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Effect of *in vitro* digestion and fermentation on antioxidant capacity of weight loss foods and Maillard reaction products content

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A B S T R A C T

Cereal snacks and meal replacement shakes are gaining popularity as part of a low-calorie diet. However, some concerns have been risen in relation to their nutrient content and industrial processing. Here we analyzed 74 products, including cereal bars, cereal cakes and meal replacement shakes. We measured furosine and 5-hydroxymethyl-furfural (HMF) due to their relation with industrial processing, mainly thermal treatment, as well as antioxidant capacity after *in vitro* digestion-fermentation. Most of the products reported a high sugar content, including also large concentrations of HMF and furosine. Small differences were found on antioxidant capacity, although chocolate addition tended to increase the antioxidant power of products. According to our results, antioxidant capacity released after fermentation is higher, which points out to the importance of gut microbes in releasing potentially bioactive compounds. Additionally, we have found alarmingly high concentrations of furosine and HMF, which calls to research into new technologies for food processing to minimize their generation.

1. Introduction

Consumer awareness about their own health and its relation with diet has been constantly increasing for the last years (Klerks, Román, Verkerk, & Sanchez-Siles, 2022). Therefore, the food market is moving towards healthier choices with consumers asking for products that are not only good for them but also eco-friendly (Topolska, Florkiewicz, & Filipiak-Florkiewicz, 2021). Additionally, consumers are looking for products that are convenient which, taken together with their health concern, has made the "healthy" snack market to gain popularity (Nielsen, 2022). According to Klerks et al. (2022), this shift has translated into a higher consumption of some functional products at the expense of regular snacks.

Among these so called "healthy" snacks we can find that cereal bars are taking up much of the market since they are presented as individual portions, they are small and can be easily carried, and thus an easy choice for having a snack at any point during the day (de Melo et al., 2020). Nevertheless, cereal cakes and meal replacement shakes are also becoming popular, though the latter are usually consumed as part of low-calorie diets (Zurita-Ortega et al., 2020). Cereal bars and cakes are usually seen as healthy or at least healthier than regular snacks since they often provide fair amounts of fiber or proteins (Klerks et al., 2022). However, it has also been found that most of the cereal products offered in supermarkets are not low in saturated fats and also have high sugar (added) content (Curtain & Grafenauer, 2019). In addition, although in general these products could make an important contribution to the recommended daily intake of some trace elements, it was also found that they can contain large concentrations of toxic elements (Zurita-Ortega et al., 2020).

On the other hand, another concern that has been pointed out is their high degree of processing (Klerks et al., 2022; Parra-Murillo et al., 2021). This is going to lead not only to a high salt content (Zurita-Ortega et al., 2020), but also to the formation of potentially toxic compounds such as those derived from the Maillard reaction or chemical browning (Pastoriza de la Cueva et al., 2017; Rufián-Henares, Delgado-Andrade, & Morales, 2006). Briefly, the Maillard reaction happens while heating

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together reducing sugars and molecules with free amino groups such as proteins (Rufián-Henares, Guerra-Hernández, & García-Villanova, 2002). Maillard reaction can be monitored via determination of some of the intermediate compounds that appear such as furosine or 5-hydroxymethyl-furfural (HMF), commonly called thermal treatment markers (Pérez-Burillo, Jiménez-Zamora, Párraga, Rufián-Henares, & Pastoriza, 2019). The end product of the reaction are polymers called melanoidins (Rufián-Henares, Guerra-Hernández, & García-Villanova, 2006) which have showed some health-promoting effects such as antioxidant (Morales & Jiménez-Pérez, 2004) or prebiotic (Tagliazucchi & Bellesia, 2015). Therefore, by monitoring Maillard reaction, the manufacturing process could be optimized as to reduce as much as possible the formation of potentially toxic compounds and decrease of the nutritional value of foods (Delgado-Andrade, Rufián-Henares, & Morales, 2007).

Regardless, due to their popularity among consumers, cereal bars have been the focus of different studies, most of the trying to improve their health effects by adding new ingredients such as almonds (de Ramos, Pertuzatti, Gomes, Santana, & de Brito, 2020), stevia leaves (Silva et al., 2019), spirulina (Lucas et al., 2019), tempeh (de Melo et al., 2020), pre- and probiotics (Pereira et al., 2019), berries (Smith et al., 2019), coffee (Lara et al., 2018), or quinoa (Kaur, Ahluwalia, Sachdev, & Kaur, 2018).

Taking all this information into consideration, the aim of the paper was to report on the presence of furosine and HMF (as markers of Maillard reaction development) of 74 commercial weight loss products sold in Spain (including cereal bars, cereal cakes and meal replacement shakes) and the impact of consumption of these foods on HMF exposure, due to its potential toxicological relevance. In addition, we assessed their antioxidant capacity after going through a step of *in vitro* gastrointestinal digestion and colonic fermentation.

2. Materials and methods

2.1. Chemicals

2.1.1. Reagents needed for in vitro digestion and fermentation

Pancreatine from porcine pancreas was acquired from Alpha Aesar (Kandel, Germany). Tryptone, cysteine, resazurin, sodium dihydrogen phosphate, sodium sulfide, porcine bile acids, pepsin and salivary alpha-amylase were purchased from Sigma-Aldrich (Darmstadt, Germany).

2.1.2. Reagents needed for antioxidant capacity assays

Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), sodium acetate, TPTZ (2,4,6-Tri(2-pyridyl)-s-triazine), iron (III) chloride hexahydrate, gallic acid, Folin-Ciocalteu® reagent and hydrochloric acid. All the reagents for the analysis of antioxidant capacity were obtained from Sigma Aldrich (Darmastad, Germany).

2.1.3. Reagents needed for thermal treatment indicators

Furosine and 5-hydroxymethylfurfural as standards and acetonitrile were purchased from Sigma Aldrich (Darmastad, Germany).

2.2. Samples

Seventy-four products marketed in Spain were selected and bought at several supermarkets in Granada, Spain. We acquired each of the products from three different places so as to have products from different batches. We included 35 different cereal bars, 33 different cereal cakes and 6 different meal replacement shakes. Among cereal cakes, 14 were rice-based, 14 wheat-based, 3 barley-based and 2 were made of a cereal mix. Thirty-four products had chocolate as a major ingredient, 20 bars and 14 cakes. Cereal bars and cereal cakes were grounded to simulate mastication using an Ultraturrax (model T25, IKA, Spain) and stored at −80 °C until analysis.

2.3. Protein, fiber and sugar content of cereal products and shakes

Protein, fiber and sugar content is reported according to that expressed in the nutritional label of each product.

2.4. Thermal treatment indicators: Furosine and HMF

Furosine analysis was carried out following the protocol described by Rufián-Henares, Guerra-Hernández, and García-Villanova (2013). Samples were hydrolyzed with 7.95 M HCl (0.125 mg of sample/4 mL of HCl) at 120 °C for 23 h in a Pyrex screw-cap tubes. Nitrogen gas was bubbled into the solution for 2 min. 500 µL were passed through a Sep-pack C_{18} cartridge (Waters, Milford, MA, USA) prewetted with 5 mL of methanol and 10 mL of ultrapure water and then eluted with 3 mL of 3 mol/L HCl. HCl was evaporated in a rotary evaporator and the precipitate resuspended in 5 % acetonitrile. Fifty microlitres of the solution were analysed by ion-pair RP-HPLC. The HPLC system was an Accela 600 equipped with a PDA detector set at 280 nm, a quaternary pump and an autosampler (Thermo Scientific, USA). A RP C18 column was used. The mobile phase consisted on 20 % acetonitrile with 1.022 g of sodium heptanosulphonate and 0.1 % of formic acid. A calibration curve in the range of 0.01–100 mg/L was used. Results are expressed as mg of fusosine/Kg of sample.

HMF analysis was performed following the protocol described by Rufián-Henares, Garcia-Villanova, and Guerra-Hernandez (2001). Samples were suspended in ultrapure water and clarified using the Carrez solution (Carrez I potassium ferrocyanide, 15 % w/v, Carrez II zinc acetate, 30 % w/v). The solution was filtered through a 0.22 um nylon filter after which the sample was ready for HPLC injection. The HPLC system was the same used for furosine analysis. The flow rate was 1 mL/min of acetonitrile 5 % with the PDA set at 284 nm. A calibration curve in the range of 0.01 to 100 mg/L was used. Results were expressed as mg/Kg of sample.

2.5. In vitro digestion

Samples were *in vitro* digested following the protocol described by Brodkorb et al. (2019). Five grams of each sample were weighed (in triplicate) into 50 mL centrifugation tubes. Five milliliters of simulated salivary fluid with 150 U/mL of alpha-amylase were added into the tube carrying the sample and kept at 37 °C for 2 min. Secondly, 10 mL of simulated gastric fluid with 4000 U/mL of gastric pepsin were added to the mix, the pH lowered to 3 and kept at 37 °C for 2 h. Finally, 20 mL of simulated intestinal fluid with 200 U/mL of pancreatin and 20 mM bile salts were added into the tube, the pH increased to 7 and kept at 37 °C for 2 h. Enzyme activity was halted by immersion in ice for 15 min. Tubes were centrifuged, the supernatant (fraction available for absorption at the small intestine) collected and the pellet (fraction not digested that would reach the colon) used for *in vitro* fermentation.

2.6. In vitro fermentation

In vitro fermentation was carried out following the protocol described by Pérez-Burillo et al. (2021). Fecal samples were collected from 3 healthy donors (BMI = 21.3–23.8 and they had not taken antibiotics in the last 3 months). Fecal material was pooled to account for inter-individual variability. In vitro fermentation was carried out at 37 °C for 20 h, in oscillation. For this purpose, 0.5 g of the pellet obtained after *in vitro* gastrointestinal digestion were used, as well as 10 % of the supernatant. Fermentation medium composed of peptone (14 g/L, cysteine 312 mg/L, hydrogen sulfide 312 mg/L and resazurin 0.1 % v/v) was added to the fermentation tube at a volume of 7.5 mL. A fecal inoculum mas made from fecal material by mixing it with phosphate buffered saline (PBS) at a concentration of 33 %. Two milliliters of inoculum were added to the fermentation tube. Afterwards, nitrogen was bubbled into the tube until reaching anaerobic conditions

(transparent solution as opposed to pink when oxygen is dissolved). After 20 h at 37 °C, microbial activity was halted by immersion in ice for 15 min and tubes were centrifuged to collect the supernatant (fraction available for absorption at the large intestine), which was stored at −80 °C until further analysis. Blanks carrying water instead of sample were included in the *in vitro* digestion as well as in the *in vitro* fermentation to account for enzymes and other solutions activity.

2.7. Antioxidant capacity

Antioxidant capacity was studied in both the fraction obtained from *in vitro* digestion (potentially absorbable in the small intestine) as well as in the fraction obtained after *in vitro* fermentation (potentially absorbable in the large intestine).

2.7.1. FRAP assay

A previous protocol was followed to study the iron reducing capacity samples (Benzie & Strain, 1996). It was performed using a microplate reader (FLUOStar Omega, BMG Labtech, Germany). Twenty µL of sample were placed in duplicate in a 96-well plate and mixed with 280 µL of freshly prepared FRAP reagent (25 mL of 0.3 mM sodium acetate pH 3.6, 2.5 mL of 20 mM ferric chloride and 2.5 mL of 40 mM TPTZ). The antioxidant reaction was monitored for 30 min at 37 °C and the calibration curve ranged from 0.01 to 04 mg of Trolox/mL. Results were expressed as mmol Trolox equivalent/Kg food.

2.7.2. ABTS assay

The assay was performed as described previously but with adaptations to a microplate reader (Re et al., 1999). Samples were mixed with a fresh 1:1 solution of 7 mM ABTS and 2.45 mM potassium persulfate. The reaction was measured colorimetrically at 730 nm wavelength in duplicate using a 96-well plate (20 µL and 280 µL of ABTS solution). The antioxidant reaction was monitored for 30 min at 37 °C. Trolox was used as standard to create daily calibration curves for the ABTS solution in the range of 0.01 – 0.4 mg/mL. Antioxidant capacity was expressed as mmol Trolox equivalent/Kg of sample.

2.7.3. Folin-Ciocalteu assay

We followed a previously published protocol and adapted it to a microplate reader (Singleton & Rossi, 1965). 30 µL of sample were added in duplicate to each of the 96 wells of a plastic plate. This was mixed with 190 µL of bidistilled water, 15 µL of Folin-Ciocalteu reagent and 60 µL of 10 % sodium carbonate solution. The calibration curve was prepared with gallic acid with a concentration that ranged from 0.1 to 2.5 mg/mL. The antioxidant reaction was monitored for 60 min at 37 °C. The results were expressed as mg gallic acid equivalent/Kg of food.

2.8. Statistical analysis

Statistical differences were computed using unpaired Kruskall Wallis test with a 95 % confidence. Raw *p*-values were adjusted for multiple hypothesis testing according to Benjamini and Hochberg method. A multivariate Principal Component Analysis (PCA) was carried out to explore differences between groups. Spearman's rank correlation coefficient was calculated to study possible correlations between variables. Statgraphics Plus software, version 5.1 and R version 3.6 were used to performed all the statistical analysis.

3. Results

3.1. Nutritional label. Protein, fiber and sugar content of cereal products and shakes

We first assessed the concentration of protein, sugar and dietary fiber reported on the nutritional label (Fig. 1), since they have an effect on the development of the Maillard reaction (Delgado-Andrade et al., 2007). Sugar content was in general rather high, with values between 42 % and 13 %; there were only some cereal cakes that reported low sugar content, between 2.8 % and 0.5 %. As average, cereal bars had 31.4 g of sugar/100 g, cereal cakes (except those with low content) had 26.4 g of sugar/100 g and shakes had 29.3 g of sugar/100 g. Protein content was also quite high, with values ranging from 31.3 % to 7.6 %. Cereal bars showed an average protein content of 14.3 g/100 g, cereal cakes of 6.79 g/100 g and shakes of 27.1 %. On the other hand, fiber content was between 0.9 % and 19 %. Cereal bars had an average fiber content of 7.36 g/100 g, cereal cakes of 3.93 % and shakes of 10.1 %.

3.2. Thermal treatment markers: Furosine and HMF

All three types of products showed similar values of furosine and HMF, with no significant differences between them (Fig. 2**B**). However, there were large differences within each group. In cereal bars, furosine values ranged between 32.9 and 977.5 mg/Kg, cereal cakes showed values between 29.0 and 1411.4 mg/Kg whereas in shakes, furosine ranged between 105.7 and 490.6 mg/Kg (Fig. 2**A**). Cereal cakes exhibited the largest average furosine content with 312 mg/Kg, whereas bars and shakes showed slightly lower average concentrations.

On the other hand, HMF was found, on average, in higher concentration in shakes (70.7 mg/Kg) than in bars (32.4 mg/Kg) or cakes (16.2 mg/Kg). Still, wide differences within group were presented again. Cereal bars showed HMF values between 2.0 and 124.6 mg/Kg, cereal cakes between 0.9 and 46.2 mg/Kg and shakes between 3.6 and 219.6 mg/Kg (Fig. 2**A**). To put these figures into context, cocoa was found to generate between 0 and 4000 mg/kg of HMF depending on the roasting degree (Maldonado-Mateus, Pérez-Burillo, Lerma-Aguilera, Hinojosa-Nogueira, Ruíz-Pérez, Gosalbes, & Pastoriza, 2021), tea leaves were found to contain around 2 mg/kg (Pérez-Burillo et al., 2019), coffee from 300 to 1900 mg/L, cereal products including bread, biscuits or cereal breakfast from 6.6 to 241 mg/kg or honey from 0.19 to 41 mg/kg (Rufián-Henares & de la Cueva, 2008).

Since the development of Maillard reaction is related to sugar and protein content, we also calculated the Spearman's rank correlation coefficient between thermal markers and sugar or protein concentration (data not shown). HMF concentration slightly correlated with sugar content and furosine with protein content. However, none of these correlations were significant and the correlation coefficient was below 0.4.

Finally, we set out to study whether the addition of specific ingredients would influence the formation of furosine and HMF. However, we were only able to study those products for which we had the product with no added ingredients other than the base cereal. Therefore, we could only use cakes for this purpose (Fig. 2**C**). Regarding rice cakes, only those added with flavor and white chocolate showed significantly ($p < 0.05$) lower furosine than the cereal base cake. HMF on the other hand, showed similar values across samples and only the one added with white chocolate showed significantly lower values than the cereal base. However, although not significantly, cakes added with flavor showed the highest value. Regarding wheat cakes (Fig. 2**C**), the ones added with chocolate and chocolate plus flavor showed significantly ($p < 0.05$) higher furosine content. Only cakes added with chocolate plus flavor showed significantly higher HMF content.

3.3. Antioxidant capacity

Antioxidant capacity was measured in two fractions: i) the supernatant obtained after *in vitro* digestion, which would represent the fraction potentially absorbable in the small intestine; ii) the supernatant obtained after *in vitro* fermentation, which would represent the antioxidants that remain or are produced by gut microbial metabolism and are potentially absorbable in the large intestine. Three different assays were used to assess antioxidant capacity: ABTS, Folin-Ciocalteu and FRAP.

We first performed a PCA to observe the distribution of the samples

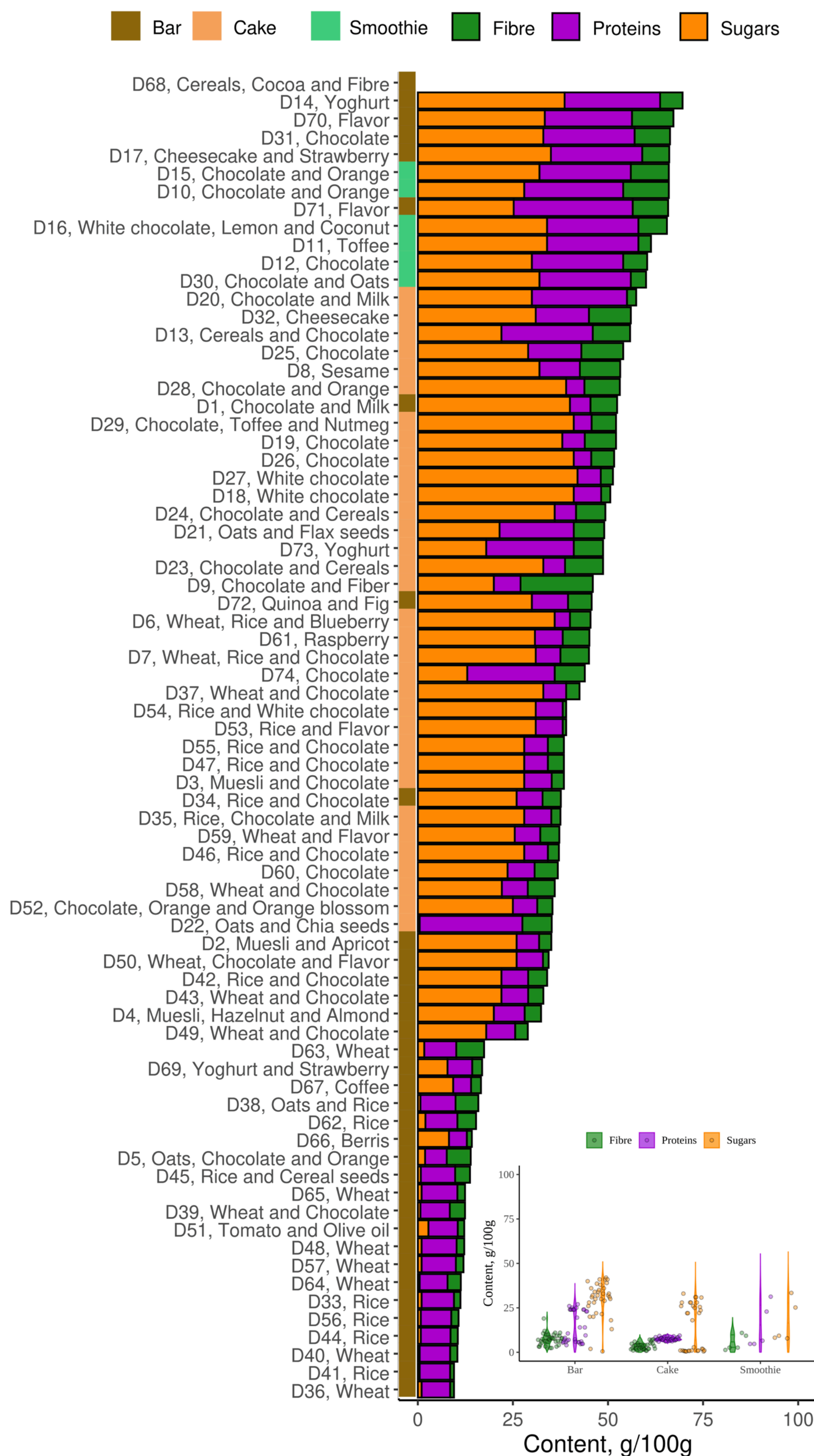

Fig. 1. Nutrient content of the different products as reported in the label (g/100 g of product). Note that there was no nutritional information for one of them. Subplot shows average content of each type of product expressed in g/100 g of product.

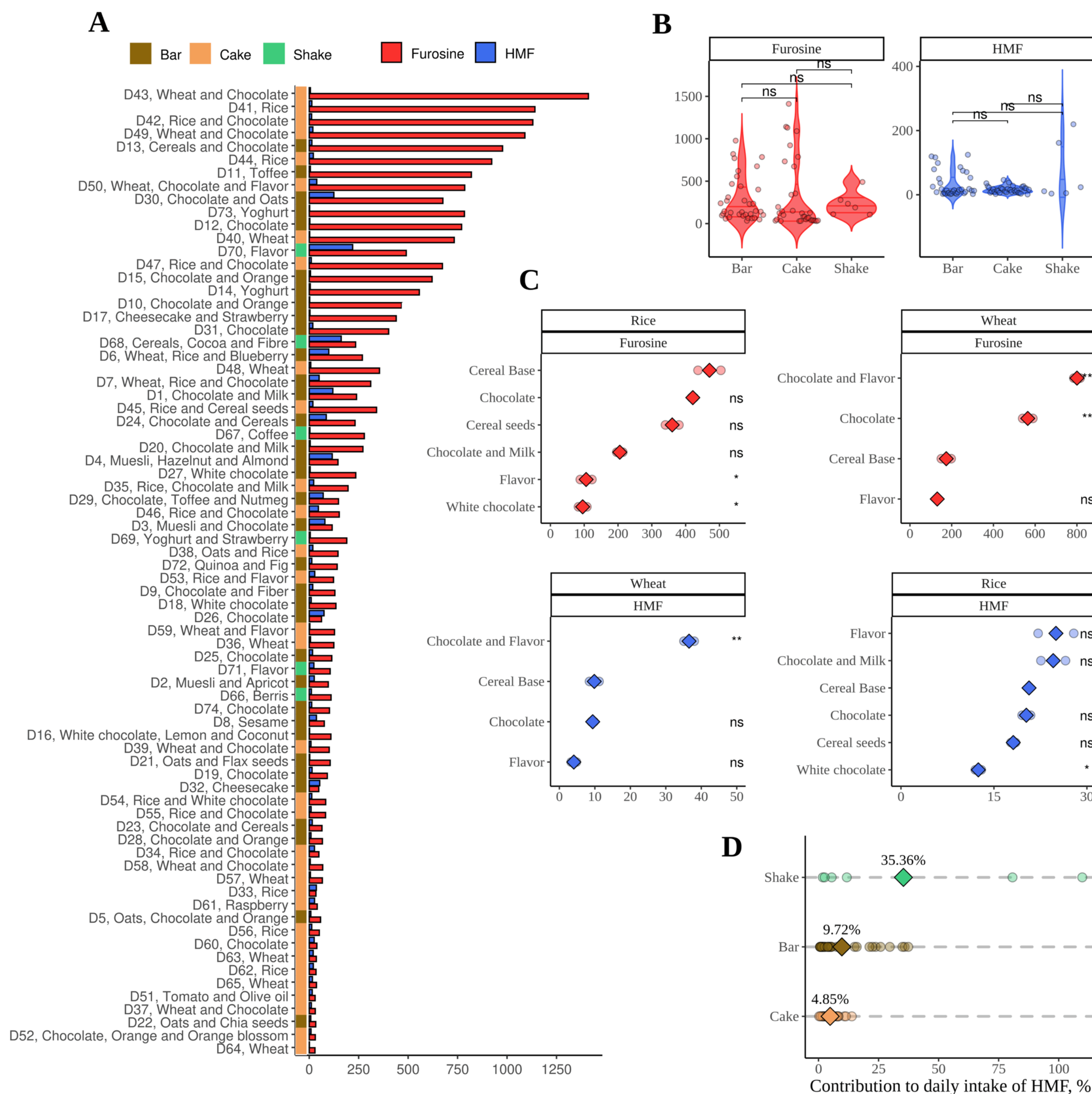

Fig. 2. Furosine and HMF content. Panel A shows the content for each product analyzed. Panel B shows the content by type of product. Panel C shows furosine and HMF of rice and wheat cakes with different ingredients. Panel D shows the contribution of the products analyzed to the daily intake of HMF estimated as 10 mg/day. Statistical comparisons were made using unpaired Kruskal-Wallis test at 95 % confidence using as reference group in panel C "cereal base". Statistic labels: ns – not significant; * - $p < 0.05$; ** - $p < 0.01$.

according to their antioxidant capacity. Antioxidant values from digestion and fermentation separated well in the ordinate plot (Fig. 3A). However, when we looked within digestion (Fig. 3B) or fermentation (Fig. 3C), samples did not cluster in any specific manner.

Overall, antioxidant capacity obtained after *in vitro* gut microbial fermentation was significantly ($p < 0.05$) higher than that obtained after digestion (Fig. 4A) irrespective of the method used. However, on average, very little differences were observed between bars, cakes or shakes (Fig. 4B). Only cereal bars showed significantly higher antioxidant values than cereal cakes when using FRAP as antioxidant assay.

As we did with thermal treatment markers, here we studied whether specific ingredients could increase antioxidant capacity of cereal cakes. For these calculations we used total antioxidant capacity, that is the sum of antioxidant capacity released after digestion and fermentation. Regarding rice cakes (Fig. 4C), chocolate and flavor increased antioxidant capacity when using Folin-Ciocalteu and FRAP as assays, although these increases were only significant ($p < 0.05$) for FRAP. The ingredients cereal seeds and chocolate plus milk had, however, opposite behaviors in Folin-Ciocalteu and FRAP: while seeds increase (not significantly) the antioxidant capacity measured via Folin-Ciocalteu, they did the opposite when measured via FRAP. Chocolate plus milk did the opposite, and was significantly lower with Folin-Ciocalteu but significantly higher with FRAP. No significantly differences were found using ABTS though, surprisingly, plain rice cake showed the highest value. Regarding wheat cakes (Fig. 4D), the ingredient flavor prompt the lowest antioxidant values across all three assays, though it was not

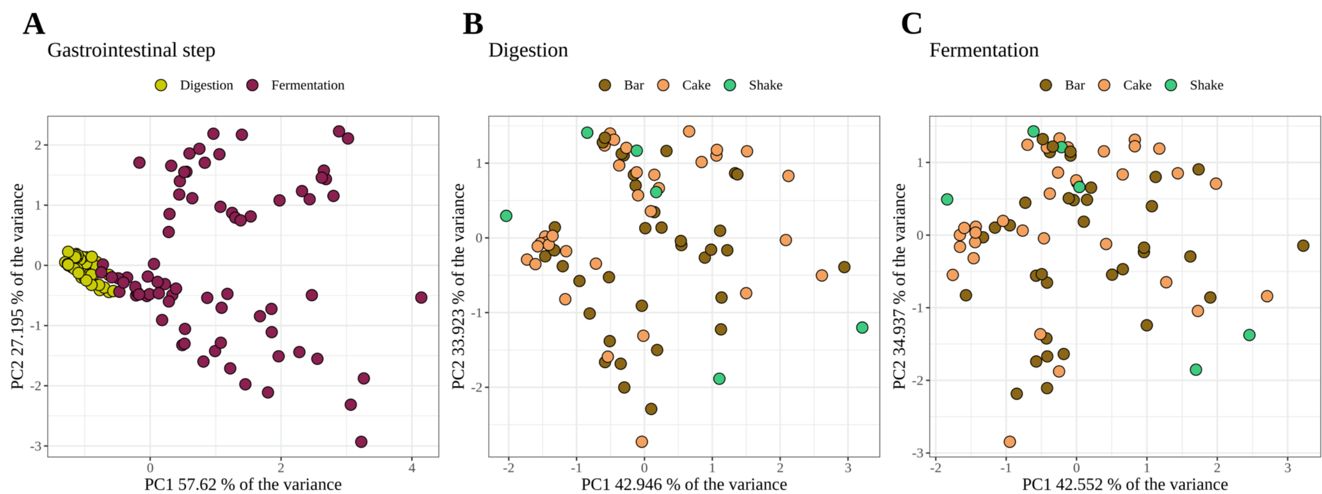

Fig. 3. Principal Component Analysis. Panel A samples are grouped according to whether they came from *in vitro* digestion or *in vitro* fermentation. Panel B only samples from *in vitro* digestion were considered. Panel C only samples from *in vitro* fermentation were considered.

significant when using Folin-Ciocalteu. Overall, the addition of extra ingredients did not seem to increase the antioxidant capacity, at least significantly. Here, chocolate did not have the same effect as before, and when added together with flavor, produced significantly lower antioxidant capacity as measured via FRAP. However, this same ingredient produced somewhat higher values via Folin-Ciocalteu or ABTS, though not significantly.

Thirty-six samples (21 cereal bars and 15 cereal cakes) had chocolate as one of their ingredients. Since cocoa is known to possess certain antioxidant power due to its polyphenol content (Ioannone et al., 2015), we set out to study whether adding chocolate or not influence antioxidant capacity of the final product. As can be seen in Fig. 5**A**, only when using FRAP as antioxidant assay differences were statistically significant ($p < 0.05$) with chocolate added samples, showing statistically higher values. The antioxidant capacity was significantly higher in both bars and cakes when chocolate was used as ingredient, but only according to the FRAP assay (Fig. 5**B**).

4. Discussion

We analyzed the presence of thermal treatment indicators and antioxidant capacity after *in vitro* gastrointestinal digestion and *in vitro* gut microbial fermentation of 74 products including cereal bars, cereal cakes and meal replacement shakes. Cereal bars and cereal cakes have recently gain popularity among consumers since they are usually perceived as healthier than traditional snacks and as such, they have been taking their place in the market (Klerks et al., 2022). However, meal replacement products are only indicated during low-calorie diets (Zurita-Ortega et al., 2020). Since some concerns have been raised about the composition and processing of these products, we set out to analyze the presence of furosine and HMF during processing and/or storage of these products along with potential antioxidant capacity.

As stated before (Curtain & Grafenauer, 2019), most of the products disclaimed a rather high added sugar content ranging between 13 and 42 %, and have become one of the major sources of sugar intake (Bandy, Scarborough, Harrington, Rayner, & Jebb, 2021). On the other hand, most of the studied products reported a quite high protein content, and a fair amount of fiber. The latter could contribute to deal with the so called “fiber gap” (Jones, 2014) but focusing on those products with low sugars.

As commented above, concerns about product processing have arisen and not only in relation to the purely technological processes, but also to the addition of food additives or selection of raw materials (Klerks et al., 2022) which, for instance, could lead to excessive content in toxic mineral elements (Zurita-Ortega et al., 2020). Here though, we focused on the presence of furosine and HMF as consequence of processing and/or storage (Delgado-Andrade, Rufián-Henares, & Morales, 2006; Pérez-Burillo et al., 2019). HMF and furosine concentrations varied widely between samples which, as has been reported before for other snacks, is likely due to different recipes and different technological conditions used by different manufacturers (Mesías, Delgado-Andrade, & Morales, 2019). In the case of wheat cakes, the addition of chocolate with or without additional flavor seemed to increase the production furosine and HMF. Chocolate addition could provide extra sugars, enhancing both the Maillard reaction and caramelization. In fact, chocolate is a good source of HMF produced during cocoa roasting (Maldonado-Mateus et al., 2021). The flavor added together with chocolate was 5-Methyl-2-hepten-4-one, a compound used in foods to simulate hazelnut flavor. Its free carbonyl group could take part in the Maillard reaction and therefore favoring it. The combined action of these two ingredients could explain the higher HMF and furosine concentrations found for these samples. However, the other flavor used in wheat cakes was 3-Methylbutyl acetate (also known as isoamyl acetate) and responsible for banana flavor. The lack of a carbonyl group available for Maillard reaction could explain why in this case HMF and furosine values were lower. However, in the case of rice cakes, results are not as easily explained since plain rice cake showed the highest furosine value whereas the addition of white chocolate or flavor the lowest. Here, the flavor added was “yoghurt” which can be obtained with different molecules making reasoning even harder. HMF values from rice cakes showed that only the one added with white chocolate presented significantly lower values. This is in agreement with furosine results. In general, the lack of significant correlations between HMF-sugar content and furosine-protein content could be related with the heterogenicity of the foods studied and the different thermal treatment performed by the manufacturing companies. Similar results were obtained in the analyses of breakfast cereals or cookies, where differences in composition and processing methods mask the potential correlations that could be obtained (Delgado-Andrade et al., 2007; Rufián-Henares et al., 2006).

In general, HMF was found at important concentrations in most products analyzed. HMF has proven in animal models to metabolize into sulphoxymethylfurfural, which has showed genotoxic (Pastoriza de la Cueva et al., 2017) and hepatotoxic (Mesías et al., 2019) activities. Considering the potentially toxic effects of HMF, we thought appropriate to estimate how much of this molecule can be found within a serving size of each product, as well as contextualize it within the estimated HMF daily consumption. Accordingly, we consider a serving size of 30 g for cereal bars (1 bar), 30 g for cereal cakes (4 units) and 50 g for meal

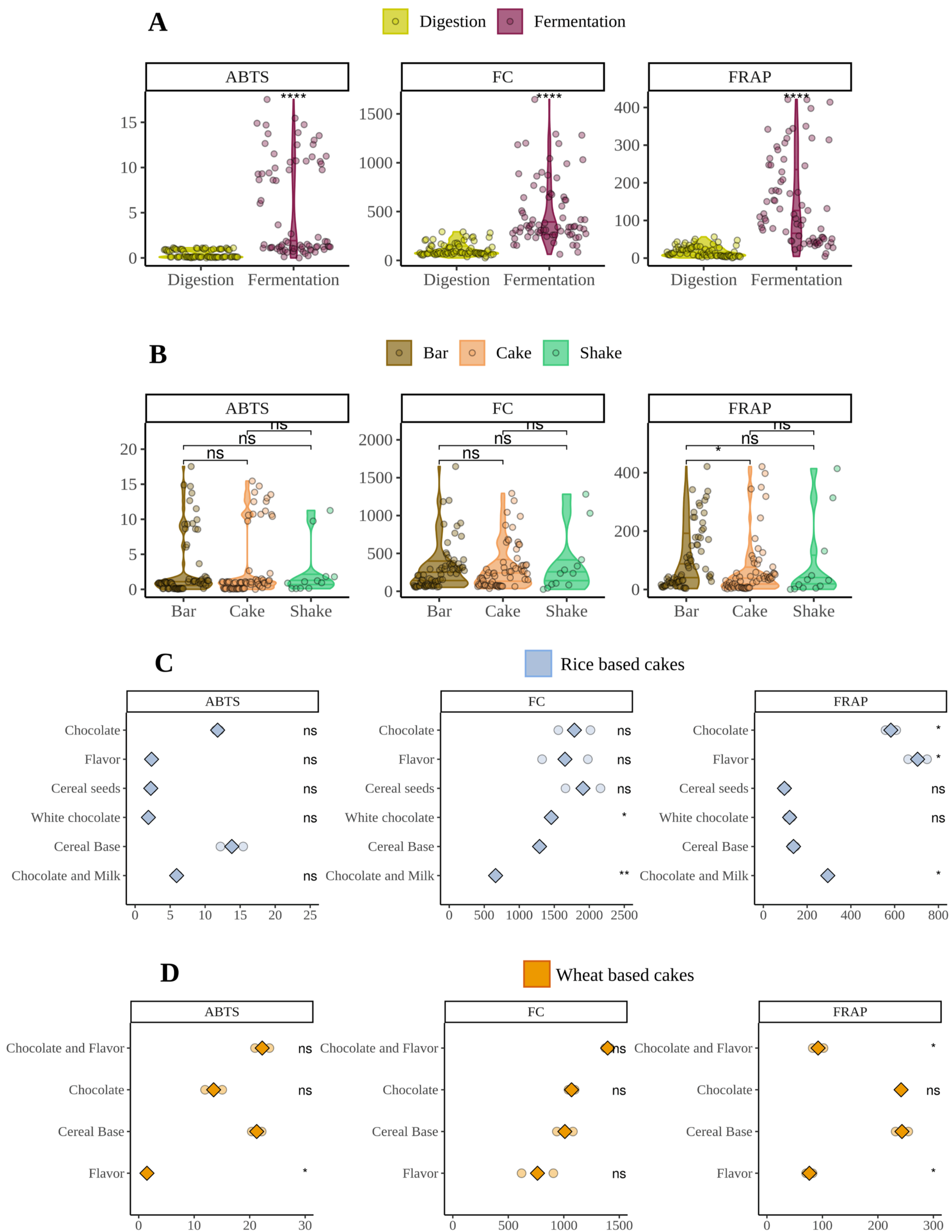

Fig. 4. Antioxidant capacity of analyzed products. Panel A shows antioxidant capacity released during each processing step. Panel B shows antioxidant capacity according to the type of product. Panel C shows antioxidant capacity of rice cakes with different ingredients. Panel D shows antioxidant capacity of wheat cakes with different ingredients. Statistical comparisons were made using unpaired Kruskal-Wallis test at 95 % confidence using as reference group in panel A “Digestion” and in panels C and D “cereal base”. Statistic labels: ns – not significant; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$; **** - $p < 0.0001$.

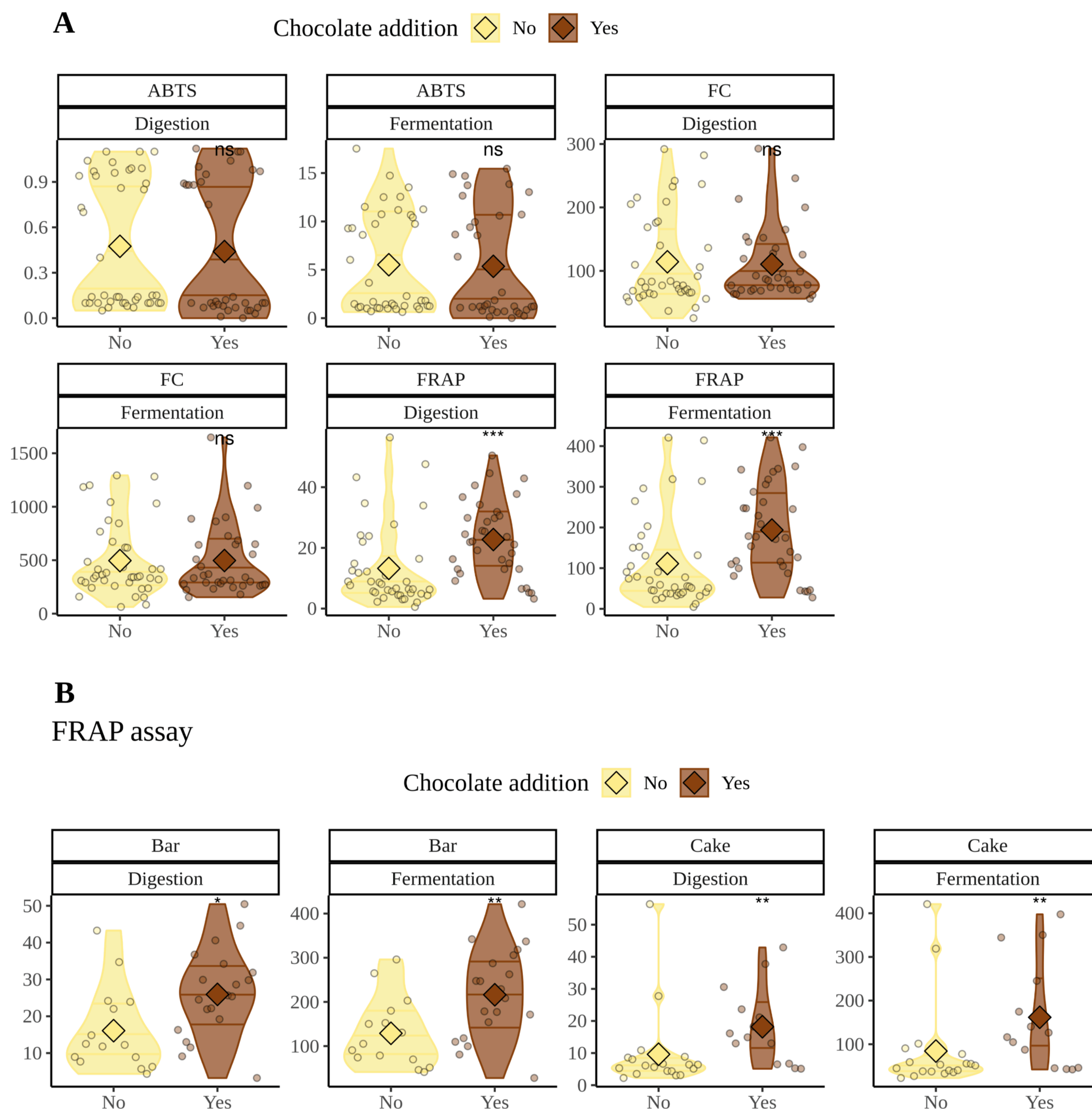

Fig. 5. Antioxidant capacity of analyzed products according to chocolate content. Panel A shows antioxidant capacity obtained for each assay during either *in vitro* digestion or fermentation. Panel B shows antioxidant capacity via FRAP assay for bars and cakes during *in vitro* digestion and fermentation. Statistical comparisons were made using unpaired Kruskall-Wallis test at 95 % confidence using as reference group in both panels panel A "No (no chocolate addition)". Statistic labels: ns – not significant; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$; **** - $p < 0.0001$.

replacement shakes (1 sachet) and a HMF daily intake of 10 mg/day, which was estimated by Rufián-Henares and de la Cueva (2008) for the Spanish population. We found that shakes could make the highest average contribution with 35.36 % of the daily intake (just for one shake), whereas cakes made the lowest with 4.85 % (Fig. 2**D**). Cereal bars showed an average contribution of 9.72 %. Those high values obtained for shakes were due to two samples that had extremely high HMF concentrations, which could make up for 75–100 % of the estimated HMF daily intake with just one shake. Those especially high HMF values found in replacement shakes could be due to their high protein, sugars but also added B vitamins. Therefore, although these are rather complete products (nutritionally speaking), the potential health risks derived from HMF exposure should be also considered. This presents as a great opportunity for food industry to improve their manufacture process so HMF or other potentially harmful substances can be reduced.

Antioxidant capacity, on the other hand, did not show much differences across products and, generally, only those with added chocolate or flavors in some cases showed significantly higher antioxidant capacity than the rest. These results fall within the expected at least up to some point. Cocoa has been reported in several occasions as product rich in polyphenols, known antioxidants (Maldonado-Mateus et al., 2021; Urbańska & Kowalska, 2019). In fact, these antioxidants have a strong reducing capacity (Di Mattia, Sacchetti, Mastrocola, & Serafini, 2017), which could explain the statistically significant relationship between

cocoa addition of weight loss foods and their antioxidant capacity measured by the FRAP method (Fig. 5**A and 5B**).

On the other hand, we also observed how the antioxidant capacity released during *in vitro* gut microbial fermentation was significantly higher than that released during *in vitro* digestion. This could be due to several reasons. These products are made of cereals, which could not be fully digested due to their content in vegetable cells and fiber. During fermentation though, they can be metabolized by gut microbes, helping to release molecules that otherwise would be trapped within vegetable structures (Miglio, Chiavaro, Visconti, Fogliano, & Pellegrini, 2008). Secondly, most polyphenols reach the colon where they can be metabolized by gut microbes (Kawabata, Yoshioka, & Terao, 2019). It has been demonstrated that some phenolic metabolites are actually more antioxidant than their parent compounds (Chen et al., 2020), though it is also true that some other are less antioxidant. These results suggest the first scenario in which gut microbes are predominantly producing metabolites with higher antioxidant capacity.

5. Conclusions

Most of weight-loss commercial products sold in Spain them reported a rather high added sugar content, which should be taken into consideration by consumers but also by industry to come up with alternatives to reduce their content. High concentrations of HMF were found in these products, which has the potential to exert some genotoxic activity. This compound is related to the technological process of manufacture and therefore, again this presents itself as an opportunity for industry to improve their processes by monitoring the appearance of Maillard compounds. On the other hand, these products showed potential to release a fair antioxidant power during gut microbial fermentation, which could contribute to intestinal health and improve oxidative stress, although animal or human interventions would be needed to provide a certain answer to that.

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CRediT authorship contribution statement

Sergio Pérez-Burillo: Investigation, Methodology, Data curation. **Daniel Hinojosa-Nogueira:** Formal analysis, Investigation. **José Ángel Rufián-Henares:** Validation, Formal analysis. **Silvia Pastoriza:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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