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Effects of Growth Medium Variation on the Nutri-Functional Properties of Microalgae Used for the Enrichment of Ricotta

Running head: Growth Medium Variation Effect on Microalgal Bioactivity and Ricotta Enrichment

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SUMMARY

Research background. Microalgae represent an emergent sustainable source of bioactive compounds such as antioxidants, vitamins, minerals, and polyunsaturated fatty acids that can ameliorate the nutritional characteristics of foods. The biochemical composition of microalgae could be modulated by varying the culture conditions to enhance the accumulation of biomolecules of interest. The aim of this work is to optimise the nutri-functional properties of two microalgae with potential utility for food-application.

Experimental approach. *Nannochloropsis gaditana* L2 and *Chlorella* sp. SM1 were screened for growth, biochemical composition, and radical scavenging activity employing four different growth media (Algal, BG-11, f/2, and Conway) with different nutrients composition. Additionally, the feasibility of using *Chlorella* sp. SM1 cultivated in BG-11 medium, in a Mediterranean under-investigated dairy product “ricotta cheese” and its effect on the sensory attributes was investigated.

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Results and conclusions. Nitrate- and phosphate-rich media (BG-11 and Algal) enhanced the biomass productivity. However, the highest lipid productivity (23.10 mg/(L·day); 11.86 mg/(L·day) for SM1 and L2 respectively) and carbohydrates content (34.79 %; 44.84 % for SM1 and L2 respectively) were obtained with the nitrate-deficient f/2 medium. Regardless of the used medium, the lipidic profile of *Chlorella* sp. SM1 and *Nannochloropsis gaditana*.L2 remained adequate for different applications with the presence of C16-18 as main fatty acid (>50 %). Significant increase in oleic acid (C18:1) content was recorded in response to nitrogen deficiency, being the highest in SM1 in f/2 medium (34 %). Nitrogen deficiency was also found to enhance phenolic compounds (48.8 GAE/(mg/g); 35.1 GAE/(mg/g) for SM1 and L2 respectively) and carotenoids contents (2.2 mg/g; 2 mg/g for SM1 and L2 respectively). Due to its interesting antioxidant potential, *Chlorella* sp. SM1 was used to enrich the ricotta cheese product at different concentrations (0.2 %, 1 % and 1.5 %). The sample with 0.2 % was found to give the most appreciated product.

Novelty and scientific contribution. This study permitted the production of an innovative ricotta cheese using *Chlorella*, as a functional ingredient, without altering the manufacturing diagram while maintaining acceptable sensorial characteristics. The biochemical composition of the used strains varied depending on the culture media's composition, which permitted the accumulation of phytonutrients of interest.

Key words: *Nannochloropsis gaditana*; *Chlorella* sp.; ricotta cheese; growth media; antioxidant; nutritional profile; sensory evaluation

INTRODUCTION

One of the biggest changes in the modern world diet has been in the quality of food consumption, for that, much focus has been placed on "green biomolecules" sources as terrestrial plants and algae. They are considered as highly efficient "biofactories" which produce mainly primary metabolites (lipids, proteins, carbohydrates) and secondary metabolites (carotenoids, polyphenols, terpens, *etc*.). Plant derived secondary metabolites including antioxidants, have been widely studied for their potential to reduce risk of illness and enhance the strength of the defense of the human body against pathologies such as neurodegenerative and cardiovascular diseases (*1*). Antioxidants, from green sources including polyphenols, vitamins, and carotenoids are becoming of great importance to replace the synthetic ones such as butylated hydroxytoluene (BHT).

Microalgae are gaining considerable interest worldwide due to their uniqueness biomass composition extremely rich in functional ingredients especially carotenoids, vitamins, minerals,

proteins and long-chain polyunsaturated fatty acids. Microalgae have no need of arable land, and can be cultured massively in controlled ponds or photobioreactors, which make them advantageous over terrestrial plants (*2*). Although most microalgae are photoautotrophic, several strains are capable to use different carbon sources to grow heterotrophically which may improve growth performance and biomass concentrations (3).

Since several essential molecules must be provided to the food ration, microalgae were reported to represent an excellent choice for consumers who are looking for tasty foods with no harmful effects (*4*). This should replace the conventional forms of bioactive phyto-compounds marketed as tablets, capsules, or powders (*5,6*).

In the last years, several researchers have incorporated microalgae biomass in conventional food recipes to improve their basic nutritional value. Furthermore, expectations of microalgae supplementation become economically promising for the food industry considering first the low environmental impact, and secondly the fact that consumers give importance to the relationship between diet and health (*7*). The main microalgae enrichment concerned products like gluten-free bread (8), cookies (*9, 10*), bread (11), yogurts (*12*), pasta (*13*) , and biscuits (*14*). This increasing number of application using microalgae in foods takes advantages from the diversity of microalgae and their variable biochemical composition in relation with both the mode and the medium of culture (*15*). It has been demonstrated that nutrients (macro and micronutrients) strongly affect the biochemical composition of microalgae (*16*). The composition of culture media is among the main factors affecting bioactive compound accumulation in the algal biomass (*17*) . Thus, Science is still trying to domesticate novel microalgae strains to enhance their growth performances and improve the overall biochemical composition, which is exclusively assessed at the cultivation level. The best alternative to evaluate the effect of nutrients availability on microalgae growth and biochemical composition, in laboratory scale, is variation of the culture media formulations.

In this context, the aim of this work is to evaluate the enrichment of a traditional dairy product, ricotta cheese, by microalgae using a commercial sample of *Arthrospira platensis* (syn*. Spirulina platensis)* as reference. It was proposed to firstly optimise the antioxidant ability and the nutritional properties of two microalgae with potential utility for food application namely *Chlorella* sp. and *Nannochloropsis gaditana,* by varying the growth media, and secondly to assess microalgae addition to enhance functional properties and estimate the acceptability of the new product designated as “Ricottalgue”.

MATERIALS AND METHODS

Algal strains and culture conditions

The experimental used strains were previously isolated from two different saline sites situated in the northern part of Tunisia (North Lake Lagoon: 36°49'25.6"N 10°12'36.4"E, salinity: 33.8 psu, and Monastir lagoon: 35°46'18.0"N 10°46'34.4"E, salinity: 44.4 psu), maintained in LIP-MB laboratory **(**Laboratoire d'Ingénierie des Protéines et des Molécules Bioactives, Tunis, Tunisia) and identified microscopically and molecularly as *Chlorella* sp.SM1 (KM401849) and *Nannochloropsis gaditana*.L2 (KT932831) (18*,19*). The commercial *Spirulina* sample used in the sensory assay was provided by Bio-Gatrana Laboratories (Gatrana Sidi Bouzid, Tunisia), referenced as TN BIO 001, N° SP01018 and identified as *Arthrospira platensis*.

The culture was carried in four different medium enriched with artificial seawater: Algal medium (20), BG-11 medium (21), f/2 medium (22), and Conway medium (23). The nutrient composition of each medium is detailed in Table S1 and all the chemicals were purchased from Sigma-Aldrich SARL (Saint-Quentin Fallavier, France). All cultures were firstly grown in 500 mL capacity Erlenmeyer flasks then mass cultures were transferred to 2 L glass reactors (0.07 m diameter, 0.5 m length) with the respective growth media. All experiments were conducted in batch mode and under controlled conditions of light intensity (200 µmol/*(*m^2·s) with a photoperiod of 14:10 (light/dark), a temperature of 23 °C and continuously aerated (0.2 L/(L·min). The absorbance was determined colorimetrically at 750 nm (Shimadzu 1240 UV-VIS, Japan). Cell counts were performed every 3 rd day under optical microscope (Nachet Pegase) using malassez counting chamber (depth 0.200 mm Hecht Assistant, Germany). Algal biomass concentration (*γ*) was measured gravimetrically after drying a centrifuged culture sample at 105 °C for 24 h.

Biomass concentration: γ/(g/L)= $\frac{\mathrm{m1-m2}}{\mathrm{Vi}}$ /1/

where m_1 corresponds to the initial weight, m_2 is the weight of the biomass after drying and V_i is the initial volume.

Different growth parameters were determined as follow (24):

Biomass productivity (mg/(L·day)= $\frac{dx}{dt}$ /2/

where dx= $x_f - x_0$; dt: number of days.

Specific growth rates: $\mu max = \frac{1}{dt} \times ln\frac{xf}{x0}$ /3/

where x_0 and x_f are the mean dry biomass concentration at the times T_0 and T_f, respectively.

Biomass generation time: $Td = \frac{ln2}{\mu max}$ /4/

Preparation of microalgae extracts

The biomass was harvested at the beginning of the stationary phase of culture and washed twice by centrifugation at 13000 ×*g* for 5 min (MPW-350R, Warsaw, Poland). After harvesting, the microalgae biomass was dried using a regular oven BINDER at 60 °C (ED115UL, Germany) for 24 h until there was no change in weight on further drying. Microalgae biomass was thereafter macerated in ethanol (1/15: *m*/*V*) for 3 hours at room temperature in darkness and under agitation (Thermo-Scientific-2871, Waltham, MA, USA). The extraction was repeated several times until the supernatant was colorless. The extraction method was selected and adjusted on the base of the results obtained by Maadane et *al.* (*25*) on different microalgae biomass, which permitted to obtain an efficient extraction of biomolecules. The extracts were filtred (Whatman® membrane filters 0.2 µm, Merck) and the solvent was removed by rotary evaporation (IKA RV-10, IKA ® -Werke GmbH & Co. KG, Germany) at 40 °C. All concentrated extracts were weighed and stored at −20 °C until use.

Biochemical composition

Total lipids were measured according a modified version of Kochert's method (*26*). Briefly, the dried biomass was ground with an equivalent mass of alumina for 5 min. To the biomass-alumina mixture, a methanol-chloroform (2:1 *V*/*V*) solution was added. The mixture was then centrifuged (2054 ×*g* for 5 min). The pellet underwent three additional extractions. To the final supernatant, 158 mM HCl (Sigma, cat. no. H1758) and 0.015 % $MgCl_2$ (Sigma, cat. no. 63068) were added. The lower phase containing the lipid fraction was extracted using a pipette pastor and transferred to a new pre-weighed tube.

Lipid productivity (LIPp) was determined as follow:

LIPp= Lipid content (%)×Biomass production. /5/

Fatty acid were transesterified based on EN ISO 5509 (Boron trifluoride method) (2000) (*27*). The resulting fatty acids methyl esters (FAMEs) composition were determined by gas chromatography (CP-3800 GC,Varian, USA) equipped with 30 m SUPELCOWAX 10 capillary column (0.32 mm of internal diameter and 0.25 µm of film thickness). The injector (split 1:50) and detector (flame ionization) temperatures were kept constant at 250 °C. The oven temperature program started at 200 °C for 8 min, then increased up to 230 °C at 5°C/min and maintained constant at that temperature for 16 min. Helium was used as the carrier gas, and kept at a constant flow rate of 1.3 mL/min. Percentages of FAMEs were calculated as percentage of the total fatty acids present in the sample, determined from the peak areas. The 1, 2-diheptadecanoyl-sn-glycero-3-phosphocholine (17:0) was

used as an internal standard. Fatty acid content was calculated according to the European Standard EN 14103 (*28*).

Carbohydrates were determined using the conventional phenol-sulfuric acid method developed by Dubois *et al.* (*29*) with glucose as standard. The sample was mixed with 72 % sulfuric acid (Merck) at 30 °C for 1 h. The mixture was autoclaved (BKM-Z12N, Biobase, China) for 1 h at 120 °C after dilution of the sulfuric acid to 4 %. To the liquid fraction was suddenly added an equivalent volume of a 5 % phenol solution (Merck) and 1 mL of concentrated sulfuric acid. Then the mixture was heated to 100 °C for 5 min. After 30 min at room temperature the absorbance was read at 480 nm.

Relative proteins were estimated from the difference between total dry ash-free biomass and the sum of lipid and carbohydrate contents (*19*).

Phenolic content

Phenolic content was determined using folin-ciocalteu reagent based on the slightly modified method of Singleton and Rossi (*30*), using gallic acid as a standard. Briefly, 125 µL of extracts sample (1mg/mL) was mixed with equal volume of Folin-Ciocalteu (Sigma-Aldrich, Merck) reagent, 1 mL of 7 % sodium carbonate (Na_2CO_3) and the volume was made up to 3 mL by adding distilled water. The mixture was mixed and incubated for 90 min in the dark. Absorbance of the mixtures was measured at 760 nm and results were expressed as mg gallic acid equivalents per g dry extract (GAE)/(mg/g).

Carotenoid content

Carotenoid content of algal extracts (at concentration of 1 mg/mL in ethanol) was estimated spectrophotometrically according to Lichtenthaler and Buschmann (*31*) method. Aliquots of the extracts were prepared at concentration of 1 mg/mL in ethanol. Absorbances were measured at 470, 648 and 664 nm, and carotenoid content was calculated using the Lichtenthaler equations (*25, 31*).

$Chla=13.36\times A_{664}-5.19\ A_{648}$ /6/

$Chlb=27.43\times A_{648}-8.12\ A_{664}$ /7/

$Carotenoids\ total = (1000\times A_{470} -1.63\times chla-104.96\times chlb)/221$ /8/

DPPH radical scavenging assay

The antioxidant capacity of the samples was evaluated by their ability to scavenger the DPPH (2,2-diphenyl− -picryl-hydrazyl-hydrate) (Sigma-Aldrich) at various concentrations. The method used was that of Kumar et *al.* (*32*) with slight modifications. To 0.2 ml of different methanolic extracts (1, 2, 3, 4 and 5 mg/mL) was added 1 mL of 0.2 mM (in absolute ethanol) DPPH solution. Absorbance was

measured at 517 nm after 30 min incubation in the dark against methanol as blank. DPPH radical scavenging capacity (%) was calculated as follow:

$$\text{Scavenging capacity (\%)} = (A \text{ control}-A \text{ sample})/A \text{ control}) \times 100 \quad /9/$$

Where *A* control is the absorbance of the control (DPPH), and *A* sample is the absorbance of the tested sample (with DPPH). The absorbance of extracts sample in methanol (without DPPH) was determined in order to subtract the absorbance of colored extracts.

The IC_{50} (Inhibition Concentration at 50 %) was calculated by linear regression, and expressed in milligram by millilitre. BHT (butylated hydroxytoluene) was used as reference standard.

Ricotta cheese production

The control ricotta cheese sample has been prepared by heating whey (FarmCheese, Manouba, Tunisia) obtained from cow milk, which was previously coagulated with rennet, to 45 °C adding salt and continuing heating in large open kettles until the temperature reached 80 to 85 °C. At that point, a suitable food grade acidulant (citric acid) was added to reduce the pH to 6.0 and induce coagulation of the proteins. The curd particles floats to the surface of the hot liquid and is scooped off and placed in perforated recipient. At this level microalgae biomass were added at 0.2 %, 1 %, 1.5 % (*m*/*m*: microalgae biomass: coagulated whey) between different layers in order to obtain homogeneous product. The samples were left to drain, and cool overnight.

Sensory analysis

Hedonic evaluation of the ricotta supplemented with the biomass of *Chlorella* sp.SM1 obtained from BG-11 medium and commercial *Arthrospira platensis* biomass, as well as the control sample was performed based on protocol previously described by Batista et *al.* (*9*). Sensory evaluation was carried in appropriate room at 25 °C and lighting in an open sitting condition while respecting the international standards (ISO 8589:2007) (*33*). The main purpose of the study was clearly explained to the individuals who had to sign an informed consent in order to express their agreement to participate in this research program. Each panel member was trained how to score different characteristics. Ricotta samples were served a day after being cooked in random order to 30 individuals, (26 female and 4 male) aged between 23 to 45 years old, whom were asked to evaluate the samples for the following attributes : color, odor, taste, texture, global appreciation (5 levels from “very pleasant” to “very unpleasant”). Panelists were also asked, whether, they would buy the ricotta they tested (from “would certainly buy” to “certainly wouldn't buy”).

Statistical analysis

The results of three replicates from each sample were used for statistical analysis and the values were expressed on dry weight extract as value ± the standard deviation .Origin Pro 8.0 software (OriginLab Corporation, Northampton, MA, USA) (*34*) was used. Results were compared among culture conditions and strains by one-way ANOVA test in conjunction with Tukey's test, at a significance level of 95 % ($p<0.05$).

RESULTS AND DISCUSSION

Growth performance of microalgae

Effects of different media-compositions on growth parameters of *Chlorella* sp.SM1 and *Nannochloropsis gaditana*.L2 are shown in Table 1 and Fig. S1. The two strains followed conventional shape of growth (Lag, exponential, and stationary phase). It can be observed that the BG-11 medium resulted in a higher growth performance for *Chlorella* sp.SM1 with $1.97E^{+08}$ cells/mL after 17 days. Regarding *Nannochloropsis gaditana*.L2 the highest number of cells was obtained in the Algal medium ($1.42E^{+08}$ cells/mL). Results mentioned in Table 1, confirm that *Chlorella* sp.SM1 and *Nannochloropsis gaditana*.L2 achieved a higher growth rates and biomass productivities in BG-11 and Algal media. The biomass productivity was significantly different between both strains when growing under the same culture medium (Table 1). However, among the selected growth-media, f/2 has given the highest lipid productivities. Biomass productivities found in this work were higher than those reported by George et *al.* (*35*) for *A. falcatus* cultivated in BG-11 and BBM medium (6.14 mg/(L·day); 1.6 mg/(L·day) respectively). Xia et *al.* (*36*) mentioned comparable biomass and lipid productivities (75.4 and 21 mg/(L·day), respectively) for *Desmodesmus* sp. cultivated in modified BG-11 medium.

Table 1

By comparing the chemical composition of the different growth media used in this study (Table S2), richness in macronutrient (Nitrogen (N) and phosphorous (P)) influences significantly microalgae cultivation. Thus, it is obvious that the presence of higher concentrations of N and P in Algal and BG-11 media is important for the cell growth, while their limitation leads to poor growth ability and higher lipid productivity. These results are similar to those of Jazzar et *al.* (*19*) who suggested that N and P are important for cell division and protein accumulation. N is converted inside algal cells into a useable form: nitrite that is reduced to ammonium, which in turn produces glutamine responsible of protein production. This justifies the lower protein contents obtained in N-deficient f/2 medium (Fig. 1).

Nannochloropsis gaditana.L2 reached its highest biomass productivity and cell growth in Algal medium; containing the highest P concentration, (fivefold higher than BG-11 medium). Indeed, excess phosphorus concentration results in excess of ATP and NADPH synthesis, carrying energy for cell functions, which in turn, enhance growth and biomass production (*37*).

Accordingly, previous studies have demonstrated that lipid and biomass production could be affected by N: P ratios in culture media. The N: P ratios for f/2 and Conway medium used here were (29:1; 7:1, respectively), which seems the suitable ones for higher lipid production. While N: P ratios of 16:1 and 98:1 (Algal and BG-11 respectively) would induce optimal cell growth. This least phenomenon was reported by Chia et *al.* (*38*) who mentioned a higher lipid production in *chlorella vulgaris* at N: P ratios of 10:1 but not at 100:1.

Micronutrients such as magnesium (Mg), sulphur (S), and iron (Fe) play a significant role in the growth of microalgae (*39*). Previous studies have reported the contribution of iron (Fe) on microalgae growth as one of most important trace metals involved in oxidation–reduction of photosynthesis pathway. Magnesium is a constituent of chlorophyll, playing a key role in O_2/CO_2 utilization in photosynthesis. Sulphur is well involved in cell division and lipid accumulation. Wong et *al.* (*40*) have reported a lower rate of growth in *Chlorella vulgaris* when cultivated under Iron-deficiency coupled with lower amount of Mg and S. The lower Mg and Fe concentrations are registered in f/2 and Conway medium, while S, for its part, is totally absent in Conway medium.

Biochemical characterization

Primary metabolites

The impact of varying the growth media (BG-11, Algal, Conway, and f/2) on *Chlorella* sp.SM1 and *Nannochloropsis gaditana*.L2 biomass composition is illustrated in Fig. 1. The biochemical composition of the two strains varied significantly in response to nutrients availability in the growth media. *Chlorella* sp.SM1 accumulated higher lipid and carbohydrate contents in f/2 medium (Fig. 1A). The same behaviour was reported for *Nannochloropsis gaditana*.L2 (Fig. 1B). Regarding protein contents, the highest level was estimated in *Chlorella* sp.SM1 (67.0 %) cultivated in BG-11, while *Nannochloropsis gaditana*.L2 accumulated the highest protein content in Algal medium (58.7 %). Protein from microalgae are well appreciated for human consumption due to the high level of essential amino acid. Matos et *al.* (*41*) mentioned an average protein content of 40 % in six different microalgae biomass using a nitrogen-to-protein (N-to-P) conversion factor of N×4.78 through Kjeldahl method. It should be noted that the estimates for the crude protein include other nitrogen compounds which in

general are expected to account for around 10 % of the total nitrogen found in microalgae. The same team (*41*) registered a lower lipid (8.1 % and 15.6 %) and carbohydrate contents (18.6 % and 16.7 %) in *Nannochloropsis gaditana* and *Nannochloropsis oculata* cultivated in f/2 medium. *Chlorella* sp.SM1 recorded 2 fold increase in lipids in comparison with *Chlorella vulgaris* cultivated in several growth media (*40*) and an interesting carbohydrate content compared with *Chlorella* sp. cultivated under nitrogen limited condition (*41*).

Regarding culture media, the findings herein reported, indicated that N-deficient medium, particularly f/2 is suitable to enhance lipid and carbohydrate accumulation. While with higher nitrate and phosphate concentrations (in Algal and BG-11 medium), higher protein content could be obtained (*19, 40*).

Fig. 1

Carotenoids and phenolic compounds

Unicellular microalgae are known to be an interesting source of antioxidants, including skeleton carbon compounds (as carotenoids and phenolic compounds), that play an important role in scavenging reactive oxygen species (ROS) generated during photosynthesis. In order to investigate the effect of nutrients availability on the accumulation of antioxidants in the studied microalgae, total carotenoid and phenolic content of *Nannochloropsis gaditana*.L2 and *Chlorella* sp.SM1, obtained from different culture media (Algal, BG-11, f/2, Conway) were determined. Total carotenoids and phenolic content differed significantly among the tested media for the both strains (Table 2). Briefly, it can be deduced that carotenoids and phenolic contents were enhanced by f/2 and Conway media. The lowest antioxidants content, however, were marked in BG-11 and Algal medium. This suggests that both strains accumulated larger amount of antioxidants when cultivated under nitrate limitation (f/2 and Conway medium). Previous works have reported the effect of modulating nitrate availability on the accumulation of carotenoids in some microalgae species such as *Dunaliella* and *Haematococcus* well known by grabbing significant percentage of secondary metabolites under stress nutrient (*41*). Moreover, it is important to note that a limited number of paper have described the phenolic content in microalgae, especially where it is associated to a nutrient stress. For a better discussion of our results, the Table 2 further summarizes a comparison of carotenoids and phenolic content reported for some *chlorophycae* strains. Regardless of the effect of the different growth media compositions, both phenolic and carotenoid contents of the analysed extracts were comparable to those earlier reported (*15,25,43*) .Nevertheless, when comparing the results herein reported with other studies, it should be taken into consideration that the content and composition of carotenoids as well as phenolic compounds are typically influenced by other stress factors such as extracting solvents (*25*), UV-stress

(*44*), or metal stress (*45*). Taking altogether, it would be interesting in the future to cultivate the strains under investigation in two stages. In the first stage, microalgae could be cultivated in N- and P-rich Algal or BG11 medium to ensure a good biomass productivity. In the second stage, cells should be transferred, at the end of their exponential growth phase, to N- and P-deficient f/2 medium for better secondary metabolites accumulation.

Table 2

DPPH radical scavenging activity

The samples were assessed for antioxidant activity using the DPPH as a stable free radic*al.* The different inhibition capabilities of the methanolic extracts obtained from *Nannochloropsis gaditana*.L2 and *Chlorella* sp.SM1 at different concentrations and issued from the different growth media (BG-11, Algal, Conway, and f/2) are illustrated in Fig. 2. The IC_{50} of the extracts were calculated and compared with the standard antioxidant BHT. The values are reported in Table S2.

As seen in Fig. 2 both strains showed the ability to reduce the DPPH, which considerably increased with increasing concentrations. The most important scavenging ability was obtained with f/2 methanolic extract of *Chlorella* sp.SM1 that exhibited 80 % as inhibition percentage at 5mg/mL, up from only 58 % for *Nannochloropsis gaditana.*L2 in the same growth medium. The antioxidant activity of the genus *Chlorella* has been emphasized in previous work, which reported that *Chlorella* species possessed an interesting DPPH radical scavenging activity (*43,46,47*).

The concentrations of inhibition (IC_{50}) ranged between 4.49±0.05 mg/mL and 2.54±0.30 mg/mL for *Nannochloropsis gaditana*.L2 and between 1.97±0.07 mg/mL and 1.15±0.27 mg/mL for *Chlorella* sp.SM1 (Table S2). The lowest values of IC_{50} were obtained in f/2 medium. The tested extracts possessed a radical scavenging activity lower than the BHT that was used as positive control, exhibiting an IC_{50} of 0.56 mg/mL.

Regarding the effect of medium, the antiradical activity did not differ significantly between extracts of Algal, BG-11, and Conway for both strains. However, it was significantly higher in N-deficient f/2 medium. Thus, it is clear that when reducing the Nitrate content of the medium to a moderate level (from 1.5 g/L in BG-11 medium to 0.99 g/L and 0.074 g/L in Algal and Conway media respectively), the inhibition percentages of *Chlorella* sp.SM1 and *Nannochloropsis gaditana*.L2 methanolic extracts remained apparently not affected as depicted in Fig. 2. The Nitrate deficient level in f/2 medium presented a stressed condition for the evaluated strains, which has been translated in a significant increase in the ability of scavenging DPPH at different concentrations. Elevated antioxidant activity under low nitrate concentration was previously recorded in nine microalgal species (*48*).

Fig. 2

Fatty acid profiles

The quality of lipids obtained from each strain cultivated in the different media (Algal, BG-11, Conway, f/2) was assessed by gas chromatography (GC) after transesterification of all extracts. The variation in the percentages of PUFA (polyunsaturated fatty acids), MUFA (monounsaturated fatty acids) and SFA (saturated fatty acids) are summarized in Table 3.

The two strains showed a constant composition in terms of palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) independently of the medium used. An exception was noticed for *Nannochloropsis gaditana*.L2 in Algal medium, where linolenic acid (C18:3) was not detected. It is also notable that nutrient variation influences outstandingly the fatty acid composition. Higher proportions of SFAs and MUFAs were recorded in Conway and f/2 medium ($P<0.05$), whereas PUFAs were significantly higher in BG-11 and Algal medium.

Elevated MUFAs contents were registered under N-deficient conditions, and the highest MUFA accumulated is the oleic acid (C18:1) observed in *Chlorella* sp.SM1 (33.9 %) in f/2 medium. Moreover, the sum of all identified fatty acids (FAs) ranged from 71.5 % for *Nannochloropsis gaditana* L2 in Algal medium to be the highest for *Chlorella* sp.SM1 in f/2 medium (93.5 %). The overall fatty acid profiles found in this work corroborate with those previously published by Mendes et *al.* (*49*) for *Chlorella* sp., and with those reported by Jazzar et *al.* (*19*) for *Chlorella sorokoniana* and *Neochloris* sp. Fatty acid variations reported in this work was also in agreement with previous studies that suggested an increase in fatty acid content in response to nitrogen deficiency (*50, 51*) accompanied by an inflation in oleic acid content.

Table 3

Ricotta enrichment and Sensory evaluation

Making a successful fresh ricotta cheese is still an art because of the crucial requirements for suitable texture and flavour (*52*). Editing manufacturing diagram steps is considered critical, and even more if an additional ingredient is to be added to the original recipe. For this purpose, several assays were made at laboratory scale in order to adjust the manufacturing diagram and then move to small-scale assays. It was finally decided to add microalgae biomass equitably between different layers of the ricotta when filling the recipients. The obtained ricotta samples with microalgae biomass had an original appearance with attractive color that differs with green tonality of used microalgae (Fig. S2). Formulations assays were carried-out with *Chlorella* sp.SM1 biomass obtained from the BG11 medium that represents a good compromise between biomass productivity and bioactivity results. This enrichment was compared to a commercial *Arthrospira platensis* as the strain is a reference in

food industry, and to better ascertain the impact of pigment colors in the sensory assays. These two strains are recognized as safe (GRAS) and are already widely used in food formulations (*6, 9*). Ricotta samples at 0.2 %, 1%, 1.5 % of *Chlorella sp.*SM1, 1.5 % of *A. platensis* and 1.5 % mixture of both *Chlorella* sp.SM1 and *Arthrospira platensis* were therefore produced as shown in Fig. 3. A sensory tasting sessions performed by INSAT researchers was carried out.

Fig. 3

Fig. 3 shows the average scores for the main sensory attributes (color, odor, flavor, texture) as evaluated by the panel. The least appreciated sample was 1.5 % *Spirulina platensis.* In fact, the panel showed more preference for the 0.2 % *Chlorella* sp.SM1 and the control one, in term of global appreciation. Concerning the color, the tasters have classified positively the samples with microalgae biomass (0.2 % and 1% *Chlorella* sp.SM1) and the 1.5 % mixture as the least appreciated one.

The consumers have always been sensitive towards the taste and odor when evaluating a product even before health-benefit consideration. Using higher concentration of microalgae marked a distasteful impact and the corresponding samples have been the least appreciated in term of odor and flavor.

Several Products enriched with microalgae have been already sensory tested, and globally appreciated (*6*). However, in some cases, the high level of addition (2 %) has negatively influenced the flavor parameter and led to a negative global appreciation (*13*).

In the comments section, the tasters mentioned that the 1.5 % *spirulina platensis* ricotta presented a very unpleasant fish odor and taste, which lasted in the after-taste feeling. In fact, the ricotta with 0.2 % *Chlorella* sp.SM1 was considered the most equilibrated in terms of flavor followed by the 1 % *chlorella* sp.SM1.

The texture was estimated manually by stirring the sample with a spoon. As seen in Fig. 3, the samples demonstrate a significant change from a texture point of view due to the addition of microalgae (>0.2 %). This change in the texture was positively received by the panelists and reached the scale 4, corresponding to “pleasant” texture. Actually, this change could be related to the fact that standard ricotta possesses, usually, a very soft texture with a tendency to crumble very easy. Microalgae biomass has brought a positive overall structural effect.

Fig. 4 presents the answers given by the tasters in relation to the buying intention. 63% and 53% of the tasters “would probably buy” the ricotta with 0.2 % and 1 % *Chlorella* sp. SM1, respectively. 26 % of the tasters “would certainly buy” and 6% “would probably buy” the 1.5 % *Chlorella* sp.SM1 ricotta. The 1.5 % *Spirulina platensis* ricotta was the least appreciated with 26 % of the tasters mentioning that “certainly wouldn't buy” and 34 % “probably wouldn't buy”. This simulation is of great

importance helping to predict a product's possible future commercialization since it gives a perspective of the potential consumer's acceptance.

Fig. 4

CONCLUSIONS

This work investigated the feasibility of using microalgae to enrich and enhance the nutritional properties of Ricotta, a Mediterranean dairy product made from cheese whey. This approach can be considered as an innovative application that allowed obtaining an innovative product. The variation of growth media showed the role of nitrogen-deficient medium like f/2 or Conway to boost the antioxidant activity of microalgae while keeping basal nutritional benefits. The cultivation of *Chlorella* sp.SM1 and *Nannochloropsis gaditana.*L2 in f/2 and Conway media permitted to reach high-quality PUFA accumulation. The highest antioxidant activity, carotenoids and phenolic contents were also obtained with these two media. The formulation of the enriched Ricotta was successfully made without altering either the flow diagram, or the sensorial acceptability of the product especially at low incorporation level (0.2 %). The addition of microalgae biomass to fresh Ricotta revealed an appreciative impact on the sensory attributes of the final product designated as "Ricottalgue". Based on the obtained results, the attractiveness of artisanal cheap dairy product could be enhanced using microalgae as natural and healthy ingredient.

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CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

SUPPLEMENTARY MATERIALS

All supplementary materials are available at www.ftb.com.hr.

AUTHORS' CONTRIBUTION

SK performed experiments, interpretation of results and writing. I.B and N.B participate in performing experiments and data processing. M.M, I.S Conception and supervision. N.K Designing experiments, revision and supervision.

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Table 1. Growth rates, generation time, biomass productivity, and lipid productivity of *Chlorella* sp.SM1 and *Nannochloropsis gaditana*.L2, cultivated in four different growth media

	Growth rate/day^{-1}	Generation time/day	Biomass productivity/ (mg/(L·day)	Lipid productivity/ (mg/(L·day)
Chlorella sp. SM1				
Algal	$(0.33\pm0.01)^{aA}$	$(2.13\pm0.03)^{aA}$	$(128.86\pm12.69)^{aA}$	$(13.15\pm0.30)^{aA}$
BG-11	$(0.34\pm0.01)^{aA}$	$(2.04\pm0.03)^{aA}$	$(133.64\pm16.36)^{aA}$	$(7.84\pm0.69)^{bA}$
f/2	$(0.10\pm0.01)^{bA}$	$(7.05\pm0.74)^{bA}$	$(71.50\pm8.27)^{bA}$	$(23.10\pm0.93)^{cA}$
Conway	$(0.10\pm0.01)^{bA}$	$(7.19\pm0.71)^{bA}$	$(58.13\pm12.45)^{bA}$	$(17.98\pm1.58)^{dA}$
Nannochloropsis gaditana.L2				
Algal	$(0.49\pm0.05)^{aB}$	$(1.43\pm0.15)^{aB}$	$(91.18\pm23.28)^{aA}$	$(4.04\pm0.04)^{aB}$
BG-11	$(0.47\pm0.05)^{aB}$	$(1.49\pm0.16)^{aB}$	$(73.97\pm26.37)^{aB}$	$(4.77\pm0.09)^{aB}$
f/2	$(0.22\pm0.05)^{bB}$	$(3.40\pm0.79)^{bB}$	$(34.06\pm5.72)^{bB}$	$(11.86\pm1.35)^{bB}$
Conway	$(0.20\pm0.05)^{bB}$	$(3.78\pm0.96)^{bB}$	$(33.22\pm5.30)^{bB}$	$(7.85\pm1.15)^{cB}$

Means followed by the same small letter in the column did not differ significantly between culture media for each strain, and means followed by the same capital letter in the column did not differ significantly between strains under the same culture medium based on Tukey's test ($p>0.05$).

Table 2. Comparison of carotenoids and phenolic content reported for different microalgae extracts under different culture conditions with the present study

Strains	Nutrient modulation	Other culture conditions	*w*(carotenoids)/(mg/g)	*w*(phenolics as GAE)/(mg/g)	Refrences
*Chlorella sp.*SM1					
	Algal	Flow rate (0.2 L/(L·min of air); Luminosity (μmol/(m^2·s); Photoperiod (14:10 light/dark); Temperature (23 °C)	(0.89±0.11)^{aA}	(27.76±2.26)^{aA}	Current study
	BG11		(0.76±0.04)^{aA}	(10.17±0.53)^{bA}	
	f/2		(2.23±0.32)^{bA}	(41.42±2.69)^{cA}	
	Conway		(2.17±0.04)^{bA}	(48.81±4.47)^{cA}	
*Nannochloropsis gaditana.*L2					
	Algal		(0.61±0.06)^{aB}	(20.82±3.50)^{aB}	
	BG11		(0.76±0.05)^{aA}	(15.04±1.07)^{aB}	
	f/2		(1.97±0.19)^{bA}	(52.72±7.62)^{bA}	
	Conway		(1.55±0.44)^{bB}	(35.09±3.36)^{cB}	
Chlorella vulgaris					
	Control : 5mM (N) 0.25mM (P)	Flow rate (0.25 v/v min of air); luminosity (125μmol photons /(m^2·s ^{2}) Photoperiod (12:12 light/dark)	3.8	3.3	*(15)*
	P-Lim : 0.01mM		0.9	1.8	
	N-Lim : 0.2mM		0.4	1.3	
Tetraselmis suecica					
	Control : 5mM (N) 0.25mM (P)		2.5	3.3	
	P-Lim : 0.01mM		1.3	2.8	
	N-Lim : 0.2mM		0.5	1.5	
Chlorella elipsoida					
	Control : 10mM (N)	Flow rate (0.03% CO_2 in air); Luminosity (200 Wm^{-2}) or (400 Wm^{-2}) under N-lim; Temperature (25 °C±3)	6.5	0.9	*(53)*
	N-Lim		30.4	4.31	
Haematococcus pluvialis					
	Control : 6.3mM (N)	Flow rate (0.05 v/v min of air); Luminosity (850 μmol/(m^2·s ^{2}); Temperature (25°C)	0.7*	--	(*54*)
	N-Lim : 1.5mM		10*	--	

Means followed by the same small letter in the column did not differ significantly between culture media for each strain, and means followed by the same capital letter in the column did not differ significantly between strains under the same culture medium based on Tukey's test ($p>0.05$). Nitrogen limitation (N-Lim); Phosphorus limitation (P-Lim), -- Not determined, *Astaxanthin

Table 3. *w*(fatty acids)/% present in *Chlorella* sp.SM1 and *Nannochloropsis gaditana*.L2, cultivated in different media (Algal, BG-11, Conway, f/2)

	Chlorella sp.SM1				*Nannochloropsis gaditana*.L2			
	Algal	BG-11	Conway	f/2	Algal	BG-11	Conway	F/2
C14 :0	nd	0.5	0.9	0.7	nd	nd	0.8	0.8
C16 :0	20.3	17.2	33.4	30.9	20.5	17.9	30.4	27.7
C16 :1	0.3	nd	nd	1.7	4.4	3.4	2.2	2.3
C18 :0	nd	nd	nd	nd	2.8	2.3	3.8	3.3
C18 :1	7.0	17.9	29.6	34.0	4.5	3.1	26.2	24.5
C18 :2	32.9	35.8	17.1	18.2	42.5	35.9	20.5	23.2
C18 :3	8.6	5.7	7.7	7.9	nd	10.8	8.2	8.6
C20 :0	2.5	1.1	0.3	0.7	nd	3.5	0.2	0.4
C22 :1	nd	nd	0.2	0.2	nd	nd	0.2	0.2
C24 :0	2.3	nd	0.2	nd	nd	nd	0.2	0.3
SFA	25.1^{aA}	18.8^{bA}	34.8^{cA}	32.3^{dA}	23.3^{aA}	23.7^{aB}	35.4^{bA}	32.5^{cA}
MUFA	7.3^{aA}	17.9^{bA}	29.8^{cA}	35.9^{dA}	8.9^{aB}	6.5^{bB}	28.6^{cA}	27.0^{dB}
PUFA	41.5^{aA}	41.5^{aA}	24.8^{bA}	26.1^{cA}	42.5^{aA}	46.7^{bB}	28.7^{cB}	31.8^{dB}
ω3	8.6	5.7	7.7	7.9	nd	10.8	8.2	8.6
ω6	32.9	35.8	17.1	18.2	42.5	35.9	20.5	23.2
ω3/ω6	1 :4	1 :6	1 :2	1 :2	nd	1 :3	1 :2	1:3

Same small letter in a line did not differ significantly between culture media for each strain, and same capital letter in a line did not differ significantly between strains under the same culture medium based on Tukey's test ($p>0.05$). nd: not detected, SFA (saturated Fatty Acids); MUFA (Monounsaturated Fatty Acids); PUFA (Polyunsaturated Fatty Acids); ω3 (Omega-3 fatty acids); ω6 (Omega-6 fatty acids).

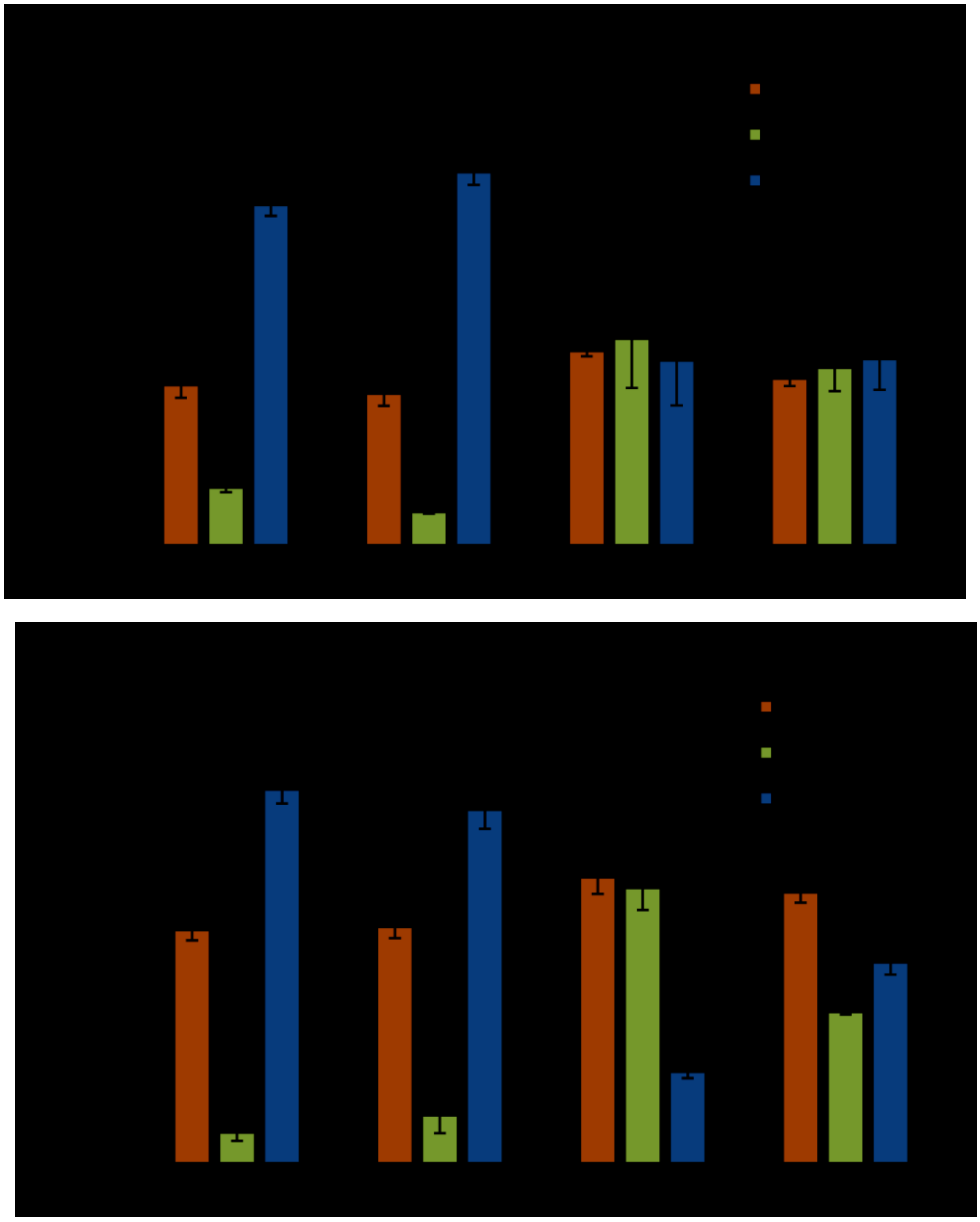

Fig. 1. Biochemical composition of (A) *Chlorella* sp.SM1 and (B) *Nannochloropsis gaditana*.L2 cultivated in four culture media (Algal, BG-11, f/2, and Conway).The same small letters indicate a non-significant difference between culture media for each strain, and the same capital letters indicate a non-significant difference between strains under the same culture medium based on Tukey's test ($p>0.05$).

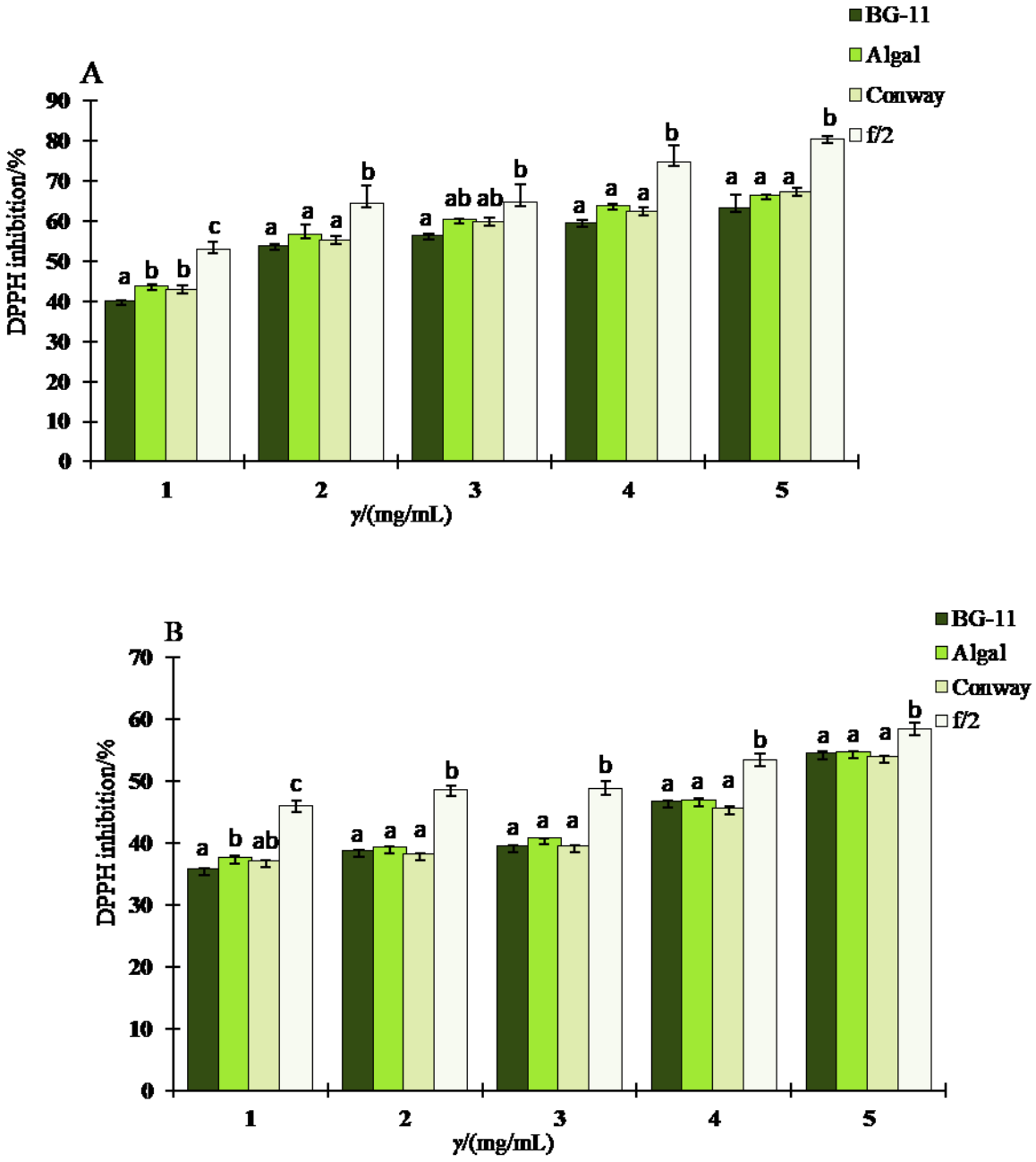

Fig. 2. DPPH radical scavenging activities in different methanolic extracts of (A) *Chlorella* sp.SM1 and (B) *Nannochloropsis gaditana*.L2.The same small letters indicate a non-significant difference between culture media for each strain at each concentration, based on Tukey's test ($p>0.05$).

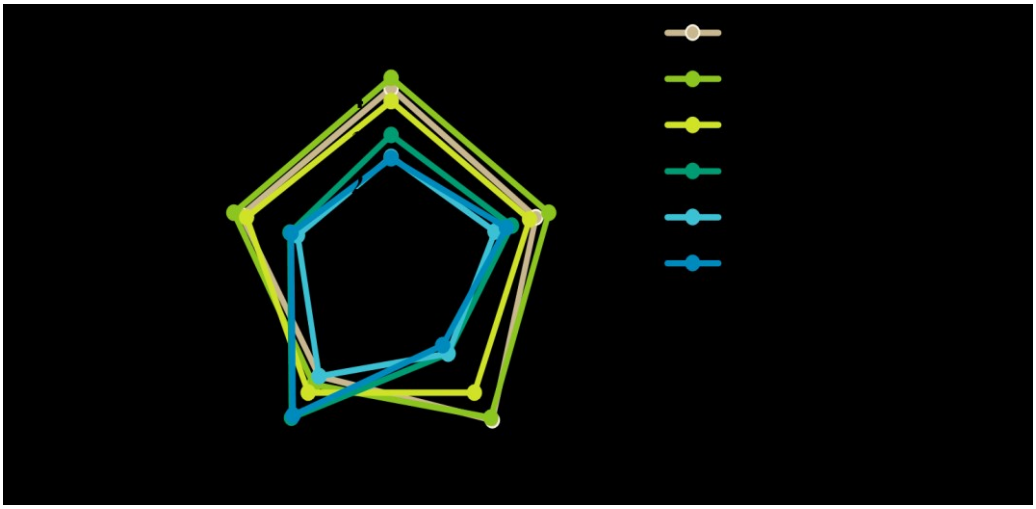

Fig. 3. Sensory evaluation results (n=30) of Control and ricotta samples with 0.2 %, 1 %, 1.5 % of *Chlorella* sp.SM1, 1.5 % of *Spirulina platensis* and 1.5 % equal mixture of *Chlorella* sp.SM1 and *Spirulina platensis.*

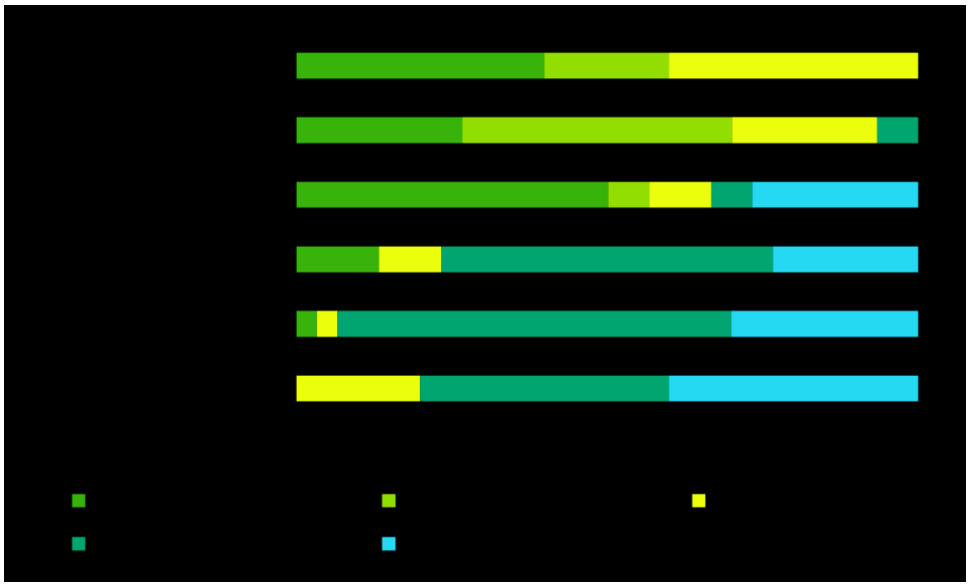

Fig. 4. Panel tasters buying intention (n=30) regarding control, *Chlorella* sp.SM1, and *Arthrospira platensis* ricotta cheese samples.