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Microalgal biomass — a bio-based additive: evaluation of green smoothies during storage

[1]*Sahin, O. I. and [2]Ozturk, B.

[1]*Department of Chemical Engineering, Faculty of Engineering, Yalova University, Yalova, Turkey*
[2]*Department of Chemical and Process Engineering, Institute of Science, Yalova University, Yalova, Turkey*

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Abstract

Microalgae biomass addition to food has been studied for its nutritional fortification. The present work investigates the impact of microalgae (*Chlorella vulgaris* and *Dunaliella salina*) addition, in terms of quality characteristics, during a 28-day storage at 5°C. As much as 2.5% (w/v) of *C. vulgaris* and *D. salina* were separately added to fresh green smoothies (spinach, green apple, and cucumber) as food additive. Without any thermal application during storage at 5°C, the changes in pH, total soluble, solid contents, titratable acidity, microbial loads, phenolic contents, antioxidant activity, and sensory characteristics were determined. The addition of microalgae biomass, either *Chlorella* or *Dunaliella*, was found statistically significant, but this addition did not make a significant difference during the 28-day storage. Compared to control samples (at day 0; 163.16 mg GAE/100 g and 2.56 mmol GAE/100 g), *Dunaliella* biomass affected green smoothie more positively on total phenolic (at day 0; 395.79 mg GAE/100 g) and antioxidant activity (at day 0; 5.54 mmol GAE/100 g), than *Chlorella* biomass (at day 0; 384.21 mg GAE/100 g and 4.22 mmol GAE/100 g). Also, a shelf-life study on 28-day storage at 5°C found that *Dunaliella*-added smoothies were more preferred by the panellists, while *Chlorella*-added samples exhibited off-odour and off-flavour through storage. Smoothie supplementation with 2.5% microalgae biomass caused a decrease in the initial microbial load. Due to this reduction, it can be said that microalgae supplementation as an additive was effective, and microalgae-added samples were shown below the "microbiologically consumable level" throughout the 28-day shelf-life study.

Introduction

Fruits and vegetables are good sources of non-nutrient compounds, especially vitamins A and C, fibre, electrolytes like magnesium and potassium, as well as phenolics and antioxidants. Bioactive metabolites known as phytochemicals have also been suggested as an active agent in human health (Elmadfa and Meyer, 2016); and because of their high content of phytochemicals, consuming fruits and vegetables is important for health. However, fruit and vegetable consumption are far from the recommended intake in most countries that have dietary recommendations (Slavin and Lloyd, 2012). Smoothies—a 'ready-to-drink' concept—are prepared from fresh or frozen fruit and/or vegetables with other ingredients such as yogurt, milk, grains, etc.; and they are a low-effort way to pack more raw vegetables and fruits into a diet, and could be a solution to the lack of micronutrients due to the insufficient fruit and vegetable consumption.

Regarding the positive effects on human health, there is an increasing concern about new functional ingredients and foods. This concept is based on food design and processing with functional ingredients that improve nutritional value. Algae have been used in traditional gastronomy in Asian countries, and has recently been adopted in Western countries as a functional bioactive ingredient. For the last decades, marine-derived nutrients, and other marine and freshwater bioactive have gained growing importance for their profound potential as functional food ingredients, due to their positive biological and functional assets (Shahidi, 2004; Slavin and Lloyd, 2012). Among the most promising sources for functional food ingredients are microalgae.

Microalgae biomass is a noteworthy supplement of biologically active compounds which contains almost every vitamin, high bioavailable protein, essential amino acid, polysaccharide, polyunsaturated fatty acid, essential mineral, as well as pigments such as carotenoids, phycobiliproteins, and chlorophylls (Batista *et al.,* 2013; Graça *et al.,* 2018). Therefore, microalgae biomass and their

*Corresponding author.
Email: isahin@yalova.edu.tr

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metabolites have become an interesting innovative additive for functional food production. Many novel healthy food productions with microalgae biomass are now used in the food industry worldwide. However, resistance to the acceptance of microalgae-incorporated novel foods is a profound issue; that is why in traditional foods like sauces, desserts, biscuits, pastas, and breakfast cereals, enrichment is preferred as a mechanism to deliver those nutraceuticals. So far, several food products such as pastas (Lemes *et al.,* 2012; El-Baz *et al.,* 2017), biscuits (Gouveia *et al.,* 2007; 2008a), chocolates (Şahin, 2019), puddings, gelled desserts (Gouveia *et al.,* 2008b), mayonnaises, and salad dressings (Gouveia *et al.,* 2005; Raymundo *et al.,* 2005) have been developed.

Microalgae are extremely heterogeneous, regarded as a unicellular, photoautotrophic, eukaryotic or prokaryotic group of organisms, and commercial production of this microbial biomass is limited to a few species, such as *Arthrospira* (*Spirulina*), *Chlorella*, and *Dunaliella*. These genera are the most interesting microalgae with great potential for worldwide industrial cultivation (Pulz and Gross, 2004). The microalga *C. vulgaris* is promoted as a good source of protein, as well as carotenoids, lutein, and especially chlorophyll, which have health benefits such as preventing atherosclerosis, decreasing cell degeneration, and improving the immune system and skin rashes (Kulkarni and Nikolov, 2018).

The other microalgae, *D. salina*, synthesises large amounts of carbohydrates, β-carotene, glycerol, vitamin C, and vitamin A under high salinity, irradiance, and nutrient-limiting conditions (Ben-Amotz and Avron, 1983; Borowitzka *et al.,* 1990; Borowitzka and Siva, 2007). *Dunaliella salina* has a great capacity for molecular improvement of recombinant proteins (Borowitzka and Siva, 2007; Yaakob *et al.,* 2014), thus decreasing the risk of lung, oesophagus, pancreas, stomach, breast, skin, colon, and ovarian cancer (Caporgno and Mathys, 2018).

As functional food production is the main concern for both the food industry and scientific research, recent studies have concluded that the consumption of fruit and vegetable juices such as smoothies can improve the health of consumers (Maeda, 2013). As thermal processing can reduce the bioactive content of smoothies, the main problem with fresh smoothies is their limited shelf-life due to spoilage susceptibility and quality degradation. For that reason, the aim of the present work was to study the effect of two algal biomasses—*C. vulgaris* and *D. salina*—as additives of bioactive compounds and to maintain the sensorial and microbial quality of green fresh vegetable smoothies throughout storage at 5°C for 28 d.

Materials and methods

Fresh raw materials were purchased from a local supermarket, and all solvents and reagents were supplied by Merck (Germany).

Algal biomasses and samples

Chlorella biomass was obtained through the cultivation of *C. vulgaris* in a Basal Bold medium at 25 ± 2°C for 25 d, and *Dunaliella* biomass was obtained through the cultivation of *D. salina* in Johnson's medium at 20 ± 2 for 30 d. Biomasses were then freeze-dried after harvesting at Food Biochemistry and Biotechnology Laboratory, Chemical and Process Engineering Department, Yalova University.

Fresh raw materials were sanitised with 75 mg. L^{-1} NaClO for 2 min, and then rinsed with tap water for 1 min. All vegetables and fruits were then cut and blended (Blender, Tefal, France). The green smoothie composition was 50% spinach, 33% green apple, and 17% cucumber.

Following a preliminary sensorial evaluation on the addition of microalgal biomass, *C. vulgaris* and *D. salina* biomasses were added at a rate of 2.5% to the green fresh smoothies.

Physicochemical analysis

Three replicates were performed for each analytical measurement. The pH, titratable acidity (TA), and total soluble solid content (SSC) of green smoothies were analysed as previously described by Li *et al.* (2015). The pH of the samples was determined using a digital pH meter (Ohaus, Starter-2100, USA), and the SSC of the smoothies was determined at 20 ± 1°C using an Abbe refractometer (Shanghai Precision and Scientific Instrument Co., Shanghai, China) and expressed as °Brix. TA was determined by titration with 0.1 mol L^{-1} NaOH (T50, Metter Toledo, Milan, Italy), and expressed as % (g ascorbic acid 100 mL^{-1}) (Wang *et al.,* 2014).

The microalgal biomasses and the 'control' smoothies were analysed for moisture, total protein, fat, and ash as per AOAC methods (AOAC, 2000), and the total amount of carbohydrates was calculated by differences.

Total phenolic content and antioxidant activity (CUPRAC) assay

Total phenolic content was determined following the method of Singleton *et al.* (1999). The

method was applied by measuring absorbance at 765 nm, after adding 1 mL of Folin-Ciocalteu reagent and 2 mL of 7.5% Na_2CO_3 to 0.2 mL of extract at a 2 $mg.mL^{-1}$ concentration, stirred in 7 mL of deionised water, and incubated at room temperature for 2 h. The total antioxidant capacity was determined following the method of Apak *et al.* (2004) with slight modifications. The solutions of 10 mM $CuCl_2 \bullet 2H_2O$ and 1 M NH_4CH_3COO were prepared in pure distilled water. Neocuproine (Nc) solution at 7.5 mM concentration was daily prepared in absolute ethanol. After mixing 1 mL of Cu(II), Nc, and NH_4CH_3COO solutions, respectively, 1 mL of sample solution was added, and the final volume was completed to 4.1 mL with pure distilled water. The solution was left to stand for 30 min to achieve equilibrium. Absorbance measurement of the resulting cuprous-neocuproine complex was performed at 450 nm. The calibration curve was made using gallic acid as standard, and the results were expressed as mmol gallic acid equivalent per 100 g of sample. All spectrophotometric measurements were performed with a Shimadzu 1280 (Kyoto, Japan) UV-vis spectrophotometer (± 0.001 units of absorbance, 10 mm light path).

Microbiological analysis

To determine the mesophilic (TM), psychrophilic (TP), enterobacteria (EB), and yeast and mould (YM) counts, the total plate count method was used according to Castillejo *et al.* (2016) and Wang *et al.* (2014). For each sample, a 10-fold dilution series was prepared and spread-plated on plate-count modified agar (PCA) (Merck, Darmstadt, Germany) for mesophilic and psychrotrophic aerobic bacteria and incubated at 30°C for 72 h and 5°C for 7 d, respectively; violet red bile dextrose agar (Merck, Darmstadt, Germany) for enterobacteria and incubated at 37°C for 48 h; Rose Bengal agar (Merck, Darmstadt, Germany) for yeast and mould and incubated for 3 - 5 d at 22°C. All microbial counts were reported as log colony forming units per gram of product (log CFU mL^{-1}). Each of the three replicates was analysed in duplicate.

Organoleptic properties

Analysis was performed according to international standards (ASTM, 1986) with 58 assessors (28 women and 30 men, aged 15 - 55 years). A five-point scale of sensorial assessment was randomly and blindly scored for visual appearance, flavour, and colour (5: 'I like it very much' to 1: 'I do not like it very much').

Statistical analysis

The data were analysed with one-way ANOVA using Minitab 19 Software (vs. 19, Minitab Inc, Pennsylvania, USA). Statistical significance was assessed at 95% level ($p < 0.05$), and comparisons were performed by Tukey HSD. Data graphics were obtained using Design-Expert Software (Stat-Ease, V11). Each measurement was carried out in three replicates, and expressed as mean ± standard deviation.

Results and discussions

Physicochemical analysis

The chemical compositions of dried microalgae biomasses are given in Table 1. The pH, total soluble solid content (SSC), total acidity (TA), total phenolic content (TPC), total antioxidant activity (TAC), total mesophilic count (TM), total psychrophilic count (TP), Enterobacteriaceae count (EB), and yeast and mould count (YM) during storage are illustrated in Table 2.

The pH, SSC, and TA were the most important and basic quality assessments for fruits and vegetables, which have low pH (2 - 5) because they are rich in organic acids, and the total titratable acidity depends on these organic acid compositions, which vary due to food processing and storage. The

Table 1. Chemical composition of microalgal biomasses.

	Chlorella vulgaris	***Dunaliella salina***	**Green smoothie**
Moisture (%)	5.83 ± 0.08	6.16 ± 0.00	89.98 ± 0.02
Ash (%)	9.85 ± 0.02	7.68 ± 0.06	1.16 ± 0.04
Protein (%)	51.75 ± 0.09	42.81 ± 0.00	2.25 ± 0.09
Lipid (%)	16.09 ± 0.45	7.50 ± 0.37	0.51 ± 0.24
Carbohydrate* (%)	16.48	35.85	6.10
TPC (mg GAE/g)	41.42 ± 0.00	53.25 ± 0.00	1.63 ± 0.17
TAC (mmol GAE/g)	0.12 ± 0.02	0.18 ± 0.06	0.026 ± 0.01

*Calculated by difference.

Table 2. Physicochemical and microbiological data of green smoothies.

Day	Sample	pH	SSC	TA	TPC	TAC	TM	TP	YM
0	*C-S*	3.94 ± 0.01^{Ca}	8.24 ± 0.00^{Ca}	4.77 ± 0.01^{Ae}	163.16 ± 0.17^{Ca}	2.56 ± 0.01^{Ca}	6.90 ± 0.01^{Ae}	5.69 ± 0.01^{Ae}	4.84 ± 0.01^{Ae}
	Chl-S	4.26 ± 0.01^{Aa}	8.75 ± 0.00^{Ba}	4.59 ± 0.01^{Ce}	384.21 ± 0.41^{Ba}	4.22 ± 0.01^{Ba}	6.38 ± 0.01^{Be}	5.30 ± 0.01^{Be}	4.11 ± 0.01^{Ce}
	Dun-S	4.12 ± 0.01^{Ba}	10.30 ± 0.00^{Aa}	4.65 ± 0.01^{Be}	395.79 ± 0.26^{Aa}	5.54 ± 0.01^{Aa}	6.11 ± 0.01^{Ce}	5.00 ± 0.01^{Ce}	4.43 ± 0.01^{Be}
7	*C-S*	3.87 ± 0.01^{Cb}	8.21 ± 0.00^{Ca}	4.85 ± 0.01^{Ad}	157.89 ± 0.21^{Cb}	2.41 ± 0.02^{Ca}	6.92 ± 0.01^{Ad}	5.72 ± 0.02^{Ad}	4.88 ± 0.01^{Ad}
	Chl-S	4.20 ± 0.01^{Ab}	8.72 ± 0.00^{Ba}	4.64 ± 0.01^{Cd}	373.68 ± 0.01^{Bb}	3.95 ± 0.01^{Ba}	6.43 ± 0.01^{Bd}	5.39 ± 0.02^{Bd}	4.17 ± 0.01^{Cd}
	Dun-S	4.07 ± 0.01^{Bb}	10.31 ± 0.00^{Aa}	4.71 ± 0.01^{Bd}	383.69 ± 0.02^{Ab}	5.13 ± 0.02^{Aa}	6.20 ± 0.01^{Cd}	5.15 ± 0.02^{Cd}	4.47 ± 0.01^{Bd}
14	*C-S*	3.69 ± 0.01^{Cc}	8.16 ± 0.01^{Cb}	4.93 ± 0.01^{Ac}	147.38 ± 0.01^{Cc}	2.07 ± 0.01^{Cb}	6.95 ± 0.01^{Ac}	5.75 ± 0.02^{Ac}	4.92 ± 0.01^{Ac}
	Chl-S	4.15 ± 0.01^{Ac}	8.68 ± 0.01^{Bb}	4.70 ± 0.01^{Cc}	353.68 ± 0.03^{Bc}	3.81 ± 0.03^{Bb}	6.49 ± 0.01^{Bc}	5.47 ± 0.03^{Bc}	4.27 ± 0.01^{Cc}
	Dun-S	4.01 ± 0.01^{Bc}	10.29 ± 0.01^{Ab}	4.77 ± 0.01^{Bc}	363.16 ± 0.02^{Ac}	4.45 ± 0.02^{Ab}	6.25 ± 0.01^{Cc}	5.25 ± 0.01^{Cc}	4.54 ± 0.00^{Bc}
21	*C-S*	3.60 ± 0.02^{Cd}	8.10 ± 0.01^{Cc}	5.01 ± 0.01^{Ab}	126.32 ± 0.02^{Cd}	1.72 ± 0.02^{Cc}	6.99 ± 0.02^{Ab}	5.81 ± 0.01^{Ab}	4.96 ± 0.02^{Ab}
	Chl-S	4.09 ± 0.02^{Ad}	8.63 ± 0.01^{Bc}	4.75 ± 0.01^{Cb}	332.63 ± 0.01^{Bd}	3.68 ± 0.01^{Bc}	6.55 ± 0.02^{Bb}	5.54 ± 0.02^{Bb}	4.38 ± 0.01^{Cb}
	Dun-S	3.93 ± 0.02^{Bd}	10.20 ± 0.01^{Ac}	4.85 ± 0.01^{Bb}	336.84 ± 0.03^{Ad}	3.74 ± 0.04^{Ac}	6.34 ± 0.01^{Cb}	5.38 ± 0.01^{Cb}	4.61 ± 0.01^{Bb}
28	*C-S*	3.51 ± 0.02^{Ce}	8.02 ± 0.01^{Cd}	5.12 ± 0.01^{Aa}	114.75 ± 0.04^{Ce}	1.32 ± 0.05^{Cc}	7.02 ± 0.01^{Aa}	5.87 ± 0.01^{Aa}	5.02 ± 0.01^{Aa}
	Chl-S	3.98 ± 0.02^{Ae}	8.59 ± 0.01^{Bd}	4.81 ± 0.01^{Ca}	320.00 ± 0.03^{Be}	3.52 ± 0.03^{Bc}	6.65 ± 0.03^{Ba}	5.60 ± 0.02^{Ba}	4.49 ± 0.02^{Ca}
	Dun-S	3.85 ± 0.02^{Be}	10.04 ± 0.01^{Ad}	4.91 ± 0.01^{Ba}	326.32 ± 0.04^{Ae}	3.27 ± 0.05^{Ac}	6.43 ± 0.01^{Ca}	5.44 ± 0.01^{Ca}	4.69 ± 0.02^{Ba}

SSC: soluble solid content, °Brix; TA: titratable acidity, g ascorbic acid.100g-1; TPC: total phenolic content, mg GAE.100g-1; TAC: total antioxidant capacity, mmol GAE.100g-1; TM: total mesophilic count, log CFU mL^{-1}; TP: total psychrophilic count, log CFU mL^{-1}; and YM: yeast and mould count, log CFU mL^{-1}. All results were given as mean ± standard deviation. Different uppercase superscripts denote significant difference ($p \leq 0.05$) among smoothie types, and different lowercase superscripts denote the significance of sampling day (storage day) among smoothie types (algae addition).

SSC is significantly influenced by the effects of processing and storage conditions. The physicochemical changes during storage were found to be statistically homogenous between smoothie types, and that the added algae type had a significant effect on the results ($p < 0.05$). Throughout the storage period, the acidity increased, and the pH levels decreased. Algae biomass fortification caused the pH of the smoothies to be slightly increased. Similarly, no absolute pH changes were observed in unheated fresh green smoothies after 24 d at 5°C (Castillejo *et al.,* 2018a; 2018b), or in another study with smoothies after 43 d at 4°C (Wang *et al.,* 2012). Control samples showed a pH alteration throughout storage of 11%, while it was 6.5% for both *Chlorella* and *Dunaliella* samples, and the change rate for TA was 7% for control, 5.5% for *Dunaliella*-added, and 4.8% for *Chlorella*-added samples. It can be said that the addition of *Chlorella* affected the pH and TA results more positively than *Dunaliella* addition during the shelf-life study. Nevertheless, smoothies fortified with *Chlorella* biomass were negatively scored by panellists (see sensory data). The SSC of microalgae-added smoothie samples was found to be higher than control samples, with a particular decrease after day seven. The moisture content of the product was consistent with the results obtained by Fradique *et al.* (2010). The best SSC alteration result was determined in *Chlorella*-added samples, with a 1.8% decrease during storage. In the study of Rabelo *et al.* (2013) with microalgae having similar properties to the present work, it was determined that microalgae biomass increased water retention capacity in the enriched food formulations.

Phenolic content and antioxidant activity

Phenolic compounds are derived from plant secondary metabolites and are the main components affecting the antioxidant activity of plants. Polyphenols have anti-inflammatory, anti-atherosclerotic, and anti-carcinogenic health benefits, and are excellent sources of natural antioxidants. To the best of our knowledge, no microalgae have been investigated for their contribution of phenolic content to antioxidant activity in algae. In this case, although there is no concrete scientific evidence for the correlation between total antioxidant activity and the content of phenolic substance in the algae, increased antioxidant activity was detected in biomass-added smoothie samples with high phenolic content (Table 1).

As seen in Table 2, the addition of microalgal biomass increased TPC values up to three times, and TAC values up to two times as compared to control samples. *Dunaliella*-added smoothies, due to their higher bioactive content of biomass, showed the highest TPC and TAC values.

Phenolic content may be affected by

processing and storage. During storage, changes in total phenolic content (TPC) and total antioxidant activity (TAC) were noticed (Figure 1). TPC and TAC values were significantly affected by microalgae types and storage time. TPC values showed a decreasing trend through the 28-day shelf-life study; meanwhile, antioxidant activity decreased to an important degree on the 14th and 21st days of storage for all samples. This decreasing trend was also reported in previous studies for red, green, and purple smoothies (Di Cagno *et al.,* 2011; Castillejo *et al.,* 2017; González-Tejedor *et al.,* 2017).

Microbiological analysis

Several processing methods are used for preparing smoothies which can influence their shelf-life and quality. During storage, the factors of microalgae, storage time, and their interactions had significant effects on microbial growth.

Smoothie supplementation with 2.5% microalgae biomass caused a decrease in the initial number of microorganisms, but did not show a statistically significant effect on microbial growth during storage. As seen in Figure 2, a decrease in TM, TP, and YM counts was noted. *Chlorella* supplementation decreased TM by 8% (0.52 log CFU mL^{-1}), TP by 5% (0.39 log CFU mL^{-1}), and YM by 14.5% (0.73 log CFU mL-1) as compared to the control samples at day 0. *Dunaliella* supplementation also resulted in an alteration of 11% in TM (0.79 log CFU mL^{-1}), 10% in TP (0.69 log CFU mL^{-1}), and 8% in YM (0.41 log CFU mL^{-1}). The decrease in YM is thought to be due to the higher carbohydrate content of *Dunaliella* biomass despite having the highest antioxidant activity. There was a total absence of EB in all microalgae-added smoothies, including the control samples. Meanwhile, microbial loads in smoothies were found to be higher than found in previous studies with green and multi-fruit smoothies (Picouet *et al.,* 2016; Castillejo *et al.,* 2016; 2018a), and slower microbial growth was observed after 28-day storage at 5°C. Compared to previous untreated smoothie studies (Formica-Oliveira *et al.,* 2017; González-Tejedor *et al.,* 2017), it can be said that microalgae supplementation was

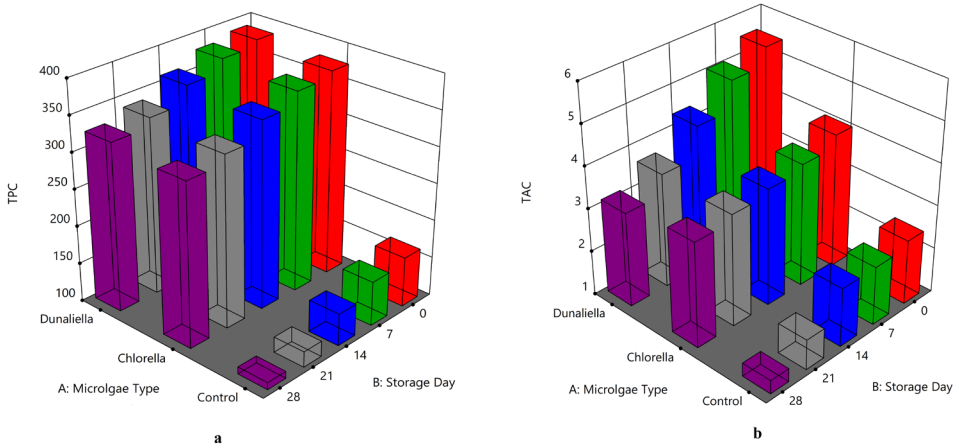

Figure 1. Total phenolic content (TPC) (a), and total antioxidant activity (TAC) (b) of green smoothies throughout the shelf-life study.

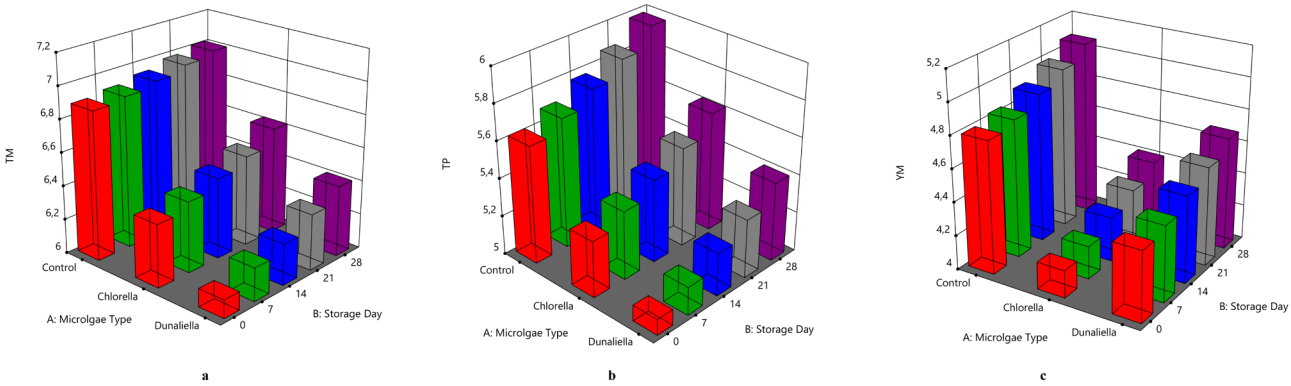

Figure 2. Total mesophilic count (TM) (a), total psychrophilic count (TP) (b), and yeast and mould count (YM) (c) of green smoothies throughout the shelf-life study.

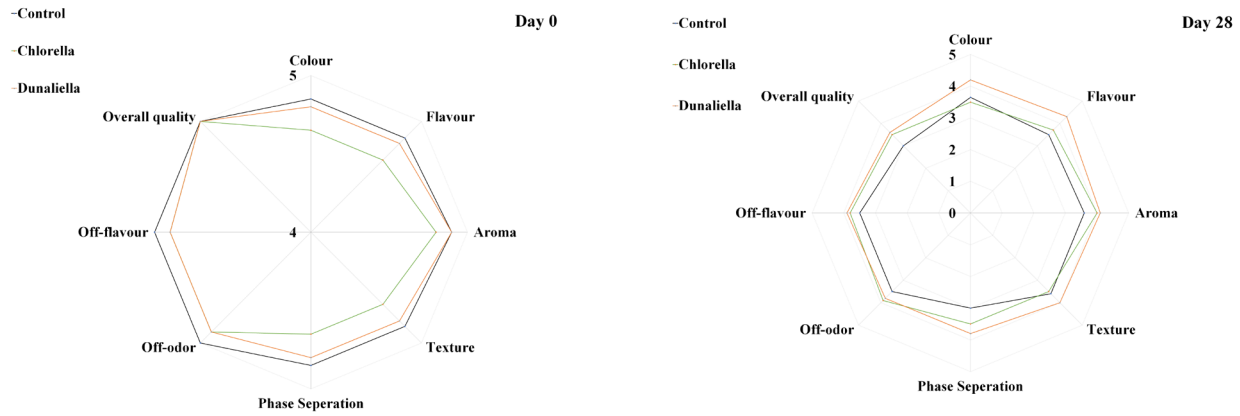

Figure 3. Sensorial evaluation of green smoothies at day 0 (a), and day 28 (b).

effective, and microalgae-added samples were shown below the 'microbiologically consumable level' throughout the 28-day shelf-life study.

Sensorial analysis

The organoleptic results were grouped according to properties of colour, flavour, aroma, texture, phase separation, off-odour, off-flavour, and overall quality (Figure 3). The addition of microalgal biomasses caused sensory attribute differences. Panellists preferred *Dunaliella*-added samples for total acceptability both, during and after 28-day storage.

Flavour may be the quality criteria that is most affecting consumers preference. The addition of *Chlorella* was found to be insufficient in terms of flavour, and scored lower than the control samples. *Dunaliella* biomass has a high water-retention capacity because of its high carbohydrate content, and because of this, the phase separation event was observed at the lowest rate in Dunaliella-added smoothie samples. Cano-Lamadrid *et al.* (2018) found that smoothies of the highest antioxidant activity were determined to have the worst flavour and aroma; however, in a previous study (Sun-Waterhouse *et al.,* 2014), smoothies had the highest phenolic content (78%) and were given the highest overall ratings for sensorial evaluation.

General acceptance refers to the first impression of the product as a whole by the consumer (appearance, aroma, colour, and flavour). In this sense, properties such as storage in refrigerated conditions, physicochemical parameters, and phenolic content were able to maintain the sensory quality of the product in relation to the data previously shown.

Conclusion

Recent studies have shown that food fortification with microalgal biomass improves techno-functional properties such as colour, texture, and water activity stability during shelf-life. During the 28-day shelf-life study at 5°C, the addition of algae to untreated fresh green smoothie samples generally had a positive effect. It was also found that *D. salina* and *C. vulgaris* biomasses increased the sensorial properties and microbiological quality of fresh green smoothies. Total microbiological loads showed a decreasing trend, with the addition of *D. salina* and *C. vulgaris* biomasses was more effective on the yeast and mould content. In addition, *D. salina* biomass was found more successful in terms of both sensorial acceptance and phenolic content.

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