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Additional Information

EVALUATION OF STRATEGIES FOR PRESERVATION OF MICROALGAE *Chlorella*

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ABSTRACT

The biomass obtained from microalgae, such as *Chlorella*, is used to make dietary products, supplements and pharmaceuticals. However, microalgae are produced very far from consumption places. One of the most usual distribution forms is as a dry product, a process that entails high production costs and leads to the loss of certain nutritional properties. Therefore, the aim of this study was to evaluate alternative preservation strategies for microalgae *Chlorella* other than dehydration and freezing. To that end, sterilization, acidification and packaging material were analyzed during two months of storage under different temperature and light exposure conditions. The results showed that color was modified considerably by sterilization, regardless of light exposure and type of package, whereas citric acid preserved color, especially at low storage temperatures. Furthermore, the study shows that acidification with 3.5% of citric acid and vacuum packaging are the recommended treatment for microalgae, without the need for cold storage.

KEYWORDS: microalgae, Chlorella, sterilization, acidification, packaging

type, storage temperature.

PRACTICAL APPLICATIONS

Stabilization of microalgae *Chlorella* from production to consumption places could increase the possibilities of commercialization of this product, recently labeled 'superfood' by the UN Food and Agriculture Organization. In order to preserve all their nutritional properties for at least 2 months, acidification with 3.5% of citric acid and vacuum packaging are the recommended treatments, without the need for cold storage.

1. INTRODUCTION

Mass production of microalgae was first carried out by Germany during World War II in order to obtain lipids to be used as source of biofuel. After World War II, microalgae biomass started to be considered as a supplement able to replace conventional animal or vegetable proteins in direct consumption by cattle or humans, shortening the inefficient protein food chain. Thus, from 1948 onwards, a group of scientists at Carnegie Institution of Washington performed the first systematic studies, establishing the scientific fundamentals of massive culture of microalgae. The aim was to use green microalgae *Chlorella* for large scale food production (Burlew, 1953).

Chlorella, which belongs to the phylum Chlorophyta, is a green microalga with a diameter of 2–10 μm. It contains a single chloroplast, is unicellular, coccoidal,

and nonmotile and is widely present in fresh, brackish and marine water (Nurachman et al., 2015).

Microalgae biomass has been described as a 'superfood' by the UN Food and Agriculture Organization (FAO) due to its high nutritional content, concerning not only protein levels (approx. 50% of dry matter, easily digestible due to a very complete amino acid profile), but also other essential components for metabolism, such as minerals, vitamins, antioxidants and polyunsaturated fatty acids, especially Omega 3 and 6 (Buggypower, 2016).

It is a source of ß-glucan, which is an active immunostimulator, reducer of blood lipids, and a free radical scavenger (Sanghvi & Lo, 2010). In addition, its contents include 1-4% chlorophyll, a pigment that detoxifies heavy metals and pesticides from the body (Se-Kwon, 2015).

The presence of polyunsaturated fatty acids (PUFAs) in microalgae should also be highlighted: Two of the most abundant fatty acids are linoleic acid (LA) and alpha-linolenic acid (ALA). Arachidonic acid Omega 6 can be synthesized by humans from LA and essential Omega 3 acids, such as DHA and EPA, from ALA (Solana, Rizza & Bertucco, 2014). Traditionally, they have been obtained from fish and fish oils, but safety issues have arisen due to the accumulation of toxins in fish (Sanghvi & Lo, 2010). Microalgae may be considered as the initial EPA and DHA producers in the marine food chain, reaching higher EPA and DHA contents than other possible sources, such as certain fishes or soybean (Adarme-Vega, Lim, Timmins, Vernen, Li, & Schenk, 2012).

Additionally, microalgae have a beneficial impact on the environment, since they release oxygen and consume 1.87 kg of CO_2 per kg of dry biomass produced (Buggypower, 2016; Solana et al., 2014).

Despite the above advantages, the production of microalgae entails several problems, due to high installing and operating costs, difficulties in controlling the culture conditions, bacterial contamination or alien algae and instability of light supply and weather (Yen, Hu, Chen, Ho, Lee & Chang, 2013). Moreover, production takes place far away from consumption sites, adding to the challenge of preserving their beneficial features during transport and ensuring an adequate shelf life to guarantee successful commercialization. Currently, they can be found in specialized markets in a dehydrated format only, further increasing production costs.

Recently, Misra, Koubaa, Roohinejad, Juliano, Alpas, Inácio, Saraiva & Barba (2017) have reviewed the landmarks in the historical development of 21st century food, focusing on the importance of the emerging technologies to reduce the consumption of energy and improve the sustainability of the food chain. These technologies can broadly be divided into thermal (e.g. ohmic heating) and non-thermal (e.g, high-pressure processing, electro technologies and irradiation). However, all these techniques required considerable investments in the acquisition of new equipment, hindering their extension. Furthermore, according to this review, thermal processes remain the most prevalent technologies even today. Low temperature preservation/cold storage of food is known since prehistoric times, when people kept food products in caves, aware that food could be preserved for longer periods in

cold places (Gavroglu, 2014). On the other hand, the introduction of heat in food processing allowed for the preservation of food during long sea voyages, as used for instance by the French army of Napoleon Bonaparte thanks to the first developments by Nicholas Appert, published in his 1812 work "The Art of Preserving Foods for Many Years" (Featherstone, 2012).

Similarly, it is well known that acidification of foods to pH levels of 4.6 or below avoid the growth of the microorganisms responsible for foodborne diseases, such as botulism. Hence, Title 21 of the Code of Federal Regulations, Part 114 (21CFR114) regulates acidified foods (FDA USA Food & Drug Administration, 2016). However, even when manufactured under proper conditions of acidification and sanitation, food products may still be spoiled by yeasts and molds. In order to prevent this, acid and acidified foods are usually heated to 180ºF and packaged hot to kill yeast and mold spores on the products and in the container and cap (Rushing & Curtis, 1993).

Regarding the influence of light exposure in the degradation of different nutritional components, several studies have confirmed that storage in dark places increases the shelf life of food products by preventing oxidative and hydrolytic degradations (Caponio, Bilancia, Pasqualone, Sikorska & Gomes 2005; Brothersen, McMahon, Legako, & Martini, 2016).

Therefore, the combination of different preserving methods (e.g., pH, a_w, salt content, storage temperature, etc.) enhances the benefits provided by each of those methods separately due to a synergistic preservation effect. In this regard, the use of hurdle technology is considered as a key to future food preservation (Leistner, 2000; Rahman, 2016) and its state of art was recently

reviewed (Singh & Shalini, 2016). Concerning the preservation of microalgae Chlorella, the study of how these hurdles could contribute to extend their shelf life may open new chances to safely distribute this product from the production to the consumption place.

Considering all the above, the aim of this study was to assess different preservation strategies (thermal sterilized treatment, packaging material, freezing or cooling, reduced pH and light exposure) for microalgae produced by a factory located in Madeira in order to make transport to their sales outlets more viable. To that end, moisture content, pH, water activity, salt content, changes in color and microbial growth were analyzed during two months of storage.

2. MATERIALS AND METHODS

2.1. Raw Material

Microalgae samples were delivered frozen in polyethylene bags by the Buggypower Company, which owns a production plant in Madeira Island (Portugal), where microalgae are grown in vertical photobioreactors. In turn, dehydrated green and red commercial algae were used to compare their characteristics with microalgae. For that purpose, the commercial algae were rehydrated using an algae:water ratio of 5:500 (w/w) for 20 minutes as per the manufacturer's recommendation. Then, algae were drained.

2.2. pH Adjustment of Microalgae

In order to reduce the pH of microalgae samples, citric acid was added at different concentrations (2.5% or 3.5%) to check the effect of this preservative on shelf life.

2.3. Thermal Treatment of Microalgae

Microalgae were sterilized in an autoclave at 115ºC for 15 minutes to extend their shelf life.

2.4. Packaging and Storage of Microalgae

Samples packaged in jars or in bags of bi-oriented polyamide/polypropylene thermal resistant material (Bolsemack S.L.) were vacuum-sealed and subsequently stored at room temperature, whereas samples stored at 4ºC or -18ºC were sealed without removing the air in the headspace of the package. Furthermore, in samples stored at room temperature, the influence of light was also controlled by leaving part of them in dark chambers and exposing the rest to light. Table 1 provides a description of all the combinations used in the storage of microalgae along with the notation for each case. Microalgae samples were stored for 2 months. For each of the thirteen preserving treatments shown in Table 1, eighteen package units were prepared in order to have enough samples for all the analysis performed in different days of the storage period. Therefore, a total amount of 234 units of preservation were considered. 20 g. of microalgae were placed in each package unit.

TABLE 1. Description and notation of the treatments for stabilization of microalgae

STORAGE CONDITIONS	DESCRIPTION	NOTATION
FRIDGE (4ºC)	BAG- 3.5% citric acid – Without thermal treatment	Bag 3.5 4C
	BAG – 2.5% citric acid – Without thermal treatment	Bag 2.5 4C
	BAG – Without citric acid – Without thermal treatment	Bag 4C
	JAR- Without citric acid – Without thermal treatment	Jar 4C
ROOM TEMPERATURE – WITHOUT LIGHT	BAG - 3.5% citric acid - Without thermal treatment	Bag 3.5 RT
	BAG - 2.5% citric acid - With thermal treatment	Bag 2.5 TT RT
	BAG – Without citric acid – With thermal treatment	Bag TT RT
	JAR - Without citric acid – With thermal treatment	Jar TT RT
ROOM TEMPERATURE – WITH LIGHT	BAG – 3.5% citric acid – Without thermal treatment	Bag 3.5 RT L
	BAG – 2.5% citric acid – With thermal treatment	Bag 2.5 TT RT L
	BAG – Without citric acid - With thermal treatment	Bag TT RT L
	JAR - Without citric acid - With thermal treatment	Jar TT RT L
FREEZER (-18ºC)	BAG- Without citric acid – Without thermal treatment	Bag -18C

2.5 Analytical Determinations

Analytical determinations of moisture content, pH, a_w and color measurements were performed on day 0, 30, 45 and 60 after packaging. Additionally, microbiological analysis of mesophyllic aerobics, molds and yeasts were performed to analyze the evolution of microalgae each 30 days during two months. The determination of the effect of citric acid on the pH of microalgae was performed initially. By contrast, the analysis of sodium chloride was performed at the end of the study period. All determinations were carried out in triplicate. Thus, initially and after 45 days of storage, 3 different packaging units were used to perform the analysis of the aforementioned parameters, whereas after 30 and 60 days of storage 6 packaging units were used, since

microbial counts were also registered. A methodological description is provided below:

2.5.1 MOISTURE

Moisture determination was performed based on an adaptation of method 934.06 (AOAC, 2000): The sample was heated under -0.8 bar of pressure and 60ºC in a vacuum oven (JP Selecta model Vaciotem-T), and the loss of weight was used to calculate the moisture content of the sample.

The purpose of using a vacuum oven is to minimize cold spots as well as to vent moisture from inside air (Nielsen, 2010). The moisture content results were given in g of water per g of microalgae.

2.5.2 WATER ACTIVITY (a_W)

Measurements were performed with a water activity meter (Aqualab, model 4TE) at 25 °C.

2.5.3 pH

Measurements were performed in duplicate using a pH meter with a contact electrode (METTLER TOLEDO SevenEasy model), previously calibrated with buffer solutions of pH 4.00 and 7.00 at 25 ° C.

2.5.4 SODIUM CHLORIDE (x_S)

Measurements were carried out in triplicate with a Chloride Analyzer (CORNING, model 925) using a combined acid buffer solution as a support

electrolyte that maintained the correct pH for the complete cycle of titrations and a colloid to prevent precipitation. This equipment was previously calibrated with a standard solution of 200 mg /L ClNa at 25 ° C.

Aliquots of microalgae samples were diluted at a 1:200 (w/w) ratio and titrated in the equipment. The sodium chloride results were given in g of ClNa per g of microalgae.

2.5.5 OPTICAL PROPERTIES

Surface color was measured with a spectrocolorimeter (Minolta, model CM-3600d) by registering reflectance and using the small lens. The spectrocolorimeter was calibrated and measurements were performed in triplicate over each surface of samples placed in cuvettes of 1-cm thickness. Color was recorded using the CIE-L*a*b* uniform color space (L*a*b*) considering the observer 10^0 and the illuminant D65.

2.5.6 MICROBIOLOGICAL ANALYSIS

Microbiological analysis of mesophyllic aerobic, molds and yeasts were performed on the different treatments of treated microalgae at day 30 and day 60 of storage. The serial dilutions of samples were seeded using the pour plate technique by duplicate. Aerobic mesophylls were analyzed using plate count agar (Scharlau, 01-161-500, Barcelona, Spain) and incubated at 35±2.0°C for 48 hours. Molds and yeasts were analyzed using Oxytetracycline Sabouraud Agar Base (Scharlau, 01-275-500, Barcelona, Spain) with a sterile

Oxytetracycline selective supplement (Scharlau, 06-115LYO1, Barcelona, Spain) and incubated at 25±2.0°C for 5 days.

For aerobic mesophylls, plates containing between 25 and 250 colonies were considered (FDA, 2001), while in the case of yeasts and molds, plates containing between 0 and 30 colonies were counted. The results were expressed as log CFU/g.

2.6 Statistical Analysis

The statistical software Statgraphics Centurion XVI was used to evaluate the statistical significance of the different treatments applied to the preservation of microalgae, storage time and different conditions of storage. Interactions of the factors studied were analyzed with a significance level of 95 % ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Initial Characterization of Algae

Table 2 shows the moisture (x_w), sodium chloride content (x_s), pH and water activity (a_w) results of microalgae *Chlorella* and the two dehydrated commercial algae that were rehydrated for analysis. The results show similar values of water activity in all cases, while water content was higher in rehydrated algae due to the process itself. Furthermore, pH was higher in Green algae. In terms of salt content, red algae showed the lowest value, despite the fact that all algae came from seawater, where salt concentration is around 3.5% (Park et al., 2011). The differences in salt content were also shown on the label of

commercial algae since the Green type had 1.6 g of salt/g of dry matter and the Red type, 0.7 g of salt /g of dry matter.

TABLE 2. Initial characterization of algae

Type of algae	pH	a_w	x_w (g water/g algae)	x_s (g salt/g algae)
Green algae (commercial)	7.60	0.9938	0.9192±0.0009[b]	0.02107±0.00016[b]
Red algae (commercial)	6.97	0.9973	0.9425±0.0036[c]	0.00311±0.00016[a]
Microalgae (*Chlorella*)	5.75	0.9814	0.8406±0.0007[a]	0.02033±0.00019[b]

Equal letters indicate homogeneous groups.

3.2. Effect of Citric Acid on the pH of Microalgae

One of the classic strategies used in food preservation is the reduction of pH. Most foodborne pathogens cannot grow at a pH less than 4.4 (Montville & Matthews, 2009). Foods with lower pH values (below 4.5) are not easily altered by bacteria, being more sensitive to alteration by yeasts and molds (Casp & Abril, 2003). Therefore, in this study, preliminary experiments were performed using citric acid to achieve a pH below 3.5 to ensure greater stability of microalgae. The results were fitted to equation 1 ($R^2 = 0.9967$). As can be seen, with 2.5% of citric acid, samples reached a pH lower than 4.5. Thus, this was the first concentration of citric acid chosen to extend the shelf life of microalgae with mild thermal treatment. It was also decided to work with a concentration of 3.5 % citric acid to see the influence of a lower pH on the stability of microalgae.

$$pH=5.6221\cdot[\%\ \text{Added citric acid}]^{-0.278} \quad \text{(equation 1)}$$

In general, no factor affected the pH, with the exception of the initial value of citric acid added, as expected. More specifically, samples of microalgae without citric acid at room temperature obtained a pH of 6.27±0.13 and 5.9±0.2 at refrigerated/frozen temperature. Regardless of temperature, adding 2.5% of citric acid to stored microalgae lead to pH values of 4.1±0.3 and adding 3.5% of citric acid lead to pH values of 3.52±0.03. No influence of storage time was registered.

3.3 Moisture Content and Water Activity

The water content values of microalgae as a function of the storage temperature, citric acid addition, exposure to light, packaging material and thermal treatment are shown in Figure 1. In all cases, there was a reduction in the amount of water compared to the initial values of microalgae. In addition, the most remarkable fact was that the higher the citric acid concentration, the lower the moisture content in microalgae, which is coherent with the increase of solids in the samples, although no differences between both concentrations of citric acid were observed for refrigerated storage. Overall, there was a slight decrease in moisture content over time, probably due to water permeability of the packaging material. Thus, glass material, impermeable to water vapor, was better able to maintain moisture content. In fact, the slight difference in moisture content of microalgae packaged in jars or in bags when stored at room temperature was not detected when the microalgae were refrigerated, since permeability depends on temperature. On the other hand, at room temperature, exposure to light had no significant impact on moisture content.

At freezing temperatures, moisture loss in samples was slightly lower than in samples stored under refrigerated conditions.

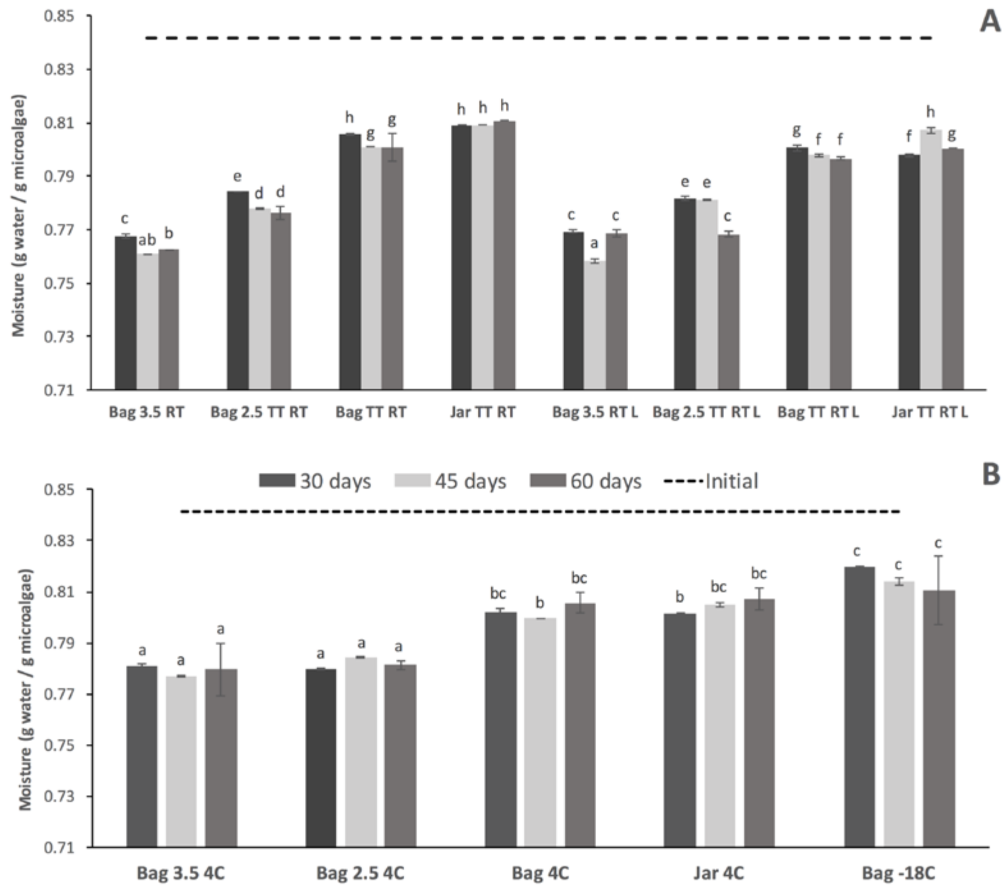

FIGURE 1. Moisture content of treated microalgae as a function of storage temperature and time. **A:** Storage at room temperature. **B:** Frozen or refrigerated storage. The notation indicates whether the microalgae were stored in bags or jars, with 3.5% (3.5) or 2.5% (2.5) citric acid and at room temperature (RT), 4 ºC (4C) or -18ºC (-18C). TT refers to cases in which microalgae were subjected to thermal treatment in an autoclave at 115ºC for 15 minutes and L indicates that the microalgae were exposed to light during the storage time.

Despite the differences in moisture content of microalgae depending on the addition of citric acid, water activity remained quite similar to the raw material for all cases (0.976±0.004).

3.4 Sodium Chloride Content (x_s)

In general, after two months of storage there was an increase in salt content (2.21±0.06 g of NaCl/100 g of microalgae) with respect to the values of raw material (2.03±0.02 g of NaCl/100 microalgae), in coherence with the loss of moisture content mentioned previously, with no significant differences between the treatments considered.

3.5 Optical Properties

Figures 2 shows the location of the coordinates b* and a* in the chromatic plane for microalgae stored at room or refrigerated/frozen temperatures.

It is worth noting that the thermal treatment increased a* and b* coordinates, which implied a browning of the microalgae and a change in the quadrant position of samples (from the second to the first) in the chromatic diagram. Both coordinates decreased at room temperature over storage time. With regard to citric acid, it significantly reduced coordinate a* initially, leading to a more intense green color. This greenish color was maintained in microalgae stored at refrigerated/frozen temperature, disappearing when they were stored at room temperature. In fact, the highest values of hue were for microalgae not treated with citric acid and stored at low temperature. Therefore, thermal treatment was the main factor behind the browning of microalgae as has been observed in previous studies: Chlorophyll is barely detectable after treatment with temperatures higher than 40ºC in kiwifruit pulp (Schwartz, Núñez, Muñoz & María, 1999). It is also important to bear in mind that heating caused the EPA and DHA content to significantly decrease (Hadipranoto, 2005). Exposure

to light did not lead to a change in color of microalgae. Consequently, they can be considered suitable for distribution in transparent packaging. Moreover, no differences in color of samples placed in bags or jars were detected, so it seems plastic bags are both easier and cheaper to use.

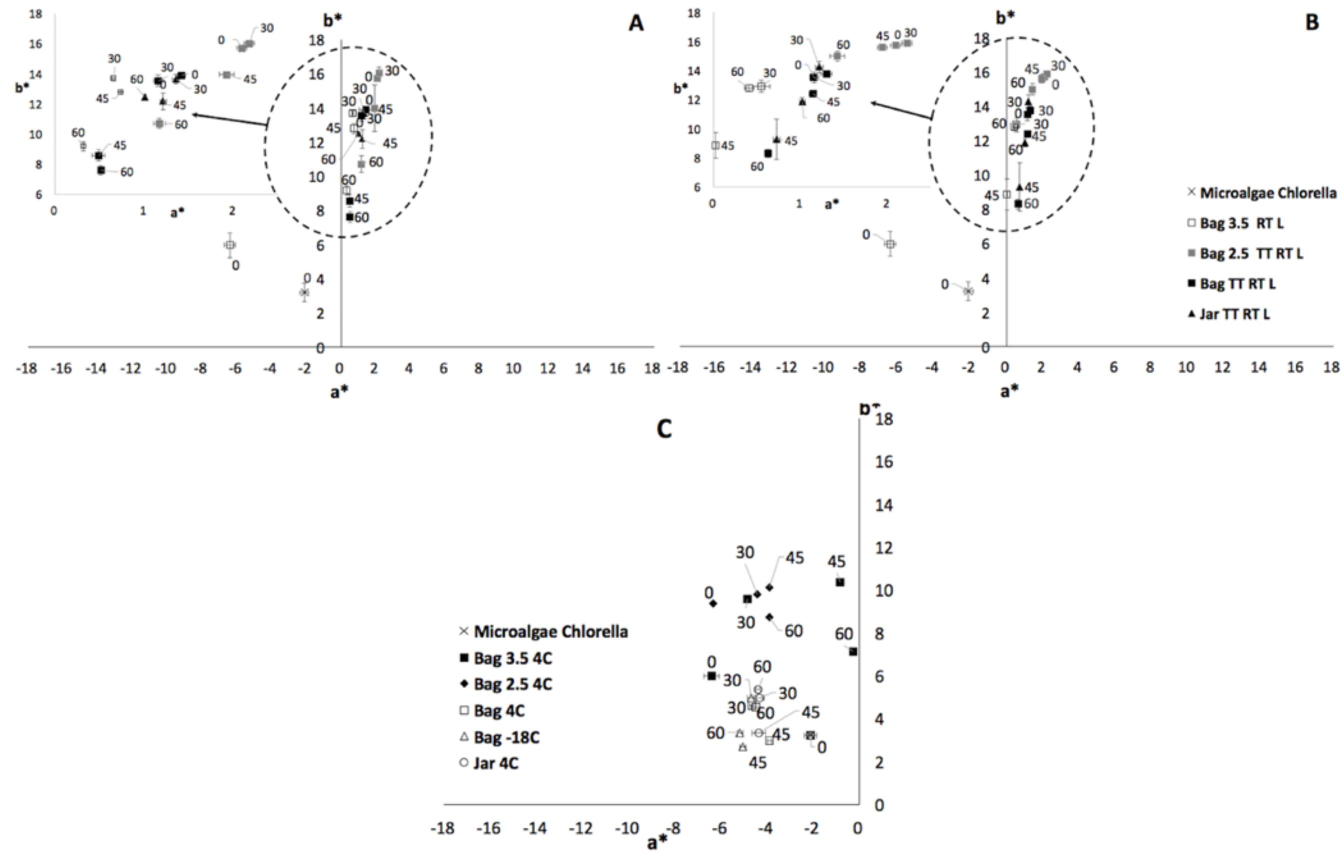

FIGURE 2. Chromatic planes b*-a* of treated microalgae stored at room temperature without exposure to light (**A**) or with light (**B**) and at refrigerated/frozen temperature (**C**). Numbers in the labels of each symbol represent the days of storage.

The luminosity results for treated microalgae are shown in Figure 3. Overall, this parameter changed less at room temperature than at refrigerated/frozen temperature. It is likely that the thermal treatment applied was responsible for this homogeneity. Moreover, the addition of citric acid at room temperature contributed to a higher luminosity at the end of storage. No significant differences were found concerning the impact of light exposure. In the case of microalgae stored at refrigerated/frozen temperature, luminosity was very similar to the raw material values up to the 30th day of storage. However, this was followed by a significant decrease in this parameter, except for microalgae treated with citric acid. The packaging material did not affect the luminosity values.

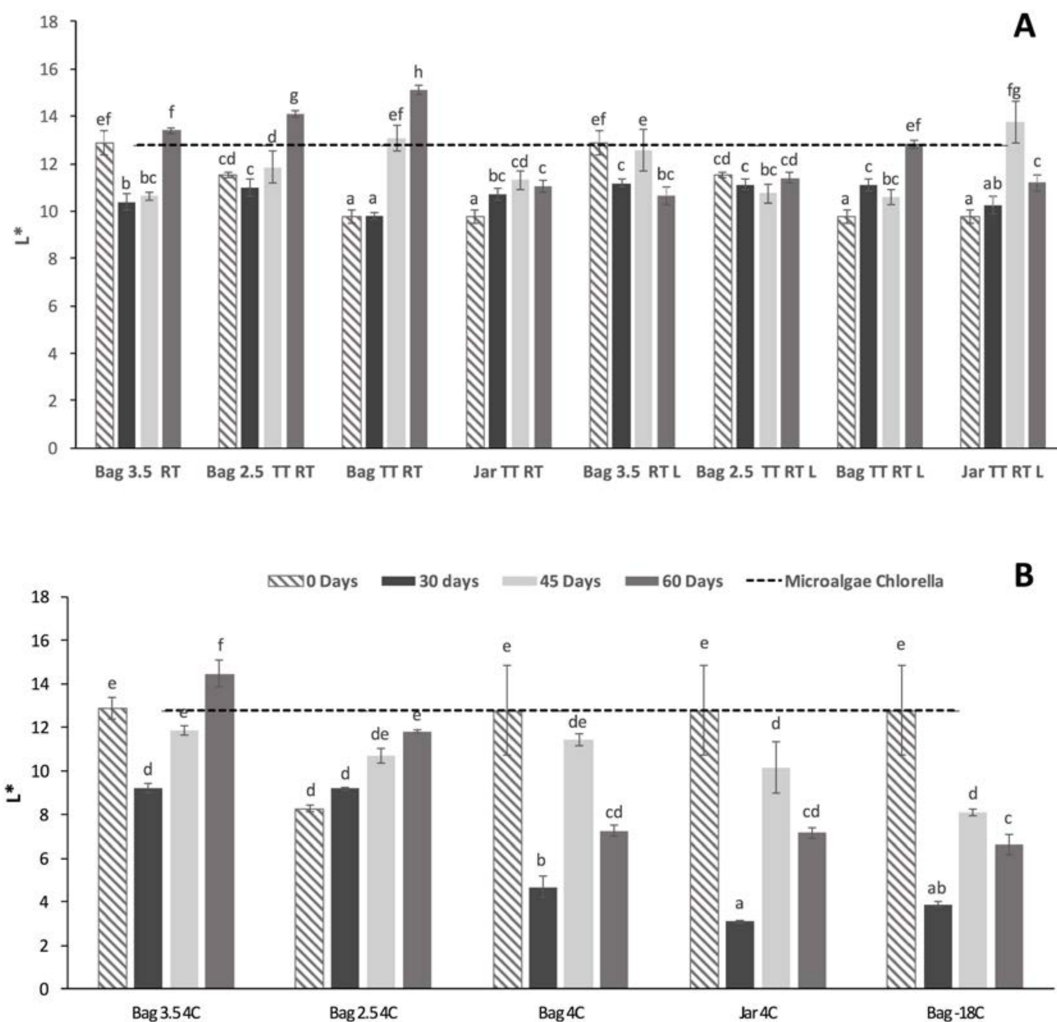

FIGURE 3. Luminosity of treated microalgae over time at room temperature (**A**) and at refrigerated/frozen temperature (**B**). Dashed lines represent the initial values for raw material. The notation indicates whether the microalgae were stored in bags or jars, with 3.5% (3.5) or 2.5% (2.5) citric acid and at room temperature (RT), 4 ºC (4C) or -18ºC (-18C). TT refers to cases in which microalgae were subjected to thermal treatment in an autoclave at 115ºC for 15 minutes and L indicates that the microalgae were exposed to light during the storage time.

3.8 Microbiology Counts

The aerobic mesophyllic, molds and yeasts counts of treated microalgae are represented in Figure 4. In the absence of legislation about these products, the acceptable limits for both types of microorganisms laid down in the Spanish regulations concerning hygiene standards for the elaboration, distribution and

marketing of processed foods (BOE 12-1-2001, R.D. 3484/2000, 29-12-2000) have been followed. More specifically, the threshold for aerobic mesophylls on the expiry date was $1x10^6$ CFU/g and the threshold for molds and yeasts was $1x10^2$ CFU/g, based on the criterion for fruits and vegetables recommended by Pascual & Calderón, 2000.

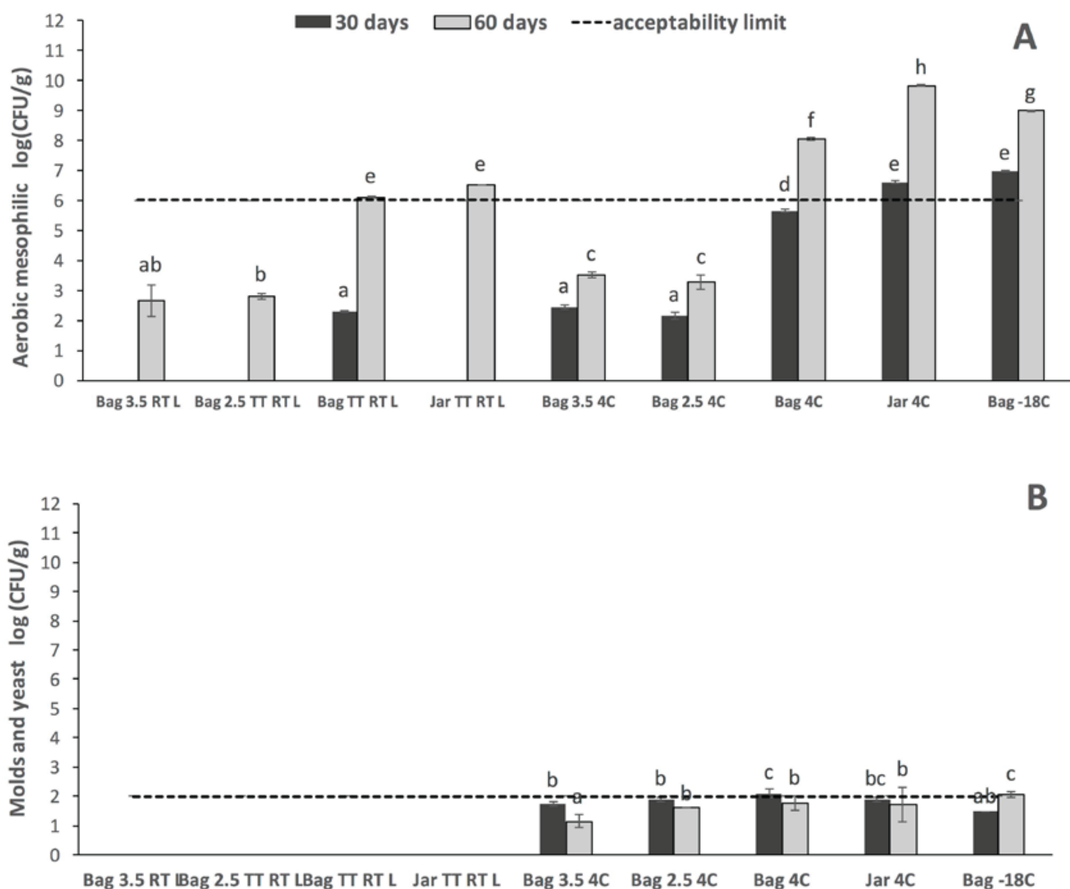

FIGURE 4. Microbiological content of treated microalgae as a function of storage temperature and time. **A:** Aerobic mesophilic counts. **B:** Molds and yeast counts. The notation indicates whether the microalgae were stored in bags or jars, with 3.5% (3.5) or 2.5% (2.5) citric acid and at room temperature (RT), 4 ºC (4C) or -18ºC (-18C). TT refers to cases in which microalgae were subjected to thermal treatment in an autoclave at 115ºC for 15 minutes and L indicates that the microalgae were exposed to light during the storage time.

According to our results, the addition of citric acid significantly reduced the growth of aerobic mesophylls in samples during the storage period analyzed.

However, no significant differences in growing aerobic mesophylls between the two tested citric acid levels were detected in microalgae stored at room temperature. In non-acidified samples, the acceptable limit was exceeded after 30 to 60 days of storage, even in thermally treated samples. This could be connected with the initial pollution level in the raw material or to the presence of spores. It should be pointed out that low storage temperatures did not slow down aerobic mesophyllic growth. In addition, the absence of oxygen in containers stored at room temperature would be responsible for the slower growth of microorganisms.

The thermal treatment applied reduced the growth of molds and yeast during the analyzed storage time. Again, low temperatures (4 and -18ºC) did not reduce the development of this type of microorganism, although the established threshold was not exceeded. Finally, in this case, citric acid did not improve the stability of microalgae. As mentioned previously, molds and yeast are less sensitive to pH reduction, with their minimum pH value for proper growth ranging from 1.5 to 3.5 (Casp & Abril, 2003).

To sum up, although thermal treatment could lead to a reduction of molds and yeasts, it is not clear whether this response is related to the absence of oxygen, since all thermally treated samples were vacuum packaged. Therefore, the addition of 3.5% of citric acid would be the recommended treatment to guarantee the maximum shelf life from a microbiological point of view.

4. CONCLUSIONS

Thermal treatment considerably changed the color of this product regardless of light exposure and type of packaging, whilst citric acid preserved it, especially at low storage temperatures. In terms of microbial stability, acidification with 3.5% citric acid and vacuum packaging would be the recommended treatment for microalgae, without the need for cold storage. In conclusion, the best way to transport and distribute microalgae would be acidification and vacuum packaging in bi-oriented polyamide/polypropylene bags stored at 4ºC.

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