Utilisation of the marine microalgae *Nannochloropsis* sp. and *Tetraselmis* sp. as innovative ingredients in the formulation of wheat tortillas

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Abstract

Powdered biomass of *Nannochloropsis* sp. and *Tetraselmis* sp. were used as innovative ingredients in wheat tortillas at flour substitution levels of 0.5-3.0%. Incorporation of microalgae into the tortilla formulations led to increased protein and fat content. The content of phenolic and carotenoids was also higher in microalgae-enriched tortillas, especially for those enriched in *Nannochloropsis* sp. at a flour substitution level of 3.0%. Not only the phenolic content but also the antioxidant capacity of the tortillas was higher after microalgae incorporation. Bioaccessible polyphenols were also higher in microalgae-containing tortillas as well as the antioxidant capacity of the enzymatic digestive extracts. No major differences in physical parameters (besides colour) were observed, and the overall acceptance of the microalgae-enriched tortillas assessed after a sensorial analysis was comparable to that of the wheat-only controls. Moreover, the purchase intention of the products as well as the acceptability index suggested that the tortillas would have a good acceptance. Several reports highlighted the nutritional value of microalgae. The production of microalgae and their utilisation as novel ingredients in innovative foods will increase the sustainability of the food industry and promote health.

Keywords: Marine biotechnology, novel foods, enriched foods, phytochemicals, antioxidant capacity.

1. Introduction

Microalgae represent an innovative biological source of highly nutritional and biologically active compounds such as β-carotene, astaxanthin, essential amino acids, polyunsaturated fatty acids, polyphenols, and phycobiliproteins such as phycocyanin and phycoerythrin [1]. Their high nutritional value led to an increased interest in the development of foods enriched in microalgae and the number of microalgae-containing foods launched into the market is increasing every year [2].

Baked goods are ideally suited as delivery vehicles for bioactive compounds because they are widely and easily consumed and they are considered as safe and nutritious by many different cultures, facilitating their consumption. Selection of a suitable food vehicle is important not only to facilitate consumption but also to aid resistance of bioactivity to food processing. In this sense, baked products are not exposed to strong pH or temperature variations and demonstrated to aid bioactivity resistance previously [3]. Marine algae-derived polyphenols are one of the top trends in the food industry [4] and several baked goods enriched in algal polyphenols and other bioactives have been developed. These include bread [5], biscuits [6], crackers [8], breadsticks [8], and many others. Moreover, a recent study suggested that consumers consider baked products as the best delivery vehicle for microalgal biomass, considered as safe, nutritious and sustainable [9]. The production of baked goods enriched in microalgae is not limited to scientific publications as there are numerous products currently being commercialised worldwide including *Spirulina filled crackers* (Lee Biscuits, Malaysia) and *Algen crackers* (Evasis Edibles, Austria).

Most of the products developed so far assessed the effect of *Arthrospira* and *Chlorella* strains on the products quality, and other microalgae such as *Nannochloropsis* and *Tetraselmis* have been overlooked. These are marine strains that have potential utilisation as food because of their high nutritional value. The latter is being commercialised by the company Fitoplancton Marino SL (Cadiz, Spain) to accentuate the marine flavour of foods after being authorised by the European Food Safety Authority (EFSA) as a Novel Food in accordance with Article 3(1) of Regulation (EC) No 258/97 [10]. So far, these strains have been incorporated into bread, breadsticks, and crackers [11–14], and there are no reports on how these “novel” microalgae affect the quality of other wheat-based products such as tortillas. Indeed, up to the best of the authors’ knowledge, there are no reports on the effect of microalgae incorporation into tortillas. Wheat-flour tortillas are widely consumed in South, Central, and

North America and are becoming more popular than bagels, croissants, muffins, or any other kind of ethnic bread in the United States (US) – currently, tortillas represent the second most sold bakery product in the US [15].

The aim of the current study was to assess the effect of incorporating dry biomass of the microalgae *Tetraselmis* sp. and *Nannochloropsis* sp. into wheat tortillas on their physicochemical, nutritional, and bioactive properties. The content of polyphenols was also determined after processing and after a simulated gastrointestinal digestion. To assess not only the nutrient content of a food but also the bioaccessibility of such compounds is of key importance while formulating new products. Moreover, a secondary aim of this study was to assess the overall acceptance of the formulated tortillas using a sensorial analysis.

2. Materials and methods

2.1 Microalgal biomass production

Both strains were produced at the pilot-plant facilities of the Cajamar Foundation in Almería, Spain. Briefly, the inocula were maintained at 23 ± 2 °C, pH 8.0 ± 0.1, and 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ in batch mode until a concentration of 1 $g \cdot L^{-1}$ was achieved. Once the inocula achieved the desired concentration, production was first scaled-up to a volume of 300 L using three (100 L each) pH-controlled outdoor bubble column photobioreactor and then to a pilot-scale tubular photobioreactor of 3,000 L described in previous studies [16]. All the reactors were located inside a greenhouse. The culture media consisted of seawater supplemented with commercial chemicals as described in previous studies [17]. Microalgal biomass was harvested by centrifugation and dehydrated by freeze-drying using an industrial scale freeze-drier FD-80 model (Cuddon Engineering, Marlborough, NZ). The temperature was maintained at less than 35 °C during the freeze-drying process. The dehydrated biomass was vacuum-sealed and stored at 4 °C until further use.

2.2 Tortilla preparation

Tortillas were produced following a traditional homemade procedure at the pilot plant facilities of IRTA Fruitcentre (Lleida, Spain). Briefly, ingredients listed in Table 1 were mixed together with an AM-700 bread dough mixer (Orbegozo, Murcia, Spain) using a dough hook. Doughs were prepared in triplicate using 200 g of Hacendado® wheat flour (Mercadona, Spain) in each replicate. Mixing time was 4 min at

the first speed and 3 min at the second speed until the doughs were fully developed. After mixing, the doughs were covered with a plastic film and left to rest for 15 min. Doughs were further divided into 25.0 ± 0.5 g balls, which were further covered with a plastic film, proofed for 10 min, and sheeted to 2.0 mm. Tortillas were baked on a griddle for 1 min on each side, cooled, packed into Ziploc bags, and stored at room temperature.

Flour substitution levels ranged between 0.5 and 3.0%, which is the common microalgae-concentration in microalgae-enriched commercial products (Lafarga, 2019). Control wheat-only tortillas were termed as C0.0 while tortillas formulated with *Nannochloropsis* sp. and *Tetraselmis* sp. at four substitution levels of 0.5-3.0% were termed as N0.5-N3.0 and T0.5-T3.0, respectively. All the ingredients (Hacendado® brand) were purchased from Mercadona (Valencia, Spain).

2.3 Physical quality

Surface colour recordings were taken in triplicate using a Minolta CR-200 chroma meter (Minolta Inc., Tokyo, Japan) and the D65 illuminant, which approximates to daylight. CIE values were recorded in terms of *L** (lightness), *a** (redness, greenness), and *b** (yellowness, blueness) which were used to calculate chroma (*Ch*) and difference from the control (*ΔE*) as described previously [18]. Moisture content of the tortillas was carried out using AACC method 44-15.02 and was measured on days 0 (approximately 5-6 h) after cooking and on day 1 (approximately 24 h after cooking). Water activity (a_w) of all samples was measured using an AquaLab meter (Decagon Devices Inc., WA, USA) and approximately 2 g of ground sample. Three measurements were taken for each formulation on days 0 and 1. Finally, the pH of 1 g of ground tortilla added to 10 mL of distilled water was measured using a Basic 20 pH meter (Crison Instruments SA, Barcelona, Spain). Measurements were carried out in triplicate for each formulation on days 0 and 1.

2.4 Chemical characterisation

Total nitrogen was determined by combustion using an induction furnace coupled with a thermal conductivity detector following AOAC 972.43 and using 6.25 as the conversion factor to calculate protein. Briefly, approximately 50 mg of grinded and sieved (0.8 mm) sample were loaded into an Elementar Rapid N (Hanau, Germany) nitrogen and protein analyser that incinerated the samples at 960 °C and measured the produced nitrogen-containing gases using a thermal conductivity detector. Carbon dioxide was used as the carrier gas. The ash content was determined by dry ashing according

to ISO 2171:2007 and crude fat by Soxhlet extraction following AACC Method 30-25.01. Ash was determined gravimetrically from 5 g of grinded and sieved (0.8 mm) sample by incineration using platinum dishes and 900 °C. For the determination of crude fat, petroleum ether was used as the solvent and the residue was dried until a constant weight at 100 °C. Carbohydrate content was calculated by difference. Protein, ash, lipid, and carbohydrate content were determined at day 0. The total phenolic content (TPC) was determined by the Folin Ciocalteu method following the modifications described previously [14]. Phenolic compound extraction was carried out using methanol 70% (v/v) at a sample:methanol ratio of 1:4 (w/v). Samples were homogenised using a T-25 ULTRA-TURRAX® disperser (IKA, Staufen, Germany) operating at 1,100 × g for 30 s and immediately placed on a stirrer at room temperature for 2h. Samples were then centrifuged using a Sigma 3-18 KS centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) operating at 19,350 × g for 20 min and TPC was determined spectrophotometrically using a GENESYS™ 10S-UV Vis spectrophotometer (Thermo-Fisher Scientific, MA, USA). Determinations were conducted in triplicate and TPC values were expressed as mg of gallic acid equivalents per 100 g of sample on a dry weight basis. Standard curves were prepared daily and TPC of the samples was determined at day 0 and day 1.

Total chlorophyll content (TChC) and total carotenoid content (TCC) were calculated as described previously [19]. Briefly, 1.0 g of sample were mixed with 10 mL of ethanol and were thermosonicated using a TI-H 20 stainless steel ultrasonic bath (Elma Schmidbauer GmbH, Singen, Germany) operating at 130 kHz and 250 W for 30 min at 65 °C. After thermosonication, samples were centrifuged using a Sigma 3-18 KS centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) operating at 19,350 × g for 10 min. TChC and TCC were determined spectrophotometrically using a GENESYS™ 10S-UV Vis spectrophotometer (Thermo-Fisher Scientific, MA, USA) using the equations:

$$TChC\ \left(\frac{mg}{g}\right) = (Ch_a + Ch_b) \cdot \frac{V}{W} \cdot 100$$

$$TCC\ \left(\frac{mg}{g}\right) = (1000 \cdot A_{470} - 2.8 \cdot Ch_a - 85.9 \cdot Ch_b) \cdot \frac{V}{245 \cdot W} \cdot 100$$

where V is the volume of extract, W is the weight of sample, A_{470} is the absorbance measured at 470 nm, and Ch_a and Ch_b are the concentration of chlorophyll *a* and chlorophyll *b*, respectively, calculated as:

$$Ch_a\left(\frac{mg}{L}\right) = 15.65 \cdot A_{666} - 7.34 \cdot A_{653}$$

$$Ch_b\left(\frac{mg}{L}\right) = 27.05 \cdot A_{653} - 11.21 \cdot A_{666}$$

where A_{666} and A_{653} are the absorbance of the extracts measured at 666 or 653 nm, respectively. Results are expressed as mg/ 100 g. TCC determinations were conducted at day 0 and day 1.

2.5 Antioxidant capacity and bioaccessibility of antioxidant compounds

Antioxidant capacity was determined using both the ferric ion reducing antioxidant power (FRAP) and the (DPPH) scavenging activity assays. Determinations were conducted in triplicate using the same extract used for the determination of TPC, described in the previous section. Antioxidant capacity determinations were carried out on days 0 and 1. Results were expressed as mg of ascorbic acid equivalents per 100 g of sample on a dry weight basis. Standard curves were prepared daily. To assess bioaccessibility after a simulated gastrointestinal digestion the INFOGEST method was used [20] after some minor modifications described previously [21]. A blank was prepared using only distilled water instead of sample and following the same procedure. Determinations of TPC, TCC, and antioxidant activity were performed after the intestinal phase as described above for tortillas on day 0.

2.6 Sensorial analysis

Sensory evaluation was undertaken by 35 semi-trained panellists (19 women, 16 men) who would be willing to purchase microalgae-containing foods recruited from IRTA Fruitcentre in Lleida, Spain. Sensory evaluation was conducted in a sensory laboratory with separate booths. Briefly, samples were placed on white polystyrene plates labelled with random codes and presented to the consumers in a randomised order. A 60-s time laps was employed between each sensory palate to reduce sensory fatigue. Sensorial analysis was conducted in three different days at the same time each day and with a wheat-only control each day. Each panellist assessed all the samples and was asked to indicate her or his opinion on the flavour, texture, and overall acceptability using a 9-point hedonic scale: 1, dislike extremely; 2, dislike very much; 3, dislike moderately; 4, dislike a little bit; 5, neither like nor dislike; 6, like a little bit; 7, like moderately; 8, like very much; 9, like extremely. The acceptability index was calculated as described in previous studies [22]. Finally, purchase intention was assessed using a 5-

point hedonic scale: 1, certainly would not buy; 2, probably would not buy; 3; I don't know; 4, probably would buy; 5, certainly would buy.

2.7 Statistical analysis

Results shown are the average of three independent experiments. Differences between measurements were analysed using analysis of variance (ANOVA) with JMP 13 (SAS Institute Inc., USA). A Tukey pairwise comparison of the means was conducted to identify where sample differences occurred. The criterion for statistical significance was in all cases $p<0.05$. To identify relationships between parameters, bivariate Pearsons' correlation analysis was carried out.

3. Results and discussion

3.1 Effect of microalgae incorporation on physicochemical attributes

Colour, which is a key parameter in baked products with a striking effect on food acceptance, was significantly affected by microalgae incorporation (Figure 1). The parameter *L** denotes lightness and values ranging between 0 and 50 indicate dark while values between 51 and 100 indicates light. In the current study, microalgal concentration and *L** values were negatively correlated (R^2=0.938 and 0.865 for *Nannochloropsis* sp. and *Tetraselmis* sp., respectively; $p<0.05$), indicating a darker colour in samples with higher microalgal concentration. This is in line with that observed in previous studies using these same strains in bread [14], and can be attributed to the high content of pigments in microalgal biomass, mainly chlorophylls and carotenoids. The TChC of *Nannochloropsis* sp. and *Tetraselmis* sp. was 6.6 ± 0.1 and 3.5 ± 0.1 mg/g of dry biomass, while the TCC was 0.16 ± 0.05 and 0.24 ± 0.09 mg/g of dry biomass, which is in line with previous reports [23,24]. *Ch* is a quantitative indicator or colourfulness and indicates the intensity of a colour. *Ch* values also were negatively correlated to *Nannochloropsis* sp. concentration (R^2=0.875; $p<0.05$) and the *Ch* value of T3.0 was significantly lower than that of the control C0.0 ($p<0.05$). Microalgal strain affected *Ch* values, being higher for tortillas containing *Nannochloropsis* sp. than for those enriched with *Tetraselmis* sp. for all the studied concentrations ($p<0.05$) probably caused by a higher content of chlorophylls in the former. This means that the green colour of the tortillas was more intense in those containing *Nannochloropsis* sp. when compared to those containing *Tetraselmis*. Moreover, *ΔE* combines the change in *L**, *a**, and *b** values to quantify the colour deviation from a standard reference sample, in this case, wheat-only tortillas. Those samples with *ΔE* higher than 3 display a visible colour deviation [18]. In the current study, all the

samples had *ΔE* values higher than 3, suggesting that colour changes were visible to the human eye (Figure 1). Moisture values were between 19.3-26.4 at day 0 and 16.9-24.9% at day 1. Moisture, pH, and a_w values were not affected by microalgal biomass (Figure 1), suggesting a similar stability and shelf-life of the control and microalgae-enriched tortillas, although this would need to be assessed in further studies.

3.2 Effect of microalgae incorporation on macromolecular composition

Microalgal biomass commercialised for food applications is generally commercialised as "rich in protein" [2]. The protein content of *Nannochloropsis* sp. and *Tetraselmis* sp. biomass was 32.04 ± 0.12 and 33.39 ± 0.09 g/100 g on a dry weight basis, respectively. The protein content of the control and microalgae-enriched tortillas is listed in Table 2. Overall, microalgae incorporation led to an increased protein content in the tortillas (p<0.05). Both the concentration of *Nannochloropsis* sp. and *Tetraselmis* sp. were positively correlated to protein content (R^2=0.979 and 0.982 respectively; p<0.05) and no differences were observed in protein content when using one strain or the other. Although selected strains are not among the group of microalgae with the highest protein content, still their protein content is higher than that of wheat flour [25]. The observed increase was not surprising as similar results were reported previously when incorporating microalgae at similar concentrations in doughnuts [26], pasta [27], and other products [22].

The lipid content of microalgae, especially of those within the phyla Haptophyta, Bacillariophyta, and Ochrophyta, varies within the range 18.6-21.3% on average [28]. The total lipid content of selected strains was 6.85 ± 0.07 and 8.05 ± 0.35% for *Nannochloropsis* sp. and *Tetraselmis* sp., respectively. Selected strains are especially interesting as food ingredients not because of their high lipid content but for their fatty acid profile, which includes long chain fatty acids [29]. Results obtained for lipid content are comparable as those observed for protein composition, with higher lipid content in microalgae-enriched tortillas than in the control (p<0.05; Figure 2). However, in this case, strain also affected lipid content with slightly higher values for tortillas containing the biomass of *Tetraselmis* (p<0.05). Similarly, microalgae incorporation also led to higher ash content (p<0.05). The ash content of the dried biomass was 15.95 ± 0.35 and 17.73 ± 0.67% for *Nannochloropsis* sp. and *Tetraselmis* sp., respectively. Flour substitution with microalgae also led to increased mineral content, mainly potassium and iron in breadsticks [8].

3.3 Antioxidant capacity and bioaccessibility of antioxidant compounds

The TPC and TCC of the samples are shown in Figure 2. Incorporation of microalgae led to both higher TPC and TCC when compared to the control (p<0.05). Higher biomass concentrations led to higher TPC up to 18.52 ± 0.15 and 17.40 ± 0.15 mg/100 g when *Nannochloropsis* sp. and *Tetraselmis* sp. were present at a flour substitution level of 3.0%, the maximum studied. Similar results were observed for the TCC, with maximum values of 0.62 ± 0.11 and 0.89 ± 0.10 mg/100 g for *Nannochloropsis* sp. and *Tetraselmis* sp. respectively. Results are in line with previous papers that increased the phenolic and/or carotenoid content of foods using microalgae [13,19,30,31]. Moreover, the effect of storage on the TPC and TCC of the samples was also determined, observing a significant reduction in both after storage during 24 h at room temperature (p<0.05). Still, the TPC and TCC of microalgae-enriched tortillas were higher than those of the controls (p<0.05).

The contents of both, polyphenols and carotenoids, in foods is of key importance. The beneficial roles of polyphenols and carotenoids in the prevention and treatment of numerous diseases or disorders including stroke, atherosclerosis, hypertension, diabetes, male infertility, cancer, or osteoporosis have been widely reported [32,33]. Their health-promoting properties have been linked to their high antioxidant capacity. In this sense, the effect of microalgae incorporation on the antioxidant capacity of the formulated foods was determined and results are shown in Figure 3. Incorporation of microalgae into wheat tortillas led to increased antioxidant capacity when assessed using both the DPPH and FRAP assays (p<0.05). Higher biomass concentration led to increased antioxidant capacity. When *Nannochloropsis* sp. biomass was introduced into the recipe, positive correlations were observed at day 0 between TPC and antioxidant capacity assessed using both FRAP and DPPH methods (R^2=0.923 and 0.979 respectively; p<0.05). The same correlation was observed when biomass of *Tetraselmis* was used as an ingredient (R^2=0.905 and 0.963 respectively; p<0.05). Moreover, TCC was positively correlated to antioxidant capacity when assessed using the FRAP assay (R^2=0.862; p<0.05) and TPC was positively correlated to antioxidant capacity when assessed using the DPPH method (R^2=0.863; p<0.05). Results are in line with previous reports that demonstrated that microalgal biomass contain important amounts of polyphenols and carotenoids, which contribute significantly to their antioxidant capacity [34,35].

Recent studies suggested that disruption of the cell wall of microalgae before incorporation into foods leads to increased antioxidant capacity [31]. The reason for this is a higher extraction efficiency of antioxidants, which are contained inside the microalgal cell. Indeed, it is generally accepted that cooking softens cell tissues facilitating the release of phytochemicals [36] and previous reports suggested that *in vitro* laboratory methods may underestimate the physiological antioxidant capacity [37,38]. Gastrointestinal simulations are useful for the estimation of pre-absorptive events such as stability and bioaccessibility of bioactive compounds present in food matrices. Several methods have been reported with significant differences in the type of the different digestive phases studied, composition and concentration of digestive fluids, and also in the length of incubation time of samples in each digestive stage [36], making it difficult to compare between different reports. The current study assessed the bioaccessibility of polyphenols and carotenoids as well as the antioxidant capacity of the enzymatic extracts using the INFOGEST method, an international consensus recently developed by the COST Infogest network [20]. Results, shown in Figure 4 demonstrate that the amount of bioaccessible polyphenols after a simulated gastrointestinal digestion is higher than that expected after extraction with organic solvents, in this case, methanol (p<0.05). The higher phenolic content in the enzymatic extracts also led to a higher antioxidant capacity assessed using both FRAP and DPPH methods (p<0.05). Microalgae-containing tortillas showed a higher phenolic content when compared to the wheat-only controls, and no major differences were observed between samples enriched in biomass of *Tetraselmis* sp. or *Nannochloropsis* sp. This results contrast with previous reports that suggested a higher antioxidant capacity of baked products enriched in *Tetraselmis* when compared to *Nannochloropsis* sp. [7]. This could be partially attributed to different phytochemical content of the microalgal biomass utilised in both studies, as culturing and operational conditions can largely affect the molecular composition of microalgal biomass. The different thermal treatments utilised in both studies as well as the differences in food matrices could have also affected the release of bioactive compounds contained within microalgal cells in a different way.

3.4 Sensorial analysis

Formulated tortillas were also evaluated in terms of sensorial attributes. Overall, microalgae-enriched tortillas were well accepted by consumers with flavour and texture scores close to 7 (like moderately) and overall acceptance scores close to 6 (like a little bit) – Figure 5. No differences between the control and microalgae-enriched tortillas were observed in any of the three scores assessed. Although marine

microalgae have an intense “marine” flavour, *Nannochloropsis* sp. and *Tetraselmis* sp. at the concentrations studied herein did not negatively affect key sensorial attributes. This is important as unlike Japanese consumers, who consider functional foods a distinct food where the importance of their health benefits exceeds the importance of their sensory attributes, western consumers do not seem to be willing to compromise taste for health [39]. Several strategies such as the use of organic solvents to remove compounds responsible for unwanted odour or flavour have been studied [40] and previous reports suggested that the marine flavour of microalgae could be an opportunity if used, for example, to prepare fish based culinary preparations [41]. Indeed, dried biomass of *Tetraselmis chuii* is currently being commercialised to accentuate the marine flavour of foods. Moreover, colour has a striking effect on consumers expectations [42] and in the current study, colour was significantly affected by microalgal pigments (Figure 1). Indeed, several panellists commented that the green colour of the tortillas was “not pleasant”. The intense (generally green) colour of microalgae has been suggested as an inconvenient for food applications previously [43]. This is even more common in western cultures where, although some green baked products are commercially available, they are uncommon. To mask the intense colour of microalgae is complicated because of their high content of pigments, namely chlorophylls. The use of delivery vehicles that are naturally green, such as broccoli soup, has been used as a strategy to minimise the effect of chlorophylls on the colour of the end product [44] as well as incorporating microalgae into dark products, for example, chocolate biscuits [45]. In addition, the production of microalgae that has limited or no chlorophyll content is also an option that is gaining increased attention in the food industry. White, yellow, and light green variants of *Chlorella vulgaris* are commercially available (https://www.algenuity.com/chlorella-colours).

Despite their green colour, the purchase intention of the products ranged within 3.5 and 4.0 (assessed using a 5-point hedonic scale where 5 meant “would certainly buy”) suggesting that the product would have a good acceptance if commercialised. Moreover, the acceptability score of microalgae-enriched tortillas ranged between 59-75%, which is acceptable as it is necessary to achieve an acceptability index greater than 70% for a product to be accepted in terms of sensorial characteristics. A recent study reported that the awareness of average Spanish consumers about microalgae and the health benefits associated to their consumption is low, but are generally considered as safe, nutritious, and sustainable food ingredients [46]. In that same study, the authors demonstrated that increasing consumers’ knowledge about microalgae and their environmental and health promoting advantages could be used

as a strategy to increase purchase intention of microalgae containing products. It is important to highlight that results reported in the sensorial evaluation conducted in the current study must be taken with caution as the ideal would be to assess sensorial attributes with a larger group of consumers. Also, it would be interesting to see the acceptance of these products in countries like Mexico or the US, where tortillas are more commonly consumed than in Spain [15].

4. Conclusions

Incorporation of *Tetraselmis* sp. and *Nannochloropsis* sp. into wheat tortillas allowed obtaining an innovative product rich in protein and in biologically active compounds namely polyphenols, carotenoids, and chlorophylls. Tortillas were characterised by an increased greener hue and a darker colour, which was not well accepted by consumers. However, in the current study, the overall acceptance of the microalgae-enriched tortillas as well as their purchase intention scores were comparable to those of the wheat-only controls. Moreover, tortillas contained a higher content of bioaccessible polyphenols than what could be expected based on *in vitro* determinations. This was in line with previous reports that suggested that *in vitro* determinations after extraction using organic solvents may underestimate the physiological antioxidant capacity of foods. Despite their broad applicability and potential, *in vitro* methods do not fully mimic what happens during digestion and further *in vivo* studies will be conducted to validate *in vitro* results. Moreover, a sensorial analysis using a larger group of panellists would be interesting as well as assessing the effect of increasing consumers´ awareness about the health benefits of the product.

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Declaration of competing interest

Authors declare that they have no conflict of interests.

Statement of informed consent, human/animal rights

An informed consent was obtained from all individual participants in the sensorial analysis. Sensory research procedures followed were in accordance with the ethical standards of IRTA and with the Code of Ethics of the World Medical Association.

Table 1. Formulated control and microalgae-containing tortillas.

Ingredients	C0.0 (control)	N0.5-N3.0[a]	T0.5-3.0[b]
Wheat flour (g)	100.0	97.0-99.5	97.0-99.5
Salt (g)	2.0	2.0	2.0
Olive oil (mL)	20.0	20.0	20.0
Water (mL)	50.0	50.0	50.0
Baking powder (g)	0.6	0.6	0.6
Microalgal biomass (g)	0.0	0.5-3.0	0.5-3.0

[a] **Tortillas formulated using dried biomass of *Nannochloropsis* sp. at a flour substitution level of 0.5-3.0%.**

[b] **Tortillas formulated using dried biomass of *Tetraselmis* sp. at a flour substitution level of 0.5-3.0%.**

Table 2. Macromolecular composition of control and microalgae-enriched tortillas

Sample	Protein (g/100 g)	Ash (g/100 g)	Lipid (g/100 g)	Carbohydrate (g/100 g)
C0.0	10.98 ± 0.13 ^{e}	1.55 ± 0.01 ^{f}	20.85 ± 0.08 ^{e}	66.47 ± 0.23 ^{a}
N0.5	11.14 ± 0.09 ^{Ad}	1.68 ± 0.02 ^{Ad}	21.50 ± 0.08 ^{Ad}	65.73 ± 0.35 ^{Ab}
N1.0	11.18 ± 0.03 ^{Ad}	1.70 ± 0.00 ^{Ad}	22.11 ± 0.11 ^{Ac}	64.89 ± 0.32 ^{Ac}
N1.5	11.32 ± 0.12 ^{Ac}	1.78 ± 0.01 ^{Bc}	22.14 ± 0.10 ^{Abc}	64.35 ± 0.24 ^{Ac}
N2.0	11.56 ± 0.08 ^{Ac}	1.83 ± 0.02 ^{Bc}	22.35 ± 0.20 ^{Bb}	63.51 ± 0.37 ^{Ad}
N2.5	11.69 ± 0.02 ^{Ab}	1.98 ± 0.01 ^{Ab}	22.45 ± 0.06 ^{Bb}	62.73 ± 0.12 ^{Ae}
N3.0	11.84 ± 0.01 ^{Aa}	2.11 ± 0.04 ^{Aa}	23.02 ± 0.18 ^{Ba}	62.63 ± 0.23 ^{Ae}
T0.5	11.14 ± 0.03 ^{Ab}	1.61 ± 0.06 ^{Ae}	21.61 ± 0.03 ^{Ad}	65.61 ± 0.07 ^{Ab}
T1.0	11.27 ± 0.11 ^{Abc}	1.72 ± 0.01 ^{Ad}	21.89 ± 0.05 ^{Ac}	64.95 ± 0.09 ^{Ac}
T1.5	11.40 ± 0.01 ^{Ac}	1.89 ± 0.03 ^{Ac}	22.05 ± 0.11 ^{Ac}	63.02 ± 0.28 ^{Ad}
T2.0	11.59 ± 0.06 ^{Ab}	1.94 ± 0.00 ^{Ac}	23.01 ± 0.15 ^{Ab}	63.03 ± 0.22 ^{Ad}
T2.5	11.62 ± 0.03 ^{Ab}	2.01 ± 0.02 ^{Ab}	23.15 ± 0.01 ^{Ab}	62.89 ± 0.19 ^{Ad}
T3.0	11.89 ± 0.10 ^{Aa}	2.10 ± 0.01 ^{Aa}	23.70 ± 0.19 ^{Aa}	61.81 ± 0.31 ^{Be}

Different capital letters indicate differences between microalgal strains and different lower-case letters indicate differences between microalgae concentration for the same strain. The criterion for statistical significance was $p<0.05$.

Figure 1. Control and microalgae-enriched tortillas.

Abbreviations: *L**, lightness; *Ch*, Chroma; *h*˚, hue angle; a_w, water activity; C0.0, control wheat-only tortillas; N0.5-N3.0: tortillas containing the biomass of Nannochloropsis sp. at a flour substitution level of 0.5-3.0% (w/w) respectively; and T0.5-T3.0: tortillas containing Tetraselmis sp. at a flour substitution level of 0.5-3.0% (w/w) respectively. Results shown are the average of three independent experiments ± S.D. Different letters indicate significant differences between samples. The criterion for statistical significance was $p<0.05$.

Figure 2. (A) Total phenolic content and (B) Total carotenoid content of the control and microalgae-enriched tortillas.

Abbreviations: TPC, total phenolic content; TCC, total carotenoid content; C0.0, control wheat-only tortillas; N0.5-N3.0, tortillas containing the biomass of *Nannochloropsis* sp. at a flour substitution level of 0.5-3.0% (w/w) respectively; and T0.5-T3.0, tortillas containing *Tetraselmis* sp. at a flour substitution level of 0.5-3.0% (w/w) respectively. Results shown are the average of three independent experiments ± S.D. Different capital letters indicate significant differences between microalgae concentration and different lower case letters indicate significant differences between microalgal strains.

Figure 3. Antioxidant capacity assessed using the (A) DPPH and (B) FRAP assays of control and microalgae-enriched tortillas.

Abbreviations: C0.0, control wheat-only tortillas; N0.5-N3.0, tortillas containing the biomass of *Nannochloropsis* sp. at a flour substitution level of 0.5-3.0% (w/w) respectively; and T0.5-T3.0, tortillas containing *Tetraselmis* sp. at a flour substitution level of 0.5-3.0% (w/w) respectively. Results shown are the average of three independent experiments ± S.D. Different capital letters indicate significant differences between microalgae concentration and different lower case letters indicate significant differences between microalgal strains.

Figure 4. (A) Total phenolic content and antioxidant capacity assessed using the (B) FRAP and (C) DPPH methods after a simulated gastrointestinal digestion

Abbreviations: C0.0, control wheat-only tortillas; N0.5-N3.0, tortillas containing the biomass of *Nannochloropsis* sp. at a flour substitution level of 0.5-3.0% (w/w) respectively; and T0.5-T3.0, tortillas containing *Tetraselmis* sp. at a flour substitution level of 0.5-3.0% (w/w) respectively. Results shown are the average of three independent experiments ± S.D. Different capital letters indicate significant differences between microalgae concentration and different lower case letters indicate significant differences between microalgal strains.

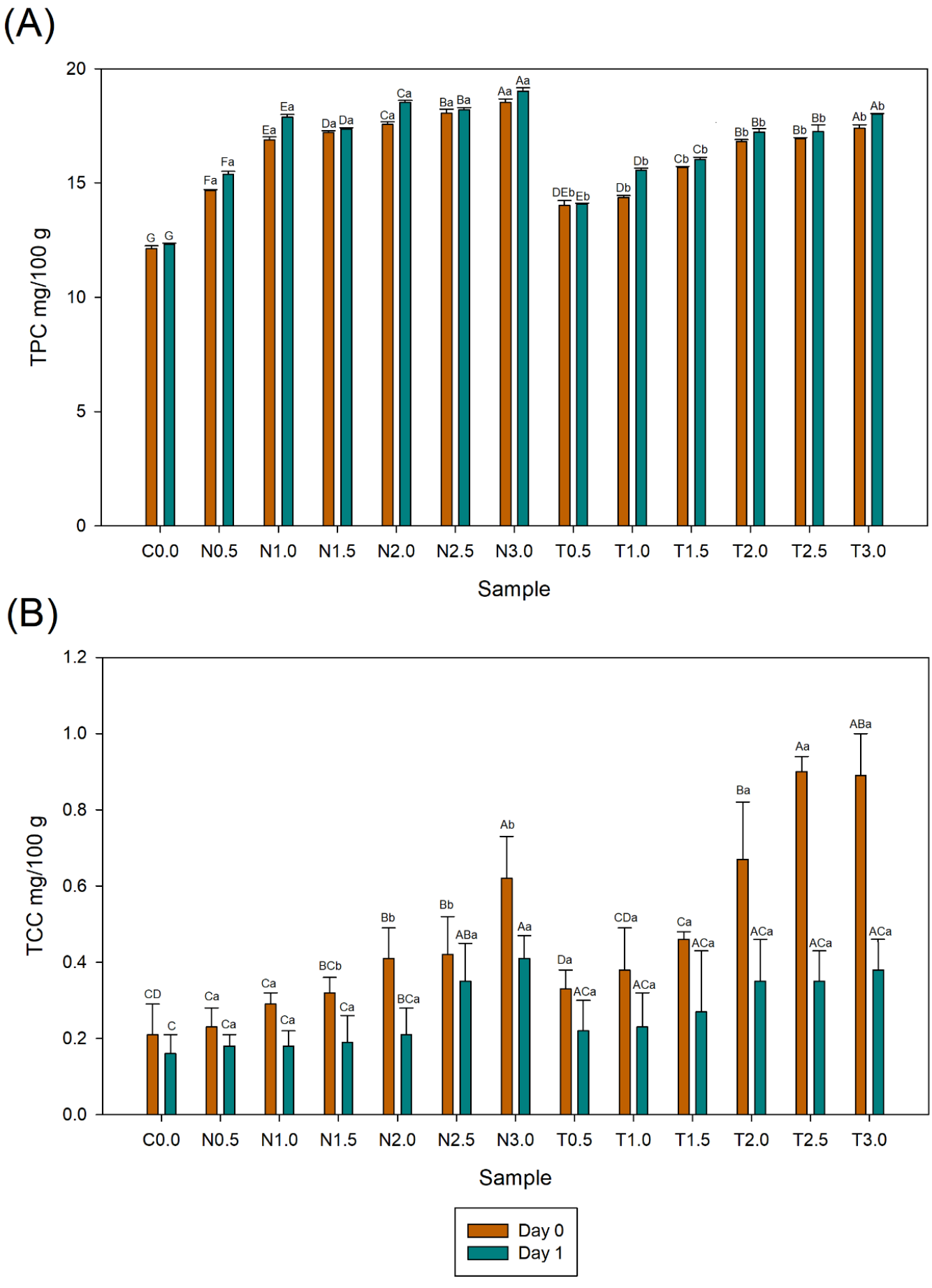
Figure 5. Sensorial analysis: (A) Flavour score, (B) texture score, (C) overall acceptance score and (D) purchase intention score.

Abbreviations: C0.0, control wheat-only tortillas; N0.5-N3.0, tortillas containing the biomass of *Nannochloropsis* sp. at a flour substitution level of 0.5-3.0% (w/w); and T0.5-T3.0, tortillas containing *Tetraselmis* sp. at a flour substitution level of 0.5-3.0% (w/w). Flavour, texture, and overall acceptance scores were assessed using a 9-point hedonic scale (from 1: dislike extremely to 9: like extremely) while purchase intention was assessed using a 5-point hedonic scale (from 1: certainly would not buy to 5: certainly would buy).

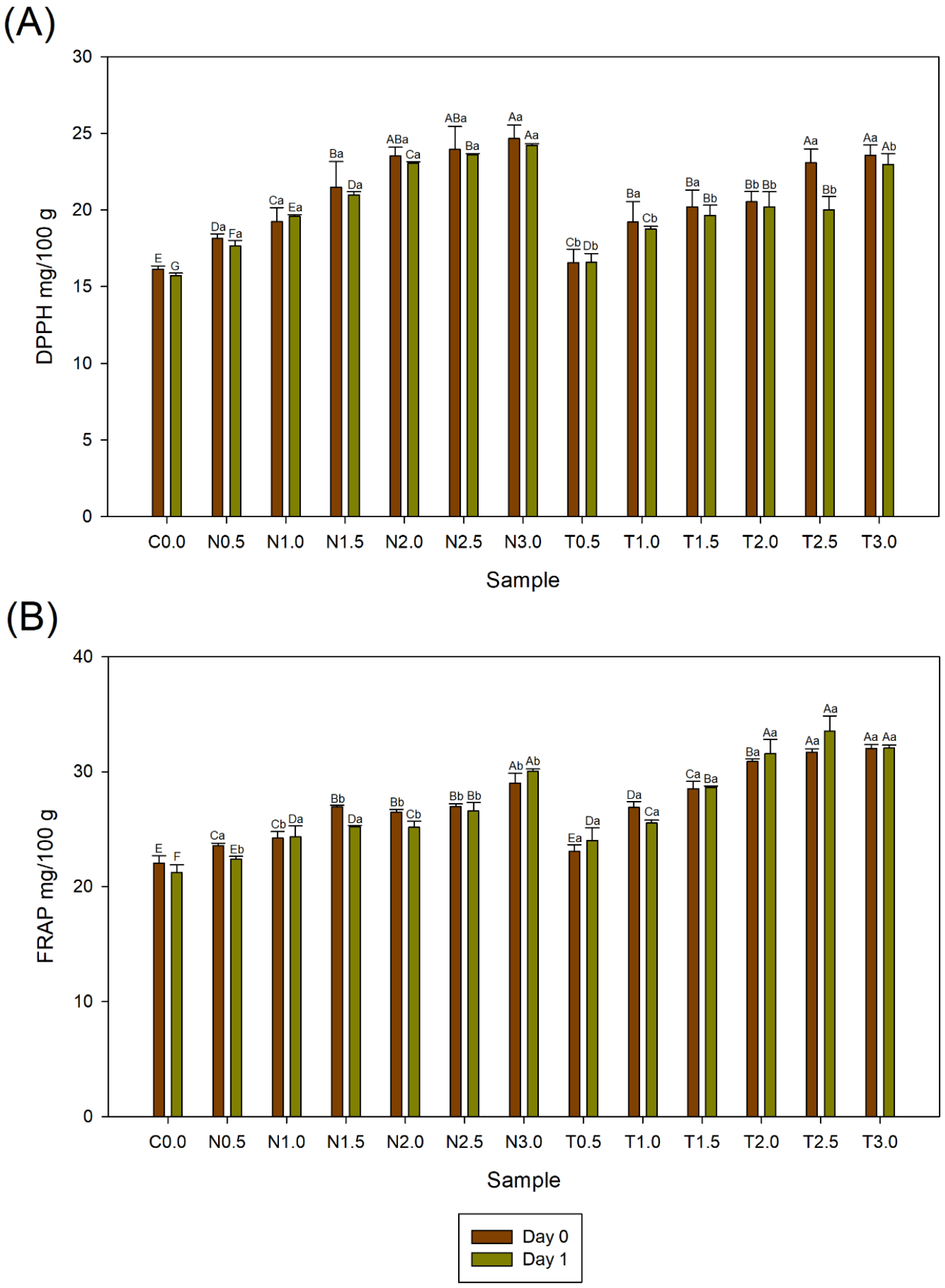
Figure 1


C0.0
L* = 77.6 ± 0.9 a
Ch = 24.4 ± 0.2 a
h° = 173.8 ± 0.7 a
pH = 9.9 ± 0.0 a
aW = 0.88 ± 0.00 a
N0.5
L* = 65.2 ± 1.6 b
Ch = 18.0 ± 0.3 b
h° = 157.9 ± 0.4 b
pH = 10.0 ± 0.1 a
aW = 0.87 ± 0.01 a
N1.0
L* = 58.6 ± 1.4 c
Ch = 16.1 ± 0.1 c
h° = 154.6 ± 0.3 c
pH = 9.9 ± 0.1 a
aW = 0.88 ± 0.00 a
N1.5
L* = 53.7 ± 0.8 d
Ch = 14.9 ± 0.0 cd
h° =153.7 ± 0.2 cd
pH = 10.1 ± 0.1 a
aW = 0.88 ± 0.00 a
N2.0
L* = 51.2 ± 0.6 de
Ch = 13.6 ± 0.0 de
h° = 152.5 ± 0.2 de
pH = 10.1 ± 0.1 a
aW = 0.88 ± 0.01 a
N2.5
L* = 47.9 ± 1.5 ef
Ch = 12.9 ± 0.4 ef
h° = 152.4 ± 0.4 de
pH = 9.9 ± 0.2 a
aW = 0.87 ± 0.01 a
N3.0
L* = 41.4 ± 0.7 g
Ch = 10.4 ± 0.3 g
h° = 151.4 ± 0.4 ef
pH = 9.9 ± 0.1 a
aW = 0.86 ± 0.01 a
T0.5
L* = 66.0 ± 0.9 b
Ch = 12.3 ± 0.6 ef
h° =155.0 ± 0.5 c
pH = 10.0 ± 0.1 a
aW = 0.88 ± 0.00 a
T1.0
L* = 62.5 ± 1.0 bc
Ch = 13.1 ± 0.3 ef
h° = 152.4 ± 0.3 de
pH = 10.1 ± 0.1 a
aW = 0.90 ± 0.02 a
T1.5
L* = 53.2 ± 2.2 d
Ch = 10.4 ± 0.8 g
h° = 149.0 ± 0.8 g
pH = 9.8 ± 0.6 a
aW = 0.89 ± 0.02 a
T2.0
L* = 63.8 ± 1.6 b
Ch = 12.3 ± 1.6 ef
h° = 154.9 ± 1.2 c
pH = 9.9 ± 0.1 a
aW = 0.89 ± 0.01 a
T2.5
L* = 54.3 ± 1.5 d
Ch = 11.7 ± 0.2 fg
h° =149.9 ± 0.3 fg
pH = 9.8 ± 0.1 a
aW = 0.87 ± 0.01 a
T3.0
L* = 47.0 ± 2.5 f
Ch = 8.1 ± 0.3 h
h° = 146.5 ± 0.3 h
pH = 9.7 ± 0.1 a
aW = 0.88 ± 0.01 a

62 **Figure 2**


(A)
TPC mg/100 g
20
15
10
5
0
G G
Fa Fa
Ea Ea
Da Da
Ca Ca
Ba Ba
Aa Aa
DEb Eb
Db Db
Cb Cb
Bb Bb
Bb Bb
Ab Ab
C0.0 N0.5 N1.0 N1.5 N2.0 N2.5 N3.0 T0.5 T1.0 T1.5 T2.0 T2.5 T3.0
Sample
(B)
TCC mg/100 g
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CD C
Ca Ca
Ca Ca
BCb Ca
Bb BCa
Bb ABa
Ab Aa
Da ACa
CDa ACa
Ca ACa
Ba ACa
Aa ACa
ABa ACa
C0.0 N0.5 N1.0 N1.5 N2.0 N2.5 N3.0 T0.5 T1.0 T1.5 T2.0 T2.5 T3.0
Sample
Day 0
Day 1

64 **Figure 3**


(A)
DPPH mg/100 g
0
5
10
15
20
25
30
E G
Da Fa
Ca Ea
Ba Da
ABa Ca
ABa Ba
Aa Aa
Cb Db
Ba Cb
Ba Bb
Bb Bb
Aa Bb
Aa Ab
C0.0 N0.5 N1.0 N1.5 N2.0 N2.5 N3.0 T0.5 T1.0 T1.5 T2.0 T2.5 T3.0
Sample
(B)
FRAP mg/100 g
0
10
20
30
40
E F
Ca Eb
Cb Da
Bb Da
Bb Cb
Bb Bb
Ab Ab
Ea Da
Da Ca
Ca Ba
Ba Aa
Aa Aa
Aa Aa
C0.0 N0.5 N1.0 N1.5 N2.0 N2.5 N3.0 T0.5 T1.0 T1.5 T2.0 T2.5 T3.0
Sample
Day 0
Day 1

67 **Figure 4**

68

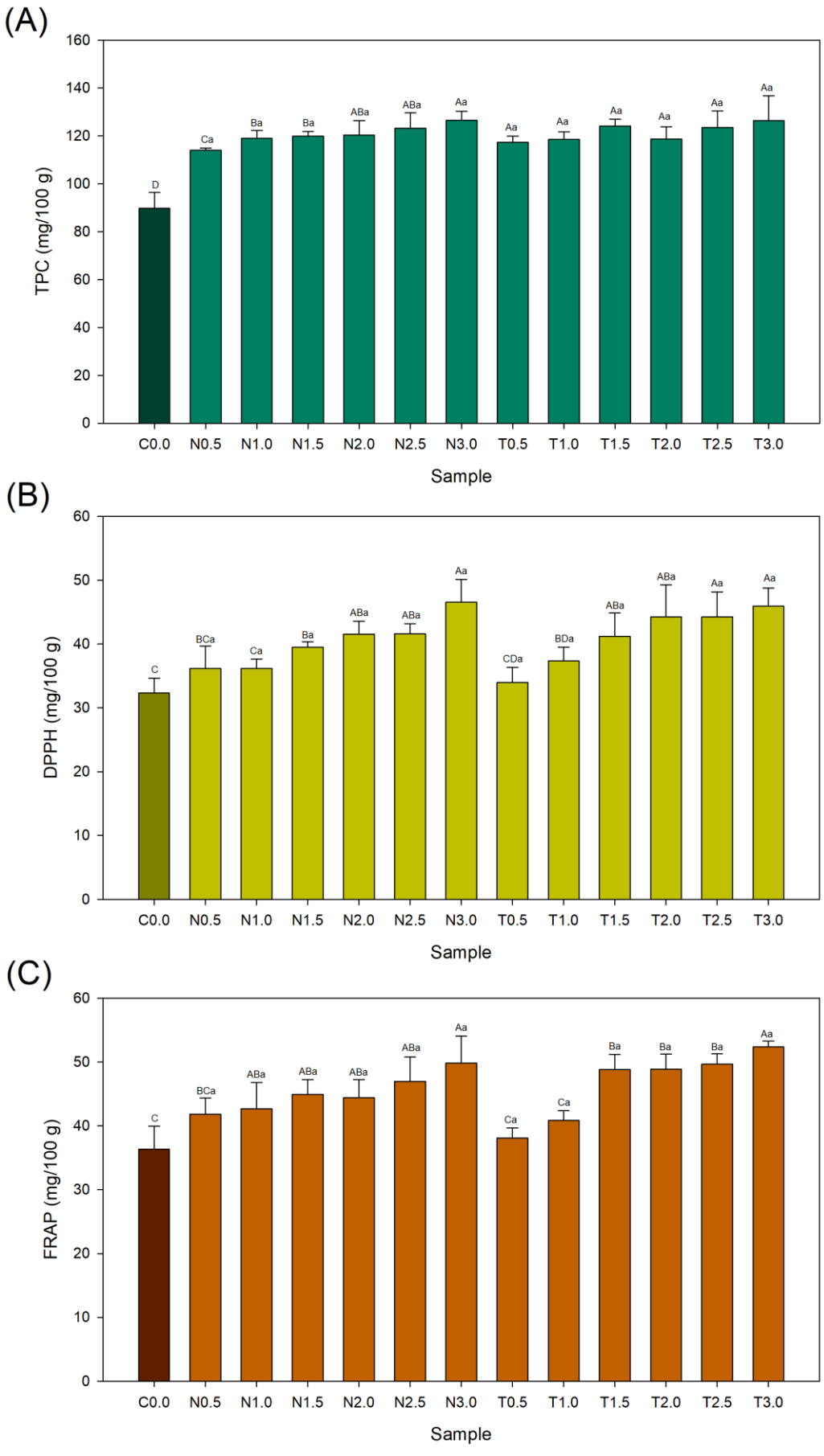
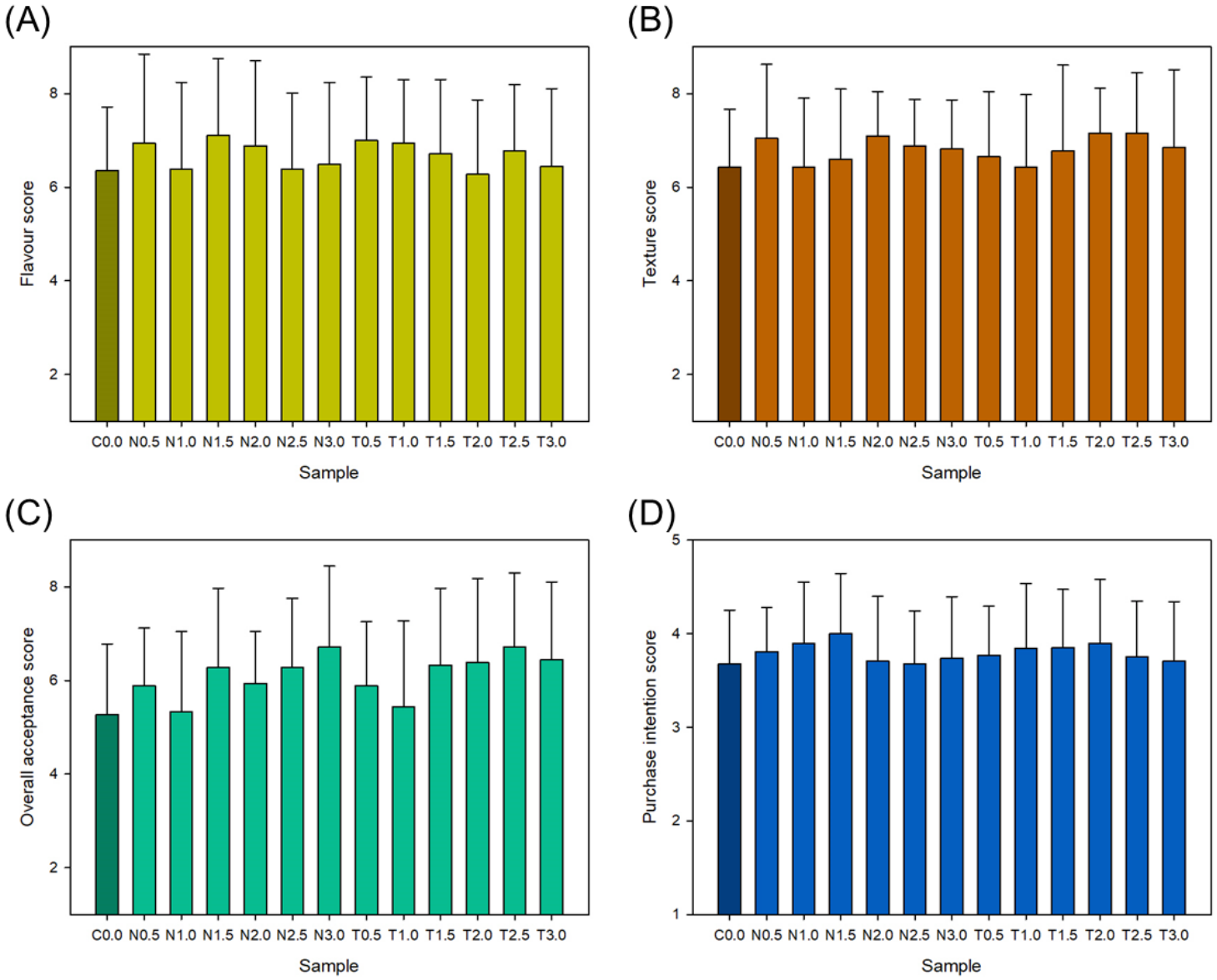


Figure 5


(A)
Flavour score
2
4
6
8
C0.0 N0.5 N1.0 N1.5 N2.0 N2.5 N3.0 T0.5 T1.0 T1.5 T2.0 T2.5 T3.0
Sample
(B)
Texture score
2
4
6
8
C0.0 N0.5 N1.0 N1.5 N2.0 N2.5 N3.0 T0.5 T1.0 T1.5 T2.0 T2.5 T3.0
Sample
(C)
Overall acceptance score
2
4
6
8
C0.0 N0.5 N1.0 N1.5 N2.0 N2.5 N3.0 T0.5 T1.0 T1.5 T2.0 T2.5 T3.0
Sample
(D)
Purchase intention score
1
2
3
4
5
C0.0 N0.5 N1.0 N1.5 N2.0 N2.5 N3.0 T0.5 T1.0 T1.5 T2.0 T2.5 T3.0
Sample

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