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Microalgae biomass as an alternative ingredient in cookies: Sensory, physical and chemical properties, antioxidant activity and *in vitro* digestibility

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ABSTRACT

Microalgae can be regarded as an alternative and promising food ingredient due to their nutritional composition, richness in bioactive compounds, and because they are considered a sustainable protein source for the future. The aim of this work was to evaluate microalgae (*Arthrospira platensis* F & M-C256, *Chlorella vulgaris* Allma, *Tetraselmis suecica* F & M-M33 and *Phaeodactylum tricornutum* F & M-M40) as innovative ingredients to enhance functional properties of cookies. Two biomass levels were tested and compared to control: 2% (w/w) and 6% (w/w), to provide high levels of algae-bioactives. The cookies sensory and physical properties were evaluated during eight weeks showing high color and texture stability. Cookies prepared with *A. platensis* and *C. vulgaris* presented significantly ($p < 0.05$) higher protein content compared to the control, and by sensory analysis *A. platensis* cookies were preferred. Besides, *A. platensis* also provided a structuring effect in terms of cookies texture. All microalgae-based cookies showed significantly higher ($p < 0.05$) total phenolic content and *in vitro* antioxidant capacity compared to the control. No significant difference ($p < 0.05$) in *in vitro* digestibility between microalgae cookies and the control was found.

1. Introduction

Microalgae can be considered an innovative and promising food ingredient, rich in nutrients such as high value proteins, long-chain polyunsaturated fatty acids, carotenoids, vitamins, minerals, and phenolics as well as other bioactive molecules [1]. Different companies are currently investing in this innovative microalgae-based food sector, such as Terravia (ex-Solazyme, USA), currently producing and commercializing algae food ingredients such as protein isolates and culinary oils (http://terravia.com/). Another example is Dulcesol Group (http://en.dulcesol.com/), leader in baked products and pastries sector in Spain, which has also invested in a microalgae production unit for developing a healthy baked product line with *Chlorella* incorporation [2–3]. Moreover, other companies are starting to pay attention to this area and according to Credence Research Report [4] the international algae products market is expected to reach US$ 44.7 billion by 2023, growing at a compound annual growth rate of > 5.0% in the 2016–2023 period. Nutraceuticals dominate the algae products market followed by Food & Feed applications [4].

However, Europe lacks a gastronomy tradition and consumer history with microalgae (in contrast with some South-East Asia countries), which makes effective marketing and consumer acceptability of microalgae based products more difficult [5]. In fact, the use of microalgae as a food source is still poorly developed in Europe, which has been mainly attributed to three major factors: i) technical difficulties related to their cultivation and high production costs; ii) low demand in European countries compared to Asian markets; iii) strict European legislation regarding Novel Foods [5].

In the last years some works have been published on innovative and healthy food products integrated with microalgal biomass such as pasta, biscuits and vegetarian “mayonnaises” and gelled desserts [6–12]. Although the bioactive properties of microalgae biomass and/or of its extracts has been extensively demonstrated (*e.g.* [1,13]), only few studies deal with bioactivity of microalgae-based foods and their response to different processing steps [10,14]. There is a lack of knowledge on how food processing conditions influence digestibility, bioavailability and bioactive properties of microalgae functional ingredients in different food matrixes.

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Cookies are considered a convenient nutritious dense snack food, widely consumed by European citizens from all age groups. There is a tendency for research and innovation in this market segment, which promotes the inclusion in cookies of healthy ingredients, such as antioxidants, vitamins, minerals, proteins and fibers [15–16]. The inclusion of microalgae biomass in cookies has been previously reported for coloring purposes with *Chlorella vulgaris* [7], omega-3 fatty acids supplementation with *Isochrysis galbana* [8], and antioxidant activity with *Spirulina* [11–12].

The aim of this work was to study microalgae addition to enhance functional properties of this baked food matrix, especially at high biomass incorporation levels. It was intended to use significantly higher concentrations than the ones found in commercial algal products (typically below 1% w/w), in order to provide higher levels of bioactive compounds, while not compromising sensorial acceptability and digestibility. Four microalgae strains were tested - *Arthrospira platensis* F & M-C256, *Chlorella vulgaris* Allma, *Tetraselmis suecica* F & M-M33, and *Phaeodactylum tricornutum* F & M-M40.

A. platensis (commonly known as "spirulina"), consumed by human populations since ancient times [17] and *C. vulgaris*, *C. luteoviridis* and *C. pyrenoidosa* have been consumed in the EU for several decades and are thus authorized as food in the European Union [18–19]. *A. platensis* has been widely consumed as nutritional supplement due to its associated health benefits, such as high protein (up to 60%), vitamin B12, γ-linolenic acid (GLA) and phycocyanin content [20]. *Chlorella* is also rich in protein, as well as pigments and glucans which can act as immunostimulants [21–22].

Tetraselmis chuii has recently been authorized for commercialization as novel food ingredient through an application by the company Fitoplancton Marino S.L. (Cadiz, Spain) [23]. In the present study, another species belonging to the same genus, *T. suecica*, was used. This marine chlorophyte is characterized by high content in polyunsaturated fatty acids and α-tocopherol [24].

P. tricornutum is a marine diatom which has not yet been submitted to novel food application. Nevertheless, it was included in the present study considering its high content in eicosapentaenoic acid (EPA 20:5 ω3) as well as in fucoxanthin, a carotenoid associated with antioxidant, anti-diabetes and anti-obesity effects [25–26]. Moreover, previous *in vitro* toxicity tests by Niccolai et al. [27] showed no adverse effects of methanolic and aqueous extracts of these biomasses on *Artemia salina*.

2. Materials and methods

2.1. Microalgae strains and biomass production

Arthrospira platensis F & M-C256 and *Tetraselmis suecica* F & M-M33 biomasses were provided by Archimede Ricerche S.r.l. (Camporosso, Imperia, Italy) and *Phaeodactylum tricornutum* F & M-M40 was produced at the facility of Fotosintetica & Microbiologica S.r.l. (Sesto Fiorentino, Florence, Italy). *A. platensis* F & M-C256, *T. suecica* F & M-M33, and *P. tricornutum* F & M-M40 were cultivated in GWP®-I [28] or GWP®-II photobioreactors [29–30] in semi-batch mode, then the biomasses were harvested by centrifugation, frozen, lyophilized, powdered and stored at − 20 °C until analysis. *A. platensis* F & M-C256 biomass was washed with tap water to remove excess bicarbonate before being frozen. *Chlorella vulgaris* Allma biomass was obtained from Allma Microalgae (Lisbon, Portugal). The two marine strains (*T. suecica* F & M-M33 and *P. tricornutum* F & M-M40) were cultivated in F medium [31], while *A. platensis* F & M-C256 was cultivated in Zarrouk medium [32]. The biochemical composition of the different biomasses, determined as reported in Abiusi et al. [33], is presented in Table 1.

2.2. Cookies preparation

Cookies were prepared according to a previously optimized formulation [7–8], using wheat flour, sugar, baking powder, margarine, and microalgae biomass, as indicated in Table 2. A control, without microalgae incorporation was also prepared and further analyzed. Batches of 150 g were prepared, yielding around 10 cookies per batch. The ingredients were mixed in a food processor (Bimby, Vorwerk), kneading 15 s at speed 4. The cookies were then molded into 46.5 mm diameter and 5 mm height circles disks and baked at 110 °C for 40 min. After cooling, sample cookies were stored at room temperature in hermetic containers, protected from light. Physical analyses (color, texture, and a_w) were performed after 24 h, and after 8 weeks storage. Some of the cookies batches were immediately crushed to powder (using an electric mill) and frozen to be used for chemical composition, antioxidant capacity and *in vitro* digestibility analyses.

Table 1
Biochemical composition of the four microalgae biomasses used in the experiments (%, dry weight). Results are expressed as average ± standard deviation (n = 3).

	Protein (%)	Carbohydrate (%)	Lipid (%)	Ash (%)
A. platensis F & M-C256	68.9 ± 1.0	12.8 ± 0.2	10.7 ± 0.6	6.1 ± 0.1
C. vulgaris Allma	56.8 ± 2.7	5.9 ± 0.3	16.9 ± 2.8	9.3 ± 1.5
T. suecica F & M-M33	40.2 ± 0.5	10.2 ± 0.2	28.5 ± 1.2	15.7 ± 0.2
P. tricornutum F & M-M40	38.8 ± 0.1	11.0 ± 0.7	19.3 ± 1.7	14.8 ± 0.1

Table 2
Cookie formulations (%, w/w). F1 - control cookie formulation; F2 - 2% algae cookie formulation; F3 - 6% algae cookie formulation.

Ingredients	F1 (control)	F2	F3
	g/100 g	g/100 g	g/100 g
Wheat flour	49	47	43
Sugar	20	20	20
Margarine	20	20	20
Water	10	10	10
Baking powder	1	1	1
Microalgae	0	2	6

2.3. Cookies analyses

2.3.1. Color analysis

The color of cookies samples was measured instrumentally using a Minolta CR-400 (Japan) colorimeter with standard illuminant D65 and a visual angle of 2°. The results were expressed in terms of L*, lightness (values increase from 0 to 100%); a*, redness to greenness (60 to −60 positive to negative values, respectively); b*, yellowness to blueness (60 to −60 positive to negative values, respectively), according to the CIELab system. Chroma, C^*_{ab} (saturation), and hue angle, h°_{ab}, were also calculated, as defined by: $C^*_{ab} = [(a^{*2} + b^{*2})]^{1/2}$; $h^\circ_{ab} = \arctan(b^*/a^*)$. The total color difference between sample cookies along storage time (up to eight weeks), as well as between raw and cooked samples, was determined using average L*a*b* values according to: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. The measurements were conducted under the same light conditions, using a white standard (L* = 94.61, a* = −0.53, b* = 3.62), under artificial fluorescent light at room temperature, replicated ten times for each formulation sample (one measurement per cookie), as well as for the control, 24 h and 8 weeks after preparation.

2.3.2. Texture analysis

The cookie texture was measured using a texturometer TA.XTplus (Stable MicroSystems, UK) in penetration mode with a cylindrical aluminum probe of 2 mm diameter plunged 3 mm at 1 mm s^{-1}. The resistance to penetration, or hardness, was measured by the total area below the force *vs.* time curve, corresponding to the penetration work

(N.s). Measurements were repeated ten times for each formulation sample (one measurement per cookie), as well as for the control, 24 h and 8 weeks after preparation.

2.3.3. Water activity (a_w) determination

The cookie water activity (a_w) was determined using an HygroPalm HP23-AW (Rotronic AG, Switzerland), at 20 ± 1 °C. Measurements were repeated four times for each sample (crushed powder), as well as for the control, 24 h and 8 weeks after preparation.

2.3.4. Proximate chemical composition determination

Cookie moisture content was determined gravimetrically using an automatic moisture analyzer PMB 202 (aeADAM, Milton Keynes, UK) at 130 °C, until constant weight.

Total ash content was determined gravimetrically by incineration at 550 °C in a muffle furnace.

Crude protein was determined by the Kjeldhal method according to the AOAC 950.36 official method for baked products [34]. The determined total nitrogen content was multiplied by a conversion factor of 5.7 to obtain the cookie crude protein content.

The cookie crude fat content was determined according to the procedure used for cereals and derived products in the Portuguese standard method NP4168 [35]. This procedure is based on the hydrolysis of the bonds between lipids, proteins, and carbohydrates by using hydrochloric acid, ethanol and formic acid, followed by filtration and extraction with *n*-hexane in a Soxhlet extractor for 6 h. The crude fat residue was determined gravimetrically, after solvent evaporation in a rotary evaporator and oven drying.

All chemical composition analyses were repeated, at least in triplicate, and were performed after cookie preparation.

2.3.5. Phycocyanin, phenolics and antioxidant capacity determination

Phycocyanin content was determined in *A. platensis* cookie, and respective dough samples, according to the method developed by Boussiba & Richmond [36] modified by Reis et al. [37]. This method is based on the extraction of these water soluble pigments with phosphate buffer at pH 7, 0.1 M at low temperatures and spectrophotometric quantification at 620 nm (*C*-phycocyanin) and 650 nm (*C*-allophycocyanin).

For total phenolic content determination, extracts were prepared according to the procedure used by Hajimahmoodi et al. [38]. The total phenolic content in the extracts was determined according to Rajauria et al. [39], using the Folin Ciocalteu assay. Results were expressed in gallic acid equivalents (mg GAE g^{-1}) of dry microalgae biomass and cookies, through a calibration curve with gallic acid (0 to 500 μg mL^{-1}).

The antioxidant capacity of the cookies and microalgae samples was assessed by direct quencher procedure, as optimized by Serpen et al. [40–41] for cereal products, using Ferric Reducing Antioxidant Power (FRAP) as quantification method. Two blank assays, one without sample and another without reagents were also performed. Standard calibration curves were made using Trolox standard solutions that were submitted to the same FRAP protocol. The antioxidant capacity of the samples was expressed in terms of mmol of Trolox Equivalent Antioxidant Capacity (TEAC) per kilogram of sample. Analyses were repeated in triplicate and performed after cookie preparation.

2.3.6. In vitro digestibility tests

The cookies and microalgae biomasses *in vitro* digestibility (IVD) was assessed by the Boisen & Fernández method [42]. Microalgae biomass and cookie samples were weighed (1 g, particle size ≤ 1 mm) and transferred in 250 mL conical flasks. To each flask, phosphate buffer (25 mL, 0.1 M, pH 6.0) was added and mixed, followed by HCl (10 mL, 0.2 M) and pH was adjusted to 2.0. A freshly prepared pepsin water solution (3 mL; Applichem, Darmstadt, Germany) containing 30 mg of porcine pepsin (0.8 FIP-U/mg) was added. The flasks were incubated at 39 °C for 6 h with constant agitation (150 rpm). After, phosphate buffer (10 mL, 0.2 M, pH 6.8) and NaOH solution (5 mL, 0.6 M) were added to each sample and pH was adjusted to 6.8. A freshly prepared pancreatin ethanol:water solution (10 mL, 50:50 v/v) containing 500 mg of porcine pancreatin (42362 FIP-U/g, Applichem, Darmstadt, Germany) was added to each sample. The flasks were incubated again at 39 °C, 150 rpm, for 18 h. A reagent blank without sample was also prepared. The undigested residues were collected by centrifugation at 18,000 × *g* for 30 min and washed with deionised water. This procedure was repeated twice and the final supernatant was filtered on glass-fiber membranes (47 mm Ø, pore 1.2 μm). The pellet and membranes were dried at 80 °C for 6 h, and then at 45 °C until constant weight.

The IVD (%) was calculated from the difference between the initial biomass and the undigested biomass (after correction for the blank assay) divided by the initial biomass and multiplied by 100. Analyses were repeated in triplicate.

2.3.7. Sensory analysis

Sensory analysis assays were performed for cookies with *C. vulgaris* and *A. platensis* (2% and 6%). An untrained panel of 41 people, 9 males and 32 females, with ages between 18 and 60, evaluated the cookies in terms of color, smell, taste, texture, global appreciation (6 levels from "*very pleasant*" to "*very unpleasant*"). The buying intention was also assessed, from "*would certainly buy*" to "*certainly wouldn't buy*" (5 levels). The assays were conducted in a standardized sensory analysis room, according to the standard EN ISO 8589 [43].

2.4. Statistical analysis

Statistical analysis of the experimental data was performed using STATISTICA from StatSoft (version 8.0), through variance analysis (one way ANOVA), by the Scheffé test – *Post Hoc* Comparison at a significance level of 95% ($p < 0.05$). All results are presented as average ± standard deviation.

3. Results and discussion

The cookies with microalgae biomass incorporation presented visually attractive and unusual appearances (Fig. 1). Innovative green tonalities varied, depending on the microalga used, from a blueish-green (*A. platensis*) to a brownish-green (*P. tricornutum*). The microalgae cookies presented an average diameter of 46.8 ± 0.5 mm and an average thickness of 7.5 ± 0.3 mm while the control cookie dimensions were slightly higher (47.9 ± 1.5 mm diameter, 8.3 ± 0.5 mm thickness).

3.1. Color stability

The results obtained for the cookie color parameters, lightness (L*), greenness (a*), yellowness (b*), chroma (C*) and hue (h°) are presented in Fig. 2. Regarding the lightness parameter L*, a reduction in luminosity with increasing algae concentration can be observed.

An increase in microalgae concentration has also led to lower values of the chromatic parameters a* and b* (in modulus), thus lower chroma (C*), while the hue remains practically constant for each sample (100° to 120°, between yellow and green, depending on the sample).

These results may seem unexpected, considering that in Fig. 1, the cookies with 6% algae seem to have more intense green color. In previous studies, a similar effect was found for *C. vulgaris* [7] and *Isochrysis galbana* [8] cookies, where a reduction in a* and b* parameters upon increasing microalgae biomass concentration from 0.5% to 3.0% (w/w) was observed. This effect may be related to a higher pigment degradation with the baking process or with a pigment saturation effect, above certain algae concentrations.

Cookies with 2% *C. vulgaris* and *T. suecica* presented the highest a* values (in modulus) and intermediate b* values (22.8–25.3) (Fig. 2),

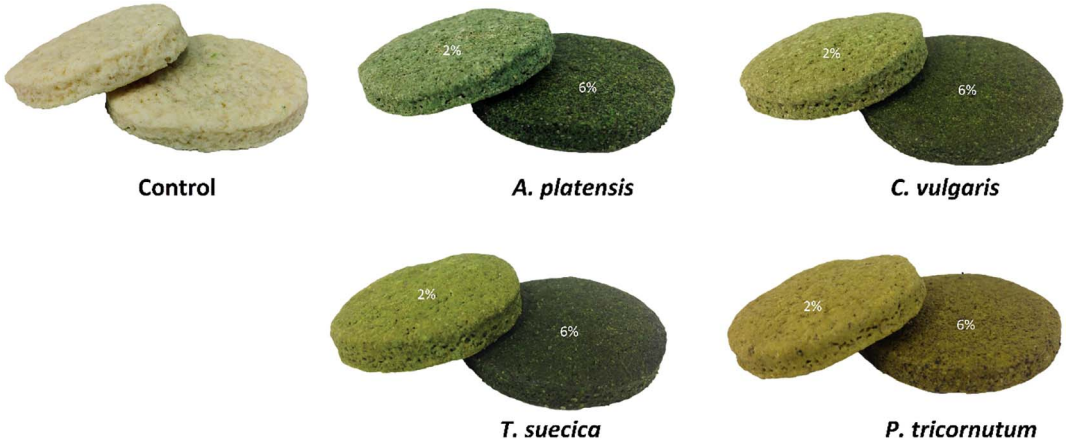

Fig. 1. Control cookie and cookies with 2% (w/w) and 6% (w/w) microalgae biomass.

which is in agreement with the high chlorophyll content that characterizes chlorophyte algae [1]. *A. platensis* cookies presented tonalities similar to the chlorophyte cookies, although with less intensity (lower a* and b* values, in modulus), reflecting the lower chlorophyll and carotenoid content generally present in this alga [44]. On the other hand, *P. tricornutum* cookies presented low a* values (in modulus) and the highest b* values, resulting in a hue angle of 100°, closer to yellow (90°) than to green (180°). These results should be related to the

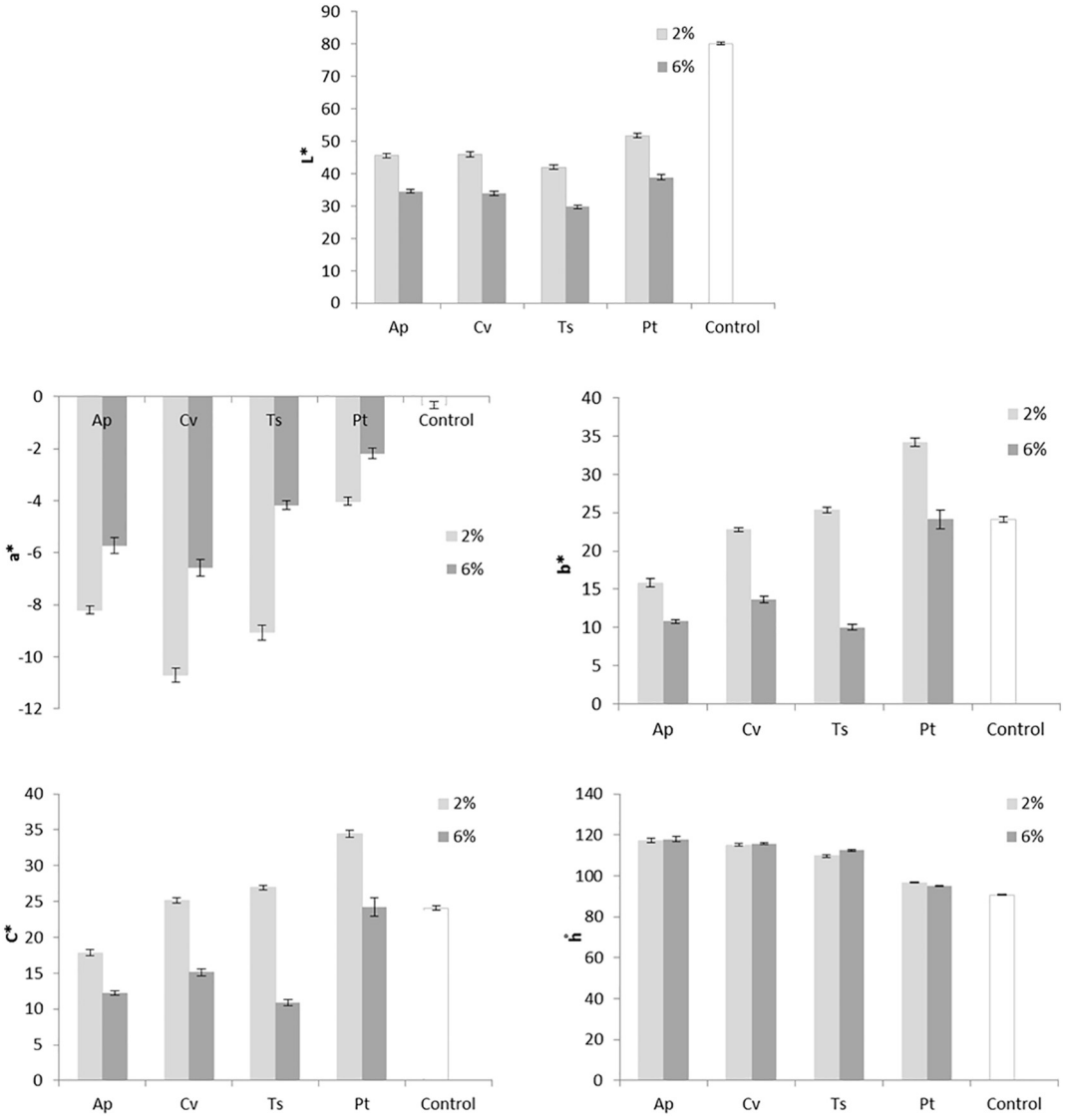

Fig. 2. Color parameters, L*, a*, b*, C* and h° of cookies with 2% and 6% (w/w) microalgae biomass incorporation, in week 0 (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*). Results are expressed as average ± standard deviation (n = 10).

Table 3
Total color variation (ΔE*) between cooked and raw cookie samples and color stability along conservation time (ΔE* in relation to week 0).

Total color difference (ΔE*)		Raw *vs.* cooked	Week 1 *vs.* week 0	Week 2 *vs.* week 0	Week 3 *vs.* week 0	Week 4 *vs.* week 0	Week 8 *vs.* week 0
Control		7.63	0.84	0.86	1.23	1.55	1.89
A. platensis	2%	16.01	0.60	0.66	1.16	1.63	1.86
	6%	15.58	0.73	0.89	0.94	0.94	0.77
C. vulgaris	2%	11.22	0.70	1.17	0.96	0.74	1.12
	6%	12.58	0.75	1.26	1.11	1.32	3.13
T. suecica	2%	15.93	1.02	1.73	2.43	2.49	2.78
	6%	10.85	1.83	2.12	2.40	3.80	4.69
P. tricornutum	2%	18.97	1.50	2.03	2.48	2.37	4.19
	6%	23.63	1.31	2.57	2.37	3.35	5.42

presence of fucoxanthin, a carotenoid usually present in high concentrations in this marine diatom [25].

Table 3 presents the total color differences (ΔE*) between baked and raw (dough) sample cookies. Microalgae cookies show significantly color differences upon baking (ΔE* = 19–24). These differences result mainly from a general increase in luminosity (probably associated to water evaporation) and an accentuated hue angle tonality decrease in the case of *P. tricornutum* (results not shown), which should be related to pigment loss upon baking.

The color stability along conservation time can also be observed in Table 3 through the calculation of total color difference of each sample with time in relation to week 0. In all cases ΔE* is lower than 5 (except for *P. tricornutum* 6% in week 8: 5.42) which means that the cookie color differences are not detected by normal human vision [45]. Therefore, it can be concluded that the developed cookies present stable colorations along eight weeks of storage.

3.2. Texture stability

The cookies texture was evaluated by penetration tests, and the resulting hardness, expressed by resistance to penetration work, was calculated from the texturograms and presented in Fig. 3.

At the beginning of the study (week 0), no significant differences ($p > 0.05$) were found between the cookies with 2% algae when compared to the control (and between different algae), which means that adding 2% biomass does not prompt cookie structural changes that can alter the resistance to probe penetration. Increasing microalgae concentration from 2% to 6% causes significant ($p < 0.05$) hardness increase, from 24 to 29 N.s (2% cookies) to 37–38 N.s for 6% *C. vulgaris* and *T. suecica* cookies, to 50 N.s for *P. tricornutum* and to 63 N.s for *A. platensis* cookies.

These results confirm the findings of previous water absorption tests carried out with the same microalgae strains biomass [46], in which significantly higher ($p < 0.05$) water absorption indexes (WAI: 4.4–5.0 g/g_{alga}) and Oil Absorption Capacity (OAC: 1.8–2.2 g/g_{alga}) were obtained in relation to wheat flour (WAI: 2.1 g/g_{flour}, OAC: 1.7 g/g_{flour}). The highest WAI and OAC values were attained for *A. platensis*, followed by *P. tricornutum*, and at last, for *C. vulgaris* and *T. suecica*, which can be related to the different nature of these algae cell walls (peptidoglycan, silica and cellulose/hemicellulose, respectively). It is possible that, when microalgae are added to the cookie dough, they absorb more water and oil/fat, reinforcing the cookie internal structure. These data suggest that it would be possible to increase the water content or reduce the flour content, resulting in cookies with the same texture properties than the control cookie.

These results are also in agreement with previous studies where it was observed a linear increase in cookies hardness with *C. vulgaris* [7] and *I. galbana* [8] at concentrations from 0.5% to 3.0%. Singh et al. [12] also observed that increasing the content of *A. platensis*, from 1.6 to 8.4%, had positive effect on the hardness of sorghum flour biscuits. The same "*texturing*" or "*structuring*" effect of microalgae has been described also in other type of food products, such as fresh pastas with *A. maxima* and *C. vulgaris* [9].

The evolution of cookies hardness with time can also be observed in Fig. 3. The cookies did not present significant ($p > 0.05$) changes in hardness after eight weeks of storage, except for *A. platensis* cookies.

3.3. Water activity

Water activity, a_w, is an important physical parameter regarding conservation of low moisture cookies, particularly for the maintenance of a crispy texture [47]. At a_w values below 0.5, no microbial proliferation occurs. Lipid oxidation reactions, can be accelerated at high a_w by increased mobilization of reactant molecules, although it is also recognized that very low water contents in fat-containing foods (*e.g.* cookies with 3–5% moisture and 20% fat) are conductive to rapid oxidation since substrates and reactants become more concentrated [48].

Fig. 4 presents the results of a_w for the microalgae cookies along eight weeks. The control cookies presented an average a_w value of 0.29 without significant differences with time ($p < 0.05$). Microalgae cookies presented more variable behavior regarding a_w values, with a tendency for a_w to increase along time. Overall, it should be noted that for all samples, a_w values were below 0.5, after eight weeks storage, these a_w variations did not promote any appreciable modification on texture stability (Fig. 3).

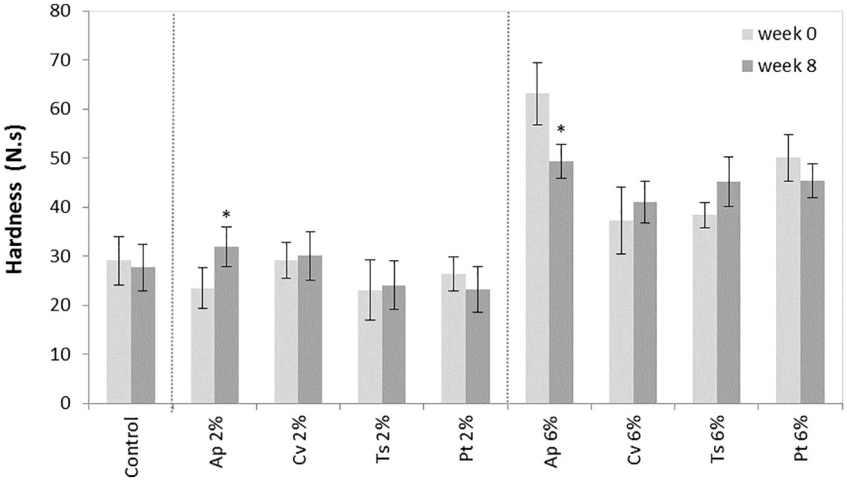

Fig. 3. Texture, expressed by penetration work (hardness, area N.s), of cookies with 2% and 6% (w/w) microalgae biomass incorporation, along time (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*). Results are expressed as average ± standard deviation (n = 10). Samples marked with * showed significant ($p < 0.05$) differences from week 0 to week 8.

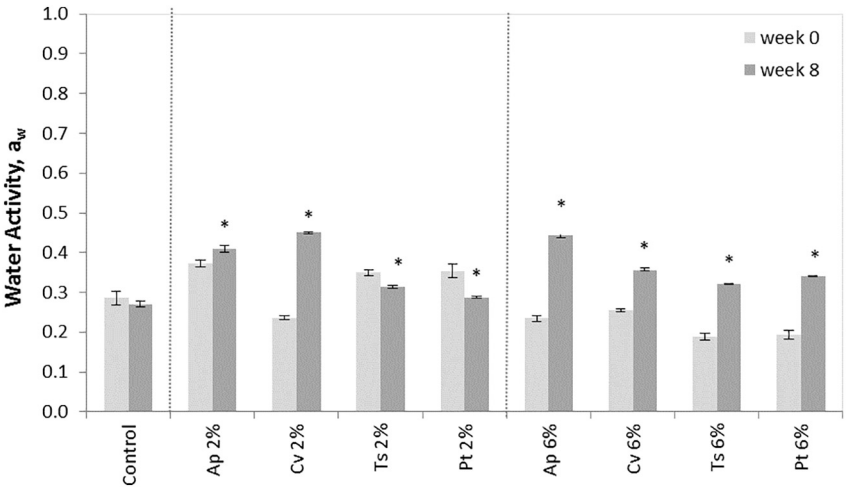

Fig. 4. Evolution of water activity (a_w), of cookies with 2% and 6% (w/w) microalgae biomass incorporation, along time (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*). Results are expressed as average ± standard deviation (n = 4). Samples marked with * showed significant ($p < 0.05$) differences from week 0 to week 8.

Table 4
Proximate chemical composition (g/100 g) of cookies with 2% and 6% (w/w) microalgae biomass incorporation. Results are expressed as average ± standard deviation (n = 3). Different letters in the same column correspond to significant differences ($p < 0.05$).

		Moisture (g/100 g)	Total ash (g/100 g)	Crude fat (g/100 g)	Crude protein (g/100 g)	Carbohydrates* (g/100 g)	Energy value (kcal/100 g)
Control		3.8 ± 0.2^{ab}	2.7 ± 0.2^{a}	16.1 ± 0.1^{a}	4.9 ± 0.5^{a}	72.6	454
A. platensis	2%	3.8 ± 0.1^{ab}	2.6 ± 0.4^{a}	16.1 ± 0.5^{a}	6.1 ± 0.2^{abc}	71.4	455
	6%	5.0 ± 0.2^{d}	2.3 ± 0.1^{a}	16.1 ± 0.1^{a}	7.8 ± 0.3^{de}	68.7	451
C. vulgaris	2%	3.2 ± 0.1^{a}	2.3 ± 0.1^{a}	16.3 ± 0.2^{a}	5.9 ± 0.5^{abc}	72.4	460
	6%	4.8 ± 0.3^{cd}	2.6 ± 0.1^{a}	16.9 ± 0.4^{a}	8.0 ± 0.6^{e}	67.7	455
T. suecica	2%	3.4 ± 0.2^{ab}	2.4 ± 0.2^{a}	16.1 ± 0.1^{a}	5.2 ± 0.1^{a}	73.0	457
	6%	3.3 ± 0.1^{a}	3.2 ± 0.1^{a}	16.3 ± 0.4^{a}	6.9 ± 0.4^{cd}	70.4	456
P. tricornutum	2%	3.9 ± 0.1^{ab}	2.3 ± 0.2^{a}	16.1 ± 0.1^{a}	5.1 ± 0.2^{ab}	72.6	456
	6%	4.3 ± 0.2^{bc}	3.0 ± 0.1^{a}	16.2 ± 0.1^{a}	6.6 ± 0.4^{bc}	70.0	452

* Carbohydrates were calculated by difference.

3.4. Proximate chemical composition

Table 4 presents the proximate chemical composition of the cookies prepared with microalgae biomass incorporation.

All cookies presented moisture values ranging from 3.2 to 5.0%, which is typical for this type of dried foods. No significant changes ($p < 0.05$) were observed on the cookies ash content upon microalgae addition (2.3–3.2%) neither on the crude fat content (16.1–16.9%).

The main chemical composition changes arising from microalgae incorporation in cookies are related to protein (Table 4). The protein content of microalgae cookies was always higher than the control cookie (4.9%). Cookies with 2% algae ranged from 5.1 to 6.1% protein while 6% algae cookies ranged from 6.6 to 8.0% protein. The highest values were attained for *A. platensis* and *C. vulgaris* cookies with protein contents around 8%. Bolanho et al. [49] found an increase of 20% in protein content of samples with 5% *A. platensis* biomass added, when compared to the control cookie, which is similar to the increase found in our study for the cookies with 2% *A. platensis* biomass (+ 24%). Furthermore, when we increased *A. platensis* content up to 6%, a + 59% increase in protein content was obtained compared to the control cookie.

3.5. Bioactive compounds and antioxidant capacity

The presence of bioactive compounds in the microalgae biomass could be associated to antioxidant potential, among other biological functions.

In the case of *A. platensis* cookies, it should be noted the presence of phycocyanin, a blue pigment with demonstrated nutraceutical properties and healthy benefits which are mainly attributed to its antioxidant activity [50]. Even after thermal treatment, the cookies presented 172 mg kg^{-1} and 363 mg kg^{-1} phycocyanin for 2% and 6% incorporation levels (data not shown), respectively, thus about 10% of phycocyanin from the microalga biomass (8.2% w/w) was still present. In previous studies [51], this pigment has been used as coloring and functional ingredient in oil-in-water food emulsions, proving also to be a powerful structuring agent.

Phenolic compounds including simple phenols, flavonoids, phenylpropanoids, tannins, lignins, phenolic acids, and their derivatives, synthesized as secondary metabolites [52] are considered as one of the most important classes of natural antioxidants and have received an increasing interest from consumers and also from food manufacturers for their health benefits [53]. Fig. 5 shows the phenolic content of microalgae cookies and microalgae biomass. *A. platensis* biomass presented the highest ($p < 0.05$) total phenolic content (19 mg GAE g^{-1}), followed by *T. suecica* (9.2 mg GAE g^{-1}), *P. tricornutum* (8.4 mg GAE g^{-1}) and *C. vulgaris* (6.4 mg GAE g^{-1}) (Fig. 5A).

The addition of microalgae results in an effective supplementation of phenolic compounds, which are practically absent in the control cookie. *A. platensis* 6% cookies presented the highest phenolic content (0.90 mg GAE g^{-1}), which is in agreement with this alga composition, followed by *P. tricornutum* 6% cookies (0.62 mg GAE g^{-1}). Both *A. platensis* and *P. tricornutum* 2% cookies also showed much higher phenolic content than the chlorophyte algae at the highest concentration (*C. vulgaris* and *T. suecica*). When submitted to baking (110 °C/40 min) Chlorophyceae algae showed high phenolic losses, in the order of 50% for *C. vulgaris* and 80% for *T. suecica*. On the other hand, *P. tricornutum* cookies showed high final phenolic content, without appreciable losses in relation to the microalgal biomass, upon baking. It is possible that the different cell wall of *P. tricornutum*, (presenting silica bands or an amorphous silica matrix according to the morphotype) [54], when compared to the other microalgae, had a higher protecting effect for

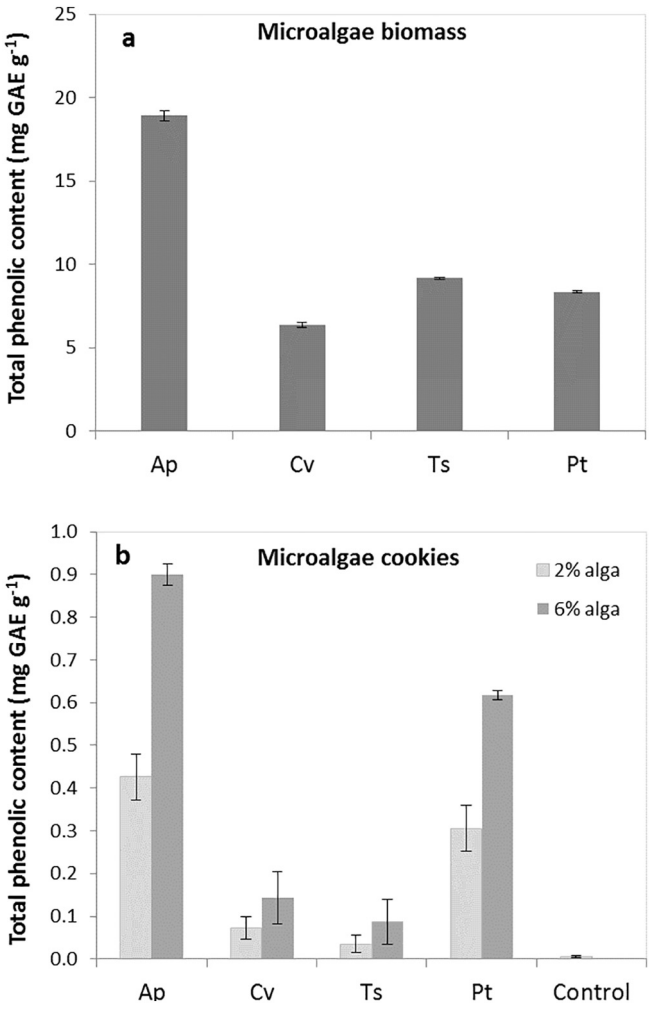

Fig. 5. Total phenolic content (expressed as gallic acid equivalents mg g^{-1} dry weight) of four microalgae strains (a) and in cookies enriched with different levels of microalgae (b) (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*). Results are expressed as average ± standard deviation (n = 3).

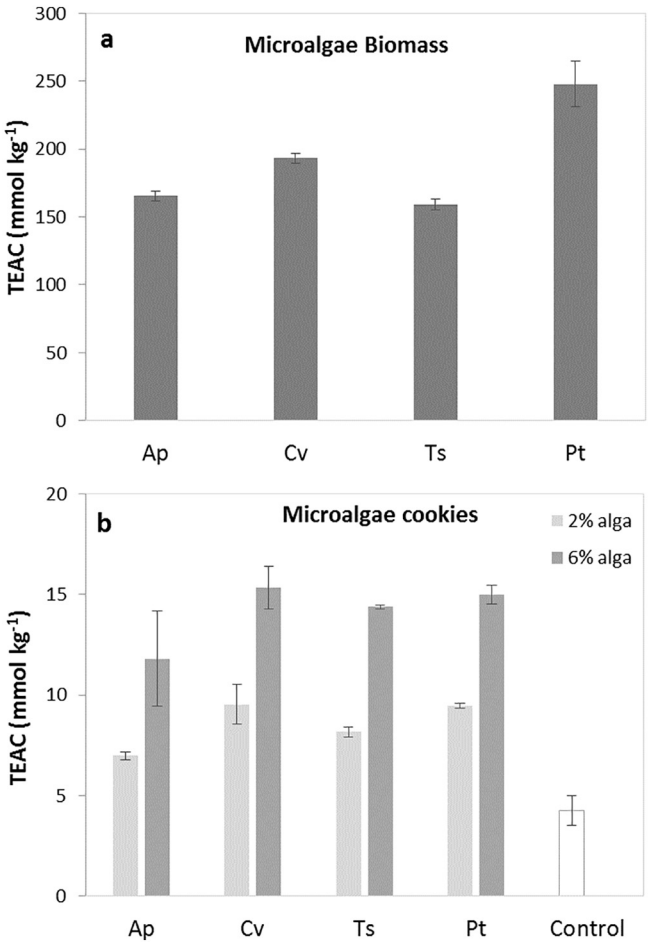

Fig. 6. Antioxidant capacity (expressed as mmol of Trolox Equivalent Antioxidant Capacity, TEAC, per kg) of four microalgae strains (a) and in cookies enriched with different levels of microalgae (b) (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*). Results are expressed as average ± standard deviation (n = 3).

phenolics from thermal degradation. Therefore, *P. tricornutum* could become an interesting functional ingredient for future developments.

Other authors have highlighted the high phenolic compounds content of *A. platensis* [55], and *P. tricornutum* [56], as well as the correlation with the antioxidant activity of these algae extracts. Bolanho et al. [49] report 12 mg GAE g^{-1} total phenolics for *A. platensis* biomass (in the present work: 19 mg GAE g^{-1}) and, for 5% *A. platensis* cookies, an increase from 1.4 to 2.3 mg GAE g^{-1} in total phenolic content when compared to the control, which is in the same order of magnitude than that found in our study for 6% *A. platensis* cookies (from 0.01 to 0.90 mg GAE g^{-1}). Tumbas Saponjac et al. [15] studied sour cherry pomace extract incorporation in cookies and obtained 0.8 mg GAE g^{-1} total polyphenols, a value close to 6% *A. platensis* cookies in the present work.

The antioxidant capacity of microalgae-enriched cookies and biomass was tested by the FRAP method (Fig. 6a and b).

P. tricornutum biomass presented the highest ($p < 0.05$) antioxidant capacity (248 mmol TEAC kg^{-1}), followed by *C. vulgaris* (193 mmol TEAC kg^{-1}), and by *A. platensis* and *T. suecica* (about 160 mmol TEAC kg^{-1}) (Fig. 6A). In addition to phenolic content, *P. tricornutum* has a high content of the carotenoid fucoxanthin, which is a valuable pigment with several biological activities, such as antioxidant activity [25–26]. Compared to other microalgae, green microalgae such as *Chlorella* and *Tetraselmis* have antioxidant activity thanks to the high content of chlorophylls (*a* and *b*) [57] and vitamin E [58], which includes compounds with antioxidant activity [59], such as tocopherols (up to 125 μg g^{-1} dw in *T. suecica* F & M-M33 [58] and tocotrienols [59]. As reported by Lanfer-Marquez et al. [60], chlorophylls are capable of inhibiting the DPPH radical. A study conducted by Siriwardhana et al. [61] also reported a high correlation between DPPH radical scavenging activities and total polyphenolic content.

Compared to the control cookie, the incorporation of microalgae led to a significant ($p < 0.05$) increase in the antioxidant capacity of all the microalgae-based cookies (even at the lowest dosage). For all the cookies it was observed a significant increase in antioxidant capacity when increasing biomass concentration from 2 to 6%, although at the same biomass concentration, no significant differences ($p < 0.05$) in antioxidant capacity were found between the four tested microalgae cookies. Overall, cookies with 2% alga showed values from 7.0 to 9.5 mmol TEAC kg^{-1} (+65% and +125% compared the control cookie, respectively) while 6% cookies showed values from 11.8 to 15.4 mmol TEAC kg^{-1} (+178% and +262% compared the control cookie, respectively).

In the case of *P. tricornutum*, there seems to be a greater loss of microalgae antioxidants upon baking. The important reduction of the antioxidant activity observed in the *P. tricornutum* cookies (compared to the value in biomass, Fig. 6A) could be attributed to the loss of pigments upon baking, in particular the degradation of fucoxanthin, an unstable molecule sensitive to light, oxygen, and high temperature [62]. In fact, it was also noticed color loss of this sample upon cooking (Table 3).

Some other authors have studied the antioxidant capacity of *A.*

platensis enriched cookies. El Baky et al. [11] and Singh et al. [12] attributed the antioxidant activity of *A. platensis* enriched-cookies to the phycobiliproteins provided by this cyanobacterium. El Baky et al. [11] observed increasing antioxidant activity for biscuits containing 0.3 to 0.9% *A. platensis* biomass. Singh et al. [12] found a linear positive correlation between *A. platensis* concentration (1.6 to 8.4%) in sorghum flour biscuits, and antioxidant activity. Our results are in agreement with the findings of El Baky et al. [11] and Singh et al. [12] considering that after baking *A. platensis* cookies (both 2 and 6%) still showed a high content of phycocyanin, supposedly responsible for the observed antioxidant activity.

3.6. In vitro digestibility

The *in vitro* digestibility analysis reproduces the chemical-enzymatic catalysis that occurs in the proximal tract of the monogastric digestive system [42]. As far as digestibility of algae is concerned, most of the literature deals with tests for macroalgae [63–65] and only few studies focus on the digestibility of microalgae [66–68]. To our knowledge, no literature is available concerning *in vitro* digestibility of microalgae-based cookies.

The *in vitro* digestibility (IVD) results are presented in Fig. 7. *T. suecica* and *P. tricornutum* microalgae biomass presented the lowest IVD (around 50%). The differences among the microalgae tested could be related to their different cell wall structure [69–71]. No significant difference in IVD between microalgae cookies and the control (IVD 87–95%) were found.

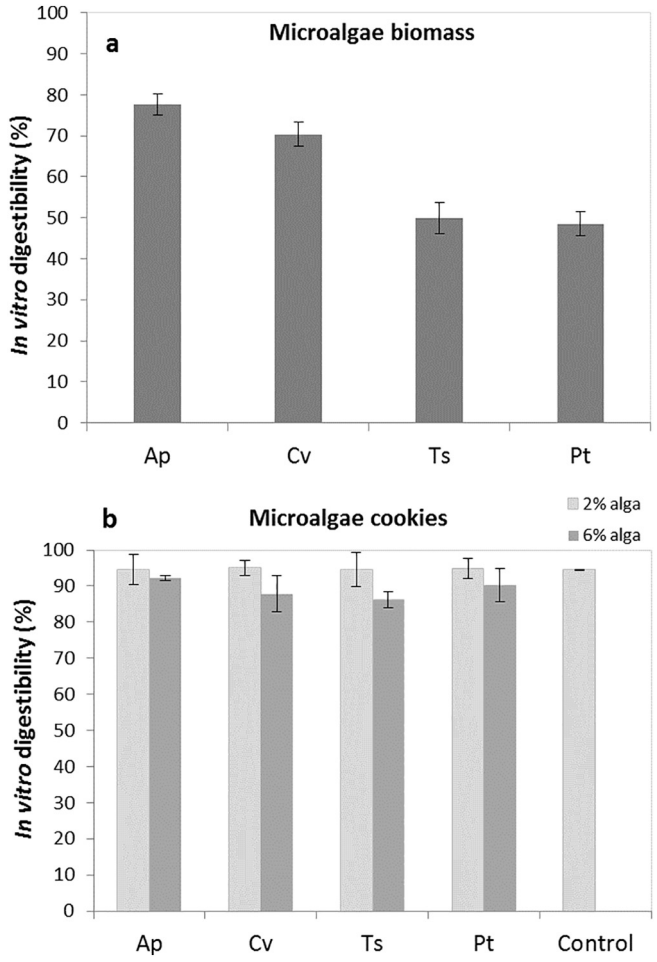

Fig. 7. *In vitro* digestibility (%) of four microalgae strains (a) and in cookies enriched with different levels of microalgae (b) (Ap – *A. platensis*, Cv – *C. vulgaris*, Ts – *T. suecica*, Pt – *P. tricornutum*). Results are expressed as average ± standard deviation (n = 3).

3.7. Sensory evaluation

At the end of this work, sensory analysis assays were carried out with *A. platensis* and *C. vulgaris* microalgae cookies, at 2% and 6% incorporation level. These species are already widely accepted as food, then they are more present in the food market compared to other microalgae. Moreover, in restricted tasting sessions by the research team, it was observed that *T. suecica* and *P. tricornutum* marine algae had a very intense fishy flavor, so they were less appreciated.

Fig. 8 represents the average scores of the sensorial parameters as evaluated by the panel. It is clear that the less appreciated sample was 6% *C. vulgaris*. Regarding color, the preferred cookie was 2% *C. vulgaris* while in terms of smell the tasters preferred the cookies with *A. platensis*. Concerning texture there were no significant differences between *A. platensis* (2% and 6%) and *C. vulgaris* 2%, while *C. vulgaris* 6% was the less appreciated. In relation to taste and global appreciation, the preferred cookie was *A. platensis* 2%, while 6% *A. platensis* and 2% *C. vulgaris* had similar scores. From Fig. 8 it can also be observed that the average of the analyzed sensorial attributes reached (at maximum) the scale 4, corresponding to "*pleasant*".

As found by many authors, the results of sensory analyses of microalgae-based products such as pasta [9–10,72], cookies [11–12,49] or yoghurt [73] reveal that these products are generally appreciated. Similar to our results, El Baky et al. [11] reported that functional biscuits supplemented with different levels of *Spirulina platensis* biomass (0.3, 0.6 and 0.9% incorporation level), were significantly acceptable for sensory parameters (color, odor/aroma, flavor, texture), global appreciation, and overall acceptability. Singh et al. [12] reported that biscuits prepared from sorghum and whole wheat flour with the addition of *Spirulina platensis* (> 7% incorporation level) adversely affected the textural and sensory attributes of flavor and graininess. Bolanho et al. [49] also found the addition of *S. platensis* biomass (2 and 5%) in the cookies decreased the sensorial acceptance when compared to the control cookie.

Fig. 9 presents the answers given by the tasters in relation to the buying intention. Forty six percent of the tasters "*would probably buy*" and 22% "*would certainly buy*" the cookie with 2% *A. platensis*, the most appreciated cookie. Also 39% and 37% of the tasters "*would probably buy*" the 6% *A. platensis* and 2% *C. vulgaris* cookies, respectively. The 6% *C. vulgaris* cookie was clearly unappreciated with 39% of the tasters referring that "*certainly wouldn't buy*" and 34% "*probably wouldn't buy*".

In the comments field, in the sensory analysis sheet, the 2% *A. platensis* cookie was considered the crunchiest, tastier and most equilibrated in terms of flavor. Regarding the cookie with 6% *A. platensis* the tasters referred that the color was too dark, but that it was very pleasant in terms of taste. The 2% *C. vulgaris* cookie, was referred as the one with most appealing color, although it presented a strange residual taste. In relation to the 6% *C. vulgaris* cookie the tasters referred that it had a very strong fishy flavor, which lasted in the after-taste feeling.

4. Conclusions

The addition of microalgae biomass as natural ingredient resulted in cookies with an attractive and innovative appearance. Innovative and stable green tonalities varied, depending on the microalga used, from a blueish-green (*A. platensis*) to a brownish-green (*P. tricornutum*). *A. platensis* provided a significant structuring effect, in terms of cookies texture. In general, increasing microalgae content from 2% to 6% resulted in a significant ($p < 0.05$) increase in the cookies total phenolic content and antioxidant capacity, while concerning digestibility no significant differences compared to the control cookie were found. *A. platensis* cookies presented the highest sensory scores, as well as high protein and phenolic content. This study suggests that microalgae-based cookies could become widely appreciated and consumed functional foods in the next future.

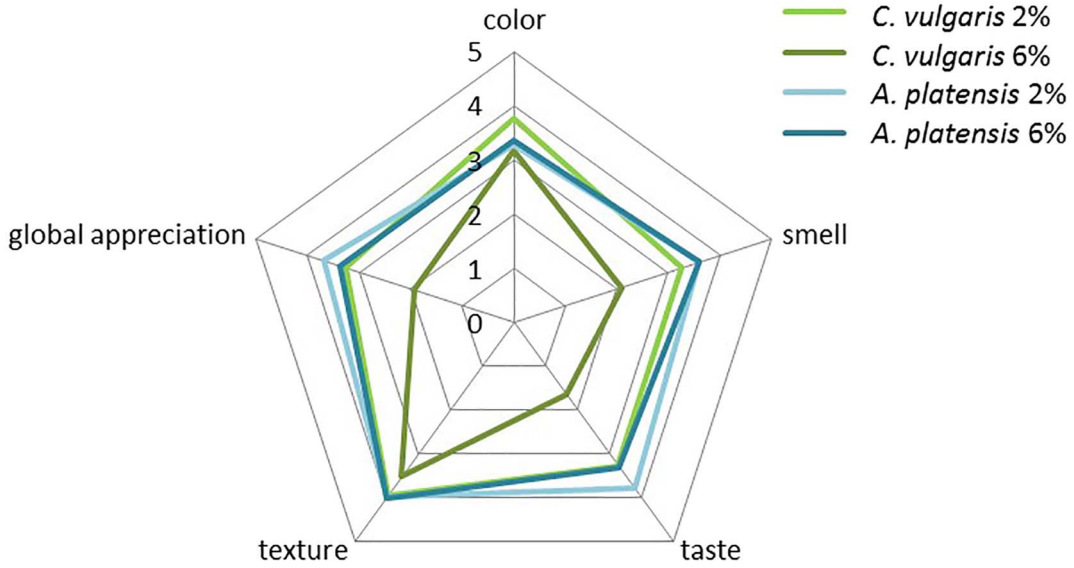

Fig. 8. Responses of the sensory analysis panel tasters (n = 40) regarding *A. platensis* and *C. vulgaris* cookies. 0 – "*very unpleasant*"; 1 – "*unpleasant*"; 2 – "*slightly unpleasant*"; 3 – "*slightly pleasant*"; 4 – "*pleasant*"; 5 – "*very pleasant*".

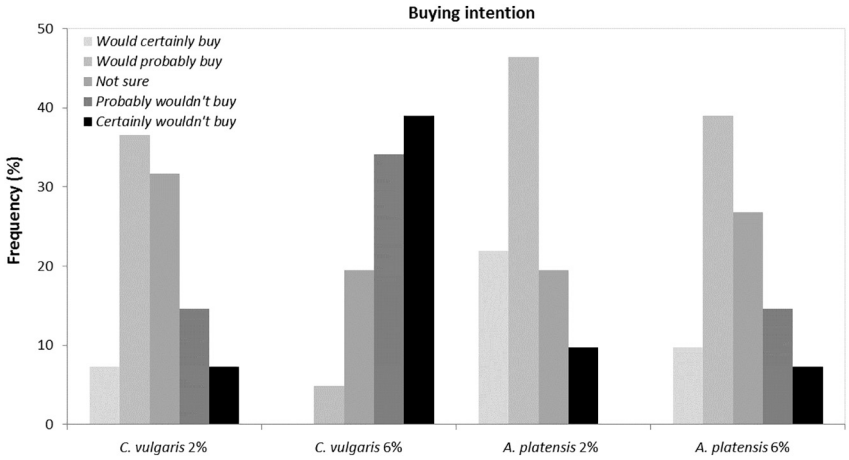

Fig. 9. Responses of the sensory analysis panel tasters (n = 40) regarding *A. platensis* and *C. vulgaris* cookies intention of buying.

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Conflict of interest

A. platensis F & M-C256, *Tetraselmis suecica* F & M-M33, and *Phaeodactylum tricornutum* F & M-M40 belong to the Microalgae Culture Collection of Fotosintetica & Microbiologica S.r.l., in which M.R. Tredici and L. Rodolfi have a financial interest; all the other authors have no conflicts of interest.

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