Impact of different sequences of mechanical and thermal processing on the rheological properties of *Porphyridium cruentum* and *Chlorella vulgaris* as functional food ingredients

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Microalgae are a promising and sustainable source for enhancing the nutritional value of food products. Moreover, incorporation of the total biomass might contribute to the structural properties of the enriched food product. Our previous study demonstrated the potential of *Porphyridium cruentum* and *Chlorella vulgaris* as multifunctional food ingredients, as they displayed interesting rheological properties after applying a specific combination of mechanical and thermal processing. The aim of the current study was to investigate the impact of a different sequence of high pressure homogenization (HPH) and thermal processing on the thickening and gelling potential of these microalgal biomasses in aqueous suspensions. Thermal processing largely increased the gel strength and viscosity of both microalgae, which was ascribed to larger and stronger aggregates as a result of partial solubilization of polymers, while subsequent HPH generally reduced the rheological properties. Interestingly, large amounts of intact cells were still observed for both microalgae when HPH was performed after a thermal treatment, irrespective of the applied homogenization pressure, implying that cell disruption was hindered by the preceding thermal treatment. Although thermal processing was regarded as the most effective processing technique to obtain increased rheological properties, the combination with a preceding HPH treatment should still be considered when cell disruption is desired, for instance to increase the bioavailability of intracellular components. Finally, biomass of *P. cruentum* showed the largest potential for use as a structuring agent, as the gel strength and viscosity in thermally treated suspensions of this microalga were about 10 times higher than for *C. vulgaris*.

Introduction

One of the main challenges for the food industry is the development of food products with improved quality, including nutritional, functional and sensory characteristics. In this context, research is seeking for natural and sustainable ingredients in the design of novel foods. Recently, microalgae are receiving more attention as functional food ingredients, since they are a promising source of several nutritional and health-beneficial components. In fact, several microalgae are rich in omega-3 long chain polyunsaturated fatty acids (ω 3-LC-PUFA), including eicosapentaenoic acid (EPA, C20:5 ω 3) and docosahexaenoic acid (DHA, C22:6 ω 3), which have proved successful in the prevention of cardiovascular diseases and contribute to brain development. Furthermore, microalgae are regarded as an interesting protein source, due to their

combination of large protein contents and favorable amino acid profiles. In addition, several other health-beneficial components are present in substantial amounts, such as vitamins, antioxidants and dietary fibers ¹⁻³. Besides, autotrophic microalgae are also considered a sustainable biomass source, due to their high photosynthetic efficiency and no competition with conventional crops for arable lands ². Even though research has mainly focused on the extraction of specific components from microalgae, the incorporation of the total dried biomass into food products would be a more sustainable approach to introduce these nutritional and health-beneficial compounds in our diet.

The use of microalgal biomass for enhancing the nutritional value of food products might affect the rheological properties, another important quality aspect of food products. Our previous screening study showed a large diversity in the rheological properties of different microalgae species in aqueous suspensions ⁴. Some microalgal biomasses showed negligible structural properties and could therefore easily be introduced in food products, without affecting the rheological properties of the enriched food product. In contrast, other microalgae species presented large viscosities and gelling properties, showing potential to be used as a multifunctional ingredient by combining health-related and structural properties in the food product ⁴. Since this approach would

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allow a reduction of conventional thickening or gelling agents, it would increase the economic feasibility of microalgae in food applications.

To alter the thickening or gelling potential of microalgal biomasses, different types of processing could be used. Previous results showed that high pressure homogenization (HPH) at 100 MPa increased the rheological properties of certain microalgae species, by disrupting the cells and allowing the released intracellular material to aggregate into larger particles. Furthermore, the combination of HPH with thermal processing often resulted in the highest viscosity of the microalgal suspensions, especially when an intense sterilization process was applied ⁴. Due to the large diversity among microalgae, specific processing strategies should be selected for a certain microalga species. Moreover, changing the sequence of mechanical and thermal processing might be a strategic approach to influence the rheological properties of microalgal suspensions. In fact, different sequences of mechanical and thermal treatments proved successful for targeting specific rheological properties of plant-based dispersions, such as vegetable based purées. Whereas the largest rheological properties for carrot and broccoli were obtained by blending followed by thermal treatment, the combination of thermal treatment and subsequent HPH was the most effective strategy for tomato purée. Differences in rheological behavior were attributed to microstructural changes of the plant material, including size and morphology of the particles as well as the disruption of the polysaccharidic cell wall ⁵. However, prediction of rheological and microstructural changes upon processing might be even more challenging for microalgal suspensions, due to their higher complexity compared to most plant materials, possessing high amounts of proteins and lipids next to polysaccharide substances ⁶.

Porphyridium cruentum and *Chlorella vulgaris* were selected in the current study, as they showed large potential as a multifunctional ingredient in our previous study ⁴. *P. cruentum* belongs to the red algae (Rhodophyta) and is mainly known for its sulfated exopolysaccharides (EPS). This microalga is considered one of the most promising microalgae species for commercial purposes, since these EPS have shown interesting bioactivities, such as thickening potential and anti-inflammatory effects ¹. In addition, biomass of *P. cruentum* shows a good nutritional profile, containing substantial amounts of EPA (C20:5 ω3), arachidonic acid (C20:4 ω6) and dietary fiber ^{7,8}. *C. vulgaris*, belonging to the green algae (Chlorophyta), is considered an important functional food, being widely sold as a food supplement or nutraceutical. Its high nutritional value is mainly attributed to high protein contents, including all essential amino acids. Moreover, this microalga contains a substantial amount of α-linolenic acid (C18:3 ω3) and it is a source of numerous minerals, vitamins and antioxidants ¹. The aim of this study is to investigate the effect of different sequences of HPH and thermal processing on the structural properties of *P. cruentum* and *C. vulgaris*. Aqueous suspensions at pH 6 were used to simulate low-acid to neutral food systems, such as vegetable based foods and dairy products. The rheological properties of the processed suspensions were

studied, as well as the microstructural changes, to obtain insight into the impact of the applied processing sequences. Combination of these results with previously reported data provides a scientific knowledge base and would allow an appropriate selection of processing treatments for the use of *P. cruentum* and *C. vulgaris* biomasses as multifunctional ingredients in food products.

Materials and Methods

Microalgal biomass

Lyophilized biomass of *Porphyridium cruentum* was obtained from Necton Phytobloom (Olhão, Portugal). Biomass of *Chlorella vulgaris* was obtained as a spray-dried powder from Allmicroalgae Natural Products (Lisbon, Portugal). The biomasses were stored in closed containers at -80 °C until use.

Characterization of the biomass composition

The composition of the biomasses was studied as described by Bernaerts *et al.* (2018) ⁶. In short, moisture content was determined using vacuum-drying and was further used in the calculation of the biochemical composition, to express all components as a percentage of total dry matter. Lipids were extracted with chloroform:methanol (1:1) and the obtained oil was quantified gravimetrically. Total nitrogen content was determined by elemental analysis and multiplied by the conversion factor of 4.78, as proposed by Lourenço *et al.* (2004) ⁹, to estimate the protein content. Storage polysaccharides (SPS) were quantified by enzymatic hydrolysis with α-amylase and amyloglucosidase, followed by quantification of the glucose content in the supernatant, multiplied by 0.9 to correct for the addition of a water molecule during hydrolysis. Ash content was determined gravimetrically, after ashing for 24 h in a muffle furnace at 550 °C.

Cell wall related polysaccharides, including extracellular polysaccharides (EPS) and cell wall bound polysaccharides (CWPS), were quantified as described by Bernaerts *et al.* (2018) ⁶. In short, EPS were extracted in saline solution, followed by ethanol precipitation, dialysis and lyophilization. CWPS were extracted from the residual biomass by disrupting the cells using ultra high pressure homogenization (UHPH) at 250 MPa, followed by removal of lipids (hexane:isopropanol 3:2), starch or floridean starch (α-amylase and amyloglucosidase) and proteins (Subtilisin A protease), and subsequent precipitation with ethanol and acetone. For quantification of EPS and CWPS, polysaccharides were hydrolyzed to monosaccharides using methanolysis and trifluoroacetic acid hydrolysis, which were quantified using high performance anion exchange chromatography combined with pulsed amperometric detection (HPAEC-PAD). Finally, CWPS and EPS content was calculated as the sum of the monosaccharides and uronic acids, including a correction for addition of a water molecule during hydrolysis. All analyses were performed in triplicate.

Preparation and processing of microalgal aqueous suspensions

Microalgal suspensions were prepared similarly as described by Bernaerts *et al.* (2017)⁴, however a different sequence of mechanical and thermal processing was applied. A schematic overview of the preparation and processing of the suspensions in the current study is presented in Fig. 1.

Preparation of microalgal aqueous suspensions Dry biomass was suspended overnight in demineralized water and pH was adjusted to 6 using HCl. Demineralized water was then added to obtain a final concentration of 8 g dry biomass per 100 g suspension (8% w/w), taking into account the moisture content of the biomass powders. A homogenous suspension was created by mixing for 10 min at 6000 rpm with a lab mixer (Silverson, L5M-A, East Longmeadow, MA, USA). The suspensions were prepared in duplicate.

Thermal and mechanical treatments To perform the thermal pretreatments, suspensions were vacuum packed in plastic bags (PET/LDPE, Dacklapack), incubated for 15 min in a water bath at 95 °C and subsequently cooled in an ice bath. Thermally treated suspensions were then mechanically treated using HPH (Panda 2K, Gea Niro Soavi, Parma, Italy), at either 20 MPa or 100 MPa for a single pass.

Separation of the serum phase

The serum phase was isolated by centrifugation of the microalgal suspensions at 25 °C. Whereas suspensions of *C. vulgaris* were centrifuged for 30 min at 10000g (J2-HS centrifuge, Beckman Coulter, Fullerton, CA, USA), sera of the more viscous suspensions of *P. cruentum* were obtained by ultracentrifugation for 60 min at 165000g (Optima XPN-80, Beckman Coulter, Fullerton, CA, USA).

Rheological measurements

Rheological analyses were performed as described by Bernaerts *et al.* (2017)⁴, with minor modifications. Preshear conditions were extended, using 30 s at 20 s⁻¹ followed by 300 s of rest. It was verified by time sweep tests that this resting time was sufficient to ensure a complete recovery of the structure for all samples. Frequency sweep tests were performed to characterize the viscoelastic behavior, using a constant shear strain of 1%. This shear strain was confirmed to be within the linear viscoelastic region for all suspensions by strain sweep tests at a constant angular frequency of 10 rad/s. The flow

behavior was studied using steady-shear measurements, in which 40 measuring points were recorded with each shear rate applied for 20 s. All other parameters were exactly the same as described in Bernaerts *et al.* (2017)⁴. All measurements were performed in duplicate.

Characterization of the microstructure

The microstructure was characterized as described by Bernaerts *et al.* (2017)⁴. On the one hand, particle size distribution was analyzed in duplicate using laser diffraction. On the other hand, the microstructure was visualized by microscopic images, using differential interference contrast (DIC) microscopy. In addition, microscopic staining was used to identify specific biopolymers. Coomassie brilliant blue was used to visualize proteins, by adding 200 µL of Bradford reagent (Sigma-Aldrich) to 0.5 mL of sample. Binding with proteins yielded a blue color, which was visualized by DIC microscopy. Acidic polysaccharides were stained by adding 10 µL of 2% acridine orange solution to 1 mL of sample. The sample was visualized under epifluorescence microscopy using an excitation filter between 460 and 495 nm (X-Cite[®] 120Q, EXFO Europe, Hants, United Kingdom).

Statistical analysis

The microalgal suspensions were processed in duplicate and for each individual sample rheological analyses were performed in duplicate. As a result, these data are presented as the mean of four measurements ± standard error.

Differences between storage modulus, loss modulus and phase angle of (un)processed suspensions, obtained from strain sweep measurements, were statistically analyzed using one-way ANOVA combined with Tukey's test for multiple comparison ($P < 0.05$) with JMP statistical software (JMP Pro 13, Cary, NC, USA). Frequency sweep and flow curves were fitted to rheological models using non-linear regression procedures with SAS statistical software (SAS 9.4, Cary, NC, USA). The estimated model parameters of different samples were statistically compared by use of 95% confidence intervals.

Results and Discussion

Biochemical characterization of the biomass

The biomass profiles of *P. cruentum* and *C. vulgaris* are presented in Table 1. These values were corrected for the

Fig. 1 Schematic overview of the preparation and processing of microalgal aqueous suspensions. Boxes with 'Untreated', 'TT', 'TT+HPH 20' and 'TT+HPH 100' indicate the moment of collecting respective samples. (HPH: high pressure homogenization)

Table 1 Biochemical composition of biomasses of *P. cruentum* and *C. vulgaris*, expressed as percentage of dry matter (%) $\pm$ standard error (n = 3). (SPS: storage polysaccharides; CWPS: cell wall polysaccharides; EPS: exopolysaccharides).

	<i>P. cruentum</i>	<i>C. vulgaris</i>
Lipids	12.6 $\pm$ 0.7	8.2 $\pm$ 0.5
Proteins	28.0 $\pm$ 0.6	45.7 $\pm$ 0.8
SPS	4.4 $\pm$ 0.1	2.1 $\pm$ 0.2
CWPS	14.0 $\pm$ 0.5	11.5 $\pm$ 0.2
EPS	2.7 $\pm$ 0.1	0.6 $\pm$ 0.1
Ash	22.3 $\pm$ 0.8	7.6 $\pm$ 0.3

residual moisture content in the biomasses of *P. cruentum* and *C. vulgaris*, being 3.36% and 0.49% respectively. The biomasses had a similar composition as different batches in our previous study ⁶. *C. vulgaris* was rich in proteins, accounting for almost half of the dry matter of the biomass (45.7%), which was also previously reported by several authors ^{10,11}. In fact, biomass of *C. vulgaris* has been commercialized as a protein source with high nutritional value, due to its favorable amino acid profile containing high amounts of essential amino acids ^{2,12}. In addition, a considerable fraction of *C. vulgaris* biomass was represented by CWPS (11.5%), while lower amounts of lipids (8.2%) and ash (7.6%) were observed. In contrast, biomass of *P. cruentum* contained a very high ash content (22.3%) and substantial amounts of lipids (12.6%). The higher ash content can be attributed to the cultivation conditions, since *P. cruentum* is a marine microalga species cultivated in a salt-rich medium, in contrast to the freshwater species *C. vulgaris*. With large amounts of calcium and iron ⁶, *P. cruentum* biomass can be considered as a good mineral source. Proteins and CWPS represented 28.0% and 14.0% of *P. cruentum* biomass, respectively, in accordance with previous reports ^{7,8}. It has been proven that large amounts of these CWPS serve as dietary fiber, another nutritional added value of this biomass ⁸. In addition, both microalgae species displayed extracellular polymers, of which the polysaccharide fraction was quantified in this study as EPS. As a consequence, both biomasses contained large amounts of structural biopolymers, which could play an important functional role in obtaining desired structural properties.

Effect of processing on the rheological characteristics

Different rheological properties of the processed microalgal suspensions were analyzed. On the one hand, the linear viscoelastic behavior was studied using small amplitude oscillatory measurements, to gain insight into the network structure of the samples. Since small deformations were applied within the linear viscoelastic region, the undisturbed structure of the suspensions could be analyzed. On the other hand, larger deformations were applied to study the flow behavior, which is more related to several food operations such as mixing, pumping and swallowing of food products ^{13,14}.

Linear viscoelastic behavior: strain sweep The linear viscoelastic behavior was studied using strain sweep and frequency sweep tests. The former measurement is typically

performed to determine the linear viscoelastic region. In addition, results obtained from strain sweep tests provide information on the network structure of the sample. The obtained strain sweep curves are presented in **Fig. 2A** and **2B**. The linear viscoelastic region could be clearly observed for all suspensions as a plateau at low shear strains, with storage modulus (G') and loss modulus (G'') independent of the shear strain. In **Fig. 2C** and **2D**, both moduli are presented for the suspensions at a constant shear strain of 1%, within this linear viscoelastic region. Whereas the absolute value of G' provides information on the stiffness of the structure, the viscoelastic behavior is determined by the ratio of both moduli, which is presented in **Fig. 2C** and **2D** as the phase angle $\delta = \tan^{-1}(G''/G')$. Since all (un)processed suspensions of *P. cruentum* presented values of $\delta < 45^\circ$, they all displayed predominantly elastic behavior. As observed in our previous study ⁴, untreated suspensions of *P. cruentum* presented substantial gelling properties, as can be seen from the large storage modulus (113 Pa) and a phase angle of 12.6° . This behavior was ascribed to its sulfated EPS, as they are known to form a weak gel structure or stable interactions resulting in the formation of large cell clusters ^{4,15}. Even though the concentration of EPS was not very high (**Table 1**), a strong network structure was observed, probably resulting from their unique molecular structure. Thermal treatment of *P. cruentum* suspensions resulted in an increased gel stiffness, as the value of G' was almost doubled. Interestingly, the gelling behavior was not influenced, since no significant differences were observed between the phase angle of the untreated and thermally treated suspensions. With a storage modulus of 208 Pa and a phase angle of 11.5° , the thermally treated suspension of *P. cruentum* can be considered as a strong gel structure. The use of HPH resulted in a drastic decrease of the network structure, given the large reduction of the storage modulus and an increased phase angle. However, the extent to which the structure was destroyed was depending on the applied homogenization pressure. While a lower pressure of 20 MPa resulted in a partial breakdown, the network structure was completely destroyed after HPH at 100 MPa. In fact, the latter suspension presented similar values of G' and G'' , resulting in a phase angle of approximately 45° . In other words, the elastic behavior was no longer predominant and the suspension cannot be considered as a structured system. As a consequence, although the use of HPH was detrimental to the network structure in *P. cruentum* suspensions, the effect was remarkably stronger when a higher homogenization pressure of 100 MPa was applied.

Different behavior was observed for suspensions of *C. vulgaris* (**Fig. 2D**). Untreated suspensions presented viscoelastic behavior, characterized by a slightly higher viscous behavior since $G' < G''$ and $\delta > 45^\circ$. In addition, the storage modulus was approximately 20 times lower than for untreated suspensions of *P. cruentum*, confirming the distinct rheological behavior of these two microalgae species. However, processing of *C. vulgaris* suspensions can be applied to enhance the viscoelastic properties, which was already shown in our previous study ⁴. In fact, the thermal treatment resulted in a

Fig. 2 A,B: Strain sweep curves at a constant angular frequency of 10 rad/s for (un)processed suspensions of *P. cruentum* and *C. vulgaris*. **C,D:** Storage modulus (G'), loss modulus (G'') and phase angle (δ) at a shear strain of 1%. Standard errors are indicated by error bars ($n = 4$). The data obtained were compared statistically (one-way ANOVA). Data points of a certain parameter that are indicated with asterisks are not significantly different (Tukey test, $P < 0.05$).

more structured system, with a storage modulus of 24 Pa and a phase angle of 30.5° . Similar to *P. cruentum*, subsequent HPH resulted in a loss of the gel stiffness. However, no significant differences were observed in G' values between HPH at 20 MPa and 100 MPa. Interestingly, the homogenization pressure influenced the gel behavior, since the elastic properties became more predominant over the viscous properties after HPH at 100 MPa. In other words, even though HPH at 100 MPa resulted in a loss of gel strength, interactions in these suspensions were more characterized by those of an elastic solid than a viscous fluid compared to thermally treated suspensions of *C. vulgaris*.

Linear viscoelastic behavior: frequency sweep The network structure was further characterized by frequency sweep measurements, to study the dependence of G' and G'' on the angular frequency. The obtained frequency sweep curves are presented in **Fig. 3A** and **3B** for *P. cruentum* and *C. vulgaris*, respectively. In addition, data of the frequency sweeps were fitted by a power law model:

$$G' = a \cdot \omega^c \quad (\text{Eq. 1})$$

$$G'' = b \cdot \omega^d \quad (\text{Eq. 2})$$

with ω the angular frequency (rad/s) and a , b , c and d model parameters. While parameters a and b provide insight into the strength of the network, parameters c and d are related with the viscoelastic behavior¹⁶. The obtained estimates for

parameters a , b and c are presented in **Fig. 3C** and **3D**. Since values of parameter d are typically more sensitive to inertia phenomena of the measuring system, they will not be discussed in this paper.

The presentation of the network strength (parameters a and b) and the gel behavior (parameter c) in **Fig. 3C** and **3D** corresponds well with the data obtained from the strain sweep (**Fig. 2C** and **2D**) and similar conclusions can be drawn as discussed in previous paragraph. Generally, most suspensions of *P. cruentum* and *C. vulgaris* displayed weak gel behavior, characterized by predominant elastic behavior ($a > b$), a low frequency dependency ($c < 0.5$) and a ratio of G''/G' in the order of 10^{-1} ^{17,18}. Thermal processing was for both microalgae species the most effective approach to create gelling properties in the biomass suspensions. In fact, whereas the strain sweep data suggested that thermal processing only affected the gel stiffness of *P. cruentum* suspensions (**Fig. 2A**), modelling of the frequency sweep curves also revealed a significant increase in gelling behavior (decrease of parameter c). Subsequent HPH resulted in decreased gelling properties in suspensions of both microalgae species, but different effects of homogenization pressure were observed. Whereas a higher pressure of 100 MPa caused a more drastic breakdown of the *P. cruentum* network structure compared to 20 MPa, the opposite was observed in

C. vulgaris suspensions. Finally, *P. cruentum* showed larger potential for use as a gelling agent than *C. vulgaris*, with its network in the thermally treated suspensions about 10 times stronger than in *C. vulgaris* suspensions.

Flow behavior The flow curves of the (un)processed suspensions of *P. cruentum* and *C. vulgaris* are presented in Fig. 4A and 4B, respectively. All suspensions presented shear-thinning flow behavior, as their viscosity decreased when shear rate was increased. This non-Newtonian flow behavior was also observed in our previous study for these two microalgae species, indicating the presence of interactions between microalgal cells or polymeric substances⁴. To describe the flow behavior quantitatively, flow curves of (un)processed suspensions were fitted to a power law model:

$$\sigma = K \cdot \dot{\gamma}^n \quad (\text{Eq. 3})$$

with σ representing the shear stress (Pa), $\dot{\gamma}$ the shear rate (s^{-1}), K the consistency coefficient ($\text{Pa} \cdot \text{s}^n$) and n the flow behavior index (-). No apparent yield stress was included in the model, since no constant values of $\log(\sigma)$ were observed at low shear rates in a $\log(\sigma)$ - $\log(\dot{\gamma})$ plot. The parameter estimates of K and n are presented in Fig. 4C and 4D, respectively.

First, it was observed that values for the consistency coefficient were higher for all *P. cruentum* suspensions than for *C. vulgaris*. Hence, next to its potential as a gelling agent, *P. cruentum*

biomass is also a more promising source as a thickening agent compared to *C. vulgaris*. Furthermore, the data obtained showed that processing can also be used to alter the thickening properties of microalgal biomasses. An increased consistency coefficient was observed after thermal processing of *P. cruentum* suspensions. Thus, the increased network structure upon thermal processing (Fig. 3C) was also contributing to the viscosity of the suspension. Interestingly, no significant change in shear-thinning behavior was observed after thermal processing, with a flow behavior index of 0.23. It should be noted that very low values of n were obtained for *P. cruentum* suspensions, indicating that the increase of the shear stress is very limited at higher shear rates. Even though no yield stress parameter was included in our model, these findings suggest that a yield stress could be present in these suspensions. Extrapolation to lower shear rates would result in estimated yield stresses below 10 Pa for *P. cruentum* suspensions that were untreated, thermally processed and treated by the combination of thermal treatment and HPH at 20 MPa. Moreover, an increased consistency coefficient and shear-thinning behavior was observed after subsequent HPH at 20 MPa, even though a decreased network structure was found in the previous paragraph. Hence, the high viscosity in this suspension cannot be ascribed to the formation of an organized

Fig. 3 A,B: Frequency sweep curves at a constant shear strain of 1% for (un)processed suspensions of *P. cruentum* and *C. vulgaris*. **C,D:** Power law models ($G' = a \cdot \omega^c$ and $G'' = b \cdot \omega^d$) were fitted to the storage modulus (G') and loss modulus (G'') as a function of the angular frequency (ω). Standard errors are indicated by error bars ($n = 4$). The estimated values of the model parameters were compared statistically by use of 95% confidence intervals. Data points indicated with an asterisk are not significantly different.

Fig. 4 A,B: Viscosity curves for (un)processed suspensions of *P. cruentum* and *C. vulgaris*. **C,D:** A power law model ($\sigma = K \cdot \dot{\gamma}^n$) was fitted to the shear stress (σ) as a function of the shear rate ($\dot{\gamma}$), resulting in a consistency coefficient K and the flow behavior index n , plotted in **(C)** and **(D)**, respectively. Standard errors are indicated by error bars ($n = 4$). The estimated values of the model parameters were compared statistically by use of 95% confidence intervals. Data points of one microalga species that are indicated with asterisks are not significantly different.

network structure. Instead, the increased viscosity could result from non-specific entanglement of disordered polymers or an increased water-binding capacity of biopolymers released by HPH^{19,20}. However, when a higher homogenization pressure of 100 MPa was applied, the consistency coefficient was drastically reduced. In addition, the flow behavior index largely increased, indicating a loss of interactions between polymers and/or particles that would provide resistance to the flow. According to Ramus and Kenney (1989), shear forces reduce the viscosity of microalgal EPS, by breaking weak interactions between copolymers²¹. The present results suggest that this phenomenon is only occurring at higher shear forces, since no loss in viscosity was observed after HPH at 20 MPa.

Thermal processing was also effective in increasing the viscosity of *C. vulgaris* suspensions. A higher consistency coefficient and a lower flow behavior index indicate the presence of stronger interactions in thermally treated suspensions. Subsequent HPH at both pressure levels resulted in a decreased consistency coefficient and a slightly reduced shear-thinning behavior, however still higher than that of the untreated suspension. These results suggest that interactions which were formed

during the thermal treatment, were partially destroyed by the mechanical process. Although interactions were occurring in the suspension that was thermally treated followed by HPH at 100 MPa (**Fig. 3D**), combination with the flow behavior illustrates that these interactions were rather weak, since they gave low resistance to higher shear stresses.

The rheological properties of suspensions can be influenced by particle properties, such as size and shape, but also by the continuous phase. In fact, Einstein's relation shows that the viscosity of diluted suspensions comprising rigid particles is determined both by the properties of the dispersed particles and the viscosity of the serum phase:

$$\eta = \eta_s \cdot (1 + [\eta] \cdot \phi) \quad (\text{Eq. 4})$$

with η the suspension viscosity (Pa·s), η_s the serum viscosity (Pa·s), $[\eta]$ the intrinsic viscosity (dL/g) and ϕ the phase volume of the suspended particles (g/dL)¹⁶. To determine the contribution of the continuous phase to the overall viscosity of the suspensions, the serum phase was separated and its flow behavior was determined (**Fig. 5A** and **5B**). The flow curves were fitted to the power law model (**Eq. 3**) and the estimated parameters are shown in **Fig. 5C** and **5D**.

Low consistency coefficients were observed for all sera of *C. vulgaris*, indicating that the solubilization of components into the continuous phase was limited. In addition, these sera presented little deviation from the Newtonian flow behavior, since flow indices of 0.83 – 0.98 were observed. In contrast, the serum phases of *P. cruentum* suspensions presented high consistency coefficients in combination with low flow behavior indices. Thermal processing resulted in a large increase of the consistency coefficient, in accordance with the suspension viscosity (Fig. 4). However, subsequent HPH at 20 MPa led to a decreased consistency and shear-thinning behavior. The high suspension viscosity can thus not be fully ascribed to solubilized polymers in the continuous phase. A drastic loss of viscosity and shear-thinning behavior was observed after HPH at 100 MPa, indicating that these high shear forces resulted in a reduced network formation between solubilized polymers in the continuous phase.

Effect of processing on the microstructure

In general, the rheological properties of processed suspensions can be partially ascribed to changes in microstructural properties. Therefore, the microstructure of the suspensions

was studied by determining volumetric particle size distributions (Fig. 6) and visualized by microscopic images (Fig. 7). For both microalgae, the untreated suspensions were characterized by a bimodal particle size distribution, with one peak (1 – 10 μm) accounting for individual cells and the other peak (10 – 100 μm) for clusters of intact cells. This was previously observed in Bernaerts *et al.* (2017)⁴ and confirmed by microscopic images in the current study. These observations demonstrate the presence of intercellular polymer interactions, probably related with the EPS in both microalgae. However, the more elastic behavior of untreated *P. cruentum* suspensions suggests that interactions are stronger in this microalga compared to those in *C. vulgaris*.

Thermal processing resulted in the formation of larger particles, with a diameter up to 200 μm . A large decrease in the volume fraction of individual cells indicates that more individual cells were introduced in cell clusters by the thermal treatment for both microalgae species. Whereas the clusters in the untreated suspensions were exclusively composed of intact cells, microscopic images suggest that the aggregates in the thermally treated suspensions also contained free cell material, especially for *C. vulgaris*.

Fig. 5 A,B: Viscosity curves for the sera separated from (un)processed suspensions of *P. cruentum* and *C. vulgaris*. **C,D:** A power law model ($\sigma = K \cdot \dot{\gamma}^n$) was fitted to the shear stress (σ) as a function of the shear rate ($\dot{\gamma}$), resulting in a consistency coefficient K and the flow behavior index n , plotted in (C) and (D), respectively. Standard errors are indicated by error bars ($n = 4$). The estimated values of the model parameters were compared statistically by use of 95% confidence intervals. Data points indicated with an asterisk are not significantly different.

Fig. 6 Volumetric particle size distribution of suspensions of *P. cruentum* and *C. vulgaris*, for untreated suspensions (Untreated), suspensions treated by a thermal treatment (TT) and by a combination of a thermal treatment and high pressure homogenization at 20 MPa (TT+HPH 20) or 100 MPa (TT+HPH 100).

To identify the nature of this material, microscopic staining was performed with specific dyes (**Fig. 8**). Coomassie brilliant blue was used to localize proteins, yielding a blue color (465 nm)²², whereas anionic polysaccharides were stained by acridine orange. In fact, acridine orange is a general dye which is also able to stain other anionic polymers such as DNA and shows two emission bands, resulting in a green (532 nm) or an orange-red color (650 nm)²³. Proteins were only observed inside the cells of *P. cruentum*, visualized as blue colored material, with the exception of a few protein groups in the surrounding cell envelope. Staining with acridine orange clearly demonstrated the presence of anionic polysaccharides between the cells, as observed from the bright orange color of the filamentous structures surrounding the cells. The orange color indicates the highly acidic characteristics of these polysaccharides²³. In fact, the acidic characteristics of EPS in *P. cruentum* have been previously described, as a result from substantial amounts of glucuronic acid residues and sulfate ester groups in the

polysaccharide chain²⁴. These observations suggest that EPS play an important role as a glue between cells to create large aggregates, resulting in increased rheological properties of *P. cruentum* suspensions. In contrast, free material in aggregates of thermally treated suspensions of *C. vulgaris* was clearly stained by the coomassie brilliant blue dye. In addition, acridine orange staining resulted in small spherical structures emitting green light. These observations suggest that proteins were solubilized during the thermal treatment and contributed to the formation of larger aggregates. Both protein and carbohydrate solubilization by thermal processing have been previously reported for microalgae^{25,26}.

The use of HPH after thermal processing resulted in a decreased particle size for both microalgae species (**Fig. 6**). In *P. cruentum* suspensions, the final particle size was between 1 and 30 μm, irrespective of the homogenization pressure. Moreover, microscopic images showed similar microstructures in the homogenized samples (**Fig. 7**). As a consequence, the

Fig. 7 Microscopic images of untreated suspensions (Untreated), suspensions treated by a thermal treatment (TT) and by a combination of a thermal treatment and high pressure homogenization at 20 MPa (TT+HPH 20) or 100 MPa (TT+HPH 100).

Fig. 8 Microscopic images of thermally treated suspensions (TT) that were stained with coomassie brilliant blue and acridine orange.

differences in rheological properties between HPH at 20 MPa and 100 MPa can be completely ascribed to the changes in serum viscosity. On the other hand, the effect of HPH on the particle size in *C. vulgaris* suspensions was dependent on the pressure level. Surprisingly, HPH at 20 MPa resulted in a larger fraction of individual cells (1 – 10 µm) and somewhat smaller cell clusters (10 – 50 µm) compared to HPH at 100 MPa, which was also observed in the microscopic images. At higher homogenization pressures, larger shear forces occur which would intuitively facilitate the disruption of aggregates. However, it is hypothesized that HPH at 100 MPa did not only break aggregates, but also resulted in disruption of individual cells. As a consequence, more intracellular material was released, which contributed to the formation of aggregates and

therefore resulted in larger particles compared to the suspension homogenized at 20 MPa. These microstructural findings support the observed changes in rheological behavior. In fact, the combination of thermal processing with HPH at 100 MPa resulted in a more elastic behavior, characterized by the lowest parameter c (Fig. 3), probably resulting from the composition and size of these aggregates. Interestingly, the processing sequence of thermal treatment followed by HPH resulted in a large fraction of individual cells, regardless of the homogenization pressure. However, when HPH was directly applied on untreated suspensions of *P. cruentum* or *C. vulgaris*, our previous study showed a substantial degree of cell disruption⁴. It can therefore be concluded that a thermal pretreatment of microalgal suspensions influences the effect of HPH on cell disruption. Spiden *et al.* (2015) also reported a reduced degree of cell disruption for *Chlorella* sp. when thermally pretreated for 10 min at 90 °C. However, scanning electron microscopy (SEM) images revealed that the thermal treatment caused solidification of the protoplast, which remained intact after subsequent HPH²⁷. Hence, the use of SEM imaging might be an interesting tool to investigate whether intact cells or solidified protoplasts were present in the current study. Nevertheless, it can still be concluded that the use of the thermal pretreatment hindered the release of intracellular material during HPH and therefore resulted in distinct rheological properties compared to HPH of untreated suspensions.

Comparison of different process sequences

To quantify the effect of different processing sequences of mechanical and thermal treatments on the rheological properties, the data obtained in the current study were compared with those of our previous study⁴. This comparison was done for the viscosity at a specific shear rate of 10 s⁻¹ and is presented in Fig. 9. Comparison of the linear viscoelastic

Fig. 9 Comparison of different sequences of mechanical and thermal processing of microalgal suspensions of *P. cruentum* and *C. vulgaris*. The normalized viscosity at a shear rate of 10 s⁻¹ is presented for untreated suspensions (Untreated), suspensions treated by a thermal treatment (TT) and a combination of a thermal treatment and high pressure homogenization at 20 MPa (TT+HPH 20) or 100 MPa (TT+HPH 100). In addition, results of Bernaerts *et al.* (2017)⁴ were included, presenting suspensions treated by high pressure homogenization at 100 MPa (HPH 100) and by a combination of high pressure homogenization and sterilization with a process value of 10°C_{F121.1°C} = 5 min (HPH 100+S).

behavior of these suspensions, as the storage modulus at an angular frequency of 10 rad/s, yielded similar conclusions (not shown). Since slightly different viscosities were observed for untreated suspensions due to batch variability, all data were normalized according to their untreated suspensions. In addition, a hypothesized schematic overview of the observed microstructural changes due to different processing sequences is shown in Fig. 10. Depending on the type, the intensity and the sequence of processing steps, different biopolymers are affected resulting in distinct microstructures. For instance, the occurrence of protein denaturation or interactions between solubilized EPS in the serum phase largely influenced the microstructural and rheological properties.

It should be noted that different intensities of thermal processing were used in the two studies. In our previous study, a sterilization process was applied for suspensions at pH 6 as a preservation step to create shelf-stable products, equivalent to 5 min at 121.1 °C. In the present study, a thermal treatment with a lower intensity was applied as a functionalization process, i.e. 15 min at 95 °C. Nevertheless, thermal processing always resulted in an increased viscosity of suspensions of *P. cruentum* and *C. vulgaris*, irrespective of the intensity of the thermal treatment. In contrast, HPH mostly decreased the viscosity of the suspensions, with large influence of the homogenization pressure. However, due to distinct biomass profiles of the two microalgae species, differences were observed in rheological behavior and microstructural changes upon processing between *P. cruentum* and *C. vulgaris*.

Untreated suspensions of *P. cruentum* presented a high viscosity, which was ascribed to their EPS causing intercellular interactions, resulting in large and stable cell clusters. Moreover, solubilization of these EPS into the continuous phase provided shear-thinning flow behavior to the serum phase. Whereas thermal processing strengthened these interactions, the use of HPH led to a drastic reduction of the viscosity due to its high shear forces. Even though the combination of thermal processing and HPH at 20 MPa resulted in the highest suspension viscosity, the mechanism behind it could not be revealed by the particle and serum characteristics. However, a drastic decrease in gel strength was observed for this suspension (Fig. 3), implying that the advantages of this processing sequence might be limited to specific applications. Therefore, it can be concluded that a thermal treatment is the most effective strategy to obtain a high viscosity in *P. cruentum* suspensions.

In contrast, HPH of untreated *C. vulgaris* suspensions resulted in a slight increase of the viscosity. As the untreated suspensions displayed only limited structural properties, disruption of the cells by HPH to release intracellular material provided structural build-up in this suspension. In fact, given the minimal contribution of the serum phase in all *C. vulgaris* suspensions, the particle phase is determining the rheological behavior for this microalga. Applying thermal processing resulted in increased rheological properties, mainly ascribed to protein denaturation and solubilization. Although a combination of HPH with sterilization led to the largest increase in viscosity, the same linear viscoelastic properties were

Fig. 10 Hypothesized schematic representation of the observed microstructural changes due to different processing sequences for suspensions of *P. cruentum* and *C. vulgaris*, as applied in the current study and in our previous study⁴. (HPH: high pressure homogenization)

observed when suspensions were immediately thermally treated (data not shown). As a consequence, these two treatments are both considered as the most effective processing strategies to obtain increased rheological properties of *C. vulgaris* suspensions.

Interestingly, even though different processing strategies could be used to obtain certain gelling or thickening properties, the microstructure is affected differently. When HPH is included as a first processing step, a substantial degree of cell disruption is achieved, which is desired in many applications. For instance, the bioavailability of nutritional components can be increased by a cell disruption step, removing the cell wall as a barrier for releasing nutrients in the gastrointestinal tract²⁸. In contrast, the use of direct thermal processing did not lead to disruption of the cells, but only resulted in solubilization of some intracellular components. Hence, taking into account the

microstructural changes might be an important factor in the selection of the most suitable processing strategy for specific food applications.

Conclusions

The current study investigated the rheological properties of aqueous suspensions of *P. cruentum* and *C. vulgaris*, two microalgae species that showed large potential as a multifunctional ingredient in our previous study. It was demonstrated that different sequences of mechanical and thermal processing resulted in distinct rheological properties. In general, HPH resulted in a reduction of rheological properties, whereas a thermal treatment was an effective processing step for structure build-up. However, combination of HPH with a subsequent sterilization process also proved successful for *C. vulgaris* suspensions. Protein denaturation and solubilization were regarded as the main mechanisms influencing the rheological properties of *C. vulgaris* suspensions, with only minimal contribution of the serum viscosity. In contrast, a large serum viscosity for all (un)processed suspensions of *P. cruentum* revealed the importance of the EPS, providing substantial contribution to the rheological properties. Microscopic staining also indicated their importance in the particle phase, providing intercellular interactions that resulted in stable aggregates. As a consequence, EPS of *P. cruentum* displayed great potential as a structuring agent and further research should be focusing on its applications, such as its use as a gelling or thickening agent in food products.

The results of the present study also demonstrate the importance of the microstructural changes upon processing. Even though HPH did not result in structure build-up, this might still be a necessary processing step for disruption of microalgal cells. Cell disruption might for instance be required to increase the bioavailability of nutrients, since the cell wall will not be digested and therefore will hinder the release of nutrients in the gastrointestinal tract. For suspensions of *C. vulgaris*, HPH could be combined with a subsequent sterilization process to obtain increased rheological properties, since this sequence resulted in equal gelling and thickening properties as a thermal treatment alone. In contrast, no effective processing strategy was found for *P. cruentum* suspensions when HPH was included. As a consequence, optimization of cell disruption treatments in relation with the rheological properties might be desired. The use of a lower homogenization pressure could possibly result in an optimal balance between a substantial degree of cell disruption and a minimal destruction of the network structure. Thermal processing can thus be considered as an effective treatment to functionalize microalgal biomass as a structuring agent. However, additional research is required on the nutritional stability of microalgal biomass during processing, especially in terms of heat sensitive components such as ω 3-LC-PUFA and carotenoids. In fact, a balance should be found between structure build-up and possible degradation of nutritional components. Nevertheless, this study proved that a short period at a high temperature is sufficient to double the gel stiffness of *P. cruentum* suspensions, possibly showing a limited

impact on the nutritional stability. Therefore, it can be concluded that *P. cruentum*, and *C. vulgaris* to a lesser extent, show large potential for application of the biomass as a multifunctional ingredient in processed food products.

Conflicts of interest

There are no conflicts to declare.

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Abbreviations

ω 3-LC-PUFA	omega-3 long chain polyunsaturated fatty acids
CWPS	cell wall polysaccharides
DHA	docosahexaenoic acid
DIC	differential interference contrast microscopy
EPA	eicosapentaenoic acid
EPS	exopolysaccharides
HPH	high pressure homogenization
SEM	scanning electron microscopy
SPS	storage polysaccharides

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