Bioaccessibility of polyphenols and antioxidant capacity of fresh or minimally processed modern or traditional lettuce (*Lactuca sativa* L.) varieties

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Abbreviations:

TPC: Total phenolic content; TCEP: Tris(2-carboxyethyl)phosphine hydrochloride; FCR: Folin-Ciocalteu's reagent; FRAP: Ferric reducing antioxidant power; UPOV: International Union for the Protection of New Varieties of Plants.

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Abstract

Modern city lifestyle is characterized by an increased demand for fresh or minimally processed foods. Lettuce (*Lactuca sativa* L.), mainly iceberg lettuce, is the main vegetable used during the manufacture of fresh-cut salads. The current study evaluated the phenolic content and antioxidant activity of ten fresh and minimally processed lettuce varieties. The phenolic content of selected lettuce samples varied significantly among varieties. Although a higher phenolic content was observed in modern lettuce varieties, when compared to the traditional ones (except for the landrace *Francès 219/855*), the antioxidant capacity of modern and traditional lettuce varieties was similar. Minimal processing followed by storage for a 7-day period led to an increased phenolic content in varieties *Rutilaï RZ*, *Abago RZ*, *Maravilla LS044*, *Francès 219/855*, *Negre borratger 386/935*, and *D'hivern LS008*, supporting the hypothesis that wounding can induce the accumulation of phenolic compounds in lettuce leaves. For example, the total phenolic content of *Francès 219/855* after processing and storage increased from 8.3 to 11.3 mg/100 g (p<0.05). Accumulation of phenolic compounds after minimal processing was not observed in all the studied samples, suggesting that this effect could be matrix-dependant. The amount of bioaccessible polyphenols was higher after minimal processing and storage. Indeed, the amount of bioaccessible polyphenols after a simulated gastrointestinal digestion of fresh or minimally processed *Pelikan* lettuce was calculated as 32.6 or 43.3 mg/100 g respectively (p<0.05), suggesting that the increased amount of polyphenols caused by processing and storage can also lead to a higher amount of bioaccessible phenolic compounds.

Keywords: lettuce, *Lactuca sativa*, antioxidant activity, minimal processing, bioaccessibility, polyphenols

1. Introduction

The World Health Organisation (WHO) recommends the consumption of at least 400 g of fruits and vegetables per day (Appleton et al. 2017) because consumption of fruit and vegetables contribute to wellness and disease prevention. Indeed, it has been associated with reduced incidence of all-cause mortality and mortality from cardiovascular diseases including coronary heart disease and stroke as well as with reduced risk of suffering from hypertension, osteoporosis, dementia, some types of cancer, and cognitive decline among other positive health outcomes (Appleton et al. 2016).

Approximately 55% of the current worlds' population lives in urban areas and this proportion is expected to increase to 68% by 2050 (FAO 2017). Modern city lifestyle is characterized by an increased demand for ready-to-eat fresh or minimally processed foods. Indeed, 33% of global fruit and vegetable product launches featured some kind of convenience claim in 2017 (MINTEL 2017). Minimal processing, which includes operations such as peeling, cutting, and dipping, does not negatively affect the nutritional properties of foods as other more intense processing strategies (Lafarga et al. 2018a). For example, Martínez-Sánchez et al. (2012) reported an increase in the content of flavonols during storage of baby-sized lettuce (*Lactuca sativa* L.) leaves. Similar results were reported by Cefola et al. (2016), who observed an increase in the content of cyaniding-3-*O*-glucoside after minimal processing and storage of radicchio (*Cichorium intybus* L.) leaves from 2.63 mg/100 g at day 0 to 3.57 mg/100 g at day 12.

Lettuce, principally iceberg lettuce, is currently the main vegetable used during the manufacture of fresh-cut salads. Lettuce is of particular interest due to its high antioxidant and phytochemical content (Malejane et al. 2018). Most relevant phytochemicals found in lettuce include vitamin B_9, vitamin C, vitamin E, carotenoids, and polyphenols. The latter refer to a group of secondary metabolites responsible for the plants' defense system

that have been described to have higher antioxidant capacity than, for example, vitamin C and E (Kim et al. 2016). Although their mechanisms of action are not yet fully understood (Fraga et al. 2019), a large number of epidemiological studies, and their associated meta-analyses, suggested that long-term consumption of diets rich in plant-derived polyphenols and antioxidants offer protection against development of diseases associated to metabolic syndrome, different types of cancer, osteoporosis, and neurodegenerative diseases (Pandey and Rizvi 2009). Chong et al. (2010) reviewed the evidence for the effects of fruit polyphenols on platelet function, blood pressure, vascular function, and blood lipids, all of them risk factors of cardiovascular diseases. The authors of that study concluded that despite the heterogeneity in the design of studies, the lack of controls, and the short intervention periods, there is evidence to suggest that flavonols, anthocyanins, and procyanindins are effective at reducing the above-mentioned risk factors. Similar conclusions were published by Williamson and Manach (2005) after reviewing 93 human intervention studies. In this case, the authors reported that despite the lack of *in vivo* biomarkers and long-term studies, for some classes of polyphenols such as isoflavones, catechins, procyanidins, or flavonols (quercetin) there are sufficient intervention studies to demonstrate short-term changes in biomarkers. Williamson and Manach (2005) also suggested the need for increasing the length of human intervention studies and to consider bioavailability and bioaccessibility in *in vitro* studies.

During the last years, the use of alternatives to iceberg lettuce such as baby leaves or other lettuce varieties has gained increased interest (Fadda et al. 2016) and plant breeders have shown special interest in increasing the phenolic and antioxidant content of lettuce to meet consumer demands (Martínez-Sánchez et al. 2012). It is important to determine the lettuce type that provides not only innovative products but also the highest content of health-promoting compounds such as polyphenols. However, the health benefits of plant

polyphenols can only be effective if they reach the relevant tissues in a dose that allows a biological effect. Bioavailability measures the amount of a certain compound that is absorbed and accessible to produce systemic effects after ingestion (Toutain and Bousquet-Mélou 2004). Some of the main factors affecting bioavailability are resistance to food processing and bioaccessibility. Minimal processing does not generally affect the nutritional value of foods. Therefore, it is of key importance to study the bioaccessibility of polyphenols, which is a measurement of the amount of these compounds that is released from the food matrix during digestion (Ribas-Agustí et al. 2017).

The aims of this study were to: (i) compare the total phenolic content (TPC) and bioaccessibility of polyphenols of ten lettuce varieties that include modern and traditional varieties, which showed potential for being used in fresh-cut salads, and (ii) assess the effect of minimal processing on the bioaccessibility of polyphenols and antioxidant capacity of the selected vegetables.

2. Materials and methods

2.1 Chemicals and reagents

Ferric chloride and methanol were purchased from Panreac (Barcelona, Spain). Gallic acid, ascorbic acid, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrochloric acid, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), potassium phosphate monobasic, potassium phosphate dibasic, calcium chloride, α-amylase (EC 3.2.1.1), pepsin (EC 3.4.23.1), 1,2- benzenedithiol, pancreatin, fresh bile, sodium hydroxide, and sodium carbonate were purchased from Sigma-Aldrich (Madrid, Spain). Folin-Ciocalteu's reagent (FCR) was purchased from VWR (Llinars del Vallès, Spain). All reagents used were of analytical grade.

2.2 Plant material: Minimal processing

Five traditional landraces and five modern lettuce varieties, shown in Figure 1, were studied. Plants were grown in open field during the winter season in Viladecans (Northeast Spain: 41º17'19.3''N 2º02'42.5''E) and were cultivated using plastic mulch and irrigated with drip tapes. Minimal processing was carried out at the pilot plant facilities of IRTA Fruitcentre, Lleida, Spain. After selection for uniformity of size, colour, and freedom from defects, the external leaves of the heads of lettuce were removed. Heads were then manually cored using a sharp knife and the internal leaves were separated and cleaned using tap water at 4 ºC. Lettuce leaves were cut into pieces of approximately 5 × 5 cm, sanitized by immersion into a 100 ppm sodium hypochlorite solution (pH 6.5, 4 ºC) for 2 min, rinsed using tap water at 4 ºC to remove residual chlorine, and centrifuged using a Marrodan PR47248 industrial scale centrifuge (Marrodan Food Technology, Navarra, Spain) at 350 rpm during 1 min. At this stage, samples were divided into two lots. One was immediately frozen at day 0 using liquid nitrogen, milled using a

MINIMOKA GR-020 grinder (Taurus Group, Barcelona, Spain), and stored at -80 ºC until further analysis (results shown in Figure 2A, Figure 3A, and Figure 4A). Although these samples were processed (cut and sanitised), as samples were immediately frozen using liquid nitrogen we can assume that processing had no effect on the studied parameters and therefore this samples will be referred as fresh lettuce. The other lot was stored as follows: approximately 100 ± 2 g of lettuce were sealed under air in 230 × 160 mm biaxially oriented polypropylene bags, commercially used for storage of minimally processed lettuce. Samples were stored for 3 days at 4 ºC in the dark and then transferred to 7 ºC for 4 days. After this period, the bags were opened and lettuce leaves were immediately frozen using liquid nitrogen, milled using a MINIMOKA GR-020 grinder (Taurus Group, Barcelona, Spain), and stored at -80 ºC until further analysis (results shown in Figure 2B, Figure 3B, and Figure 4B). As these samples were processed and stored, they will be referred as minimally processed lettuce.

2.3 Simulated gastrointestinal digestion

The methodology followed to determine bioaccessibility was the standardised static *in vitro* method described by Minekus et al. (2014) with some modifications. This method is an international consensus, which consists of three sequential stages, and was developed by members of the EU Cost Action INFOGEST, an international network joined by over 200 scientists from over 30 different countries. The method includes an oral stage (pH 7.0, α-amylase), a gastric stage (pH 3.0, pepsin), and an intestinal stage (pH 7.0, pancreatin and fresh bile). Briefly, for the oral phase, 5.0 g of lettuce were homogenised using a T-25 ULTRA-TURRAX® homogeniser (IKA, Staufem, Germany) with: (i) 3.5 mL of a simulated salivary fluid (15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2 \cdot (H_2O)_6$, 0.06 mM $(NH_4)_2CO_3$, and 1.2 mM HCl); (ii) 0.5 mL of α-amylase (1,500 U/mL); (iii) 25 µL of 0.3 M $CaCl_2$, and (iv) 975 µL of distilled water.

After incubation at 37 ºC for 2 min, the oral bolus was mixed with: (i) 7.5 mL of simulated gastric fluid (6.9 mM KCl, 0.9 mM KH_2PO_4, 25.0 mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2 \cdot (H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$, and 25.2 mM HCl); (ii) 1.6 mL of porcine pepsin (25,000 U/mL); (iii) 10 µL of 0.15 M $CaCl_2$; (iv) 200 µL of 1 M HCl – to reach pH 3.0; and (v) 690 µL of distilled water. The mixture was incubated at 37 ºC with gentle shaking (150 rpm) for 2 h. After the gastric phase, 10 mL of the mixture were collected and centrifuged at 13,523 × *g* for 15 min. The supernatant was immediately frozen with liquid nitrogen and stored at -80 ºC until further analysis. Moreover, 10 mL of the gastric chime were further mixed with: (i) 5.5 mL of simulated intestinal fluid (6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.3 mM $MgCl_2 \cdot (H_2O)_6$, and 16.2 mM HCl); (ii) 2.5 mL of pancreatin (800 U/mL, based on trypsin activity), (iii) 1.25 mL of bile (160 mM), (iv) 20 µL of 0.3 M $CaCl_2$; (v) 0.145 mL of 1M NaOH – to reach pH 7.0; and (vi) 0.585 mL of distilled water. The mixture was incubated at 37 ºC and 150 rpm for 2 h. The pH was controlled every 20 min and 1 M HCl was used when necessary to keep the pH constant at 7.0. After the intestinal phase, 10 mL of the mixture were collected and centrifuged at 13,523 × *g* for 15 min. The supernatant was immediately frozen with liquid nitrogen and stored at -80 ºC until further analysis. A blank was prepared using only distilled water instead of sample and following the same procedure. Determinations of TPC and antioxidant activity were carried out in triplicate per sample and per replicate after both gastric and intestinal phases.

2.4 Total phenolic content

The TPC was determined by the Folin-Ciocalteu method and the modifications reported by Lafarga et al. (2018b). Briefly, for the extraction, the milled lettuce samples were homogenized with methanol 70% (v/v) at a sample:methanol ratio of 3:10 (w/v) using a T-25 ULTRA-TURRAX® homogenizer (IKA, Staufem, Germany) operating at 12,000

rpm for 30 s. Extraction was performed under gentle stirring at room temperature (22 ± 1 ºC) during 120 min. The obtained mixtures were centrifuged using a Sigma-3-18 KS centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 10,000 × *g* for 20 min. The supernatant was immediately frozen using liquid nitrogen and stored at -80 ºC until further use.

The assay was performed by adding 4.3 mL of MilliQ water and 0.5 mL of FCR to 0.7 mL of extract (either methanolic extract or enzymatic digestive extract). After incubation for 5 min at room temperature in the dark, 2 mL of saturated sodium carbonate solution was added. The mixture was shaken and further incubated for 1 h at room temperature and in the dark. Absorbance was read at 760 nm using a GENESYS^{TM} 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, MA, USA). The TPC was determined in triplicate and results were expressed as mg of gallic acid equivalents per 100 g of fresh weight (FW). Standard curves were prepared daily.

2.4 Ferric ion reducing antioxidant power assay

Antioxidant activity was assessed using the ferric ion reducing antioxidant power (FRAP) and following the methodology described by Lafarga et al. (2019a). Briefly, the FRAP reagent was freshly prepared by mixing 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM hydrochloric acid, and 20 mM ferrous chloride in the proportion 10:1:1 (v/v/v). Determinations were carried out by mixing 1.4 mL of the FRAP reagent and 0.1 mL of the methanolic extract (or the enzymatic digestive extract) and after 20 min of incubation in the dark at 37 ºC and constant shaking. The absorbance was read at 593 nm using a GENESYS^{TM} 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, MA, USA). Antioxidant activity was determined in triplicate and expressed as mg of ascorbic acid equivalents per 100 g of FW. Standard curves were prepared daily.

2.5 2,2-diphenyl-1-picrylhydrazyl free radical assay

Antioxidant activity was also assessed using the DPPH assay and following the methodology described by Lafarga et al. (2019a). Briefly, the DPPH assay was performed by adding 1.4 mL of 0.1 mM DPPH· solution to 0.1 mL of the methanolic extract or the enzymatic digestive extract. After 60 min of incubation at room temperature and in the dark, the absorbance was read at 515 nm using a GENESYS^{TM} 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, MA, USA). Antioxidant activity was determined in triplicate and expressed as mg of ascorbic acid equivalents per 100 g of FW. Standard curves were prepared daily.

2.6 Statistical analysis

Results are the average of three independent experiments and were expressed as mean ± standard deviation (S.D.). Difference between samples were analysed using analysis of variance (ANOVA) with JMP 13 (SAS Institute Inc., Cary, USA). A Tukey pairwise comparison of the means was conducted to identify where sample differences occurred. The criterion for statistical significance was $p<0.05$.

3. Results and discussion

3.1 Antioxidant capacity and total phenolic content of fresh unprocessed lettuce leaves

Kim et al. (2016) recently reviewed the nutritional value and health benefits of lettuce and observed that the TPC in lettuce varies significantly among lettuce types and varieties. Indeed, the authors of that study observed that the TPC in Crisphead (iceberg) lettuce was generally lower when compared to pigmented varieties. Results were in line to those of Llorach et al. (2008), who reported the TPC of Iceberg, Romaine, Continental, Red oak leaf, and Lollo rosso lettuce as 18.2, 63.5, 125.5, 322.1, and 571.2 mg/100 g, respectively. In the current paper, the TPC of fresh lettuces varied from 3.05 ± 0.13 mg/100 g in *Maravilla LS044* to 15.02 ± 0.07 mg/100 g in *Pelikan* (Figure 2A). Similar TPC values were reported previously (Bahorun et al. 2004). A higher TPC was observed in the red leaf lettuce variety *Francès 219/855* when compared to the green lettuce types – except for *Francesca* lettuce which had the highest TPC (p<0.05). A higher phenolic content in red leaf and red Romaine lettuces when compared to green lettuce types has been reported previously (Kim et al. 2016, Llorach et al. 2008, Martínez-Sánchez et al. 2012). For example, in a study carried out by Nicolle et al. (2004), the TPC ranged from 8.4 to 12.9 mg/g in green varieties and reached 27.8 mg/g in a dry weight basis, in Red oak leaf lettuce. The lower TPC of green varieties could be caused by the absence (or lower amount) of anthocyanins, a subgroup of coloured phenolic compounds known for their high antioxidant capacity (Kim et al. 2016). Overall, modern varieties assessed in the current study had a higher TPC when compared to traditional lettuce varieties (p<0.05). This does not mean that modern varieties have a higher TPC when compared to traditional landraces, as a study with a larger number of samples would be needed to confirm these results. The TPC modern varieties ranged between 5.41 ± 0.23 and 11.89

± 0.05 mg/100 g while the TPC of traditional varieties ranged between 2.84 ± 0.06 and 6.62 ± 0.24 mg/100 g. Phenolic profile and content can also be influenced by environmental factors (Nicolle et al. 2004). However, in the current study all varieties were grown under the same conditions and in the same field.

TPC of lettuce varieties upon reception was positively correlated with FRAP ($r^2 = 0.768$; $p<0.05$) and DPPH ($r^2 = 0.910$; $p<0.05$) values. FRAP and DPPH values are shown in Figure 3A and Figure 4A, respectively. The modern variety *Pelikan* had the highest ($p<0.05$) DPPH and FRAP values, calculated as 11.89 ± 0.05 and 8.29 ± 0.05 mg/100 g, respectively. The traditional variety *Francès 219/855* also showed a relatively high antioxidant capacity before minimal processing with DPPH and FRAP values of 7.24 ± 0.11 and 6.62 ± 0.24 mg/100 g. These two varieties showed the highest TPC ($p<0.05$). Results obtained in the current study were similar to those reported previously (Szeto *et al.* 2002). However, results obtained in the current paper were lower when compared to those reported by Llorach et al. (2008), who observed FRAP and DPPH values ranging between 98.2-814.4 and 68.6-775.3 mg/100 g, respectively. The use of different lettuce varieties and field or growth conditions in both studies could affect the antioxidant activity of vegetables. The different extraction protocol used in both studies could also partially explain these differences. Not only the content of total polyphenols affects the antioxidant capacity of foods but also the type of polyphenols. Several thousands of molecules having a polyphenols structure have been identified in higher plants and algae and these may be classified into different groups. Main groups include: (i) phenolic acids, which can be subdivided into those derived from benzoic acid and those derived from cinnamic acid; (ii) flavonoids, such as quercetin and kaempferol; (iii) stilbenes, which include resveratrol; and (iv) lignans (Manach et al. 2004). Previous studies observed significant differences in the phenolic compound composition of lettuce varieties. For

example, Martínez-Sánchez et al. (2012) reported that phenolic acids were the main group in green leaf lettuce varieties, while flavonols were most abundant in red-leafed lettuce genotypes. The authors of that study also reported that anthocyanins, which are highly antioxidant pigments, which belong to the flavonol group, were only present in the red-leafed varieties. Further studies assessing the phenolic composition of the lettuce varieties studied herein would allow to better understand the influence of each phenolic group on the observed antioxidant capacity. Similar results were reported by Kim et al. (2016), who calculated the content of phenolic acids in green-leafed and red-leafed lettuce varieties as 70-94 and 35-45% respectively, and by Llorach et al. (2008). Moreover, other phytochemicals that are not phenolic compounds and are present in lettuce such as carotenoids, vitamin B_9, vitamin C, or vitamin E are also responsible for the observed antioxidant capacity and health-promoting properties of lettuce (Kim et al. 2016).

3.2 Effect of minimal processing and storage on the antioxidant capacity and total phenolic content of selected lettuce varieties

The TPC of fresh-cut lettuce varieties after minimal processing and a 7-day storage ranged between 3.99 ± 0.23 and 15.09 ± 0.08 mg/100 g for *Maravilla LS044* and *Pelikan*, respectively. Results were comparable to those obtained before minimal processing, as both varieties showed the lowest and highest TPC, respectively (p<0.05). Minimal processing and storage for 3 days at 4 ºC, followed by storage for 4 days at 7 ºC, resulted in a significant increase in the TPC of the samples (Figure 2B; p<0.05), except for the varieties *Muraï RZ*, *Pelikan*, *Francesca*, and *Carxofeta LS007*. Wounding induced the accumulation of phenolic compounds in Iceberg and Romaine lettuce leaves previously. For example, Luna *et al.* (2012) observed a 4-fold increase in the TPC of minimally processed lettuce after a 2-day storage period in air at 7 ºC. Martínez-Sánchez et al. (2012) also reported an increase in the TPC of green leaf, red leaf, and lollo rosso lettuce during

storage, at the same conditions studied in the current paper, which was especially high at days 7 and 8. It is widely accepted that wounding or physical damage can promote biochemical reactions responsible for an increased respiration rate or the production of phytochemicals including polyphenols (Saltveit, 2003). Cutting increases phenolic metabolism in lettuce midrib with the accumulation of soluble polyphenols that react to produce wound-induced tissue browning (Martinez-Sánchez et al. 2012). Results can also be partially attributed to a higher extraction efficiency caused by cell wall disruption during processing and storage. Moreover, determination of individual polyphenols using HPLC would help to demonstrate the observed increase in TPC, as the Folin-Ciocalteus' reagent can react with non-phenolic compounds leading to an overestimation of the TPC (Lafarga et al. 2019a).

Antioxidant capacity values, shown in Figure 3B and Figure 4B, were in line to those obtained for TPC. Minimal processing followed by a 7-day storage period resulted in increased FRAP and DPPH values ($p<0.05$), except for FRAP values of *Maravilla LS044* and *Carxofeta LS007*. FRAP values ranged between 1.64 ± 0.17 and 10.13 ± 0.24 mg/100 g while DPPH values ranged between 3.00 ± 0.25 and 14.07 ± 0.25 mg/100 g. In both cases, the lowest antioxidant activity was observed for sample *Maravilla LS044* while *Pelikan* showed the highest antioxidant capacity ($p<0.05$). A positive correlation was observed between TPC and antioxidant capacity after storage with r^2 values of 0.837 and 0.899 for DPPH and FRAP values, respectively ($p<0.05$). Similar results were observed by Kang and Saltveit (2002) who reported a positive correlation between TPC and antioxidant activity values with r^2 values of 0.97 and 0.95 for Iceberg and Romaine lettuce leaf tissues. In the current paper, the observed increase in antioxidant activity during storage was not the same for all the studied varieties, probably caused by different bioactivity of the polyphenols generated in by different varieties after processing. Indeed,

Kang and Saltveit (2002) observed that wound-induced phenolics generated by Romaine lettuce varieties had higher antioxidant capacity when compared to those produced in *iceberg* lettuce varieties. The same overall trend was observed when assessing the antioxidant capacity using the FRAP or the DPPH method. However, some differences were observed between both methods. For example, no differences were observed between the DPPH value of fresh and minimally processed *Abago RZ*, while the FRAP value increased from 2.02 ± 0.21 to 2.52 ± 0.14 mg/100 g (p<0.05). The observed differences can be attributed to the differences between both methods as it is not generally easy to observe a good agreement between methods (Prior et al. 2005). Both FRAP and DPPH assays are based on the ability of a molecule to transfer one electron to reduce another compound. However, the DPPH radical can be neutralised either by direct reduction via electron transfer or by radical quenching via H atom transfer (Jiménez et al. 2004). Moreover, carotenoids can interfere with the DPPH assay and the FRAP assay cannot detect compounds that act by radical quenching and assumes that the redox reaction is complete within a few minutes and this is not always true (Prior et al. 2005).

3.3 Phenolic content and antioxidant capacity of enzymatic digestive extracts of fresh and minimally processed lettuce

The current study aimed at calculating the total amount of bioaccessible polyphenols obtained after a simulated gastrointestinal digestion of fresh lettuce. As mentioned previously, the assessment of the bioaccessibility of polyphenols, which is a measurement of the amount of these compounds that is released from the food matrix during digestion, is of key importance (Ribas-Agustí et al. 2017). The main reason for this is that literature data on the phenolic content and/or composition of foods are partial and insufficient to determine dietary intakes and there is a lack of comprehensive data on intake of polyphenols – although some studies provided individual data concerning intake of

specific groups of polyphenols such as flavonols (Saura-Calixto et al. 2007). Results, shown in Figure 2A demonstrate that *in vitro* gastrointestinal digestion resulted in increased TPC in all the studied modern and traditional varieties (p<0.05). Previous studies observed increased TPC of vegetables after *in vitro* gastrointestinal digestion. For example, Chen *et al.* (2014) observed an increase in the TPC of tomatoes and apples from 30.7 and 56.9 to 93.5 and 106.6 mg/100 g, respectively. Similar results were reported for cereals (Chandrasekara and Shahidi 2012), pulses (Lafarga et al. 2019a), and vegetable-derived beverages (Lafarga et al. 2019b, Rodríguez-Roque *et al.* 2013). A positive correlation was observed between the amount of bioaccessible polyphenols and FRAP (Figure 3A) or DPPH (Figure 4A) values after *in vitro* gastrointestinal digestion of unprocessed samples with r^2 values of 0.891 and 0.795 (p<0.05), respectively. The same trend was observed after a simulated digestion of minimally processed samples. Indeed, *in vitro* digestion of minimally processed samples resulted in increased TPC in all the studied varieties (p<0.05). The content of bioaccessible phenolics in minimally processed samples ranged between 14.44 ± 0.44 and 58.96 ± 0.89 mg/100 g for *LS044* and *Francès 219/855*, respectively (Figure 2B). The increased extractability of polyphenols has been attributed to the acidic pH and the enzymatic activity at this digestive phase, which can induce the hydrolysis of polyphenols bound to other constituents of the food matrix (Rodríguez-Roque et al. 2013). The longer extraction time of enzymatic extracts obtained after the intestinal phase (4 h), if compared to values prior to digestion (2h) or to the enzymatic extracts obtained after the gastric phase (2 h), may also partially explain these findings. Therefore, we can conclude that the observed increase in antioxidant capacity after the intestinal phase of digestion can be attributed to an increased liberation of polyphenols during the gastric and intestinal phases of digestion. The higher TPC in the enzymatic digestive extracts of minimally processed samples resulted in an increased

antioxidant capacity when compared to the values observed prior to digestion (p<0.05). Indeed, FRAP values after *in vitro* digestion of fresh-cut samples ranged between 3.90 ± 0.02 and 16.36 ± 0.07 mg/100 g while DPPH values ranged between 5.49 ± 0.06 and 32.07 ± 0.62 mg/100 g.

The current paper also aimed at assessing the effect of minimal processing and storage on the amount of bioaccessible polyphenols in lettuce samples. Overall, minimal processing and storage during 7 days, at the conditions evaluated in the current study, resulted in increased bioaccessibility of polyphenols (p<0.05). For example, the TPC of the enzymatic digestive extracts obtained after the intestinal phase of digestion of fresh and minimally processed *Pelikan* lettuce was 32.65 ± 0.16 and 43.32 ± 0.29 mg/100 g, respectively (p<0.05). The same increase in bioaccessible polyphenols was observed in minimally processed *Rutilaï RZ*, *Abago RZ*, *Muraï RZ*, *Francesca*, *Francès 219/855*, *Negre borratger 386/935*, *Carxofeta LS007*, and *D'hivern LS008* when compared to the unprocessed samples at the same stage of digestion (p<0.05). However, no statistically significant differences were observed in the TPC after the intestinal phase of fresh and minimally processed *Maravilla LS044,* suggesting that the observed increase could be matrix-dependent. For this variety, the phenolic content after the intestinal phase was 14.02 ± 0.01 mg/100 g before processing and 14.44 ± 0.44 mg/100 g after minimal processing and storage. Further studies are needed in order to demonstrate this hypothesis because for all the studied varieties the antioxidant capacity of the enzymatic digestive extracts was higher after minimal processing and storage when compared to the fresh product (p<0.05). Lettuce varieties were sanitized using sodium hypochlorite. Previous studies suggested that food acidulants such as citric acid can affect the bioaccessibility of minerals (Hemalatha et al. 2005) and carotene (Veda et al. 2008). Therefore, future

studies will include the assessment of the effect of chemical sanitizers and antioxidants on the bioaccessibility and antioxidant activity of lettuce leaves.

Conclusions

The phenolic content of the lettuce varieties evaluated in the current study varied significantly among varieties, cultivars, and types. Although an overall higher phenolic content was observed in modern lettuce varieties, when compared to the traditional ones, the antioxidant capacity of modern and traditional varieties was similar. Minimal processing and storage for a 7-day period led to an increased phenolic content in some lettuce varieties, which is in line with previous studies. This trend was not observed in all the studied varieties, suggesting that the wound-induced generation of polyphenols could be matrix-dependent. Finally, the amount of bioaccessible polyphenols was higher after minimal processing and storage, suggesting that the increased amount of polyphenols caused by processing and storage can also lead to a higher amount of bioaccessible phenolic compounds.

Conflict of interests

The authors declare no conflict of interests.

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Figure captions

Figure 1. Selected modern and traditional lettuce varieties.

Definitions: A variety means a plant grouping within a single botanical taxon of the lowest known rank. Landraces are domesticated local plant varieties grown on farm by farmers who reproduce their seeds year after year. They can be distinguished by specific traits. These varieties have a high capacity to tolerate biotic and abiotic stress, resulting in high yield stability and an intermediate yield level under a low input agricultural system (Zeven, 1998). Modern varieties are those obtained after an intensive and deliberated selection during a formal breeding programme (Bitocchi et al. 2009). Variety type refers to a number of physical attributes that are common to different varieties i.e. number of leaves, thickness, undulation of margin, venation. In the figure, variety types were selected following the guidelines for the conduct of tests for distinctness, uniformity, and stability of the International Union for the Protection of New Varieties of Plants (UPOV) (UPOV 2017).

Figure 2. Total phenolic content of lettuce varieties at (A) day 0 and (B) after minimal processing followed by a 7-day storage period.

Abbreviations: TPC, total phenolic content.

Legends: Initial, Gastric, and Intestinal refer to results obtained for methanolic extracts (initial) and for enzymatic digestive extracts after the gastric or intestinal phase, respectively. Sample A-J refer to *Rutilaï RZ, Abago RZ, Muraï RZ, Pelikan, Francesca, Maravilla LS044, Francès 219/855, Negre borratger 386/935, Carxofeta LS007,* and *D'hivern LS008*, respectively. Values represent the mean of three independent experiments (mg/100 g) ± S.D. Significant differences between digestive phases are

shown in capital letters. Lower case letters indicate differences between varieties at the same digestive phase. The criterion for statistical significance was $p<0.05$.

Figure 3. Antioxidant capacity assessed using the FRAP assay of lettuce varieties at (A) day 0 and (B) after minimal processing followed by a 7-day storage period.

Abbreviations: FRAP, Ferric ion reducing antioxidant power assay.

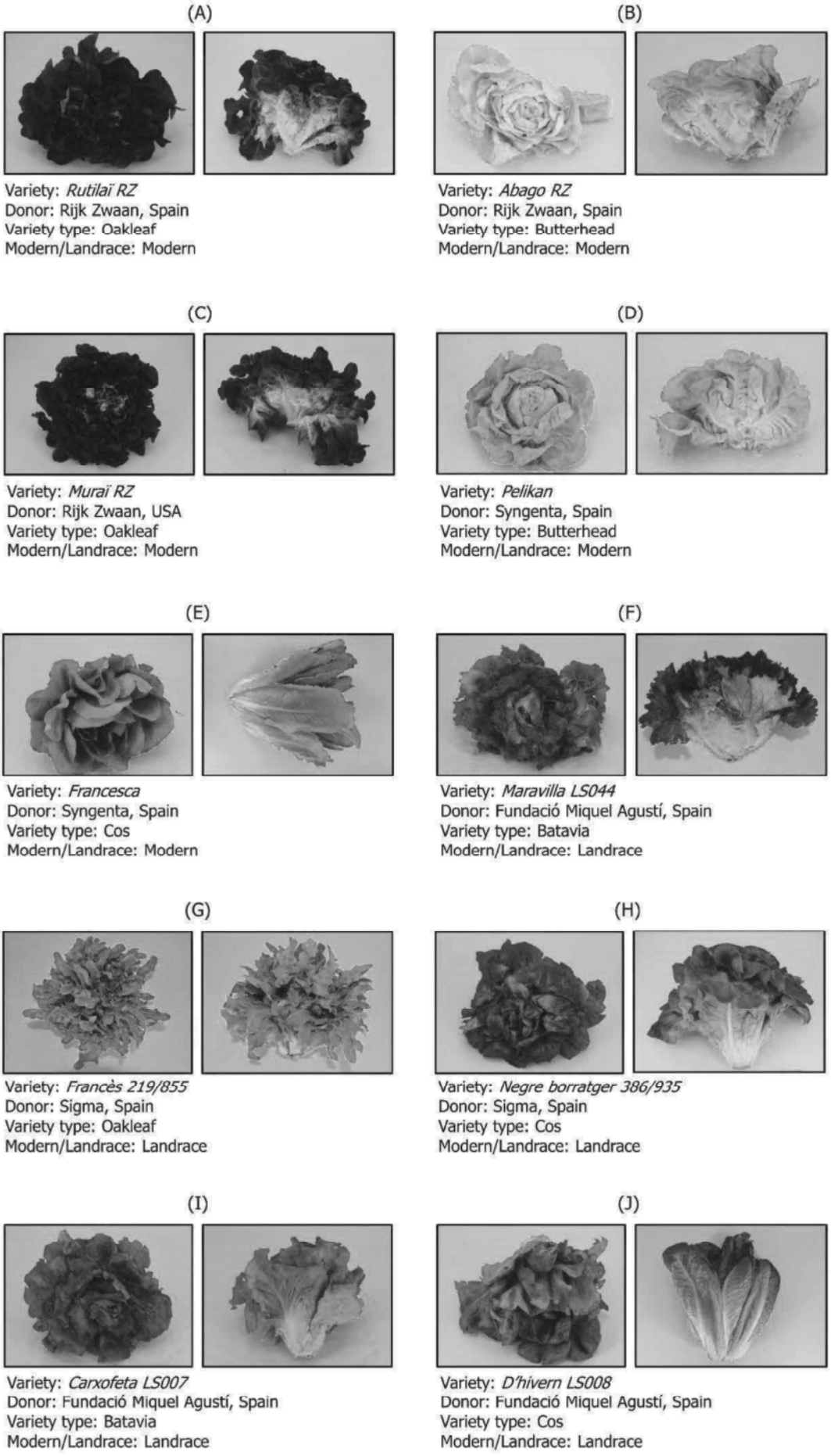
Legends: Initial, Gastric, and Intestinal refer to results obtained for methanolic extracts (initial) and for enzymatic digestive extracts after the gastric or intestinal phase, respectively. Sample A-J refer to *Rutilaï RZ, Abago RZ, Muraï RZ, Pelikan, Francesca, Maravilla LS044, Francès 219/855, Negre borratger 386/935, Carxofeta LS007,* and *D'hivern LS008*, respectively. Values represent the mean of three independent experiments (mg/100 g) ± S.D. Significant differences between digestive phases are shown in capital letters. Lower case letters indicate differences between varieties at the same digestive phase. The criterion for statistical significance was $p<0.05$.

Figure 4. Antioxidant capacity assessed using the DPPH assay of lettuce varieties at (A) day 0 and (B) after minimal processing followed by a 7-day storage period.

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl free radical assay.

Legends: Initial, Gastric, and Intestinal refer to results obtained for methanolic extracts (initial) and for enzymatic digestive extracts after the gastric or intestinal phase, respectively. Sample A-J refer to *Rutilaï RZ, Abago RZ, Muraï RZ, Pelikan, Francesca, Maravilla LS044, Francès 219/855, Negre borratger 386/935, Carxofeta LS007,* and *D'hivern LS008*, respectively. Values represent the mean of three independent experiments (mg/100 g) ± S.D. Significant differences between digestive phases are shown in capital letters. Lower case letters indicate differences between varieties at the same digestive phase. The criterion for statistical significance was $p<0.05$.

Figure 1.


(A)
Variety: *Rutilaï RZ*
Donor: Rijk Zwaan, Spain
Variety type: Oakleaf
Modern/Landrace: Modern
(B)
Variety: *Abago RZ*
Donor: Rijk Zwaan, Spain
Variety type: Butterhead
Modern/Landrace: Modern
(C)
Variety: *Muraï RZ*
Donor: Rijk Zwaan, USA
Variety type: Oakleaf
Modern/Landrace: Modern
(D)
Variety: *Pelikan*
Donor: Syngenta, Spain
Variety type: Butterhead
Modern/Landrace: Modern
(E)
Variety: *Francesca*
Donor: Syngenta, Spain
Variety type: Cos
Modern/Landrace: Modern
(F)
Variety: *Maravilla LS044*
Donor: Fundació Miquel Agustí, Spain
Variety type: Batavia
Modern/Landrace: Landrace
(G)
Variety: *Francès 219/855*
Donor: Sigma, Spain
Variety type: Oakleaf
Modern/Landrace: Landrace
(H)
Variety: *Negre borratger 386/935*
Donor: Sigma, Spain
Variety type: Cos
Modern/Landrace: Landrace
(I)
Variety: *Carxofeta LS007*
Donor: Fundació Miquel Agustí, Spain
Variety type: Batavia
Modern/Landrace: Landrace
(J)
Variety: *D'hivern LS008*
Donor: Fundació Miquel Agustí, Spain
Variety type: Cos
Modern/Landrace: Landrace

546 **Figure 2**

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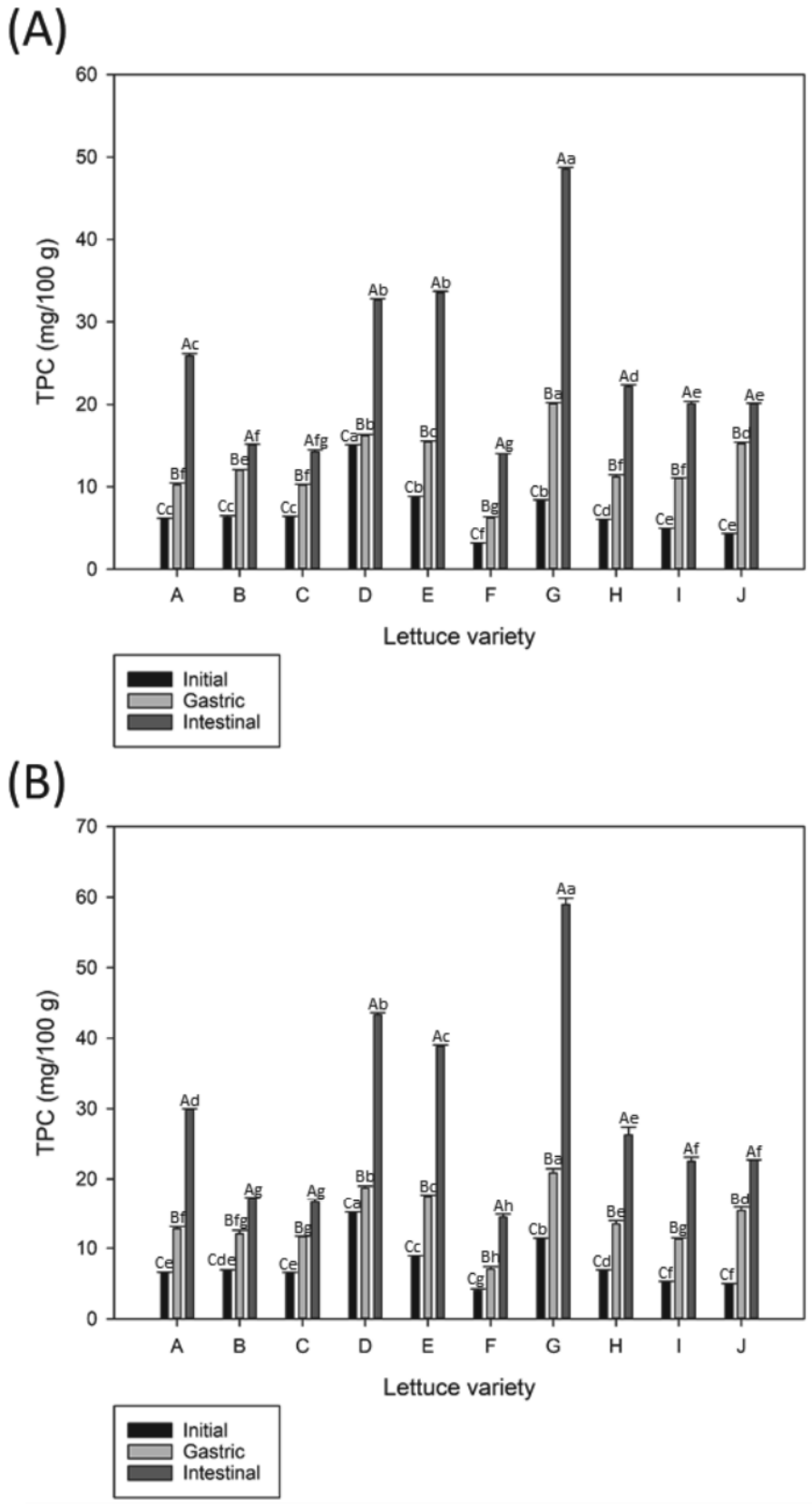


548 **Figure 3**

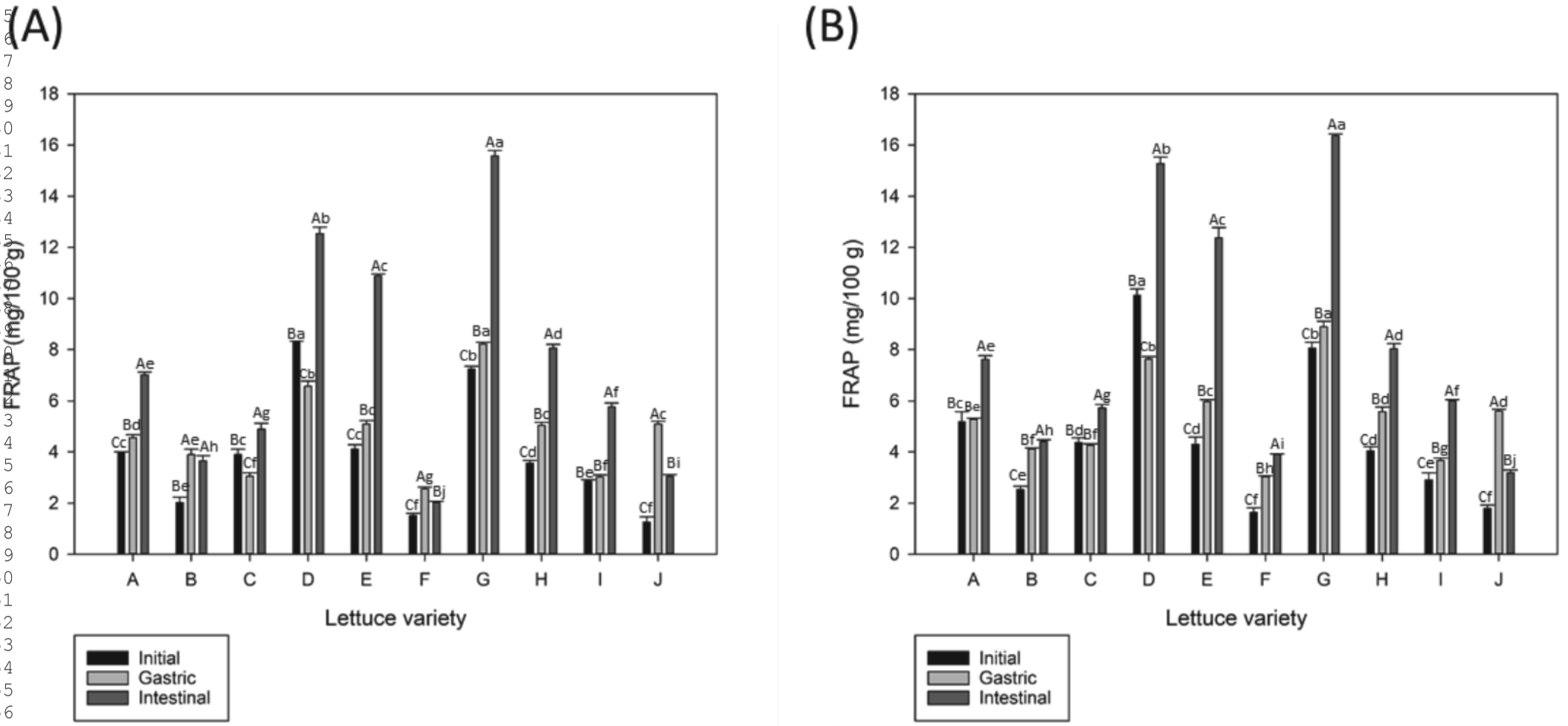
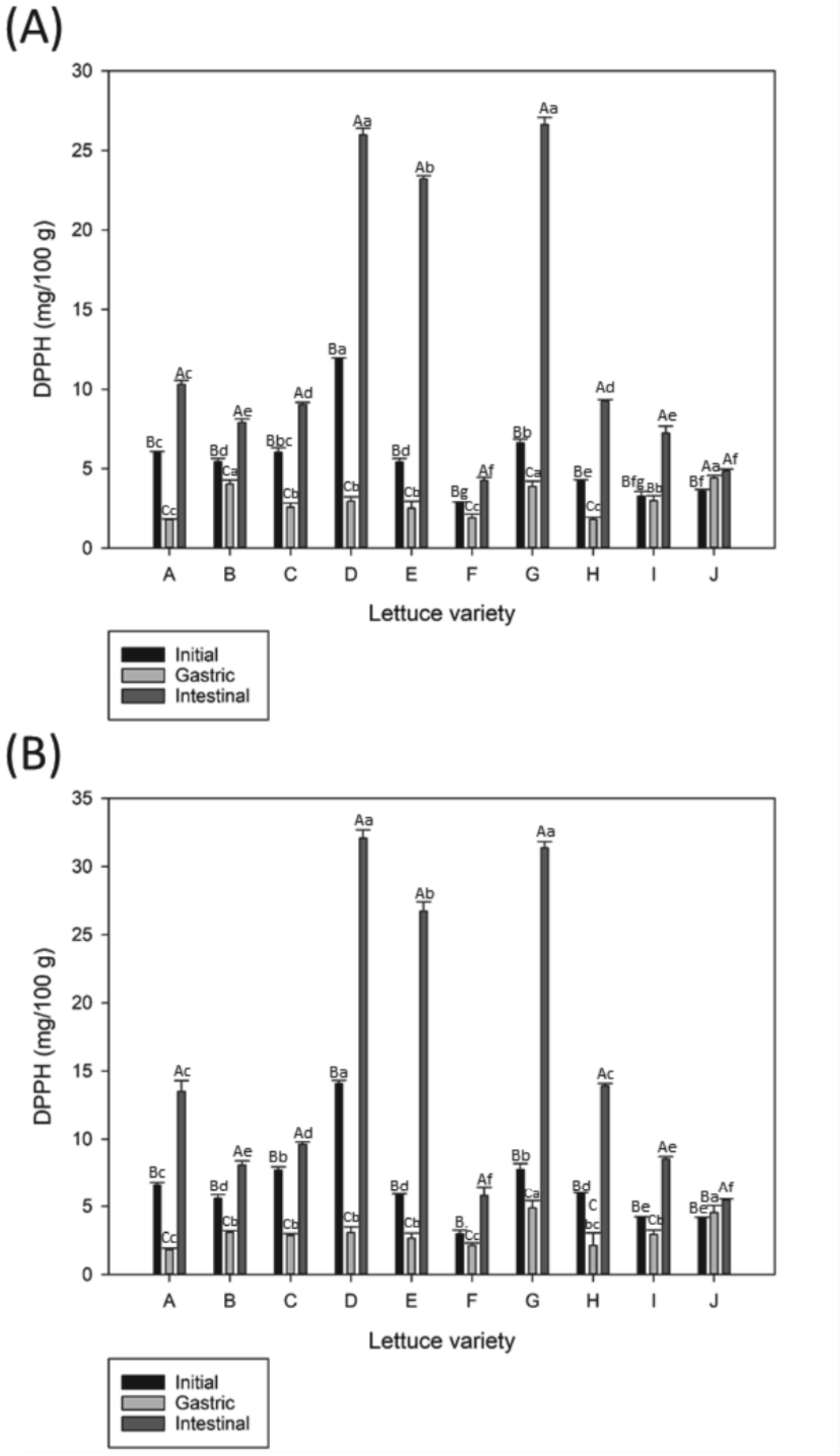

(A)
FRAP (mg/100 g)
Lettuce variety
A B C D E F G H I J
Initial
Gastric
Intestinal
(B)
FRAP (mg/100 g)
Lettuce variety
A B C D E F G H I J
Initial
Gastric
Intestinal

Figure 4


(A)
DPPH (mg/100 g)
Lettuce variety
A
B
C
D
E
F
G
H
I
J
Initial
Gastric
Intestinal
(B)
DPPH (mg/100 g)
Lettuce variety
A
B
C
D
E
F
G
H
I
J
Initial
Gastric
Intestinal