Stabilization of Arthrospira platensis with high-pressure processing and thermal treatments: Effect on physico-chemical and microbiological quality

Stabilisation of *Arthrospira platensis* with high-pressure processing and thermal treatments: effect on physico-chemical and microbiological quality

Running title: Thermal, non-thermal treatment on Spirulina

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Abstract

Arthrospira platensis (S*pirulina*) is a cyanobacterium that has been recently studied for food applications due to its high biological and nutritional value. When *A. platensis* is used as ingredient in food applications, proper treatments have to be applied in order to reduce microbial contamination. This work compared the effect of thermal treatments (sterilization at 121°C and pasteurization at 90°C) and high-pressure processing (400, 600 MPa) on the chemical, physico-chemical and microbial quality of 5% (w/v) *A. platensis* aqueous suspensions. Total antioxidant capacity, total polyphenols content, colour and pigments content were not strongly lowered/modified by the HPP treatments. HPP at 400 MPa even improved the release of C-phycocyanin from the biomass because of the breakage of cell walls. HPP treatments were comparable to pasteurization in reducing Yeasts, Coliforms, Staphylococci and total bacterial count. Conversely, sterilization was the only treatment that guaranteed the inactivation of spore-forming species but affecting the final quality.

Keywords: non-thermal processing; emerging technologies; high pressure processing; Spirulina; C-phycocyanin

Novelty Impact Statement HPP treatments were found to be a good strategy to preserve or even improve some physical and chemical properties of *Arthrospira platensis* (Spirulina), in particular antioxidant capacity, polyphenols, colour and pigments content. Furthermore, HPP treatments were comparable to pasteurization in reducing microbial cell count, while sterilization was the only treatment able to ensure the inhibition of spore-forming species.

1. Introduction

Arthrospira platensis, a cyanobacterium commonly known as S*pirulina,* is widely cultivated and studied because of its richness in bioactive compounds with high nutritional value, that can be used in several food formulations (Niccolai, Chini Zittelli, et al., 2019; Golmakani et al., 2019; Batista et al., 2017; Barkallah et al., 2017).

It can be commercialized dehydrated as a food integrator, or as an ingredient for food formulations or, as recently reported, as a booster in the fermentation process or as natural antimicrobial (Martelli, Alinovi, Bernini et al., 2020; Zheng et al., 2020,

Bancalari et al., 2020). Moreover, several bioactive or functional compounds can be extracted from *A. platensis* biomass and used as ingredients (-Martelli, Cirlini, Lazzi et al.,2020; Bancalari et al.,2021)

A. platensis is commonly cultivated in raceway ponds that is cheaper than cultivations in photobioreactors (Richardson et al., 2012) but one of its major drawbacks is the high level of microbial contamination. In fact, alterative and spoilage bacteria are reported as contaminants in these cultivations and their presence could lead to safety issues for consumers (Wang et al., 2013). For this reason, microalgal biomass cannot be marketed fresh but needs to be dehydrated in order to reduce its water activity (a_w) and avoid the potential bacterial proliferation. Otherwise, thermal or non-thermal treatments can be applied to inactivate alterative and foodborne pathogens, but, on the other hand they may cause an unwanted loss of nutritional and functional compounds (Martelli et al., 2014). One of the most promising emerging alternatives to thermal treatment is high pressure processing (HPP), that can reduce the microbial load and stabilize food products, avoiding losses of nutritional and functional compounds (Paciulli et al., 2019). Furthermore, high pressure processing could also improve the extraction of bioactive compounds by disrupting tissues, cell walls, membranes and organelles (Cox et al., 2014; Jun, 2013; Martelli, Favari, Mena et al., 2020). Reducing bacterial contamination to an acceptable level maintaining unchanged or even enhance the composition of *A. platensis* is a goal that must be reached to use it as a safe and functional ingredient in food formulations. Moreover, HPP treatment showed to have a positive effect in stabilizing the C-phycocyanin mainly in presence of proteins or hydrocolloids due to encapsulation induced by pressure (Zhang et al., 2021). In the last years, the extraction and stabilization of C-phycocyanin, an accessory photosynthetic blue-protein that is present in relatively high concentration in *A. platensis,* has been widely investigated for potential food applications (Zhang et al., 2021; Martelli et al., 2014; Martinez et al., 2017). The interest for this protein pigment has grown because the confectionary and drinks industry has a high demand in blue colorants, that are however uncommon in nature and have to be synthesized (Martelli et al., 2014) and because of the high protein yield and the relatively low cost of production of this pigment. Thus, the aims of the present study were to: i) investigate the effect of the above-mentioned treatments on: phenolic content, antioxidant capacity, chlorophyll, carotenoids and C-phycocyanin content, protein fraction and colour and ii) evaluate the efficacy of two thermal treatments (pasteurization and sterilization) and two high pressure treatments (HPP at 400 and 600 MPa for 3 min) in reducing the microbial contamination of *A. platensis* aqueous solution.

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2. Materials and Methods

2.1 Materials

Gallic acid, sodium carbonate, Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), DPPH (2,2-diphenyl-1-pirylhydrazyl free radical) and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA); Folin-Ciocalteu's phenol reagent solution was obtained from VWR (Milan, Italy). Bi-distilled water was produced by a Millipore Alpha Q purification system (Waters, Billerica, MA, USA).

Acrylamide, N,N'-methylenbisacrylamide, N,N,N,N, tetramethyl ethylene-diamine (TEMED), glycine, Sodium dodecyl sulphate (SDS), Tris aminomethane (TRIZMA® BASE), ammonium persulfate, 1,4-ditioeritrol (DTE), Bromophenol blue, glycerol, sigmamarker™ wide range protein ladder (200-6.5 kDa), Coomassie brilliant blue G250, orto-phosphoric acid (85%), aluminium sulphate, ethanol, and acetone were purchased from-Merck (Milan, Italy).

2.2 Experimental design and processing treatments

Dried *A. platensis* was kindly provided by S.A.Ba.R (Novellara, RE, Italy). One hundred grams of dried product were rehydrated in 2 L of distilled water (5% w/v) under gentle stirring at 4°C overnight. After rehydration, the suspension was divided into ten aliquots of 200 mL that were packed in sterilized bottles with an internal volume of 250 mL. The aliquots were grouped in 5 different treatment (two 200-mL aliquots for each treatment): i) raw, non-treated sample (control); ii) 400 MPa for 3 min (HPP400), iii) 600 MPa for 3 min (HPP600), iv) pasteurized at 90°C for 10 min (PAST) and v) sterilized at 121°C for 20 min (STER).

Both HPP treatments were performed with a QUINTUS FOOD PRESS QFP 35 L (Avure Technologies Inc., Middletown, OH, USA). Cold water (4 °C) was used as pressure medium; temperature during processing was not controlled but the temperature increase due to compression was not higher than 2-3 °C/100 MPa.

Sterilization was performed by means of Tuttnauer autoclave (Cavallo s.r.l., Milan, Italy), at 121°C for 20 min. Pasteurization treatment was performed by placing the samples in a temperature-controlled laboratory bath (type M418-BM, MPM instruments, Bernareggio, Italy) at 90°C for 10 min; the treatment durations of sterilization and pasteurization were respectively calculated when the inner temperature of the autoclave reached 121 ± 1°C and when the water inside the bath reached 90± 1°C after the immersion of the bottles. After both pasteurization and sterilization, the bottles were immediately cooled in a water bath and then, immediately analysed or stored at refrigerated temperature (4 ± 1°C).

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Five out of the ten bottles subjected to different treatments were analysed immediately (0 d of storage) and the other five after a short period of refrigerated storage at 4°C (6 d of storage). Two technical replicates for each analysis were performed for each sample.

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2.3 Total phenolic content determination

Total phenolic content was determined using the Folin-Ciocalteu's method, following the protocol reported by Wu et al. (2005) with some modifications. Briefly, 0.25 mL of water diluted sample (1/5, v/v) were transferred in the test tubes and added with 1 mL of Folin-Ciocalteu's phenol reagent solution (1/10, v/v in bi-distilled water) and with 2 mL of aqueous sodium carbonate (20 %, w/v). All the samples were incubated for 30 min, in the dark at room temperature. Absorbance was measured at 760 nm using a JASCO V-530 spectrophotometer (Easton, MD, USA), and water was used to adjust zero. A calibration curve was built analysing 5 different gallic acid standard solutions (10 – 100 mg GAE/L). Results were expressed as gallic acid equivalent (mg GAE/L).

2.4 Radical-scavenging activity by DPPH method

The antioxidant capacity test was performed applying the DPPH radical scavenging assay on the basis of the protocol reported by Dall'Asta et al. (2013) with slight modifications: an aliquot of 0.1 mL of the 1/5 diluted sample was putted in a test tube and added with 2.9 mL of a methanolic DPPH solution (0.05 mM). The resulting mixture was kept in the dark at room temperature for 30 minutes, then the absorbance at 517 nm was registered on a JASCO V-530 spectrophotometer (Easton, MD, USA), and methanol was used for setting the autozero. At the same time, a blank sample was prepared and submitted to the same procedure. After that, the inhibition of DPPH radical was determined on the basis of the following formula:

$$I\% = \frac{Abs_0 - Abs_1}{Abs_0} \cdot 100 \qquad (2)$$

where Abs_0 was the absorbance of the blank and Abs_1 was the absorbance of the sample. A calibration curve was constructed using Trolox as reference, preparing 5 different standard solutions in a concentration range of 0.1 – 1 mM. Then, the antioxidant capacity of samples was expressed as TEAC value (Trolox Equivalent Antioxidant Capacity; mmol Trolox eq/kg).

2.5 Quantification of chlorophylls, carotenoids and phycocyanin content

2.5.1 Sample preparation for spectrophotometric assays

To perform spectrophotometric analyses, the insoluble biomass of *A. platensis* was separated by centrifugation (6,500 g for 20 min at 4°C) from the supernatant containing water-soluble C-phycocyanin. Supernatant was extracted by pipetting and an aliquot (100 µL) was diluted by adding 900 µL of ultrapure water for C-phycocyanin quantification. The pellet containing *A. platensis* biomass was weighed and resuspended in ~900 µL of pure acetone (1:20 dilution). The suspension was vortexed at 2,000 rpm and sonicated for 15 min using a workbench bath (VWR ultrasonic Cleaner) (Hynstova et al. 2018). Extracted pigments (chlorophyll and carotenoids) were separated by centrifuging at 13,700 g for 10 min at 4°C. Supernatant was filtered by syringe filtration using 0.45 µm filters (VWR International, Milan, Italy) and 50 µL of permeate were diluted by adding 950 µL of pure acetone. Samples preparation was performed in dim light to avoid pigment degradation. Each sample was prepared by means of two independent extractions.

2.5.2 Spectrophotometric determination of the pigments

For chlorophylls and carotenoids determination, methanol was used as the blank of analysis. Concentration of chlorophyll a (Ca), chlorophyll b (Cb), total chlorophylls (Ca+b), total carotenoids (Cx+c), total chlorophylls and carotenoids (Ccc) and pheophytins (Cph) were calculated according to Hynstova et al. (2018) and Lichtenthaler (1987) using equations 3-8:

$$Ca\ (\mu g/mL) = 11.24\ A_{662} - 2.04\ A_{645} \quad (3)$$

$$Cb\ (\mu g/mL) = 20.13\ A_{645} - 4.19\ A_{662} \quad (4)$$

$$Ca + b\ (\mu g/mL) = 7.05\ A_{662} + 18.09\ A_{645} \quad (5)$$

$$Cx + c\ (\mu g/mL) = \frac{(1{,}000\ A_{470} - 1.90\ Ca - 63.14 Cb)}{214} \quad (6)$$

$$Ccc\ (\mu g/mL) = (Ca + b) + (Cx + c) \quad (7)$$

$$Cph\ (\mu g/mL) = 321.3\ A_{653} - 208.4\ A_{654} \quad (8)$$

For C-phycocyanin, milliQ water was used as the blank of analysis. Concentration of water-soluble C-phycocyanin was determined according to the method reported by de Marco Castro et al. (2019). Quantification of C-phycocyanin was performed according to equation (9):

$$C - phycocyanin\ \left(\frac{mg}{mL}\right) = \frac{A_{615} - 0.474\ A_{652}}{5.34} \quad (9)$$

where A_{615} and A_{652} are absorbances of the sample measured at 615 nm and 652 nm.

2.6 Physical properties

Colorimetric parameters were evaluated with image analysis: samples were scanned by means of a desktop flatbed scanner (Hewlett Packard Scanjet 8200, Palo Alto, CA, USA) at 236 pixels/cm (600 dpi of resolution; true colour – 24 bit), equipped with a cold cathode lamp for reflective scanning. All images were scanned at the same conditions, during image acquisition, the scanner was held in a black box to exclude surrounding light and external reflections. Flatbed scanner colour (R, G and B) was corrected as previously reported by N'Dri et al. (2010) and conversed to L* (lightness), a* (redness at positive values, greenness at negative values) and b* (yellowness at positive values, blueness at negative values). Total colour differences (ΔE) between 0 and 6 days were also calculated.

Water holding capacity (WHC) of *A. platensis* suspensions was measured similarly to Barkallah et al. (2017). Samples aliquots (~1 g) were placed in 1.5 mL tubes and centrifuged at 6,000 g per 10 min at 4°C with a benchtop centrifuge (mod. 5810R, Eppendorf, Hamburg, Germany). WHC was calculated as follows (equation 1):

$$WHC\ \left(\%\frac{w}{w}\right) = \left(\frac{W_1}{W_2}\right) \cdot 100 \quad (1)$$

where W_1 is the weight of *A. platensis* centrifuged pellet and W_2 is the weight of the original sample.

2.7 Optical microscopy observation

An aliquot of 10 µL of each sample was observed by means of an optical light microscope Nikon 80i (Tokyo, Japan) equipped with a 10X objective. Pictures of each sample were acquired by the Nis Elements software (ver. 2.10 Nikon, Tokyo, Japan).

2.8 Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS PAGE)

Sodium dodecyl sulphate (SDS) PAGE was performed according to the method of Laemmi (1970). Stacking and separating gels were casted by using a 4 and 15% acrylamide concentration, respectively. Diluted samples (proteins concentration of ~2 µg/µL) each sample were loaded in the gel wells (5 µL aliquots). Samples were run in non-reducing conditions at 150 V on a Mini-Protean II cube (Bio-Rad, Hercules, CA, USA).

SDS PAGE gels were stained with Coomassie Brilliant Blue G250 according to Kang et al. (2002), destained in several changes of distilled water and scanned with a flatbed scanner.

2.9 Microbial counts

Each sample was ten-fold (1:10 v/v) diluted, up to 10^{-6} in Ringer's solution (Sigma Aldrich, Milan) and then inoculated in specific culture media using the spread-plate technique. Total microbial count (TBC) was obtained by plating onto Plate Count Agar (PCA, Oxoid, Basingstoke, Hampshire, UK) and incubating at 37 ± 1 °C for 48 h. Chromocult® Coliform Agar (Merck, KGaA, Darmstadt, Germany) was used for the enumeration of Coliforms by incubating at 37 ± 1 °C for 24 h. Staphylococci were counted by spreading the dilutions on Mannitol Salt Agar (Oxoid, UK) and incubated at 37 ± 1 °C for 48 h. For the count of Listeria, Agar Listeria Ottaviani and Agosti medium (ALOA, Biolife Italiana, Milan) was used and plates incubated for 48 h at 37 ± 1 °C. Yeast and molds were counted on Yeast Extract Glucose Chloramphenicol Agar (YGC, Merck KGaA, Darmstadt, Germany) that was incubated at 25 ± 1 °C for 48–72 h.

For aerobic and anaerobic spore-forming bacteria, samples were heated to 80 °C for 10 min and then cooled before analysis. Then the samples were serially diluted in Ringer's solution, and plated on Tryptone Soy Agar (TSA, Merck KGaA, Darmstadt, Germany), followed by incubation for 24 h at 30 ± 1 ºC under aerobic condition for the determination of aerobic spore-forming bacteria (Abdelmassih et al., 2011). For the count of anaerobic spore-forming bacteria, 1 mL of each dilution was inoculated in the medium using the pour-plate technique and then covered with a thin layer of the same medium in order to obtain a double-layer. The plates were anaerobically incubated into the jars with the AnaeroGen sachet (Oxoid, UK), at 30 ± 1 ºC for 24 h.

2.10 Statistical analysis

Statistical analyses were performed using SPSS Statistics v. 25 (IBM, Armonk, NY, USA): one-way ANOVA and Tukey HSD post hoc test were applied to test significant differences among the treatments and storage times ($P<0.05$). Pearson correlation coefficients between total polyphenolic concentrations and antioxidant capacity values were also calculated considering a significant correlation for $P<0.05$.

3. Results and Discussion

3.1 Total polyphenolic content (TPC)

Immediately after processing, HPP treatments did not affect the amount of polyphenols if compared to the control sample. Conversely, pasteurized and sterilized samples (0 d of storage) showed significantly ($P<0.05$) lower concentrations of phenols in respect to the untreated suspension (Table 1). The loss of polyphenols in PAST and STER samples could be ascribed to the effect of the heating treatments.

The phenolic concentration of the aqueous suspension of *A. platensis* at 0 d (Table 1) was 149.49 ± 0.31 mg GAE/L, significantly higher ($P<0.05$) than after the storage period (216.48 ± 2.07 mg GAE/L).

After 6 d of refrigerated storage, control and HPP400 samples showed a significant increase ($P<0.05$) of TPC. This trend could be probably due to a release of phenolic compounds from cells; polyphenols are contained in cells and for their completely extraction the lysis of cell walls results necessary (Parniakov et al., 2015). Conversely, HPP600 samples showed a significantly lower value than HPP400 and control samples, but still a significantly higher ($P<0.05$) value than PAST and STER (Table 1). Differences observed between the two HPP treatments after the storage could be ascribed to a degradation of the compounds that react with Folin-Ciocalteau's reagent, such as C-phycocyanin (İlter et al., 2018). By increasing the pressure applied in HPP processing, the degradation of C-phycocyanin may be observed, as reported in a previous work which investigated the decomposition of this molecule at different pressure levels (50 – 600 MPa) (Li et al., 2020).

3.2 Antioxidant activity

A. platensis aqueous sample at 0 d presented an antioxidant activity of 0.472 ± 0.036 mmol Trolox eq/kg (Table 1). As already observed for TPC at 6 d, the antioxidant capacity of the control samples increased to 0.578 ± 0.027 mmol Trolox eq/kg ($P<0.05$). On the other hand, the other samples presented values comparable with those found in the control at 0 d, while after 6 d of storage, all the samples presented a lower antioxidant activity in respect to the control. In particular, the pasteurized

sample showed the statistically lowest radical scavenging capacity ($P<0.05$) (Table 1). These results reflected those observed for total polyphenolic content: a strongly positive relationship ($r = 0.81$) between the radical scavenging activity and the phenolic concentration values was found.

Along with polyphenolic compounds, several other molecules naturally present in *A. platensis* showed antioxidant properties, such as carotenoids, chlorophylls, phycocyanin and ascorbic acid (Chopra & Bishnoi, 2008). So, even if heating treatments caused a reduction of TPC as in the case of sterilized samples, the antioxidant capacity remained almost comparable in respect to the control, probably due to the presence of other compounds not belonging to the polyphenols class. The control sample, prepared at a concentration of 5 % of *A. platensis* (50 µg/mL), showed a concentration of antioxidant compounds of 0.472 ± 0.036 mmol Trolox eq/kg, corresponding to an inhibition of 12.65 ± 0.97 %, resulting indeed higher as compared to data already reported in literature. Also ethanolic suspension that contained 25 µg/mL of *A. platensis*, showed an inhibition of 10.30 ± 0.80 % (Anbarasan et al., 2011). *A. platensis* is a well-known antioxidant source: some studies reported the antioxidant properties of *A. platensis* water extracts in reducing cell death due to apoptosis caused by the presence of free radicals (as DPPH), so the authors hypothesized that these products could be applied in food as natural preservatives (Chu et al., 2010).

3.3 Physical properties of *A. platensis* aqueous dispersions

In general, all the treatments showed a significant effect on the colour of the samples, as depicted in Fig. 1. Regarding L* values, all treatments caused a significant increase ($P<0.05$) (Table 2). Increase of L* values could be due to rapid oxidation and/or pigment decomposition at high temperature, in accordance with Nouri et al. (2018).

Redness (positive a* scores) was significantly reduced ($P<0.05$) by all treatments apart from HPP400, that showed an increase if compared to the control; the extent of reduction was in the following order: PAST>HPP600>STER. Pasteurization (PAST) caused the highest decrease of a*; in particular, PAST was the only sample that showed a negative a* value, indicating a green shade of the sample, that was also visually observable (Fig. 1). Interestingly, HPP400 showed a significantly higher ($P<0.05$) a* value than HPP600.

Yellowness (positive b* scores) significantly increased with the thermal treatments ($P<0.05$), while HPP samples did not show significant differences ($P>0.05$) compared to the control. The thermal degradation of C-phycocyanin in PAST and STER probably caused a loss of blue hue towards a greener and browner colour (Fig.1).

Changes in b* and a* values were due to the effect of different processes that can cause pigments' degradation: e.g. for chlorophylls with the formation of various coloured compounds (Koca et al., 2007), and for C-phycocyanin which results in precipitation and fading of the blue pigment (increase of b*) (Chaiklahan et al. 2012). On the contrary, the HPP treatments compared to the control did not show significantly different values in terms of the b* parameter, despite the highest C-phycocyanin content was observed in HPP400 (Fig. 5) and apparently led to a bluer shade (Fig. 1), with this latter characteristic probably caused by the higher, positive a* score that led to a bluer hue.

, The highest total colour difference (ΔE) was higher for the thermally treated samples if compared to the HPP ones (Table 2). Surprisingly, the highest total colour difference (ΔE), with an evident variation of colour compared to the control, was observed for PAST and not for STER, that reported the second highest total colour difference with the control sample. On the contrary, the lowest ΔE value was recorded for HPP600 and HPP400. These results confirmed the strongest impact of the thermal treatment on colour characteristics, that still represent an important limitation of *A. platensis* in food applications (Chaiklahan et al. 2012).

During refrigerated storage, the thermally-treated samples showed a considerable stability, conversely, the control and the HPP samples showed significant variations ($P<0.05$), especially a decrease in L* and b* and an increase in a* values was observed, probably due to microbial activity and/or residual activity of enzymes.

As reported in Fig. 2, water holding capacity (WHC) of the control samples resulted 13.3 ± 0.1 %, confirming the characteristic of *A. platensis* to retain water thanks to the high protein (Benelhadj et al., 2016) and extracellular polysaccharides (EPS) content (Trabelsi et al., 2009). All the treatments caused a significant increase ($P<0.05$) in WHC values except for HPP600. The increase in WHC was probably related to gelation of *A. platensis* proteins (Chronakis, 2001) due to dissociation into subunits and unfolding of the protein molecules. As temperature increases, reactive groups are exposed favouring protein-protein or other electrostatic and hydrogen bonding interactions. Thermally treated samples (PAST and STER) presented

significant higher values ($P<0.05$) compared to high pressure treated ones. After 6 d of storage, WHC of the treatments remained the same with no significant ($P>0.05$) variations, confirming the stability of the formed network.

3.4 Pigments concentration

Concentrations of chlorophyll a (Ca), carotenoids (Cx+c) and pheophytins (Cph) are reported in Table 3. The control sample showed a relatively high Ca content (135 ± 8 µg/mL at 0 d, corresponding to 2.71 ± 0.15 mg/g dry weight), comparable to values already reported in other studies (Hynstova et al., 2018; Park et al., 2018). Conversely, Cb content of raw *A. platensis* was low (6 ± 4 µg/mL) (Table 3).

Cph, the primary degradation product of chlorophylls due to thermal treatments, was found in a concentration of 779 ± 45 µg/mL in the control. In particular, Cph content was found to be higher than Ca (Table 3) and this can be due to the initial degradation of chlorophylls during the *A. platensis* industrial drying step (Hynstova et al., 2018).

The concentration of C-phycocyanin (Fig. 3), the main phycobiliprotein present in *A. platensis,* was 760 ± 20 µg/mL in the control, corresponding to 15.1 ± 0.4 mg/g dry weight, in accordance with de Marco Castro et al. (2019).

Afterwards, the different treatments showed significant differences ($P<0.05$) in terms of pigments content (Table 3, Fig. 3). As expected, thermal treatments promoted a significant reduction of chlorophylls, pheophytin and C-phycocyanin. On the contrary, the reduction of carotenoids was only significant for STER ($P<0.05$). Interestingly, both HPP treatments did not cause a significant variation of total chlorophylls, carotenoids and pheophytin content, and this result was in line with the colorimetric analyses. This observation highlights the benefits of applying HPP treatments, compared to the thermal ones, which lead to significant denaturation of these thermolabile compounds that are known to have an important positive bioactive activity (Ariede et al., 2017; Kumar et al., 2015).

C-phycocyanin concentration was slightly, but significantly higher ($P<0.05$) for the HPP400 (Fig. 3) also if compared to the control, probably due to both an increase in extraction of this molecule from *A. platensis* cells and a limited denaturation. Contrarily, Li et al. (2020) observed a significant reduction in the C-phycocyanin content in HPP-treated *A. platensis* at 400 MPa for 3.5 min and hypothesized that its decrease was related to the denaturation promoted by the HPP treatments. The microscopic observations allowed to observed that HPP treatment induced cell breakage, although not complete (Fig. 4). This can explain the increase in the C-phycocyanin concentration in sample treated at 400 MPa. On the contrary, stronger HPP

conditions (600 MPa x 3 min) caused a marked denaturation and a consequent reduction of C-phycocyanin concentration, in accordance with Li et al. (2020), that observed a heavier C-phycocyanin denaturation at higher pressure treatments. According to its low thermal stability (Martelli et al., 2014), C-phycocyanin was also heavily denatured by PAST and STER thermal treatments that showed about 87 and 97 % of reduction, respectively. It is quite well known that solutions containing C-phycocyanin are sensitive to heat treatment already at temperature above 47°C (Chaiklahan et al., 2012) with at least 50% of reduction after 30 min at 60°C. By considering first-order rate constants reported by (Chaiklahan et al., 2012) calculated residual activities agree with experimental data.

After 6 d of storage, a general increase of pigments, although not always significant, was observed; in particular, HPP400 and pasteurized samples showed a significant increase ($P<0.05$) in the total chlorophyll and carotenoids concentration, while the control, HPP400, HPP600 samples showed a significant increase ($P<0.05$) in the C-phycocyanin concentration. This result was partly in accordance with the increase of antioxidant capacity of the control and of the polyphenols content of the control and HPP400 (section 3.4). As stated before, an increase in the pigments' concentrations could be explained by the presence of residual enzymatic activities that may cause the release of these compounds from algae cells. Previous studies demonstrated that enzymes produced by bacteria can improve the extraction of C-phycocyanin (de Marco Castro et al., 2019; Zhu et al., 2007).

3.5 Optical microscopy observation

To further investigate whether each treatment affected the microstructure of *A. platensis*, the samples were observed using light microscopy (Fig. 4). *A. platensis* is characterized by a particular morphological feature, such as the arrangement in an open left-hand helix along the entire length (Ali & Saleh, 2012). The untreated *A. platensis* (Fig. 4a) already showed some breakdown of the cell walls and the presence of cell's fragments, probably mainly related to the drying process of the biomass (CIT). In the thermally treated samples, and particularly in STER (Fig. 4e), the appearance of some aggregates can be observed. These are assumed to be caused by the sterilization process that may lead to aggregation of denatured proteins, that are the major constituent of the total biomass (Bernaerts et al., 2017). These aggregates are probably the main responsible for the higher WHC (Fig. 2). Both HPP treatments (Fig. 4b, c), affected the structure of *A. platensis* to a lesser extent, by causing a lower but observable breakdown of the cell walls, if compared to the more intact number of cells observed in the control (Li et al., 2020).

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3.6 Protein determination by SDS PAGE

Results of SDS PAGE are reported in Fig. 5. Several proteins bands were visible and the majority were present in the medium molecular weight range (30-60 kDa) (Aiello et al., 2019). Biliproteins were the most intense bands present in the low molecular weight range of the electrophoretogram (Li et al., 2020; de Marco Castro et al., 2019), which are mainly represented by C-phycocyanin α and β subunits (Benelhadj et al., 2016). Chaiklahan et al. (2011) reported that C-phycocyanin α and β subunits were characterized by a molecular weight of about 18.4 and 21.3 kDa, respectively; Rajakumar & Muthukumar, (2018) reported a molecular weight range of 19-22 kDa. Our results were consistent with these observations, as C-phycocyanin α and β subunits were found to have a molecular weight of about 18.5 and 20.5 kDa, respectively.

Other well-defined bands were observable at higher molecular weights (~44, 60, 102 kDa, corresponding to peaks 3, 4, 5 reported in Fig. 5). In particular, peak 3 (~44 kDa) is probably indicative of the presence of chlorophyll complexes (Kalaji et al., 2017) and peak 5 (~102 kDa) is probably related to a core protein linker of the phycobilisomes to thylakoid membrane (Rajakumar & Muthukumar, 2018).

In general, protein bands showed a reduction because of both thermal and non-thermal treatments. The decrease of intensity was more evident for thermally-treated samples than HPP ones; this was expected as C-phycocyanin is denatured at temperatures above 40°C (Liu et al., 2016), and it was in accordance with pigments quantification **(section 3.4)**. No observable protein bands were present in the sterilized sample.

Concerning HPP treatments, HPP400 showed a smaller reduction in protein bands than HPP600, if compared to the control sample.

3.7 Microbial counts

The result of total microbial count (TBC) on raw samples, showed a value of 5.96 ± 0.05 Log CFU/mL (Table 4), confirming the high values of microbial contamination previously observed (Morais et al., 2009; Yu et al., 2019; Martelli, Cirlini, Lazzi et al, 2021; Martelli, Marrella, Lazzi et al 2021). Regarding the treated samples, a different microbial reduction was observed depending on the treatment: pasteurization, together with HPP400 and HPP600 allowed a reduction of respectively 4.30, 4.32

and 4.96 Log CFU/mL. Sterilization achieved a significantly higher reduction of microbial load, for all the species considered (Table 4).

If compared to both HPP treatments, pasteurization was more effective on Salmonella, allowing a reduction of 4 Log CFU/mL. Despite no significant differences ($P>0.05$) were found between HPP600 and HPP400 on Salmonella, a greater reduction of 2.68 Log CFU/mL was observed in samples treated with HPP600 (Table 1). This could be due to the higher pressure, that could induce a greater denaturation of enzymes and proteins, the breakdown of the cell membrane with the consequent loss of internal substances, and a higher bacterial death (Abe, 2007).

Results on spores confirmed that neither pasteurization nor HPP treatments can completely inactivate endospore-forming strains: their effect on those bacteria count was not consistent, allowing a small reduction of only about 1 Log CFU/mL. Our results agreed with Reddy et al. (2006), who reported that bacterial spores cannot be inactivated by high pressure alone and that they can survive at pressure treatments above 1000 MPa, probably due to the thickness structure of bacterial spore coat. Under such conditions, the pressure tolerance of the endospore substantially exceeds that of the microorganism's original vegetative state (Leggett et al., 2012). From these results, HPP effect on *A. platensis* microbial count, was comparable to the effect of a high temperature pasteurization. Sterilization was the only treatment that can ensure the inhibition of spore-forming species. Some authors reported that to achieve sterilization levels, HPP treatments can be used combined with an appropriate temperature treatment or, in combination with other pressure cycle, to effectively inhibit endospore activity (Huang et al., 2014).

Regarding the analysis after 6 d of refrigerated storage, the overall microbial count (TBC) increased for all the treated samples; probably due to the presence of heat-resistant, psychrotrophic gram-positive bacteria (Fromm & Boor, 2004). Significant ($P<0.05$) increments were also found in the yeast and Staphylococci enumeration.

For *Salmonella,* a significant reduction ($P<0.05$) of concentration was found after 6 d of storage for both HPP treatments while spore forming bacteria, showed a small reduction in number for all the treatments applied.

Concerning the sterilized samples, no significative differences ($P>0.05$) were found during the storage.

4. Conclusions

In this study different thermal and HPP treatments were applied on 5% (w/v) *A. platensis* aqueous suspensions, to assess which treatments was the best compromise between the achievement of microbial safety and the preservation bioactive compounds. HPP treatments were found to be the best process in preserving the biological value and the physical properties of *A. platensis*. In particular, HPP400, even improved the release of C-phycocyanin from *A. platensis* probably because of the breakage of cell walls promoted by the mechanical effect of high pressures.

From the microbiological analysis it was observed that HPP treatments were comparable to pasteurization in reducing microbial cell count, while sterilization was the only treatment able to ensure the inhibition of spore-forming species.

These results suggest that HPP, despite being the best treatments in preserving the biological values of *A. platensis,* were not sufficient to achieve microbial stabilization. In conclusion, a combination of thermal and HPP treatments or a combination of HPP cycles, could be further investigated as a strategy to obtain a good compromise between microbiological and physico-chemical, nutritional quality. These results may be useful to design strategies to stabilize and transform food products containing *A. platensis* as ingredient.

Declarations of Interest

None

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558

559 **Table 1.** Total phenolic content and antioxidant capacity of aqueous suspension of *A. platensis* analysed after different

560 treatment at time zero and after 6 days of storage

Storage time (d)	Treatment	Total phenolic content (mg/L GAE)	Antioxidant capacity (mmol Trolox eq/kg)	~~Antioxidant capacity (I%)~~
0	Control	$149.49^{aB} \pm 0.31$	$0.472^{aB} \pm 0.036$	~~$12.65^{aA} \pm 0.97$~~
	HPP400	$144.02^{aB} \pm 5.53$	$0.424^{aA} \pm 0.026$	~~$11.36^{aA} \pm 0.70$~~
	HPP600	$146.58^{aA} \pm 2.72$	$0.437^{aA} \pm 0.015$	~~$11.71^{aA} \pm 0.39$~~
	PAST	$95.80^{bA} \pm 6.45$	$0.372^{aA} \pm 0.028$	~~$9.97^{aA} \pm 0.74$~~
	STER	$112.62^{bA} \pm 6.42$	$0.450^{aA} \pm 0.021$	~~$12.05^{aA} \pm 0.56$~~
6	Control	$216.48^{aA} \pm 2.07$	$0.578^{aA} \pm 0.027$	~~$15.50^{aA} \pm 0.72$~~
	HPP400	$205.94^{aA} \pm 5.67$	$0.461^{bA} \pm 0.011$	~~$12.35^{bA} \pm 0.29$~~
	HPP600	$142.68^{bA} \pm 0.82$	$0.427^{bA} \pm 0.004$	~~$11.45^{bA} \pm 0.12$~~
	PAST	$113.89^{cA} \pm 6.31$	$0.348^{cA} \pm 0.008$	~~$9.33^{b,cA} \pm 0.22$~~
	STER	$110.31^{cA} \pm 1.97$	$0.413^{bA} \pm 0.016$	~~$11.06^{bA} \pm 0.44$~~

561 [a-c] lower case superscripts highlight differences and/or analogies among different treatments within a column.

562 [A-B] upper case superscripts indicate a significant difference (within a column) between the same treatment analysed at 0 d and 6 d of storage

563 time.

564 Abbreviations: raw, non-treated sample (control); 400 MPa for 3 min (HPP400), 600 MPa for 3 min (HPP600), pasteurized

565 (PAST) and sterilized (STER).

566

567

568

Tabella formattata

Table 2. Colorimetric parameters (L*, a*, b*, ΔE) of *A. platensis* aqueous suspensions (5% w/v). Treatments of *A. platensis* aqueous dispersion were: fresh, non-treated *A. platensis* (control); high pressure processed (HPP) sample at 400MPa (HPP400); HPP sample at 600MPa (HPP600); 90°C x 10 min pasteurized sample (PAST); 121°C x 20 min sterilized sample (STER). Treatments were analysed at time zero (0 d) and after 6 days of refrigerated storage.

Storage time (d)	Treatment	L*	a*	b*	ΔE
0	Control	$4.06^{bA} \pm 0.92$	$6.03^{bB} \pm 1.00$	$1.25^{bA} \pm 0.28$	-
	HPP400	$9.04^{aA} \pm 0.77$	$8.67^{aB} \pm 2.07$	$1.34^{bA} \pm 0.36$	$5.85^{cA} \pm 1.36$
	HPP600	$10.31^{aA} \pm 1.09$	$0.61^{dB} \pm 0.99$	$1.73^{bA} \pm 0.29$	$8.37^{cA} \pm 0.63$
	PAST	$11.85^{aA} \pm 2.32$	$-11.25^{eA} \pm 1.58$	$13.11^{aA} \pm 3.11$	$22.45^{aB} \pm 3.54$
	STER	$11.66^{aA} \pm 2.04$	$3.39^{cA} \pm 0.35$	$12.19^{aA} \pm 1.97$	$13.60^{bA} \pm 2.73$
6	Control	$6.23^{bcA} \pm 1.35$	$12.75^{bA} \pm 1.47$	$0.32^{bB} \pm 0.50$	-
	HPP400	$5.68^{cB} \pm 0.84$	$17.08^{aA} \pm 0.88$	$-1.46^{bB} \pm 0.54$	$4.81^{cA} \pm 0.78$
	HPP600	$6.30^{bcB} \pm 1.17$	$6.80^{cA} \pm 0.87$	$0.97^{bB} \pm 0.15$	$6.08^{cB} \pm 0.87$
	PAST	$11.36^{aA} \pm 2.83$	$-11.50^{eA} \pm 1.94$	$12.23^{aA} \pm 3.35$	$27.66^{aA} \pm 3.55$
	STER	$9.67^{abA} \pm 1.60$	$1.14^{dA} \pm 0.47$	$10.67^{aA} \pm 1.29$	$15.86^{bA} \pm 1.20$

[a-c] lower case superscripts highlight differences and/or analogies among different treatments within a column.

[A-B] upper case superscripts indicate a significant difference (within a column) between the same treatment analysed at 0 d and 6 d of storage time.

Abbreviations: lightness (L*), Redness (a*), Yellowness (b*) and total colour differences (ΔE) between 0 and 6 days.

ha formattato: Inglese (Stati Uniti)

Table 3. Total content of chlorophyll a (Ca), chlorophyll b (Cb), total chlorophylls (Ca + Cb), total carotenoids (Cx+c), total chlorophylls and carotenoids (Ccc), pheophytins (Cph) of different treatments of *A. platensis* in aqueous suspension (5% w/v). Treatments of *A. platensis* aqueous dispersion were: fresh, non-treated *A. platensis* (control); high pressure processed (HPP) sample at 400MPa (HPP400); HPP sample at 600MPa (HPP600); 90°C x 10 min pasteurized sample (PAST); 121°C x 20 min sterilized sample (STER). Treatments were analysed at time zero (0 d) and after 6 days of shelf life.

Storage time (d)	Treatment	Ca (µg/mL)	Cb (µg/mL)	Ca+Cb (µg/mL)	Cx+c (µg/mL)	Ccc (µg/mL)	Cph (µg/mL)
0	Control	$135^{aA} \pm 8$	$5.9^{aA} \pm 4.1$	$186^{aA} \pm 6$	$46^{aA} \pm 4$	$233^{aA} \pm 10$	$779^{aA} \pm 45$
	HPP400	$148^{aB} \pm 5$	$3.5^{abA} \pm 0.4$	$201^{aB} \pm 6$	$47^{aA} \pm 1$	$249^{aB} \pm 7$	$820^{aB} \pm 23$
	HPP600	$142^{aA} \pm 13$	$2.8^{abA} \pm 0.3$	$192^{aA} \pm 18$	$45^{aB} \pm 2$	$237^{aA} \pm 20$	$784^{aA} \pm 73$
	PAST	$92^{bB} \pm 8$	$2.1^{abA} \pm 0.6$	$125^{bB} \pm 11$	$43^{aB} \pm 5$	$168^{bB} \pm 16$	$476^{bB} \pm 43$
	STER	$43^{cA} \pm 3$	$0.6^{bA} \pm 0.2$	$57^{cA} \pm 4$	$26^{bA} \pm 1$	$83^{cA} \pm 5$	$184^{cA} \pm 11$
6	Control	$192^{aA} \pm 21$	$5.0^{aA} \pm 2.3$	$260^{aA} \pm 30$	$60^{aA} \pm 6$	$320^{aA} \pm 36$	$1062^{aA} \pm 123$
	HPP400	$189^{aA} \pm 10$	$5.0^{aA} \pm 2.1$	$257^{aA} \pm 11$	$58^{aA} \pm 4$	$315^{aA} \pm 14$	$1017^{aA} \pm 49$
	HPP600	$185^{aA} \pm 10$	$4.6^{abA} \pm 1.8$	$251^{aA} \pm 11$	$59^{aA} \pm 3$	$309^{abA} \pm 15$	$1034^{aA} \pm 43$
	PAST	$138^{bA} \pm 7$	$1.2^{abA} \pm 0.6$	$185^{bA} \pm 9$	$60^{aA} \pm 3$	$245^{bcA} \pm 12$	$702^{abA} \pm 44$
	STER	$105^{bA} \pm 21$	$0.7^{bA} \pm 0.5$	$141^{bA} \pm 29$	$53^{aA} \pm 12$	$194^{cA} \pm 41$	$484^{bA} \pm 113$

[a-c] lower case superscripts highlight differences and/or analogies among different treatments within a column.

[A-B] upper case superscripts indicate a significant difference (within a column) between the same treatment analysed at 0 d and 6 d of storage time.

Table 4. Microbial concentration at 0 d and after 6 d of storage at refrigerated temperatures. Microbial concentration was expressed as Log CFU/ml.

Storage time (d)	Treatment	Microbial species						
		Aerobic spor.	Anaerobic spor.	TBC	Yeast	Salmonella	Coliform	Staphylococci
0	Control	2.84 ± 0.12^{aA}	2.66 ± 0.19^{aA}	5.96 ± 0.05^{aA}	4.44 ± 0.41^{aA}	4.53 ± 0.15^{aA}	5.06 ± 0.08^{aA}	4.94 ± 0.19^{aA}
	HPP400	1.88 ± 0.29^{bA}	1.83 ± 0.27^{bA}	1.66 ± 0.08^{bA}	$<1^{bA}$	2.70 ± 0.17^{bA}	nd^{bA}	1.70 ± 0.20^{bA}
	HPP600	1.81 ± 0.31^{bA}	1.70 ± 0.20^{bA}	1.00 ± 0.17^{bA}	$<1^{bA}$	1.85 ± 0.08^{bA}	nd^{bA}	1.70 ± 0.10^{bA}
	PAST	1.86 ± 0.32^{bA}	1.66 ± 0.25^{bA}	1.66 ± 0.19^{bA}	nd^{bA}	nd^{cA}	nd^{bA}	$<1^{bA}$
	STER	nd^{cA}	nd^{cA}	nd^{cA}	nd^{bA}	nd^{cA}	nd^{bA}	nd^{bA}
6	Control	1.44 ± 0.29^{aB}	2.65 ± 0.17^{aA}	6.90 ± 0.07^{aB}	5.14 ± 0.12^{aB}	2.10 ± 0.10^{aB}	6.83 ± 0.15^{aB}	6.76 ± 0.21^{aB}
	HPP400	1.86 ± 0.26^{bA}	1.85 ± 0.19^{bA}	3.40 ± 0.09^{bB}	1.18 ± 0.17^{bA}	nd^{bB}	nd^{bA}	1.83 ± 0.08^{bB}
	HPP600	1.71 ± 0.21^{bB}	1.56 ± 0.17^{bB}	2.35 ± 0.12^{bB}	1.30 ± 0.08^{bB}	nd^{bB}	nd^{bA}	1.81 ± 0.13^{bB}
	PAST	1.48 ± 0.26^{bB}	1.50 ± 0.31^{bB}	2.00 ± 0.08^{bB}	nd^{cA}	nd^{bA}	nd^{bA}	1.78 ± 0.31^{bB}
	STER	nd^{cA}	nd^{cA}	nd^{cA}	nd^{cA}	nd^{bA}	nd^{bA}	nd^{cA}

Results are shown as the mean ± standard deviation. nd, not detected

$^{a-c}$ Different lower-case superscript letters highlight significant differences ($P<0.05$) among treatments within each column

$^{A-B}$ Different upper-case superscript letters highlight significant differences ($P<0.05$) between the same treatments at 0 and 6 days of storage

Abbreviations: aerobic spore-forming bacteria (Aerobic spor), anaerobic spore-forming bacteria (Anaerobic spor.), Total Bacteria Count (TBC).

606 **Figure captions**

607 **Fig.1** Visual appearance of *A. platensis* in aqueous suspensions (5% w/v) colour characteristics at 0 and 6 d of storage time.
608 Samples were: fresh, non-treated *A. platensis* (control); high pressure processed (HPP) sample at 400 MPa (HPP400); HPP
609 sample at 600 MPa (HPP600); 90°C x 10 min pasteurized sample (PAST); 121°C x 20 min sterilized sample (STER).
610 Treatments were analysed at time zero (0 d) and after 6 days of refrigerated storage.

611

612 **Fig.2** Water holding capacity (WHC) of *A. platensis* in aqueous suspensions (5% w/v) at 0 and 6 d of storage time. Samples
613 were: fresh, non-treated *A. platensis* (control); high pressure processed (HPP) sample at 400 MPa (HPP400); HPP sample at
614 600 MPa (HPP600); 90°C x 10 min pasteurized sample (PAST); 121°C x 20 min sterilized sample (STER). Treatments were
615 analysed at time zero (0 d) and after 6 days of refrigerated storage.

616

617 **Fig.3** Concentration of phycocyanin (µg/mL) of different treatments of *A. platensis* in aqueous suspension (5% w/v).
618 Treatments of *A. platensis* aqueous dispersion were: fresh, non-treated *A. platensis* (control); high pressure processed (HPP)
619 sample at 400MPa (HPP400); HPP sample at 600MPa (HPP600); 90°C x 10 min pasteurized sample (PAST); 121°C x 20
620 min sterilized sample (STER). Treatments were analysed at time zero (0 d) and after 6 days of shelf life.

621

622 **Fig.4** Light microscopy observations (100x magnification) of *A. platensis* in aqueous suspensions (5% w/v) processed by
623 applying different treatments. (**a**) raw, control suspension; (**b**) HPP sample processed at 400 MPa x 3 min; (**c**) HPP sample
624 processed at 600 MPa x 3 min; (**d**) sample pasteurized at 90 °C x 10 min; (**b**) sample sterilized at 121°C x 20 min. **CF**: cell's
625 fragments; **CB**: cell's breakdown; **AG**: aggregates.

ha formattato: Tipo di carattere: Grassetto

626

627 **Fig.5** Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) results of *A. platensis* aqueous suspensions
628 (5 w/v) at 0 d of storage. Treatments of *A. platensis* aqueous dispersion were: fresh, non-treated *A. platensis* (control); high
629 pressure processed (HPP) sample at 400MPa (HPP400); HPP sample at 600MPa (HPP600); 90°C x 10 min pasteurized sample
630 (PAST); 121°C x 20 min sterilized sample (STER). Treatments were analysed at time zero (0 d) and after 6 days of shelf life.
631 Band 1: subunit of C-phycocyanin; band 2: subunit of C-phycocyanin; band 3: chlorophyll a complex; band 4: chlorophyll a
632 complex