to prevent sample carryover. The morphology of the samples was observed using a Primostar 3 optical microscope (Carl Zeiss, Germany). Micrographs were captured using a Jenoptik digital camera attached to the microscope and further processed using the corresponding image acquisition and analysis software. All analyses were conducted at room temperature.

Pigment extraction

Lyophilized samples had their pigments extracted overnight at − 20 °C with 100% methanol. The concentration of chlorophylls and carotenoids in the methanolic extracts was determined spectrophotometrically based on the peak absorption of each pigment, as described by Lichtenthaler and Wellburn (1983):

$$Chlorophyll\ a\ \left(\mu g\ mL^{-1}\right) = 15.65 \times A_{666} - 7.34 \times A_{653}$$

$$Chlorophyll\ b\ \left(\mu g\ mL^{-1}\right) = 27.05 \times A_{653} - 11.21 \times A_{666}$$

$$Carotenoids_{x+c}\left(\mu g\ mL^{-1}\right) = \left(1{,}000 \times A_{470} - 2.86 \times Chl_a - 129.2 \times Chl_a\right)/245$$

where A_{470}, A_{653}, and A_{666} are the absorbances at 470, 653, and 666 nm, respectively; and Chl_a and Chl_b are the concentrations, in µg mL^{-1}, of chlorophylls *a* and *b*, respectively.

Microscopic analyses, particle size class distribution, and pigment extraction efficiency were evaluated to select the optimal processing time and cell concentration of the bead milling process. Protein digestibility and VOCs were determined for comparison between the intact cells and an optimally disrupted sample.

Protein and amino acid digestibility

The protein digestibility of the lyophilized microalga was analyzed by following the workflow described by Sousa et al. (2023), which is based on the INFOGEST static in vitro digestion protocol (Brodkorb et al. 2019). Each microalga sample corresponded to 40 mg of protein. For the oreal phase, the samples were diluted (1:1 w/w) with simulated salivary fluid (pH 7.0) containing α-amylase (75 U mL^{-1} final activity) and incubated at 37 °C for 2 min. The oral bolus was then mixed (1:1 v/v) with simulated gastric fluid (pH 3.0) containing pepsin (2,000 U mL^{-1} final activity) and incubated at 37 °C with rotatory mixing for 2 h. The dilution (1:1 v/v) of the gastric chyme with simulated intestinal fluid (pH 7.0), pancreatin (100 U mL^{-1} final trypsin activity), and bile (10 mmol L^{-1} final concentration) started the intestinal phase. After 2 h of rotatory mixing at 37 °C, digestion was stopped by the addition of ice-cold 100% methanol to achieve a final concentration of 80%. A protein-free cookie was digested simultaneously as a blank to determine the enzyme background (Moughan 2005; Sousa et al. 2023). Before in vitro digestion, the activities of the digestive enzymes, the concentration of bile salts, and

the buffer capacity of individual samples were determined according to Brodkorb et al. (2019).

After methanol precipitation, the digested samples were separated by centrifugation (4,000 × *g* at 4 °C for 15 min) into soluble (digestible) and insoluble (indigestible) fractions by collecting the supernatant and pellet, respectively (Sousa et al. 2023). Both fractions were hydrolyzed in hydrochloric acid at 110 °C for 15 h. The concentration of individual amino acids in the hydrolysates was analyzed according to method 2018.06 of the Association of Official Analytical Chemists for infant formula (Jaudzems et al. 2019) adapted by Sousa et al. (2023). An ultra-high-performance liquid chromatograph coupled to an ultraviolet detector (Vanquish, Thermo Scientific) and equipped with an Acquity UPLC BEH C18 column (150 mm length, 2.1 mm internal diameter, 1.7 µm particle size, Waters) was used. In addition, the amino acid profile of the undigested microalga was analyzed following the same procedure (Jaudzems et al. 2019). Finally, the in vitro digestibility of proteins and amino acids was calculated based on the proportion of total and individual amino acids found in the soluble and insoluble digested fractions (Sousa et al. 2023).

The in vitro digestible indispensable amino acid ratio (DIAAR) was calculated according to FAO recommendations (FAO 2013). The in *vitro* digestible indispensable amino acid content was divided by the amino acid pattern of a reference protein to reflect the human dietary requirements for indispensable amino acids in (i) infants (birth to 6 months), (ii) young children (6 months to 3 years), and (iii) older children, adolescents, and adults. The DIAAS is the lowest DIAAR calculated for the sample, and the amino acid corresponding to this value is considered the first limiting amino acid.

Volatile organic compounds

The VOC profiles of the lyophilized microalga powder were determined using a 7890 B gas chromatograph (GC) coupled to a 5977 A mass spectrometer (MS) equipped with an HP-FFAP capillary column (50 m × 200 µm × 0.33 µm, Agilent, USA). The column flow was 1.44 mL min^{-1}, and the GC oven was operated at 40 °C for 6 min, heated at a rate of 10 °C min^{-1} to 250 °C, and held for 3 min. The transfer line and ion source temperature were set to 250 °C, and the MS full scan mode operated in the 40–400 m/*z* range with hydrogen as the carrier gas (hydrogen generator Cosmos MF.H2, F-DGSi, Lieusaint, France). The samples were extracted following the vacuum in-tube extraction (V-ITEX) method described by by Fuchsmann et al. (2019). A multipurpose sampler with commercial in-tube extraction (ITEX) tool equipped with a Tenax TA/Carbosieve SIII filled trap (Gerstel, Switzerland) was modified with two valves and additional flow paths as described by Fuchsmann et al. (2019) to perform the V-ITEX. The samples were incubated at 60 °C for 10 min and shaken at 500 rpm to reach equilibrium, then the gas phase of the samples was extracted under vacuum at 60 °C for 5 min. The vacuum pump (V-300, Büchi, Switzerland) was operated at 5 mbar. VOC desorption into the cooled injection system (CIS) equipped with a Tenax filled liner (both Gerstel, Switzerland) was conducted by heating the ITEX trap to 300 °C with a desorption flow of 150 mL min^{-1} for 4 min. The solvent vent flow for the CIS operation was 20 mL min^{-1}. The CIS temperature program started at 10 °C, held for 4 min during desorption, and then increased to 300 °C at a rate of 12 °C s^{-1}. After each sample, the ITEX trap was cleaned at 300 °C for 10 min under hydrogen flow. VOC identification with the MS data was conducted by comparing the MS spectra with the NIST database (Version NIST23, National Institute of Standards and Technology, Gaithersburg, USA), as recommended by the Metabolomics Standard Initiative (Sumner et al. 2007). Additionally, the retention indices listed in the NIST database were compared to the retention indices obtained during the analyses with an allowed deviation of ± 10. For this purpose, an alkane mix (Merck, Switzerland) was measured with the same analytical method as the samples.

Statistical analysis

The reported data represent the means of three independent bead milling followed by analytical experiments using *C. saccharophilum* biomass from three biologically independent cultivations. Student's t-tests were used to determine significant differences between two datasets (e.g., undisrupted vs. disrupted samples), while one-way analysis of variance (ANOVA) was conductes to determine significant differences between more than two datasets (e.g., pigment extraction as a function of time). All statistical analyses were performed at a confidence level of 95% ($\alpha = 0.05$).

Results

Cell disruption

The disruption of *C. saccharophilum* cells by bead milling using the NanoWitt-100 was evaluated by determining the particle size class distribution (Fig. 1) and pigment extraction capacity (Fig. 2) as a function of the processing time and concentration of the cell suspensions. All initial samples had particles (i.e., whole cells) with an average size of approximately 6 µm. In addition, the more concentrated samples showed cell aggregates of approximately 30 µm (Fig. 1; Supplemetary Material). At the end of the bead milling process, the size class of the majority of particles in the

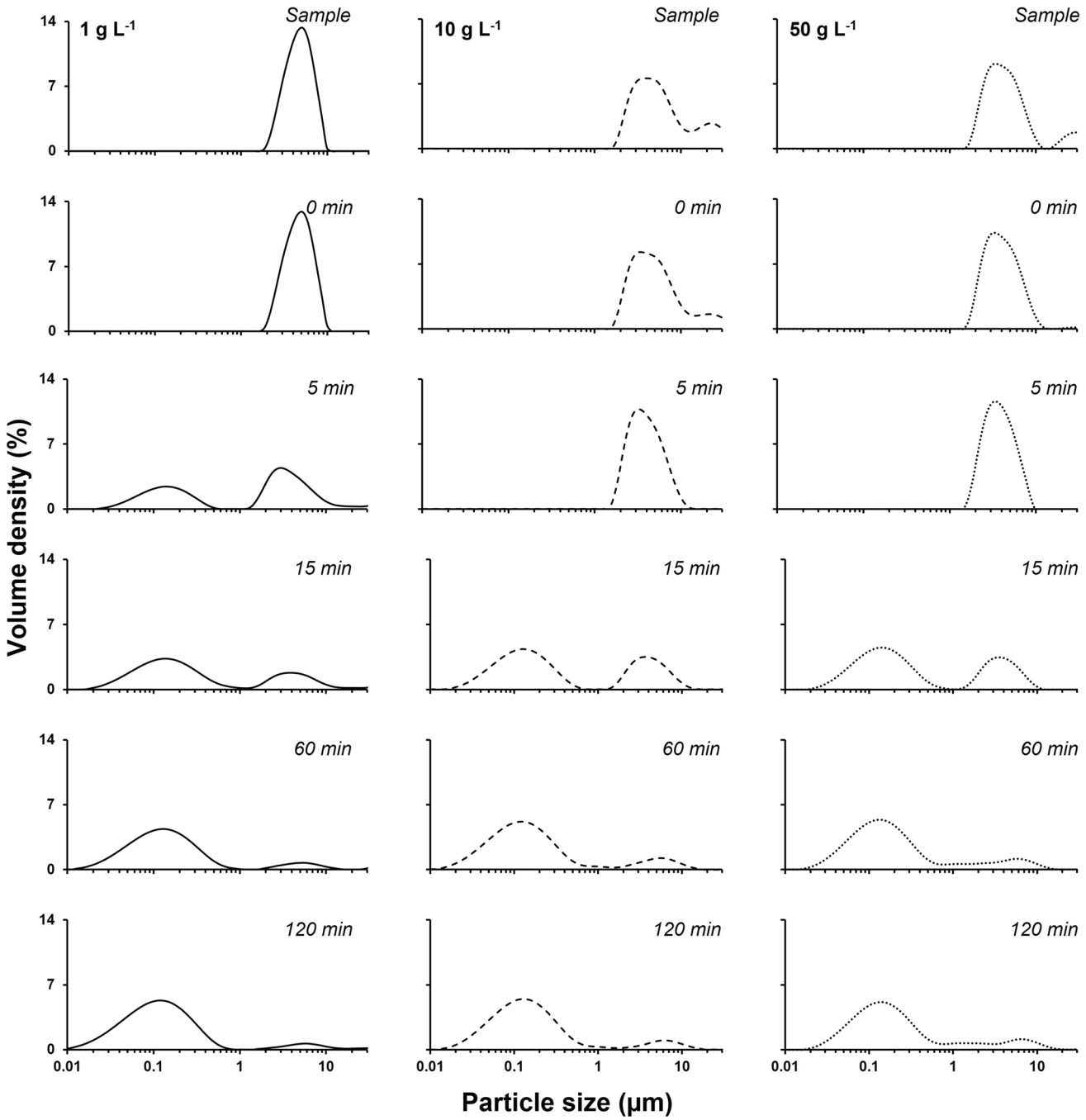

Fig. 1 Particle size distribution of *Chloroidium saccharophilum* cell suspensions at 1 (continuous lines), 10 (dashed lines), and 50 (dotted lines) g L^{-1} as a function of the bead milling time. The curves represent the average of three independent measurements ($n = 3$)

suspension (i.e., cell fragments) was around 100 nm in all samples. The changes in particle size distribution over the bead milling time were similar, except that at the concentration of 1 g L^{-1}, particles of about 100 nm were detected after 5 min, whereas in the concentrated samples, they were not detected until 15 min.

The absolute values of the pigments extracted from the dry microalgal biomass varied between treatments and were 20 to 40% lower in the 10 g L^{-1} concentration. Nevertheless, the effects of the bead milling time were observed separately for each treatment (Fig. 2). In all cases, bead milling led to a higher ability to extract pigments from dry biomass. However, this trend was not statistically significant in the 1 g L^{-1} treatment ($p > 0.05$). In the two other treatments, pigment extraction reached its maximum after 15 min of bead milling, which was almost

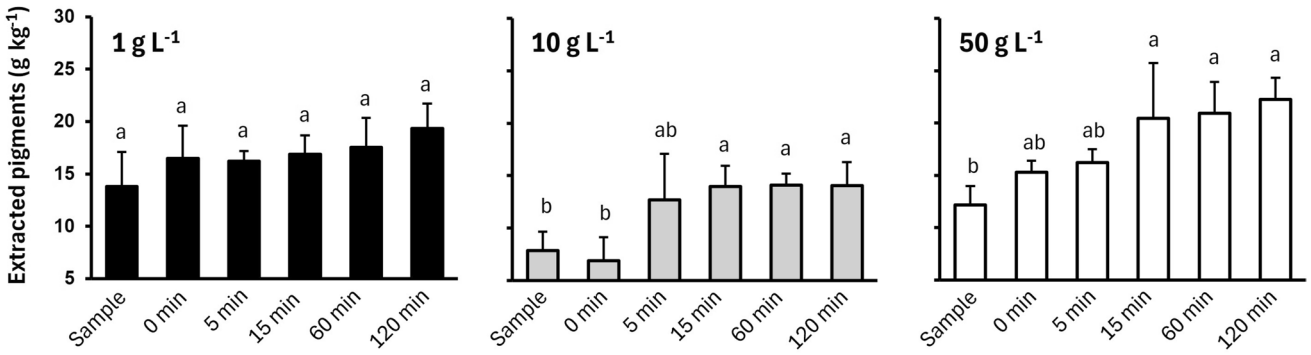

Fig. 2 Pigment extraction of *Chloroidium saccharophilum* cell suspensions at 1 (black bars), 10 (gray bars), and 50 (white bars) g L^{-1} as a function of the bead milling time. The bars represent the average of three independent measurements ($n=3$), and error bars show standard deviation (±SD). The different superscript letters above bars in the same graph represent significant differences ($p<0.05$), where a > b

twofold higher than that of the initial undisrupted samples. The excessive processing time investigated (i.e., 60 and 120 min) was not detrimental to the disruption process.

Based on the improvements from increasing the concentration of the cell suspension and minimizing the processing time, the effects of mechanical cell disruption on the protein digestibility and aroma of dry *C. saccharophilum* biomass were investigated by comparing the initial undisrupted sample to 50 g L^{-1} samples after at least 15 min of milling.

Effects on protein and amino acid digestibility

The total protein digestibility increased significantly ($p<0.001$) in the disrupted biomass compared to that in the initial sample, rising from 62.2 ± 2.28 to 99.7 ± 0.06%. The individual digestibility of all essential and non-essential amino acids increased significantly (Table 2), except for that of cysteine, which was fully digestible in the undisrupted sample (100% digestibility). Leucine was the least digestible amino acid in the undisrupted sample (33.1 ± 4.87% digestibility). By contrast, most amino acids were completely digestible in the disrupted biomass. The only exceptions were methionine, tryptophan, and tyrosine, with digestibilities of 99.5 ± 0.26%, 98.3 ± 2.92%, and 89.3 ± 1.10%, respectively.

The in vitro DIAAR of each indispensable amino acid was calculated based on its content on the biomass, their digestibility, and the human amino acid requirements for different life stages proposed by the FAO (2013). These values enable the qualitative evaluation of a protein source. All DIAAR values increased after cell disruption, except for the sulfur-containing amino acids (Fig. 3). The DIAAS represents the lowest DIAAR value and objectively evaluates the protein quality of a food rather than its quantity. The DIAAS of *C. saccharopilum* biomass was also substantially enhanced by cell disruption. It varied from 24.4 ± 3.59 to 38.4 ± 5.66 in the undisrupted sample and from 62.2 ± 0.00 to 93.7 ± 0.16 in the disrupted sample, depending on the growth phase (Table 2). The amino acid that determined the DIAAS, called the limiting amino acid, was leucine in the undisrupted sample and isoleucine or sulfur-containing amino acids (methionine + cysteine) in the disrupted sample, depending on the reference growth phase.

Effects on volatile organic compounds

The percentage distribution of all VOC classes detected in *C. saccharophilum* dry biomass increased significantly after cell disruption (Table 3). However, the extent of the increase varied among the different classes of compounds. Esters and alcohols, for example, increased by 4,200% and 530%, respectively, in the disrupted biomass, whereas amides and aldehydes increased by only 58% and 30%, respectively (Fig. 4). Acids were the most abundant VOCs regardless of cell disruption, accounting for 76.6 ± 5.35% and 58.5 ± 0.08% of the total VOCs in the undisrupted and disrupted samples, respectively. Amides were the least abundant, comprising less than 1% of the total VOCs in all treatments. Acetic acid was the most abundant VOC in absolute values, and the ketone 1H-pyrrole-2,5-dione 3-ethyl-4-methyl was the least abundant VOC in both treatments. In total, 25 different VOCs were detected, and three were found exclusively in the disrupted sample: ethyl 9,12-hexadecadienoate, hexadecaenoic acid ethyl ester, and benzofuranone,5,6,7,7α-tetrahydro-4,4,7α-trimethyl. Likewise, the number of ester compounds detected was the highest, and the number of aldehydes and amides was the lowest (Table 3). Among the aroma descriptors proposed for each compound, "fruity" was the most common. The average odor threshold of acids was higher than that of the other compounds.

Table 2 Amino acid profile (in g kg^{-1} of sample) of *Chloroidium saccharophilum* and individual amino acid digestibility (%) of undisrupted and disrupted dry biomass

Amino acid	*C. saccharophilum* FAM 27962 (g kg^{-1})	Digestibility (%)	
		Undisrupted	Disrupted
Essential			
Histidine	8.78 ± 0.08	76.6 ± 5.58	100 ± 0.00
Isoleucine	18.6 ± 0.10	44.9 ± 4.75	100 ± 0.00
Leucine	38.5 ± 0.10	33.1 ± 4.87	100 ± 0.00
Lysine	29.4 ± 0.15	73.0 ± 2.02	100 ± 0.00
Methionine	6.81 ± 0.17	96.0 ± 0.57	99.5 ± 0.26
Phenylalanine	23.6 ± 0.20	43.6 ± 2.42	100 ± 0.00
Threonine	21.9 ± 0.15	54.6 ± 2.72	100 ± 0.00
Tryptophan	7.66 ± 0.19	74.4 ± 9.37	98.3 ± 2.92
Valine	23.6 ± 0.06	48.9 ± 5.50	100 ± 0.00
Non-essential			
Alanine	32.3 ± 0.12	42.4 ± 3.05	100 ± 0.00
Arginine	29.8 ± 0.17	69.8 ± 2.66	100 ± 0.00
Asparagine	45.2 ± 0.46	93.3 ± 5.91	100 ± 0.00
Cysteine	4.94 ± 0.02	100 ± 0.00	100 ± 0.00
Glutamine	39.6 ± 0.70	64.1 ± 2.71	100 ± 0.00
Glycine	27.1 ± 0.15	75.1 ± 1.77	100 ± 0.00
Proline	25.6 ± 0.12	63.2 ± 1.91	100 ± 0.00
Serine	19.3 ± 0.17	71.9 ± 6.96	100 ± 0.00
Tyrosine	16.9 ± 0.12	39.6 ± 2.87	89.3 ± 1.10
DIAAS/limiting amino acid			
Infant (birth to 6 months)		24.4 ± 3.59/LEU	62.2 ± 0.00/ILE
Child (6 months to 3 years)		35.5 ± 5.23/LEU	79.8 ± 0.14/SAA
Older child, adolescent, adult		38.4 ± 5.66/LEU	93.7 ± 0.16/SAA

The reported data are the mean and standard deviation (± SD) of three independent measurements. DIAAS = digestible indispensable amino acid score; LEU = leucine; ILE = isoleucine; SAA = sulfated amino acids (methionine + cysteine)

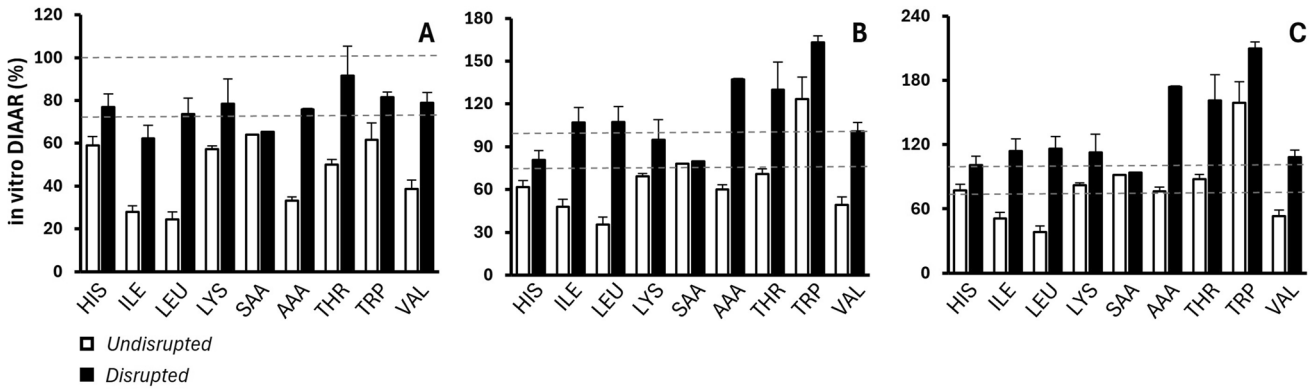

Fig. 3 Comparison of the in vitro digestible indispensable amino acid ratio (DIAAR) between whole cells (white bars) and disrupted biomass (black bars) based on the reference protein requirements for humans at 0 to 6 months (**A**), 6 months to 3 years (**B**), and older than 3 years old (**C**). The dotted gray lines show the protein quality categories according to the FAO (2013) where 75–99 is good and above 100 is excellent. The reported data are the mean and standard deviation (± SD) of three independent measurements. HIS = histidine; ILE = isoleucine; LEU = leucine; LYS = lysine; SAA = sulfated amino acids (methionine + cysteine); AAA = aromatic amino acids (phenylalanine + tyrosine); THR = threonine; TRP = tryptophan; VAL = valine

Table 3 Absolute abundance of volatile organic compounds (in arbitrary area units) of undisrupted and disrupted *Chloroidium saccharophilum* dry biomass. The aroma (www.thegoodscentcompany.com) and odor threshold (http://www.leffingwell.com/odorthre.htm) of each compound are reported

VOC	Undisrupted peak area (AAU·10^3)	Disrupted peak area (AAU·10^3)	Substantivity	Odor threshold (ppb)
Acids				
Acetic acid	1,198 ± 280	4,554 ± 170	Sharp, pungent, sour, vinegar	-
Butanoic acid	118 ± 17.3	868 ± 72.2	Sharp, acetic, cheesy, buttery, fruity	240
Hexanoic acid	4.76 ± 0.68	13.6 ± 3.46	Sour, fatty, sweaty, cheesy	3,000
Propanoic acid	919 ± 81.9	3,568 ± 134	Pungent, acidic, cheesy, vinegar	20,000
Alcohols				
1-Hexanol	12.6 ± 1.41	25.8 ± 1.04	Pungent, ethereal, fusel, oily, fruity, banana	2,500
1-Pentanol	13.4 ± 1.43	105 ± 5.66	Pungent, fermented, bready, yeasty, fusel, winey, solvent	4,000
1-Penten-3-ol	3.97 ± 0.11	57.6 ± 3.03	Ethereal, horseradish, green, radish, chrysanthemum, vegetable, tropical, fruity	400
Aldehydes				
Hexanal	68.1 ± 12.6	76.5 ± 3.36	Fresh, green, fatty, aldehydic, grassy, leafy, fruity, sweaty	4.75
Alkenes				
1-Heptadecene	61.9 ± 22.9	234 ± 0.27	-	-
8-Heptadecene	371 ± 84.3	873 ± 8.91	-	-
Amides				
Acetamide	11.7 ± 0.55	18.5 ± 0.20	-	-
Esters				
Butanoic acid ethyl ester	19.9 ± 1.96	1,963 ± 121	Fruity, juicy, pineapple, cognac	1
Butanoic acid propyl ester	2.70 ± 0.09	100 ± 4.94	Fruity, sweet, apricot, pineapple, rancid, sweaty	18–124
Ethyl acetate	27.5 ± 0.85	296 ± 19.8	Ethereal, fruity, sweet, weedy, green, grape, rummy	2,500
Ethyl 9,12-hexadecadienoate	-	120 ± 1.23	-	-
Hexadecanoic acid ethyl ester	-	38.3 ± 4.03	Waxy, fruity, creamy, milky, balsamic, greasy, oily	2,000
Hexanoic acid ethyl ester	2.17 ± 0.03	230 ± 11.3	Sweet, fruity, pineapple, waxy, green, banana	1
Pentanoic acid ethyl ester	2.12 ± 0.13	246 ± 6.49	Sweet, fruity, apple, pineapple, green, tropical	1.5–5
Propanoic acid butyl ester	5.84 ± 0.16	93.9 ± 11.9	Sweet, estery, banana, ripe, tutti frutti, cherry, rummy	25–200
Propanoic acid ethyl ester	52.8 ± 1.15	1,848 ± 93.1	Sweet, fruity, rummy, juicy, grape, pineapple	10
Ketones				
α-Ionone	3.67 ± 0.22	10.2 ± 0.02	Orris, violet, powdery, woody	-
1H-Pyrrole-2,5-dione 3-ethyl-4-methyl	0.22 ± 0.03	0.82 ± 0.07	-	-
2(4H)-Benzofuranone,5,6,7,7α-tetrahydro-4,4,7α-trimethyl	-	14.0 ± 0.93	-	-
3-Buten-2-one,4-(2,6,6-trimethyl-1-cyclohexen-1-yl)	11.2 ± 0.82	28.9 ± 0.86	Floral, woody, sweet, fruity, berry, tropical, beeswax	-
3,5-Octadien-2-one	3.57 ± 0.07	8.61 ± 0.86	Fruity, green, grassy	-

The reported data are the mean and standard deviation (± SD) of three independent measurements. All values were significantly higher in the disrupted biomass ($p < 0.05$)

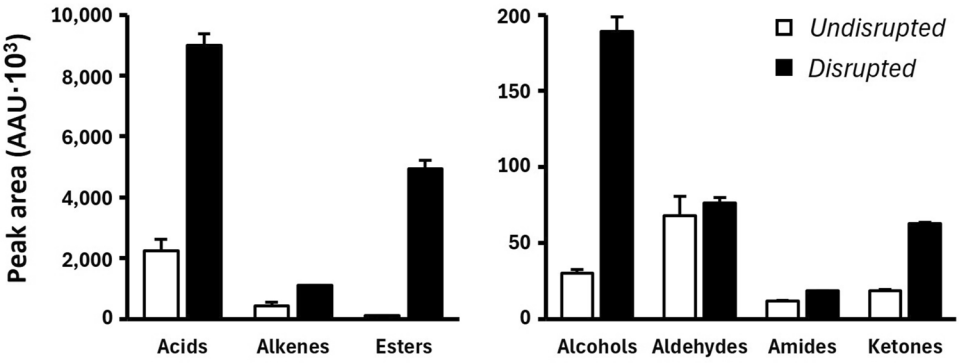

Fig. 4 Volatile organic compound (VOC) classes on the undisrupted (white bars) and disrupted (black bars) dry biomass of *Chloroidium saccharophilum*. The bars represent the average of three independent measurements ($n = 3$) and error bars show standard deviation ($\pm$ SD)

Discussion

Bead milling for microalgal biotechnology

Bead milling is a type of mechanical grinding that uses the shear forces created when beads collide with themselves and with cells to break down cell walls in a non-specific manner and release intracellular compounds. Process efficiency depends on a wide array of parameters, including the size and composition of the beads, the grinding chamber design, and the rotor or agitator speed. Previous comprehensive instrumental optimization studies have shown that the efficiency of bead milling cell disruption decreases with increasing bead size, and that increasing rotor speed and bead chamber filling improves cell disruption only up to a certain point (Montalescot et al. 2015; Zinkoné et al. 2018). The instrumental parameters selected here (flow rate of 500 mL min^{-1}, rotor speed of 4.7 m s^{-1}, bead size of 0.1–0.2 mm, and 60% bead chamber filling) were based on a literature review and on the authors' previous experience with other biological materials (frewitt.com). Still, the bead mill NanoWitt-100 can benefit from a case specific optimization of these parameters on future studies.

In addition to instrumental parameters, the morphology and composition of the cells, as well as the concentration and viscosity of the cell suspension, affect the outcomes of the disruption process (Günerken et al. 2015). Furthermore, the variety of milling equipment available and the huge diversity of microalgal species and strains have led to heterogeneous and sometimes contradictory results in the literature, even for the same species (Montalescot et al. 2015). For example, positive and negative results have been reported when increasing the concentration of a *Chlorella vulgaris* cell suspension for bead milling (Doucha and Lívanský 2008; Postma et al. 2015). As a result, drawing general conclusions or comparing findings with currently available information is challenging. In theory, the concentration of the microalgal suspension plays a crucial role in determining the efficiency of cell disruption. Moderately increasing it enhances the number of cells per unit volume. This, in turn, increases the frequency of collisions between cells and grinding beads, thereby improving disruption efficiency. Additionally, a higher concentration increases the suspension's viscosity, promoting more stable and effective bead movement. This generates stronger shear forces and facilitates cell breakage. However, excessively high concentrations can result in excessive viscosity, which limits bead mobility and reduces energy transfer efficiency, thereby negatively affecting disruption performance. High concentrations also tend to promote cell aggregation, leading to uneven disruption and reduced accessibility of intracellular components. Therefore, optimizing the concentration of the microalgal suspension is essential to achieving high disruption efficiency, maximizing downstream processing yields, and minimizing energy consumption.

From a biotechnological perspective, optimizing processing time and biomass concentration has major economic and energy-related relevance. The former determines the minimum time required to obtain the ideal cell extract, whereas the latter indicates the necessity of preconcentration steps. Using the Nanowitt-100 with the described parameters, we found that the cells of *C. saccharophilum* were efficiently disrupted after 15 min, regardless of the initial cell concentration (Fig. 1). The process was faster at a concentration of 1 g L^{-1} of cell suspension, which can be naturally achieved in cultures. Pigment extraction, used as a proxy to evaluate cell disruption, did not increase significantly in the 1 g L^{-1} sample but nearly doubled in the two other treatments (Fig. 2). Therefore, our results suggest that a preconcentration step is preferable, as concentrations of 10 and 50 g L^{-1} are not naturally achievable in green algal cultures. Similar results were found for *Chlorella* sp., *Nostoc* sp., and *Tolypothrix* sp. when lipid extraction was used as a proxy (Prabakaran and Ravindran 2011). Visual determination of cell disruption through optical microscopy or flow cytometry reported > 90% disruption of *Chlorella* cells (Doucha and Lívanský 2008; Postma et al. 2015), underlining the high disruption efficiency of this technique. Based on the disruption efficiency results obtained in this study, bead milling for at least 15 min at a cell concentration of 50 g L^{-1} was deemed optimal. Therefore, this

treatment was used as the disrupted biomass for the digestibility and VOCs comparisons. While this study primarily focused on cell disruption efficiency, other factors must be considered when evaluating bead milling as a method for microalgae downstream processing. High throughput, disruption efficiency, scalability, and low labor intensity are some characteristics that highlight the potential of implementing bead milling in microalgal biotechnology (Günerken et al. 2015). However, the high energy demand, heat generation, and nonselective distribution of compounds over the soluble and solid phases need further investigation and improvement (Günerken et al. 2013).

Protein quantity and quality

Microalgae are often considered a valuable source of protein for human and animal nutrition because of their high biomass protein content, which can reach up to 70%. The available data indicate that proteins from unprocessed microalgae are generally bioavailable for digestion (Williamson et al. 2024). This essentially depends on the structural characteristics of the microalgal cells, such as the presence and composition of cell walls. The *Chlorella*-like coccoid cells used in this study, *C. saccharophilum*, were recently reclassified as belonging to the genus *Chloroidium* (Darienko et al. 2010). Previously, they were classified as *Chlorella* species due to their morphological similarities. Both have thick, bilayered cell walls made of polysaccharide microfibrils. However, it is not clear whether *C. saccharophilum* has a trilaminar outer layer, similar to true *Chlorella* strains (Baudelet et al. 2017).

Given the presence of a thick cell wall, it has been hypothesized that downstream processing, such as bead milling cell disruption, could improve the digestibility of proteins in the biomass. In theory, the disintegration of cell walls should improve biomass digestibility. However, the results are difficult to predict because anti-nutritional factors may be released and interact with proteins or digestive enzymes, inhibiting their activity (Hammer et al. 2023). Our results revealed that bead milling led to nearly complete protein digestibility in the disrupted biomass, whereas only about 60% of the proteins in intact cells were digested. Interestingly, the most digestible amino acids in the undisrupted biomass were the sulfur-containing amino acids cysteine (100% digestibility) and methionine (95% digestibility). Further research on the kinetics of peptide formation during digestion is needed to better understand this behavior. Nevertheless, this highlights the importance of considering individual amino acid digestibility rather than total protein digestibility in nutritional analyses.

Using five different commercial products based on *Chlorella*, Wild et al. (2018) reported that cell disruption through a ball mill increased protein digestibility from 72% – 86% to 80% – 94%. The authors also demonstrated that the average protein digestibility increased from 74 to 78% following cell disruption in *Limnospira platensis*, from 54 to 79% in *Nannochloropsis*, and from 77 to 83% in *Phaeodactylum tricornutum*. Interestingly, these findings suggest that an increase in digestibility is related to cell wall robustness. For example, *L. platensis* has a thin cell wall made of peptidoglycans; *Chlorella* spp. and *Nannochloropsis* spp. have thick polysaccharide-rich cell walls; and *P. tricornutum* has a siliceous frustule (Van Den Hoek et al. 1995). However, it should be noted that cultivation and processing conditions may substantially affect microalgal cell wall thickness and resistance to mechanical disruption; this variability is due to microalgal metabolic plasticity, even within the same species (Yap et al. 2016). Therefore, these conditions must be carefully considered when comparing results across studies.

The nutritional value of a protein source depends not only on its protein content but also on its protein quality. Protein quality is determined by the composition of indispensable amino acids and their postprandial bioavailability. The digestibility and DIAAR values of all essential and non-essential amino acids increased in the disrupted microalgal biomass, and consequently, the DIAAS also increased (Table 2). Protein quality is considered excellent if the DIAAS is 100 or greater, indicating that the bioavailability of the first limiting amino acid is equal to or greater than that of the reference protein source. It is considered good if the DIAAS is between 75 and 99 (FAO 2013). Based on this classification, unprocessed *C. saccharophilum* biomass cannot be considered a good source of proteins. Bead milling disruption of *C. saccharophilum* cells made its biomass a good source of proteins (DIAAS > 75) for children, older children, adolescents, and adults. Unfortunately, no DIAAS values have been reported for microalgae for comparison purposes (Williamson et al. 2024). In our study, the undisrupted microalga sample showed relatively low DIAAR values of all essential amino acids, except for tryptophan. In contrast, the disrupted biomass showed limited availability of sulfated amino acids, especially for older children, adolescents, and adults (Fig. 3). In this case, supplementation may help achieving an excellent protein quality. Microalgae are typically exploited commercially as an ingredient or supplement in mixed meals rather than as a standalone food. Therefore, a balanced composition of essential amino acids can be achieved through the combination of complementary dietary proteins. For example, insects, which are a novel protein source like microalgae, have been proposed as a complement to increase the lysine content of cereal-based diets (Hammer et al. 2023).

Volatile organic compounds and biomass aroma

VOCs have been relatively unexplored in microalgae, although they can contribute significantly to the organoleptic characteristics of algal biomass and therefore determine its final application (Van Durme et al. 2013). Notably, aroma plays a central role in consumer acceptance. Furthermore, VOC profiles provide valuable insights into microalgal metabolism, as they originate from multiple metabolic pathways, primarily amino acid and fatty acid conversion (Hosoglu 2018). To the best of the authors' knowledge, this is the first study to investigate the impact of cell disruption through bead milling on microalgal VOC profiles. The concentration of all VOC classes increased in the disrupted biomass. In microalgae, esters can form through the action of microorganisms in the short term or through the esterification of alcohols and acids in the longer term. These compounds can be responsible for the unpleasant odor produced by some algae and have been shown to accumulate during the death phase of marine microalgae (Zhou et al. 2017). However, most esters detected in this study were characterized as having a sweet, fruity aroma (Table 3). Due to their relatively high odor thresholds, assessing whether the resulting aroma of the biomass changes significantly after cell disruption is difficult. Nonetheless, during bead milling experiments, a more pronounced grassy odor could be perceived in the disrupted samples (personal communication). It has been proposed that the poor palatability of certain algae when fed to animals could be due to their aroma (Lamminen et al. 2019). Preliminary results from Agroscope indicate that pigs seem to prefer feed mixed with microalgae (unpublished data). However, these experiments were conducted using undisrupted biomass.

Interestingly, three VOCs were detected exclusively in the disrupted sample: ethyl 9,12-hexadecadienoate, hexadecanoic acid ethyl ester, and benzofuranone,5,6,7,7α-tetrahydro-4,4,7α-trimethyl. The first two originate from the esterification of long-chain fatty acids and are commonly found in fruits and wine, whereas the latter is a benzofuran with biological activity in photosynthetic organisms (Margl et al. 2005). These compounds were unlikely produced during bead milling. Therefore, they were probably protected by the cell envelope and were not released in whole cells.

Acids were the most abundant among VOC classes determined with the described analytical method in *C. saccharophilum* undisrupted and disrupted samples, accounting for 60% to 80% of the total VOCs. These compounds have characteristic sharp, sour, and cheesy aromas that can be unappealing at high concentrations (Colonia et al. 2023). Consistent amounts of alcohols, aldehydes, amides, and ketones were detected in both undisrupted and disrupted *C. saccharophilum* biomass, accounting for less than 2% of the total VOCs. This indicates that, despite their relatively low odor threshold, these compounds have a limited influence on the overall aroma profile of this microalga. In the disrupted biomass, alkenes were almost twice as high as in the undisrupted biomass, while esters increased by more than 40-fold after mechanical cell disruption. Alkanes, alkenes, and alkynes generally have a high odor threshold and minimal impact on the final aroma (Zhou et al. 2017). By contrast, esters may contribute sweet, fruity, and sometimes ethereal and creamy notes to the final aroma. Therefore, the data suggest that bead milling cell disruption improves the aroma of *C. saccharophilum* biomass by reducing the concentration of acids and increasing the concentration of esters. However, sensory analysis and in vivo trials are still needed to confirm these findings.

Conclusion

Robust and scalable cell disruption machinery is essential for developing the microalgal biotechnology sector and implementing a biorefinery framework. We tested the bead mill Nanowitt-100 to disrupt the cells of the green microalga *C. saccharophilum* FAM27962, which is known to have a thick polysaccharidic cell wall. The optimal processing time and concentration of the cell suspension for the bead milling batch process were 15 min and 50 g L^{-1}, respectively. Under these conditions, cell disruption resulted in complete protein digestibility of the resulting biomass, compared to about 60% digestibility in the undisrupted biomass. The VOC profile shifted, with a notable increase in esters, which are closely associated with sweet and fruity aromas, compared to other compounds. Taken together, the findings highlight the potential of bead milling with the NanoWitt-100 as an effective and scalable strategy for improving the digestibility and sensory properties of microalgal biomass for food and feed applications. However, further research on sensory analysis and in vivo trials is necessary.

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Declarations

Competing interests Authors XG and CR are employed by Frewitt Ltd., the manufacturer and seller of NanoWitt-100 bead mill tested in this work.

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