TITLE

NATURAL VITAMIN B12 AND FUCOSE SUPPLEMENTATION OF GREEN SMOOTHIES WITH EDIBLE ALGAE AND RELATED QUALITY CHANGES DURING THEIR SHELF LIFE

RUNNING TITLE

Natural vitamin B12 and fucose supplementation of green smoothies

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ABSTRACT

BACKGROUND: Some algae are an excellent source of vitamin B12, of special interest for vegetarian/vegan consumers, and fucose to supplement fruit and vegetables beverages like smoothies. Nevertheless, the algae supplementation of smoothies may lead to possible quality changes during smoothie shelf life that need to be studied. Accordingly, the quality changes of fresh green smoothies supplemented (2.2%) with 9 edible algae (sea lettuce, kombu, wakame, thongweed, dulse, Irish moss, nori, spirulina and chlorella) were studied throughout 24 days at 5ºC.

RESULTS: The initial vitamin C content (238.7−326.0 mg kg^{-1} fw) of a 200 g−portion of any of the smoothies ensured a full coverage of its recommended daily intake, being still covered a 50−60% of the recommended intake after 7 days. Chlorella and spirulina−smoothies showed the highest vitamin B12 content (33.3 and 15.3 µg kg^{-1} fw, respectively) while brown algae showed fucose contents of 141.1−571.3 mg kg^{-1} fw. Such vitamin B12 and fucose contents were highly maintained during smoothies´ shelf−lives.

CONCLUSION: The spirulina supplementation of a 200 g−smoothie portion ensured a full coverage of the recommended vitamin B12 intakes with lower vitamin C degradation during a shelf−life of 17 days. Furthermore, thongweed and kombu are also considered as excellent fucose sources with the same shelf−lives.

Keywords: Seaweed, beverages, health−promoting compounds, fucoidans, phenols, antioxidants.

INTRODUCTION

Fruit and vegetables represent a rich source of phytochemicals with health−promoting properties related to preventative effects on cardiovascular diseases, cancers, hypertension and other chronic conditions such as diabetes and obesity.[1] White grapes, broccoli and cucumber have high contents of such phytochemicals such as phenolic compounds, vitamin C and other antioxidant compounds, among others.[2-4] However, fruit and vegetables consumption is below the recommended daily intake.[5] Beverages, and more recently smoothies, represent an excellent and convenient alternative to promote the daily consumption of fruit and vegetables.[6, 7] Smoothies are non−alcoholic beverages prepared from fresh or frozen fruit and/or vegetables, which are blended and usually mixed with crushed ice to be immediately consumed. Often, some smoothies may include other components like yogurt, milk, ice−cream, lemonade or tea [8].

The current consumer searches for innovative food products with new tastes, which also cover the nutritional needs together with additional health−promoting properties. ‘Fortification’ or ‘enrichment’ is the ‘addition of one or more essential nutrients to a food whether or not it is normally contained in it, for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups’.[9] Nevertheless, the actual consumer looks for food products with natural ingredients. Accordingly, fortified products with natural ingredients are attracting much attention. Vitamins B12 and C cannot be synthetized by humans so they must be ingested with food. Usual dietary sources of vitamin B12 are animal food products, but not plant food products, being such fact of crucial interest for some populations groups such as vegetarians/vegans. Some edible algae have been reported to shown large amounts of vitamin B12.[10, 11] High contents of phenolic compounds can be also found in marine algae, being phlorotannins the main phenolic group, which provide a wide range of potential biological activities (antioxidant, anticancer, antibacterial, anti−allergic, anti−diabetes, anti−aging, anti−inflammatory and anti−HIV activities) [12, 13]. Brown algae are also rich sources of fucoidans, L–fucose sulphated polysaccharides, which have several health–promoting properties such as anticancer, antioxidant, antiviral and antioxidant, among others, as recently reviewed.[12, 14] Algae have been traditionally used for culinary purposes in Asian countries although their consumption has recently spread to Western countries as bioactive ingredients included in functional foods. Algae are commonly classified into three groups based on their pigmentation: brown (*Phaeophyceae*), red (*Rhodophyceae*) and green (*Chlorophyceae*) algae. Furthermore, such scenario also promotes the creation of edible algae industries in other countries different from Asian area which quality may be excellent, and even higher for some purposes, compared to those imported dried seaweeds from East Asia [15].

The natural vitamin B12 fortification of fruit/vegetable smoothies with algae may have a high relevance in the food industry to supply to the consumer food products with natural ingredients, which covers their nutritional needs. Furthermore, such natural fortification may lead to extra health−promoting properties derived from the high phenolics and fucose contents, among other compounds, of such marine plants. However, there are no previous reports of possible side effects of algae fortification on the quality of fruit/vegetables smoothies. Accordingly, the aim of the present work was to study the main quality changes and bioactive contents of several fresh fruit/vegetables smoothies formulated with 9 different edible algae during 24 days of storage at 5ºC.

MATERIALS AND METHODS

Plant material and smoothie preparation

Fresh white grapes and cucumbers were purchased at a local supermarket and kalian−hybrid broccoli (Bimi®) was obtained from a local producer (Campo de Lorca−Juan Marín S.L.; Lorca, Murcia, Spain) in June. Plant material was transported within 1 h to the Pilot Plant at the Universidad Politécnica de Cartagena, where it was stored at 4ºC and 90−95% relative humidity (RH) until next day.

The 9 edible algae used were sea lettuce, kombu, wakame, thongweed, dulse, Irish moss, nori, chlorella and spirulina, which are described in Table 1. They were purchased from Porto−Muiños (La Coruña, Galicia, Spain). Algae were supplied as ground dried powder (200 g) in plastic bottles. Since all samples had different particle sizes, they were grinded with a mill (IKA, A 11 Basic, Berlin, Germany) using liquid nitrogen to fine powder with a measured (Scirocco 2000, Malvern Instruments; Malvern, Worcestershire, UK) average particle size of 300 µm.

Preparation of smoothies was accomplished in a disinfected cold room at 8ºC. Plant material was carefully inspected, selecting those free from defects and with similar visual appearance. Subsequently, plant material was sanitized with 75 mg L^{-1} NaClO during 2 min and then rinsed with cold tap water for 1 min. Then, cucumbers were peeled, grape berries detached from the cluster and broccoli was cut with total length of approximately 15 cm with a sharp knife. Nine different smoothies containing the different algae were prepared. The vegetables, fruit and alga proportions for preparation of smoothies were: 56.5% white grapes, 15.5% broccoli, 25.8% cucumber and 2.2% alga. A smoothie without alga was prepared as control (CTRL) containing: 57.8% grapes, 15.8% broccoli and 26.4% cucumber. The smoothie composition was selected among several formulations according to sensory pre−evaluations conducted by a sensory panel focussing on the maximum broccoli quantity in order to maximize the bioactive contents of the smoothie. Smoothies were prepared in a food processor (Robot Cook®, Robot Coupe; Vincennes, Île-de-France, France) and immediately cooled to 4ºC with an ice−water bath. Immediately after smoothie preparation, approximately 80 g of each smoothie were filled (Infantino Squeeze station, Infantino; San Diego, California, USA) under aseptic conditions into a sterile squeeze polyvinyl chloride pouch (9 cm×13 cm; 118 mL; Infantino; San Diego, California, USA). Samples were stored in darkness at 4ºC being conducted sampling times up to 24 days. Three replicates per treatment, storage temperature and sampling day were prepared. Samples of each treatment were taken on each sampling day to be analysed storing also samples for bioactive compounds at -80ºC until further analyses.

Microbial analysis

Psychrophilic, and yeast and moulds (Y+M) growth was determined using standard enumeration methods according to Castillejo *et al.*[6]. All microbial counts were reported as log colony forming units per gram of smoothie (log CFU g^{-1}). Each of the three replicates was analysed in duplicate. *Salmonella* spp., *Listeria monocytogenes* and generic *Escherichia coli* were monitored meeting the obtained results the food safety European legislation for these products.[16]

Physiochemical analyses

The total soluble solids content (SSC), pH, titratable acidity (TA) and colour of smoothies were determined as previously described.[6] The SSC of the smoothie was determined by a digital hand−held refractometer (Atago N1; Tokyo, Kanto, Japan) at 20ºC and expressed as % (g sugar equivalents 100 g^{-1}). A pH−meter (Basic20, Crison; Alella, Cataluña, Spain) was used to determine the pH. TA was determined by titration of 5 mL of smoothie plus 35 mL of distilled water with 0.1 M NaOH to pH 8.1 (T50, Metter Toledo; Milan, Lombardia, Italy) and expressed as % (g tartaric acid 100 mL^{-1}). Colour was determined using a colorimeter (Chroma Meter CR−300, Minolta; Tokyo, Kanto, Japan) calibrated with a white reference plate (light source C), 2º observer and 8−mm viewing aperture. Samples were introduced in a special glass tube mounted on a device connected to the colorimeter. Three colour readings were taken turning the tube every caption and all three measurements were automatically averaged by the device and recorded. Measurements were recorded using the standard tristimulus parameters

(L*, a*, b*) of the CIE Lab system. Total colour differences (ΔE) throughout storage compared to their respective initial values were calculated according to equations previously described.[17]

Sensory evaluation

Sensory analyses were performed according to international standards.[18] Tests were conducted in a standard room[19] equipped with 10 individual taste booths. Smoothie samples (about 30 mL) were served at room temperature in transparent plastic glasses coded with three random digit numbers. Still mineral water was used as palate cleanser. The panel consisted of 12 assessors (6 women/6 men, aged 22–70 years) screened for sensory ability (visual appearance, colour, aroma, flavour and texture). A 5−point scale of damage incidence and severity was scored for off−colours, off−flavours, off−odours, lumpiness and phase separation (5: none; 4: slight; 3: moderate, limit of usability (LU); 2: severe; 1: extreme). Visual appearance, aroma, flavour, texture and overall quality were assessed using a 5−point hedonic scale of acceptability (5: excellent; 4: good; 3: fair, LU; 2: poor; 1: extremely bad).

Vitamin C

The ascorbic (AA) and dehydroascorbic (DHA) acids were measured as previously described.[20, 21] Briefly, 5 g ground frozen (-80ºC) sample was placed into a 25−mL Falcon tube and 10 mL of cold (4ºC) buffer (0.1 M citric acid, 0.05% EDTA, 4 mM sodium fluoride and 5% MeOH) were added. The mixture was homogenised (UltraTurrax T25 basic, IKA; Berlin, Germany) for 10 s, filtered (four−layer cheesecloth) and the pH was adjusted (6N NaOH) to 2.35–2.40. Subsequently, 750 µL filtered (0.45−µm polytetrafluoroethylene (PTFE) membrane filters) purified extract

(Sep−Pak cartridges C18, Waters; Dublin, Leinster, Ireland) was derivatised with 250 µL of 7.7 M 1,2−phenylenediamine for 37 min in darkness at room temperature. Immediately after derivatisation, 20 µL were injected in a Gemini NX (250 mm×4.6 mm, 5 µm) C18 column (Phenomenex; Torrance, California, USA), using an HPLC (Series 1100 Agilent Technologies; Waldbronn, Baden-Württemberg, Germany) equipped with a G1322A degasser, G1311A quaternary pump, G1313A autosampler, G1316A column heater and G1315B photodiode array detector. AA and DHA were quantified using commercial standards (Sigma; St Louis, Missouri, USA). Calibration curves were made with at least six data points for each standard. AA and DHA were expressed as mg kg^{-1} fresh weight (fw). Each sample was analysed in duplicate.

Total phenolic content

Frozen samples of 1 g were placed in glass bottles and 4 mL of methanol was added. The extraction was carried out in an orbital shaker (Stuart; Staffordshire, West Midlands, UK) for 1 h at 200 rpm in darkness inside a polystyrene (PS) box with an ice bed. The extracts were transferred in eppendorf tubes and centrifuged at 15,000×*g* for 10 min at 4ºC. The supernatant was used as total phenolic content (TPC) and total antioxidant capacity (TAC) extracts.[22, 23] The TPC was determined as previously described based on, but with modifications proposed by. Briefly, 19 µL of TPC extract was placed on a flat−bottom PS 96−well plate (Greiner Bio−One; Frickenhausen, Baden-Württemberg, Germany) and 29 µL of 1 N Folin–Ciocalteu reagent was added. The latter mixture was incubated for 3 min in darkness at room temperature. Then, 192 µL of a solution containing Na_2CO_3 (0.4%) and NaOH (2%) was added. After 1 h of incubation at room temperature in darkness, the absorbance was measured at 750 nm using a Multiscan plate reader (Tecan Infininte M200; Männedorf, Meilen, Switzerland). The TPC was expressed as mg gallic acid equivalents (GAE) kg^{-1} fw. Each sample was analysed in duplicate.

Total antioxidant capacity

The extracts were analysed for TAC using the same instruments and methodology as previously described[8] using three different methods: free radical scavenging capacity with 2,2−diphenyl−1−picrylhydrazil (DPPH),[24] ferric reducing antioxidant power (FRAP)[25] and 2,20−azino−bis (3−ethylbenzothiazoline−6−sulphonicacid) (ABTS).[26] Results were expressed as mg Trolox equivalent antioxidant capacity kg^{-1} fw. Each sample was analysed in duplicate.

Vitamin B12

Vitamin B12 was determined according to a commercial microbiological kit for vitamin B12 (VitaFast, r−biopharm; Berlin, Germany). Briefly, 1 g of smoothie was mixed with 40 mL of distilled water, vortex and incubated at 95ºC for 30 min. After cooling down at room temperature, the solution was centrifuged at 32,000×*g* for 15 min at 15ºC and filtered through 0.45 µm PTFE membrane filters. Subsequently, 150 µL of vitamin B12 assay medium (available from the kit) was disposed on the wells of the microtiter plate (pre−coated with *Lactobacillus delbrueckii* subsp. Lactis (leichmannii)) supplied by the vitamin B12−kit. Then, 150 µL of the vitamin B12 extract was added and the microtiter plate was incubated at 37ºC in the dark for 46 h. Finally, the absorbance was measured at 620 nm using the Multiscan plate reader. Vitamin B12 was quantified using the vitamin B12 standard supplied by the vitamin B12−kit. The vitamin B12 was expressed as µg kg^{-1} fw. Each of the samples was analysed in duplicate.

Fucoidans/Fucose

Fucose (*L*−fucose) was determined using a commercial kit (L−fucose, Megazyme; Bray, Leinster, Ireland). Briefly, 2.5 g of smoothie was mixed with 2.5 mL 1.3 M HCl, vortex and incubated at 100ºC for 1 h. After cooling down at room temperature, 2.5 mL of 1.3 M NaOH were added, vortex and filtered through 0.45 µm PTFE membrane filters. Subsequently, 200 µL of water, 20 µL of fucose extract, 40 µL of buffer (supplied by the fucose kit) and 10 µL of $NADP^+$ solution (supplied by the kit) were placed on a flat−bottom PS 96−well plate. After 4 min of incubation at room temperature, 2 µL of L−fucose dehydrogenase suspension (supplied by the kit) was added and it was incubated at 37ºC for 1 h. Finally, the absorbance was measured at 340 nm using the Multiscan plate reader. Fucose was quantified using the *L*−fucose standard supplied by the kit. The fucose content was expressed as g kg^{-1} fw. Each of the samples was analysed in duplicate.

Statistical Analysis

The experiment was a two−factor (smoothie type×storage time) design subjected to analysis of variance (ANOVA) using Statgraphics Plus software (vs. 5.1, Statpoint Technologies Inc.; Warrenton, Virginia, USA). Statistical significance was assessed at the level p=0.05, and Tukey's multiple range test was used to separate means.

RESULTS AND DISCUSSION

Physicochemical quality

The physicochemical quality of smoothies can be evaluated based on SSC, pH, TA and colour being closely related to sensory quality.[6] Table 2 represents the effect of algae supplementation on the physicochemical quality of smoothies throughout storage. CTRL smoothie samples showed an initial high SSC of 12.4% being owed to the high content of grapes in the smoothie. A similar SSC has been also reported in other fruit−containing smoothies differing from other vegetables smoothies without fruit.[8, 27] The SSC of the smoothie was not significantly (p<0.05) changed after algae supplementation. Particularly, smoothies supplemented with brown and red macroalgae showed higher SSC (p<0.05) than those with green algae (hereinafter including both macro and microalgae). Such finding may be explained by the higher content of SSC, mainly sugars, of brown and red algae regarding green algae.[28, 29] The CTRL smoothie showed an initial pH of 4.24 allowing such acidic medium a moderate shelf life of the beverage under refrigeration conditions without the need of thermal treatments[30] which may reduce the sensory and nutritional/bioactive quality of the smoothie. Algae supplementation of smoothies led to a light pH increase up to 4.32−4.77 owed to the high mineral contents of algae, which may achieve up to 40% of total weight.[31] The CTRL smoothie showed an initial TA of 0.30% that was slightly reduced after algae supplementation according to previous slight pH increment.

In general, SSC of samples did not highly change throughout storage (<0.6 SSC units), with a particular general SSC decrease on day 3, except for sea lettuce and CTRL smoothies, being newly upregulated on days 7−10. The latter slight SSC decrease may be owed to a sugar consumption by microorganisms, which initiated to growth after such initial adaptation period to the smoothie medium according to psychrophiles data (shown later). Furthermore, no high pH and TA changes were observed (<0.3 pH and <0.28 TA units) after 24 days at 5ºC, showing sea lettuce and CTRL smoothies the lowest pH/TA variations. Similarly, no pH changes were either observed in a fresh (unheated) green vegetable puree after 43 days at 4ºC.[32] Nevertheless, smoothies supplemented with the microalgae spirulina and chlorella particularly showed TA increments of 0.35 and 0.23 units in the last 7 days of storage although such acidification was not negatively scored by the sensory panel even showing spirulina smoothie the best flavour scores after 24 days of storage (see sensory data).

The addition of algae to the green smoothie induced a decrease of luminosity (L^*) and yellowness (b^*), and an increase of redness (a^*). The microalgae spirulina and chlorella showed the highest colour changes, as expected due to their intense green colour, with ΔE of 23.0 and 20.8 on processing day, respectively (Table 2). Nevertheless, such colour changes did not negatively affect to the consumer acceptance of algae−supplemented smoothies since the sensory panel highly scored (> 4) general appearance and colour of all samples on processing day (see sensory data). On the other side, algae−smoothies showed lower colour changes (ΔE=6.8−9.8) than CTRL smoothie (ΔE=11.9) after 24 days, showing spirulina−smoothie the lowest colour differences. Therefore, the colour degradation of the smoothie due to enzymatic activity, as previously observed,[8] was reduced with the algae supplementation, probably owed to enzymatic−inhibiting compounds from such marine plants.

Accordingly, the physicochemical quality of smoothies was not highly affected after algae supplementation even showing lower colour changes compared to non−supplemented smoothie.

Microbiological analysis

The smoothie preparation included several unit operations such as peeling, cutting, blending and the addition of bioactive ingredients like algae, which may highly increase the microbial growth during refrigerated storage, limiting its shelf life and compromising its food safety. Consequently, microbial quality should be determined in such products in order to monitor spoilage microorganisms and pathogens.

Psychrophilic and Y+M loads were monitored throughout storage of smoothies at this low storage temperature and the adaptation of Y+M to grow under such acidic beverages (Table 3). The initial psychrophilic and Y+M loads of the green smoothie (3.9 and 2.9 log CFU g^{-1}, respectively) were not highly altered with the addition of algae to the smoothies, reporting increments lower than 0.3 and 0.6 log units, respectively, on processing day.

A general microbial reduction was observed in the psychrophilic and Y+M growth during storage of smoothies showing loads of 2.8−3.8 and 2.3−2.9 log CFU g^{-1}, respectively, after 7 days at 5ºC. Nevertheless, microbial loads of all smoothies were increased after day 7. Particularly, psychrophilic growth was higher in algae−smoothies compared to CTRL samples, showing brown algae−smoothies loads of 7 log CFU g^{-1} after 17 days at 5 ºC while such levels were only exceeded in red−algae after 21 days at ºC. Furthermore, CTRL and sea lettuce−smoothies showed the lowest psychrophilic loads after 24 days at 5ºC, with 4.9 and 5.3 log CFU g^{-1}, respectively. *Brassica* species, i.e. broccoli, have high glucosinolates contents,[2] which after plant cell disruption, i.e. smoothie preparation, come in contact with plant myrosinase that is previously located in separate cell compartments. The activity of myrosinase transforms glucosinolates to unstable intermediate compounds, which rearranges mainly to isothiocyanates under acidic conditions and presence of mineral ions, among other factors, instead of other breakdown products.[33, 34] High antimicrobial properties have been reported by sulforaphane, the isothiocyanate resulting from the glucosinolate glucoraphanin, one of the main glucosinolates of broccoli.[6] Therefore, the higher psychrophilic growth in all algae−smoothies may be owed to the higher pH and mineral contents regarding the CTRL smoothie without algae supplementation. Nevertheless, the lower psychrophilic growth in sea−lettuce smoothie may be explained by the lower mineral contents from this alga compared to the remaining algae (data not shown). However, such antimicrobial effect throughout storage was not observed for Y+M of CTRL smoothie, which showed the highest Y+M load, together with kombu−smoothie (low SSC and TA), of 4.9 log CFU g^{-1} after 24 days at 5ºC. Meanwhile, the remaining samples showed Y+M loads that ranged among 3.3 to 3.6 log CFU g^{-1}. The latter behaviour may be explained by the high adaptation of Y+M to grow under acidic conditions. Furthermore, the lower Y+M growth of most of algae−smoothies may be owed to the early known fungistatic properties of marine algae.[35]

Salmonella spp., *L. monocytogenes* and generic *E. coli* were monitored throughout storage, meeting the obtained results the food safety European legislation for these products.[16]

Conclusively, algae−smoothies could be stored up to 17−21 days at 5ºC showing psychrophilic loads close to 7 log units, while Y+M levels were highly inhibited (1.3−1.7 lower log units) after 24 days compared to the CTRL smoothie without algae supplementation.

Sensory analysis

As expected, the used algae concentration within smoothies led to a mild marine taste detected by the panellists (Figure 1). Irish moss and chlorella addition led to the lowest overall quality scores on processing day, mainly due to a stronger marine odour/flavour of these algae, showing their smoothies scores of 2.3/3.0 and 2.4/3.1, respectively. As depicted in material and methods section, a general 2.2% algae content was used for all smoothie formulations in order to avoid quality differences owed to different algae contents. Nevertheless, the Irish moss and chlorella contents are recommended to be reduced from the 2.2% tested in order to achieve a higher consumer acceptance. The sensory quality of the remaining algae−smoothies was highly scored (>4) showing spirulina and kombu the highest overall quality scores with 4.5−4.6. No off−flavours/odours/colours were detected among all the smoothies on processing day, showing a pleasant texture without a remarkable lumpiness justified by the appropriated blending program used with the used semi−industrial food processor.

Algae−smoothies still showed overall quality scores over the limit of acceptability (3) after 14 days at 5ºC showing Irish moss, chlorella and wakame the lowest scores of 3.1−3.2 mainly owed to low flavour and aroma scores (Figure 1). Nevertheless, overall quality of algae−smoothies was below the limit of acceptability after 17 days at 5ºC, except sea lettuce−smoothie. The latter low overall quality scores were mainly owed to the low aroma and flavour scores with remarkable off−flavours, mainly for chlorella and brown macroalgae, and increased lumpiness, which reached a score of 2.7 for wakame−smoothie. No high phase separation was observed for the smoothies with scores of 4−4.5 and 3−3.5 after 17 and 24 days at 5ºC, respectively. The overall quality of sea lettuce−smoothie was scored with 3.0 after 24 days at 5ºC (Figure 1) with similar scores to the CTRL smoothie (data not shown). The latter finding is explained by the milder marine taste of sea lettuce compared to the remaining algae.

Conclusively, the shelf life of algae−smoothies could be established in 17 days at 5ºC based on sensory and microbiological quality. Particularly, the shelf life of the lettuce−smoothie was even extended up to 24 days at ºC due to its previously discussed low psychrophilic load throughout the storage period.

Vitamin C

Ascorbic acid is stable when dry but in solutions it readily oxidises to the intermediate compound monodehydroascorbate (MDHA) through the activity of the enzyme ascorbate oxidase. Subsequently, MDHA may be converted to DHA that can be reduced newly to AA or hydrolysed to 2,3−diketogulonic acid (DKG).[36] DHA also exhibits antioxidant properties in addition to antiscorbutic activity equivalent to that of AA, contrary to the non−bioactive compound DKG.[37] Therefore, vitamin C content of fruit and vegetables has been proposed as the sum of AA and DHA.[38] The CTRL smoothie showed an initial vitamin C content of 326.0 mg kg^{-1} fw (Table 4). Similar total vitamin C contents have been reported in other fruit/vegetables fresh smoothies, also containing broccoli and grapes.[6, 27] Nevertheless, no AA was detected in the smoothie samples, contrary to previous data on vegetables smoothies.[6] The latter finding may be explained by a high AA degradation by ascorbate oxidase due to the cucumber included in our smoothie, being this vegetable, and *Cucurbitaceous* family in general, among the most abundant sources of this enzyme.[39] The role of metal ions, such as those contained in algae, in the oxidation of AA has been widely known for more than 95 years[40] explaining the mild vitamin C reduction (up to 27%) observed after algae supplementation of smoothies. Nevertheless, a 200 g−portion of the algae−smoothie with the lowest (p<0.05) initial vitamin C content (Irish moss) still ensured the recommended daily intake (RDI) of vitamin C.[41]

The vitamin C content of smoothies decreased throughout storage, showing levels 50−60% lower after 7 days at 5ºC. Latter finding may be explained since DHA is itself very unstable in aqueous solution (half−life of 6 min at 37ºC) and undergoes irreversible hydrolytic ring cleavage to the non−bioactive DKG.[42] Particularly, chlorella−smoothie showed the highest vitamin C reduction of 70% probably owed to the high content in this alga of iron, one of the main metal ions which highly induce vitamin C degradation.[43] A vitamin C degradation of approximately 90%, for all smoothies without high differences among them, was observed on day 14, regarding their respective initial levels, being such low levels maintained until the last day of storage. Likewise, high vitamin C degradation has been previously observed in fresh fruit/vegetables smoothies stored under similar low storage temperature.[6, 44, 45]

Conclusively, all smoothies covered the vitamin C recommended daily intake by the WHO while a 200 g−portion stored for 7 days at 5ºC still ensured the 50−60% of the recommended daily intake of this vitamin.

Vitamin B12

Smoothies supplemented with all macroalgae, except kombu and thongweed (undetected levels), showed similar (p<0.05) initial vitamin B12 contents of approximately 1 µg kg^{-1} fw (0.4 µg kg^{-1} fw for Irish moss) (Figure 2). Nevertheless, chlorella and spirulina−smoothies showed initial vitamin B12 levels of 33.3 and 15.3 µg kg^{-1} fw, respectively. Accordingly, chlorella and spirulina−smoothies portions of just 70 and 160 g would cover the recommended vitamin B12 daily intake.[41] Spirulina and chlorella are algae with high vitamin B12 content, as previously reported.[10, 46]

Vitamin B12 contents of previous smoothies did not change (p<0.05) throughout storage. As observed, the supplementation of the smoothie with all algae (except kombu and thongweed) may be considered as a natural tool to fortify fruit/vegetable beverages with vitamin B12, being of special interest for some populations groups such as vegetarians/vegans, elderly, individuals with disorders of malnutrition, etc. Vitamin B12 belongs to the corrinoid group and is usually restricted to cyanocobalamin although microbiological analytical method, hereby used and approved by the Association of Analytical Communities,[47] may also detect other corrinoids non−bioavailable for humans known as pseudo–vitamin B12.[48-51] Accordingly, active vitamin B12 coenzymes comprised about 60 % of total vitamin B12 in nori and chlorella supplements.[52] Accordingly, the total vitamin B12 contained in 250g−portions of chlorella and spirulina−smoothies stored for 24 days at 5 ºC represents 475 and 245% of the recommended vitamin B12 daily intake which would lead to a full coverage of the needed biologically−active B12 levels.

Fucose

Brown algae are natural sources of fucoidans, or fucans, which are naturally occurring L–fucose sulphated polysaccharides. Several health–promoting properties (anticancer, antioxidant, antiviral and antioxidant, among others) have been linked to fucoidans as previously reviewed.[12] The fucoidans composition is complex and still unclear being due to its high heterogeneity, which is influenced by the alga specie, part of the plant or even the extraction method used.[53] In this sense, each fucoidan extracted from a different specie with a specific method will be unique regarding to structure and composition, leading to differences related to biological activities. Therefore, fucoidans were indirectly studied in this work by their conversion to the fucose monomer by depolymerisation and desulphation by strong acid and high temperature. The fucose data regarding to analysed brown macroalgae–smoothies, being not detected in the remaining smoothies, showed contents of 571.3, 455.7 and 141.1 mg kg^{-1} fw for thongweed, kombu and wakame, respectively. Such contents were not changed ($p<0.05$) after 24 days at 5ºC (data not shown).

Total phenolic content and antioxidant capacity

Polyphenols from terrestrial plants are derived from gallic and ellagic acid, whereas the algal polyphenols are derived from polymerized phloroglucinol units.[12] The TPC were expressed as gallic acid equivalents due to the higher fruit and vegetables contents in the smoothie compared to algae. The CTRL smoothie showed an initial TPC of 280.2 mg gallic acid equivalent kg^{-1} fw (Table 5). Such high TPC may be owed to the high grapes content together with broccoli which are fruit and vegetable with high phenolic contents.[3, 4, 54] The initial TPC content of the CTRL smoothie was increased (p<0.05) by 69−70% after supplementation with kombu and dulse algae. Brown algae have shown higher phenolic content than red and green algae being phlorotannins the major phenolic compounds.[12] Furthermore, thongweed algae has shown the higher TPC compared to the other brown algae.[55] The alga addition and the plant cell wounding implied during smoothie preparation may generate different stresses conditions in the smoothie, which may lead to the generation of free radicals. Consequently, phenols from high source pools like thongweed may be highly used to prevent such oxidative stresses. Accordingly, thongweed−smoothie showed the lowest TPC and TAC values among brown and red algae−smoothies on processing day (Table 5).

A general TPC decrease of 20−50% after 3 days was observed in the smoothies, probably owed to the use of such phenolic compounds to counterbalance the stress generated during smoothie preparation (Table 5). According to such data, a TAC increase was observed on day 3 by the three TAC methods (Table 5). Subsequently, a general TPC increase from day 3 to day 7 was observed with increments ranging from 60−140 and 10−30% in macro and microalgae−smoothies, respectively. Such increments may be due to a phenolic biosynthesis to counterbalance the stress during processing. Therefore, similar phenolic biosynthesis has been observed in smoothies during cold storage correlated with the activation of phenylalanine ammonia−lyase (PAL) which is considered the key enzyme in the phenylpropanoid pathway.[8] Higher TPC increments were observed in thongweed and Irish moss−smoothies of 400 and 340%, respectively, from day 3 to day 7 regarding the remaining smoothies. That finding may be explained by the low TPC of latter two smoothies on day 3, which could generate a higher PAL activation sign due to such low contents of those needed antioxidants. No remarkable TPC and TAC changes were observed from day 7 to day 21 being a new general TPC decrease/TAC increase observed from day 21 to day 24. The latter second antioxidants biosynthesis may be explained by the stress generated during storage of smoothies under such low storage temperatures.

CONCLUSIONS

Main quality changes of green vegetables smoothies supplemented with 9 of the most consumed/known edible algae were determined during refrigerated shelf life. Generally, the shelf life of algae−smoothies, based on microbiological and sensory quality, was stablished in 17 days at 5ºC. Sea lettuce showed the longest shelf life (24 days) although their bioactive contents were lower than the rest of algae−smoothies. Among them, the brown algae thongweed, kombu and wakame−smoothies showed high fucose contents reporting wakame also high vitamin B12 contents. The smoothies with the microalgae chlorella and spirulina showed the highest vitamin B12 contents although the chlorella−smoothie was scored with low sensory quality and the highest vitamin C degradation during storage. Accordingly, a reduction of chlorella concentration in the smoothie formulation should be further studied for supplying a high vitamin B12 contents. Therefore, fortification of smoothies with spirulina ensured a full coverage of the recommended vitamin B12 intakes with lower vitamin C degradation regarding chlorella during 17 days at 5ºC. Among macroalgae−smoothies, thongweed and kombu are also considered as excellent fucose sources.

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TABLE AND FIGURE CAPTIONS

Table 1. Classification and details of the nine edible marine algae studied.

Table 2. Total soluble solids content (SSC, %), pH, titratable acidity (TA, expressed in %: g tartaric acid 100 g^{-1}) and total colour differences (ΔE) of fresh fruit/vegetable smoothies with or without algae fortification stored at 5 ºC (n=5±SD). Different capital letters denote significant differences ($P \leq 0.05$) among smoothies for the same sampling day. Different lowercase letters denote significant differences ($P \leq 0.05$) among sampling days for the same smoothie.

Table 3. Psychrophilic, and yeast and moulds counts (log CFU g^{-1}) of fresh fruit/vegetable smoothies with or without algae fortification stored at 5 ºC (n=5±SD). Different capital letters denote significant differences ($P \leq 0.05$) among smoothies for the same sampling day. Different lowercase letters denote significant differences ($P \leq 0.05$) among sampling days for the same smoothie.

Table 4. Vitamin C (mg kg^{-1}) of fresh fruit/vegetable smoothies with or without algae fortification stored at 5 ºC (n=5±SD). Different capital letters denote significant differences ($P \leq 0.05$) among smoothies for the same sampling day. Different lowercase letters denote significant differences ($P \leq 0.05$) among sampling days for the same smoothie.

Table 5. Total phenolic content (TPC, mg gallic acid equivalent kg^{-1}) and total antioxidant capacity (three methods; mg Trolox equivalent kg^{-1}) of fresh fruit/vegetable

smoothies with or without algae fortification stored at 5 ºC (n=5±SD). Different capital letters denote significant differences ($P\leq 0.05$) among smoothies for the same sampling day. Different lowercase letters denote significant differences ($P\leq0.05$) among sampling days for the same smoothie.

Figure 1. Sensory attributes of fresh fruit/vegetable smoothies with or without algae fortification on processing day and after 14, 17 and 1 days at 5 ºC (n=5±SD).

Figure 2. Vitamin B12 (μg kg^{-1}) of fresh fruit/vegetable smoothies with algae fortification on processing day and after 24 days at 5 ºC (n=5±SD). Different capital letters denote significant differences ($P\leq 0.05$) among smoothies for the same sampling day. Different lowercase letters denote significant differences ($P\leq0.05$) among sampling days for the same smoothie.