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ORIGINAL PAPER

Bioaccessibility and antioxidant capacity of phenolic compounds during shelf life of a new functional vegetable mix

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Abstract

Fresh-cut vegetables are minimally processed healthy products that have gained the preference of consumers in accordance to the current lifestyle. Fresh-cut industry is continuously evolving and pursuing new varieties of raw material to develop innovative vegetable products, which fulfil consumer expectations. The current study evaluated the phenolic compounds in an innovative vegetable mix (red cabbage, rocket, parsley and beet greens) as well as their antioxidant capacity and bioaccessibility during their shelf life under refrigerated storage. The initial total phenolic compound and flavonoids content in the salad mixture were 163.3 ± 5.77 mg GAE/100 g FW and 280.96 ± 25.14 mg QE/100 g FW, respectively. The initial antioxidant capacity in the salad mixture was 85.4 ± 7.9 mg TE/100 g FW for DPPH and 318.1 ± 26.5 mg TE/100 g FW for ABTS assays. After digestion, total phenolic compound and TEAC capacity at initial times showed percentage of recovery values close to the chemical extraction and suffered a decrease towards day 10 of storage. Flavonoids and DPPH capacity showed recovery percentages less than 50%, at all storage times. Regarding sensory quality, the shelf-life of the salad mixture was limited by the presence of unacceptable off-odors at day 7 but since at microbiological evaluation, the shelf-life of the product is no longer than 5 days.

Keywords Fresh-cut vegetables · In-vitro gastrointestinal digestion · Polyphenols · Bioaccessibility · Antioxidant capacity

Introduction

Epidemiological, clinical and nutritional studies strongly support the evidence that a diet rich in fruits and vegetables enhance human health by preventing or lowering risk to develop degenerative diseases including cancers, cardiovascular diseases and metabolic disorders. Natural antioxidants present in these crops, such as phenolic compounds, are considered responsible for these chemopreventive effects [1, 2]. Furthermore, the use of dietary bioactive compounds, such as phenolic compounds, has emerged as a putative nutritional or therapeutic adjunct approach for COVID-19 [3].

During minimal processing of vegetables, tissue integrity is damaged inducing an increase in carbon dioxide and ethylene evolution, water loss, alterations in flavor and aroma, and an increase in the activity of enzymes related to enzymatic browning. Furthermore, these vegetables become highly susceptible to microbial spoilage [4–6]. The sum of these factors drives to a product with a shorter shelf life compared to whole vegetable [5, 6].

Usually, the analyzed factors to determine the shelf life of a product were sensorial and microbiological quality [4]. However, in accordance with the new trends oriented to bring benefits to consumer health, it is of interest to evaluate the evolution of health-promoting phytonutrients content and their bioaccessibility during storage.

Estimations of the nutritional value of vegetal foods are usually based on native concentrations of nutrients and phytochemicals obtained by direct analysis after an extraction using aqueous-organic solvents, however, these

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chemical extractions are substantially different from enzymatic extractions, such as those of the digestive tract [7]. Only certain amounts of all the nutrients and bioactive components in food will be used effectively by the organism, and this depends on the bioaccessibility and bioavailability of these compounds [8, 9]. Bioaccessibility is defined as the fraction of a compound that is released from its matrix in the gastrointestinal tract and, therefore, could potentially be available for intestinal absorption. Bioavailability is a wider concept that includes bioaccessibility, but also absorption, metabolism, tissue distribution, and efficient bioactivity of the compound at its site of action [8, 9].

Although there are several works concerning the nutritional composition and microbiological safety of fresh-cut leafy vegetables, studies on phenolic compounds stability and antioxidant capacity evolution during shelf-life product, as well as the effect of storage on the bioaccessibility of bioactive compounds, are still scarce.

Therefore, the main objective of this study was to evaluate the evolution of phenolic compound content and their antioxidant capacity of an innovative vegetable mixture with functional properties during their shelf life under refrigerated storage, as well as to study the bioaccessibility of these antioxidant components at each storage time.

Materials and methods

Plant material and conditioning of vegetable samples

For the selection of the vegetables to include in the vegetal mix, a previous study of different vegetal products, with traditional and non-traditional ingredients in the preparation of salads, was carried out [10]. From that study, four vegetables were selected for the development of the vegetable mix in accordance with their high bioactive compounds content and antioxidant capacity: red cabbage, rocket, parsley and beet greens.

Beet greens, parsley, rocket and red cabbage were collected and maintained at 5 ± 1 °C in darkness prior to processing. Beet greens and rocket were shredded in pieces of about 1.5-cm size and 2.5-cm size, respectively. Red cabbage was cut into very thin strips (< 0.5-cm wide). Parsley leaves were separated from the stem and cut in halves or thirds. Cut vegetables were washed in chlorinated water (150 ppm) at 10 °C for 2 min. The vegetables were drained and rinsed in tap water at 10 °C for 2 min, and the surface moisture was removed with a manual salad centrifuge.

Sample preparation

The combination of the four vegetables (beet greens, parsley, rocket and red cabbage) in the salad mixture were evaluated for consumer acceptance. A total of 21 vegetable combinations were tested with respect to two attributes: overall visual quality (OVQ) and taste (data not shown). The selected salad mixture was performed with beet greens, parsley, rocket and red cabbage, in proportions of 20:20:20:40 (% w/w). Each salad mixture sample (100 g) was place in open plastic trays, covered with 15 µm polyethylene wrap (O_2 permeability 600 $cm^3\ m^{-2}\ day^{-1}$, CO_2 permeability 4000 $cm^3\ m^{-2}\ day^{-1}$ and water vapor permeability 4 $g\ m^{-2}\ day^{-1}$) and hermetically sealed. The trays were kept for 10 days in refrigerated conditions (5 °C), at a relative humidity of 95%. Samples (three trays for each time) were taken for analysis at days 0, 3, 5, 7 and 10. Each sample was analyzed with respect to the enumeration of microorganism and sensory quality during storage under refrigerated conditions. Bioactive compounds and antioxidant capacity were analyzed before and after the *in vitro* digestion of samples.

In vitro digestion model

A simulated gastrointestinal digestion of mix salad was carried out following the methodology described by Minekus et al. [11] for Gastric and Intestinal phases.

For the gastric phase, 1.5 g of salad mixture, previously homogenized with a tissue blender (Braun Type 4193, Spain) for 1 min, were placed in a 50 mL tube, to which 6.25 mL of simulated gastric fluid (SGF), 0.8 mL porcine pepsine solution (62.5 mg/mL) (P7125, Sigma-Aldrich) made up in SGF, and 5 µL of 0.15 mol/L $CaCl_2$ solution, were added. A pH of 3.0 was achieved with the addition of 1 mol/L HCl and then, distilled water was added to reach a final volume of 10 mL. The mixture was incubated at 37 °C with shaking (150 rpm) for 2 h.

After the gastric phase, 10 mL of the gastric chime were further mixed with 5.5 mL of simulated intestinal fluid (SIF), 2.5 mL of pancreatin solution made up in SIF (4 mg/mL) (P1750, Sigma-Aldrich), 1.25 mL of bile solution (24 mg/mL) (B8631, Sigma-Aldrich) made up in SIF, 40 µL of 0.15 mol/L $CaCl_2$ solution and 1 mol/L NaOH to reach pH 7.0. Then, distilled water was added to a final volume of 20 mL. The mixture was incubated at 37 °C and 150 rpm for 2 h.

At the end of each phase (gastric and intestinal), tubes were centrifuged at 18,400 x g for 10 min at 4 °C and the supernatants were recovered and stored at -20 °C until further analysis. Each fraction (gastric and intestinal supernatants) were used to analyze total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacity (by

DPPH and TEAC). Determinations were carried out in triplicate per sample.

Bioaccessibility and percentage of recovery calculations

The supernatant recovery after gastrointestinal digestion represents the bioaccessible fraction of the sample according to Fernández-Jalao et al. [12]. Bioaccessibility was defined as the percentage of a specific bioactive compound or antioxidant capacity value detected in the soluble phase after gastrointestinal digestion at day "i" in respect to the total compound content/antioxidant capacity in the sample before digestion at the same day "i" (i = 0,3,5,7,10) [8, 12], and was calculated according to Eq. 1:

$$\text{Bioaccessibility}(\%) = \frac{\text{Bioactive compound content/antioxidant capacity after intestinal phase at day i}}{\text{Bioactive compound content/antioxidant capacity in ethanolic extraction at day i}} \quad \text{(Eq. 1)}$$

Percentage of recovery was defined as the percentage of a specific bioactive compound or antioxidant capacity value detected in the soluble phase after gastrointestinal digestion at day "i" (i = 0,3,5,7,10) in respect to the initial total compound content/antioxidant capacity in the sample before digestion, and was calculated according to Eq. 2:

$$\text{Percentage of recovery}(\%) = \frac{\text{Bioactive compound content/ antioxidant capacity after intestinal phase at day i}}{\text{Bioactive compound content/ antioxidant capacity in ethanolic extraction at day 0}} \quad \text{(Eq. 2)}$$

Extraction of bioactive compounds

At each time of storage and prior to the digestion process, a chemical extraction of salad mixture was carried out to evaluate TPC, TFC and antioxidant capacity; values obtained were considered 100% for each vegetable sample. Extraction of bioactive compounds was conducted according to Viacava et al. [13] with some modifications. For chemical extraction, fresh vegetable samples were homogenized with a tissue blender (Braun Type 4193, Spain) for 1 min. A sample (3 g) was taken from the homogenate and was added to 10 mL of ethanol/water (80/20 v/v). The mixture was sonicated for 30 min and then centrifuged at 18,400 x g for 15 min at 4 °C in 50 mL plastic tubes. The supernatant was collected, and the precipitate was re-extracted twice with 10 mL of 80% ethanol, under the previously described conditions. The three supernatants were mixed and filtered using Whatman filter paper N°1. The final ethanolic extract was stored at − 20 °C to be used in the determination of TPC, TFC and antioxidant capacity (DPPH and ABTS). Extractions were performed in three different samples for each salad mixture.

Quantification of bioactive compounds

*Total phenolic content (*TPC) was determined spectrophotometrically using the Folin-Ciocalteu reagent, according to the methodology described by Mazzucotelli et al. [10]. The absorbance was measured at 750 nm in a spectrophotometer (ELx800, Biotek, USA) after 2 h of incubation. Results were expressed as mg of gallic acid equivalents (GAE)/100 g FW.

Total flavonoids content (TFC) of bioaccessible fraction or ethanolic extract was quantified by following the methodology described by Mazzucotelli et al. [10]. The absorbance was read at 496 nm (ELx800, Biotek, USA). The results were expressed as mg of quercetin equivalents (QE)/100 g FW.

Determination of antioxidant capacity

The antioxidant capacity was determined by the scavenging activity of the DPPH radical, and by the Trolox equivalent antioxidant capacity (TEAC) assay, based on the scavenging of the ABTS radical.

DPPH

The DPPH assay was conducted according to the method reported by Mazzucotelli et al. [10]. The mixture was incubated in the dark for 60 min. The absorbance was read at 515 nm in a spectrophotometer (ELx800, Biotek, USA). A blank was prepared by replacing the bioaccessible fraction (or ethanolic extract) for the corresponding solvent. The results were expressed as mg of Trolox equivalents (TE)/100 g FW.

ABTS

The ABTS assay was determined according to Dávila-Aviña et al. [14], with some modifications. To prepare the radical, 19.3 mg ABTS (Sigma, A1888) were dissolved in 5 mL distilled water, and 88 µL $K_2S_2O_8$ solution (2.45 mmol/L) were added. An aliquot of 5 µL of bioaccessible fraction (or ethanolic extract) of each sample was placed into a microplate well and 145 µL of ABTS radical were added. The mixture was kept in the dark for 5 min, and the absorbance was read at 750 nm. For the standard curve, the ABTS solution was incubated with serial dilutions of Trolox (40 µmol/L – 400 µmol/L), under the same described conditions. Results were expressed as mg TE/ 100 g FW.

Microbiological analysis

Microbial counts were determined in the salad mixture at initial time, and after 3, 5, 7 and 10 days of storage at 5 °C. For microbiological analysis, 10 g of mixed vegetables (beet greens, parsley, rocket and red cabbage, in the corresponding proportion) were homogenized with 90 mL of sterile peptone water solution (0.1 mol/L) in a Stomacher 400 Circulator Homogenizer (LAB CIMA, Buenos Aires, Argentina) for 120 s. Serial dilutions (1:10) of each homogenized sample were made in peptone water, as needed for plating. The enumeration and differentiation of microbial groups was performed by using the following media and culture conditions: mesophilic aerobic bacteria, on plate count agar, incubated at 35–37 °C for 24–36 h; psychotropic bacteria, on the same medium incubated at 5 °C for 5–7 days; total coliforms, in MacConkey agar incubated at 35–37 °C for 24 h. Molds and yeasts were incubated on yeast/glucose/chloramphenicol medium at 25 °C for 5 days. Microbial counts were expressed as log CFU/g. The microbial analysis was performed in triplicates.

Sensory quality

To determine the sensory quality of salad mixture, six trained panelists evaluated the product on day 0 (initial day) and after 3, 5, 7 and 10 days of storage at 5 °C. General appearance, color, browning, dehydration, aroma, taste and texture were the quality attributes evaluated. The intensity of each one was quantified on a continuous, unstructured intensity scale from 0 to 5. The applied cut off score for all attributes was fixed at 3, and a score below 3 in one or more attributes indicates that the sample was unacceptable.

Statistical analysis

Results are the average of three independent experiments and were expressed as mean ± standard deviation (SD). Difference between samples were analyzed using analysis of variance (ANOVA) with the R software (version 2.14.0). A Tukey pairwise comparison of the means was conducted to identify where sample differences occurred. The criterion for statistical significance was $p < 0.05$.

Results and discussion

Evolution of bioactive compounds and antioxidant capacity along storage

Bioactive compounds content during storage

TPC and TFC in ethanolic extracts of salad mixtures were measured along storage time, and the obtained results are shown in Fig. 1.

The initial total phenolic content in the salad mixture was 163.3 ± 5.77 mg GAE/100 g FW, and this value resulted higher than the reported for traditional ingredients used for the elaboration of salad mixtures. For example, in lettuce several authors reported a phenolic content around 3.5-fold lesser [13, 15], in tomato around 15-fold lesser [16, 17], and in carrot around 6-fold lesser [18, 19] than in the developed salad mixture. These results indicate that a portion of the innovative salad mixture provides a considerably higher phenolic content than a portion of a salad prepared with traditional ingredients.

During refrigerated storage of the salad mixture, slightly variations of phenolic content were detected (Fig. 1.a). A decreased of total phenolic content was observed during days 3 and 7 of storage, reaching its minimum at day-7 with a decrease of 15% respect to the beginning of the storage. At the end of the storage, total phenolic levels showed no significant difference with respect to day 0 ($p > 0.05$). The relatively slight variation in the content of compounds along storage might be due to the wounding inductive stress caused by cutting leaves. Wounding or physical damage can lead to the production of phenolic compounds in response to the stress caused by the release of certain enzymes from the cell cytoplasm [5, 20]. The amount and profile of wound-induced soluble phenolics is dependent on the type of tissue

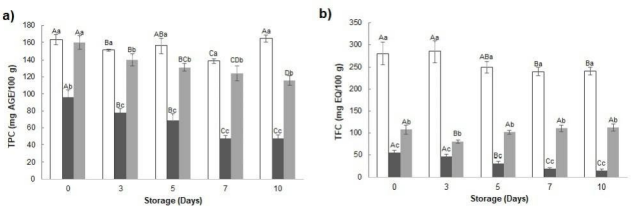

Fig. 1 Total phenolic content (a) and total flavonoid content (b) of salad mixture before (□) and after gastrointestinal digestion (gastric phase ■ intestinal phase ■) of samples stored under refrigerated conditions. Values are expressed as mean ± standard deviation. Capital letters indicate significant differences ($P < 0.05$) between storage time at same treatment (ethanolic, gastric or intestinal extracts). Lower case letters indicate significant differences ($P < 0.05$) between treatments at same storage time

and initial levels of phenolic compounds [20, 21]. Additionally, the presence of other compounds, like ascorbic acid, can justify the general stability of phenolic and flavonoid content during storage [5, 21]. In this way, Reyes et al. [21] evaluated changes in phenolic content of several shredded vegetables stored at 15 °C and found that changes in phenolic content were tissue-dependent. These authors found that while lettuce, celery, carrot, parsnips and sweetpotato showed an increase in phenolic content, other vegetables as zucchini, radish, potato and red cabbage showed a decrease in phenolic content after storage. Besides, only white cabbage showed non-significant changes in phenolic content after 2 days storage at 15 °C [21]. In the same way, phenolic content did not show important changes in minimally processed lettuce, escarole and rocket salad after 3 days of storage at 4 °C [22] and in fresh-cut celery storage during 21 days at 0 and 4 °C [23].

The initial TFC in the salad mixture was 280.96 ± 25.14 mg QE/100 g FW. This value was between 15 and 60-fold higher than reported for vegetables commonly consumed in salad mixtures as tomato, lettuce and carrot [24, 25]. Therefore, this vegetable product could represent an excellent source of bioactive compounds with high impact on the nutrition and health of consumers. Flavonoid content in salad mixture significantly varies along storage (Fig. 1.b) ($p < 0.05$). The maximum level of flavonoids was reached between days 0 and 5, with an average value of 271.95 mg QE/100 g FW, and at the end of storage, the flavonoid content was 14% below the starting value. Referring to other fresh-cut products, the same behavior was reported by Martínez-Sánchez et al. [26] in fresh-cut rocket leaves along a storage of 14 days at 4 °C. Santos et al. [5] reported that in fresh-cut aromatic herbs stored for 10 days at 3 °C, flavonoids content were stable in chives and parsley ($p > 0.05$), but suffered a 29% and 12% decrease in coriander and spearmint, respectively. Curutchet et al. [27] studied the nutritional and sensory quality in fresh-cut mints species during 21 days in refrigerated storage and found different behaviors in flavonoids content evolution between species. As observed for phenolic compounds, the induction response in flavonoid compounds due to wounding could also be tissue and cultivar-dependent.

Antioxidant capacity evolution during storage

Antioxidant capacities evolution (ethanolic extracts) in salad mixtures during storage were measured by DPPH and TEAC methods, and the obtained results are present in Fig. 2.

The initial total antioxidant capacity in the salad mixture was 85.4 ± 7.9 mg TE/100 g FW for DPPH and 318.1 ± 26.5 mg TE/100 g FW for ABTS assays. As observed

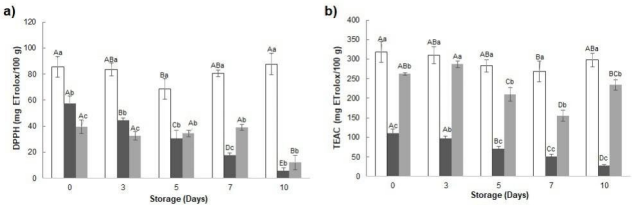

Fig. 2 Antioxidant capacity of salad mixture (a: DPPH assay, b: ABTS assay) before (□) and after gastrointestinal digestion (gastric phase ■ intestinal phase ■) of samples stored under refrigerated conditions. Values are expressed as mean ± standard deviation. Capital letters indicate significant differences ($p < 0.05$) between storage time at same treatment (ethanolic, gastric or intestinal extracts). Lower case letters indicate significant differences ($p < 0.05$) between treatments at same storage time

for TPC and TFC, these antioxidant capacity values were higher than previously reported for traditional ingredients in salad mixtures. For example, in lettuce, several authors reported an antioxidant capacity between 11 and 13.5-fold lower than in the mix [24, 28]; in onion around 3.5-fold lower [19, 24]; in tomato around 5-fold lower for DPPH and 14-fold lower for ABTS assay [14, 24, 29]; and in carrot, around 8-fold lower by DPPH and 29-fold lower by ABTS assay [19, 24].

DPPH activity values showed significant changes ($p < 0.05$) along time of storage, with values between 68.82 and 87.78 mg TE/100 g FW. The salad mixture showed a slight decrease on DPPH capacity during the first days, reaching the minimum at day 5 with a decrease of 19.4% respect to the beginning of the storage. Then, DPPH capacity increased and, at day 10, DPPH value reach similar to the initial ones ($p > 0.05$).

Regarding ABTS antioxidant capacity, results showed a similar evolution to those observed for DPPH capacity during storage. The initial ABTS value of the salad mixture was 318.10 ± 26.51 mg TE/100 g FW, decreasing at day-7 until a value 15.5% lower than the initial one. Then, no significant differences were observed between TEAC capacities at days 0 and 10 ($p > 0.05$).

As mentioned for bioactive compounds content, also evolution of antioxidant capacity of vegetables during storage is shown by other authors as dependent of each crop. For example, increases in DPPH capacity of 442%, 233%, 12%, 77%, 17% and 12% were found in celery, lettuce, parsnips, carrot, white cabbage and sweetpotato, respectively after storage; and decrease of 21%, 51% and 9% for zucchini, potato and red cabbage, respectively [21]. While Curutchet et al. [27] reported that DPPH capacity evolution in fresh-cut mints under refrigerated storage vary between species. On the other hand, in fresh-cut rocket, Martínez-Sánchez et al. [26] reported a decrease in the antioxidant capacity (by ABTS and DPPH) along storage.

In ethanolic extracts, the evaluated vegetable mix showed the best nutritional quality regarding the content of bioactive compounds and antioxidant capacity, at day 0 of storage in refrigeration, reaching the minimum values between days 5 and 7, and reaching values close to the maximum by day 10 of storage, with the exception of TFC.

In vitro digestion effect on the recovery and bioaccessibility of phenolic compounds and antioxidant capacity

The existing bioactive compounds in samples are not completely accessible for this absorption when there are consume, therefore is necessary to evaluate the real efficiency of human digestion in the extraction of this compounds [7]. Ethanolic extract were considered as the 100% of compounds and antioxidant capacity in samples.

The impact of gastrointestinal digestion on TPC is shown in Fig. 1.a. From the analysis of the recovery of phenolics after gastric digestion, it was observed that TPC values after gastric digestion in the functional salad mixture showed to be significantly lower compared to values obtained by chemical extraction, for all storage times ($p < 0.05$). At day 0, the percentage of recovery of phenolics after gastric digestion was 59% and this percentage decreased along storage, reaching 29% at day 10. TPC values found in intestinal extracts were between 1.7 and 2.5-fold higher than in gastric stage at evaluated times. At day 0, bioaccessibility of phenolic compounds were 97.7%, and the intestinal TPC value did not present significant differences between ethanolic extractions. As observed for gastric stage, after intestinal phase TPC values, bioaccessibility and percentage of recovery were decreasing along storage, reaching at day 10 a bioaccessibility and recovery around 70% (Table 1).

The effect of gastrointestinal digestion of salad mixture on TFC values is shown in Fig. 1.b. As observed for phenolic compounds, TFC values after gastric digestion showed to be significantly lower compared to values obtained by ethanolic extraction, for all storage times ($p < 0.05$). The percentage of recovery of flavonoids after gastric digestion was 19.4% at day 0, and this percentage was decreasing along storage, reaching only a 6% of flavonoids recovery at day 10. Similar to TPC, TFC values found in intestinal extracts were between 1.8 and 7.5-fold higher than in gastric stage at evaluated times. In contrast to TPC, the recovery of TFC was significantly lower in intestinal fractions in comparison with ethanolic extracts, at all times ($p < 0.05$). At day 0, bioaccessibility and percentage of recovery of flavonoids were 38.2%. Although the total flavonoid content (ethanolic extraction) decreases slightly over time, the recovery percentage after the intestinal stage remains almost constant during storage without significantly differences between storage times, with exception of Day 3, in which the TFC value of intestinal fraction was 26% lower than in the others. In addition, bioaccessibility of flavonoids slightly increase with storage time, reaching a maximum of 46.72% at day 10 (Table 1).

The fact that TPC and TFC from gastric digesta were significantly different when compared to ethanolic extraction could indicate that these bioactive compounds were not completely released from the food matrix, maybe because of an insufficient extraction time and the utilization of aqueous solvent, or that their chemical structures suffered any change under the physicochemical and enzymatic conditions of the adopted model in the current study [7]. While bioactive compounds contained in liquid matrices are promptly bioaccessible and/or able to exert certain bioactivities along the gastrointestinal tract, those contained in solid matrices must first be extracted to be bioaccessible and bioavailable [30]. The gastrointestinal tract (*in vitro* or *in vivo*) acts as an extraction system where both mechanical and chemical actions during the digestion process contribute to the extraction of bioactive compounds increasing their release throughout the different phases [30]. Physicochemical and enzymatic conditions of the gastric phase (pH 3, pepsin, 2 h of incubation) could not be highly efficient in the release of bioactive components from these vegetal matrixes, since a release between 28 and 59% of the total phenols contained in the plant samples was achieved, and between 6 and 19% of the total flavonoids. On the other hand, environmental and enzymatic conditions of intestinal phase showed to be more efficient as an extractor system for bioactive compounds. It is also possible that the additional time of extraction (plus 2 h) and/or the effect of intestinal digestive enzyme (pancreatin) on the complex food matrix, facilitated the release of phenolics bound to the matrix [7, 29]. This tendency agrees with other authors as Lafarga et al. [20], who reported higher phenolic content

Table 1 Percentage of recovery (%) and bioaccessibility (%) of bioactive compounds and antioxidant capacities

Day	TPC		TFC		DPPH		ABTS	
	% recovery	*Bioaccessibility*	*% recovery*	*Bioaccessibility*	*% recovery*	*Bioaccessibility*	*% recovery*	*Bioaccessibility*
0	97,74	97,74	38,18	38,18	46,30	46,30	82,48	82,48
3	85,72	92,82	28,59	28,20	38,31	39,11	90,19	92,49
5	80,33	83,94	36,04	40,49	40,33	50,04	66,07	74,37
7	75,85	89,33	39,20	45,90	45,74	48,43	48,74	57,71
10	70,90	70,34	39,99	46,72	14,08	13,70	73,63	78,62

and antioxidant capacities in fractions after intestinal stage in ten varieties of lettuce (fresh and minimally processed). In the same way, Kamiloglu et al. [29] reported increases in intestinal fractions in respect to gastric ones in walnut, raisin, almond and hazelnut, whereas fruits as fig or apricot showed a decreased in phenolic values at this stage. On the other hand, other authors have observed that phenolic and flavonoid content declined after duodenal phase, in respect to gastric ones [31–33] declaring that the major phenolic compounds involved are likely unstable under these conditions and could suffer irreversible structural changes.

A possible explanation is that a counter-effect may exist between the extractive effect of bioactive compounds exerted by the gastrointestinal system and the effect of pH on the structure and antioxidant capacity of that compounds [7, 34]. In this sense, some phenolics could suffer chemical changes during the transition from the acidic gastric conditions to the mild alkaline intestinal conditions, where bile salts and pancreatin could also have an impact [34]. Lucas-González et al. [35], working with co-products from persimmon fruit, observed that the global phenolic content decrease from gastric to intestinal digesta phase, although some individual phenolic compounds evidenced an increment.

In the same way, antioxidant capacity of bioactive compounds is also strongly influenced by physicochemical conditions of each phase, because these factors have an important effect on the structure of various compounds [36]. For example, phenolic compounds contain various dissociable –OH groups as part of their chemical structure, and because pH regulates their dissociation and ability to transfer hydrogen atoms or electrons, antioxidant capacity is influenced. Therefore, chemical stability and antioxidant activity of polyphenolic compounds is strongly dependent on the pH of the reaction environment [29, 36].

The influence of GI digestion on free radical scavenging activity determined by ABTS and DPPH assays is shown in Fig. 2.a and 2.b. After gastric digestion, antioxidant capacity by ABTS and DPPH followed the same behavior than TPC and TFC, with significantly lower values in gastric digesta compared to values obtained by chemical extraction, for all storage times ($p < 0.05$). The percentage of recovery of ABTS and DPPH capacity after gastric digestion was 34.8% and 67.1% at day 0, and this percentage decreased along storage, reaching 9% and 6.5% at day 10, respectively.

For ABTS capacity, values in extracts increased between 2.4 and 8.4-fold from gastric to intestinal stages at evaluated storage times. In contrast, DPPH showed a different behavior in intestinal digesta, with significant lower capacity (approximately 1.4-fold) than in gastric at days 0 and 3, but significantly higher (approximately 2.1-fold) at days 7 and 10 ($p < 0.05$).

At day 0, percentage of recovery for ABTS capacity after intestinal phase was 82.5%, and reaching the minimum at day 7, with a recovery of 48.7%. In antioxidant capacity by DPPH, between day 0 to 7, the percentage of recovery remained around 42%, and at day 10, this percentage fall to 14% of initial value (Table 1).

As mentioned above, the effect of gastrointestinal conditions is reflected in the chemical stability and bioactivity of a phenolic compound, and, consequently, in its antioxidant activity. While certain compounds could modify their structure and bioactivity at the pH of same gastrointestinal phase, others may remain unchanged throughout the digestive tract [36]. In this way, Wootton-Beard et al. [37] studied the stability of TPC and antioxidant capacity of 23 commercially vegetable juices subjected to in vitro digestion and found that the antioxidant capacity of most samples, as measured with the DPPH assay, had a slight increase after the gastric phase and a slight decrease after the duodenal phase,

Table 2 Sensory evaluation of salad mixture storage at refrigerated conditions (5 °C)

Quality attributes	Days				
	0	**3**	**5**	**7**	**10**
General appearance	5.00 ± 00	4.30 ± 0.42	3.78 ± 0.42	3.42 ± 0.54	**2.90 ± 0.95**
Color	5.00 ± 00	4.40 ± 0.73	3.78 ± 0.81	3.20 ± 0.62	**2.92 ± 0.97**
Browning	5.00 ± 00	4.43 ± 0.53	4.22 ± 0.33	3.45 ± 0.19	3.22 ± 1.13
Dehydration	5.00 ± 00	4.53 ± 0.33	3.86 ± 0.61	3.48 ± 0.55	**2.90 ± 1.01**
Aroma	5.00 ± 00	4.47 ± 0.34	3.74 ± 0.59	**2.90 ± 0.52**	**2.70 ± 1.02**
Taste	5.00 ± 00	4.63 ± 0.30	3.64 ± 0.64	3.36 ± 0.19	ND
Texture	5.00 ± 00	4.60 ± 0.42	3.56 ± 0.40	3.58 ± 0.82	ND

General appearance score: 5 = very attractive; 3 = fair; 0 = unattractive, deteriorated

Color score: 5 = characteristic; 0 = decolored

Browning and dehydration scores: 5 = none; 2.5 = moderate; 0 = severe

Aroma and taste scores: 5 = fresh, characteristic; 3 = moderate; 0 = not characteristic, oddish

Texture: 5 = turgid; 0 = soft

The score of 3 was considered the limit of consumer acceptance

Numbers in bold are scores below the acceptability limit

although there were some exceptions to this trend. Results of ABTS antioxidant capacity showed that, in contrast to DPPH, 12 juices showed significant increase in antioxidant capacity after the duodenal phase [37]. The authors therefore proposed that the observed trends are dependent to the chemical characteristics of the major phenolic compounds found in each sample.

Sensory quality

The most important factors that may describe quality for fruits and vegetables could be evaluated through different attributes, such as appearance (visual quality, color), aroma and taste (flavor) and texture [38] (Table 2). The applied cut off score for all attributes was fixed at 3, and a score below 3 indicates unacceptable sample. Even though taste and texture were above the acceptance limit still at day 7, these attributes were not evaluated at day 10 due to the microbiological quality of the samples were not safe to panelists health.

Considering that consumers often buy the first time based on appearance, maintaining a fresh appearance during storage is a critical factor to consumer acceptance. In this way, considering the attributes associated with the appearance of the product (General Appearance, Dehydration, Color and Browning), these were decreased progressively with storage time but remained above the acceptance limit even on day 7. On the other hand, it is known that crispy and crunchy textures are a desirable quality in these products, since consumers associate them with freshness, therefore factors such as flavor or texture conditioned the subsequent sales. In this vegetal product, taste and texture were above the acceptance limit ever still at day 7, and that indicate a good maintaining of these qualities along storage; however, at day 7, undesirable odors were detected by the panelists. García-Martínez et al. [39] and Neto et al. [2] found the same behavior for fresh-cut lettuce and artichoke, where the presence of off-odor was the first parameter to achieve the tolerance limit. The appearance of off-flavors in minimally process vegetables could be due to different factors. During processing of fresh-cut vegetables, mechanical wounding causes disruption of cells, which induces physiological responses such as an increase in respiration rate. Certain levels of CO_2 and O_2 could accumulate into the package leading to anaerobic respiration in vegetal tissues and favoring the growth of bacteria who shift of aerobic to fermentative metabolism [6, 40]. In consequence, a variety of volatile compounds could develop, as ethanol, acetaldehyde, CO_2, and other chemical compounds that contribute to the appearance of off-odors [6, 40].

Regarding sensory quality, the shelf-life of the salad mixture was limited by aroma, with the presence of off-odors at unacceptable levels at day 7. However, it is important to consider that the shelf life of perishable food products should always be established by combining the microbial shelf life and the sensory shelf life [41].

Microbiological quality

In general, total counts of microbiological populations on minimally processed vegetables immediately after processing range from 3.0 to 6.0 log CFU/g [41]. In the present work, the salad mixture showed initial counts around 5 log CFU/g for all study microbial populations (5.3 log CFU/g for mesophiles; 5.8 for psychrophiles; 5.1 for Enterobacteriaceae; 4.9 log CFU/g for mold and yeast) (Fig. 3). All four populations showed an increase in microbial counts along storage. Initial mesophilic count in mixed vegetables significantly increased (4 log) between days 0 and 10. The evolution of psychrophiles and Enterobacteriaceae were like that described for mesophilics, with increases of 4 and 4.5 log, respectively. Molds and yeast showed lower counts throughout storage (from 4.9 to 8.7 log CFU/g). The same behavior in microbial groups evolution under refrigerate storage were found in others fresh-cut vegetables as lettuce and kale [42, 43].

The microbial limit for consumption of fresh processed vegetables by consumers imposed by the French law is 7.7 log for total mesophilic bacteria [44]. Therefore, in the present work, when the salad mixture is processed and stored under the current conditions, the self-life of the product is no longer than 5 days.

Minimally processed vegetables have a physical structure that is susceptible to microbiological invasion. Processing frequently causes mechanical injuries of tissues, leading

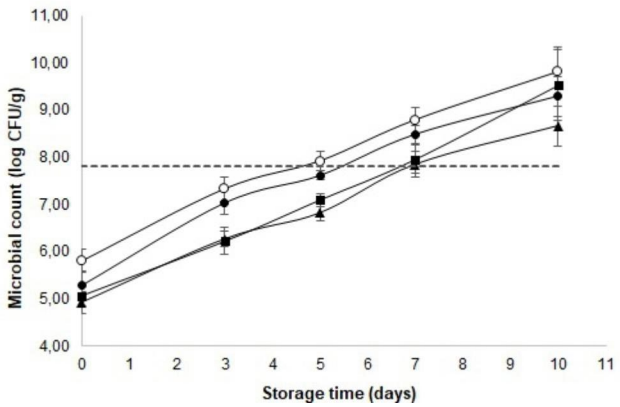

Fig. 3 Evolution of (●) mesophilic aerobes, (○) psychrotrophics, (■) coliforms and (▲) yeasts and molds (log CFU/g FW) in salad mixture store under refrigerated condition. Horizontal line indicates the shelf-life limiting number of 7.7 log CFU/g FW. Data represent the mean of three determinations and vertical bars represent standard deviation of the mean

to loss of water and formation of exudates rich in minerals, sugars, vitamins and other nutrients that favored the growth of autochthonous microbiota which accelerates the process of degradation and deterioration of these products [45, 46]. Besides microbiological activity, the presence of damaged areas results in a stress response of the produce itself, such as an increase in the respiration rate and ethylene production, resulting in faster metabolic rates. Moreover, damage leads to exposure to air, desiccation and exposure of enzymes to their substrates, all leading to quality degradation [41, 45]. Due to the sum of these factors, the shelf life of minimally processed vegetable products is shorter than that of whole products.

Conclusion

The development of this vegetable mix with high antioxidant capacity and composed by new varieties of vegetables is understood as a new innovative functional product. This mix not only brings to consumers closer to a new source of bioactive compounds, boosting their consumption but also provides add value to certain vegetable parts that are generally discarded. Moreover, it is important to determine the bioactivity of relevant compounds during the mix's shelf life in order to ensure consumers their health-promoting properties. According to results, this vegetable mix had a maximal shelf life of 5 days in refrigerated storage. Bioactive compounds and their antioxidant capacities vary in small quantities along product´s shelf life (between days 0 and 5), but percentage of recovery of these compounds and their antioxidant capacities reach their maximum value at the beginning of storage, being 97.7% for phenols, 38.2% for flavonoids, 46.4% for DPPH and 82.5% for ABTS. In all cases, these percentages decrease during the product´s shelf life reaching at day 5 values of 80.3% for phenols, 36.0% for flavonoids, 40.3% for DPPH capacity and 66.1% for ABTS capacity, in respect to total initial values. The increase or decrease in bioactive compounds content from gastric to intestinal digesta could be determined by the stability of the major bioactive compounds in each sample. Although some compounds may be stable and others undergo structural changes, the evolution of the majority compounds will impact on their bioaccessibility. In order to obtain more accurate results, in subsequent analyses, it would be necessary to characterize the extracts by HPLC to identify the bioactive compounds after each digestion stage and to analyse the relation between the antioxidant capacity detected and the chemical variations suffered by the compounds in each digestion step.

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Authors' contributions CAM was responsible for conceiving the idea and experimental design, carried the experimental work, analysis and interpretation of data and wrote the manuscript; VEIO carried out the experiments; MRA and KCDS supervised the work and corrected the manuscript.

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Data Availability All data and materials are available.

Code Availability Not applicable.

Declarations

Conflicts of interest/Competing interests There are no conflicts of interest.

Ethics approval Not applicable.

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