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Effect of *in vitro* digestion-fermentation over global antioxidant response and short chain fatty acid production of beet waste extracts in Ca(II)-alginate beads

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Abstract

The aim of the present work was to analyze the effect of *in vitro* gastrointestinal digestion-fermentation on the antioxidant capacity, total phenols and production of short chain fatty acids (SCFAs) from biocompounds derived from beet waste (leaf and stem) encapsulated in different formulations of Ca(II)-alginate beads. The encapsulated systems presented higher antioxidant capacity in the different phases (digested and fermented) than the extracts without encapsulation, making Ca(II)-alginate beads a suitable delivery vehicle. Levels of total phenolic compounds and antioxidant capacity of the fermented fraction were up to ten times higher than those of the digested fraction, boosted by the contribution of bioactive compounds from by-product of beet as well as by sugars and biopolymers. Among the formulations used, those that had excipients (sugars and/or biopolymers) presented a better overall antioxidant response than the beads with just alginate. Guar gum and sucrose lead to a promising enhancement of Ca(II)-alginate beads not only for preservation and protection but also in terms of stability under *in vitro* digestion-fermentation and production of SCFAs.

Keywords: Ca(II)-alginate beads; antioxidant capacity; short chain fatty acids; gut microbiota; digestion; fermentation; total phenols.

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1. Introduction

Several technologies have been proposed to target the waste produced from vegetables/fruits as a cheap source of valuable compounds (such as bioactives). Efforts have been made to improve the recovery of bioactive compounds from by-products, and to successfully incorporate them into ingredients and/or products. Encapsulation technology is one of the main strategies employed for protection and controlled release of bioactive compounds, allowing their recycling inside the food chain as functional additives for different products. In this way, this approach leads not only to increase healthy habits in consumers but also to improve sustainability considering environmental impacts[1].

Beet (*Beta vulgaris*) contains high concentrations of bioactive compounds that are distinctive for providing specific micronutrients in a regular diet[2,3]. Apart from having betalains that confer the red beet coloration and antioxidant properties[4], it provides several compounds with nutritional value. Particularly, beet contains phenolic compounds that are considered natural antioxidants and free radical scavengers with benefits for human health, such as anti-inflammatory, antihypertensive, antitumoral and anticholesterolemic activities[5]. An important amount of these compounds is in the peels, stems, and leaves and most of the time they become waste, usually because these parts are removed during processing or cooking. Although betalain content obtained from roots (primary part that is consumed) showed higher antioxidant activity than the obtained from by-products[6], beet stem and leaves contain compounds highly valued in the industry such as phenolic acids, flavonoids, phenolic aldehydes, among others[7]. The optimization in the recovery and protection methods of by-products to reduce biomass and environmental risks, as well as to develop added-value food, represents a necessary technological innovation for the benefit of mankind[8-10].

In order to profit from the health properties of bioactive compounds, they need to withstand the effects of food processing and their potential release from the food matrix, remaining bioaccessible in the gastrointestinal tract. Bioactive compounds from different plants are prone to oxidative degradation, and encapsulation is an effective method in improving their stability[11]. Much attention has been focused on hydrogel beads formed by food-grade biopolymers as a delivery system to protect and encapsulate some food ingredients, drugs, and bioactive compounds and/or control their release[11,12]. Alginates are widely used in the food industry due to their applications as immobilization and controlled release matrix for the encapsulation of biomaterials such as cells, probiotic bacteria, and bioactive compounds from plants[13-15]. Alginate can form hydrogels through a sol-gel transition in the presence of di- and trivalent cations such as calcium, because of the ionic crosslinking between the carbonyl groups of alginate and cations, giving rise to a three-dimensional network[16]. Under gastric conditions, Ca(II)-alginate beads were reported to shrink, under intestinal conditions to swell, and ultimately to disintegrate at the end of the intestinal phase. Depending on the formulation, different degrees of antioxidant activity were obtained throughout the gastrointestinal tract[17-22]. In recent work, we unraveled the effect of a standardized *in vitro* digestion-fermentation over Ca(II)-alginate beads synthesized with sugars and biopolymers[23]. Through several methods, it was confirmed that plain beads (with no extract) released antioxidant capacity during digestion comparable to some common foods. This capacity was even enhanced after fermentation, obtaining up to ten-fold increase in the antioxidant values and an important production of short-chain fatty acids (SCFAs). At the same time, it was established that the microstructure of Ca(II)-alginate beads slightly changed in oral and gastric phase but showed deeper changes (1-100 nm range) in intestinal fluid, where absorption takes place. In the present study, beet waste from leaves and stems, a by-

product of the vegetable processing industry, were encapsulated in Ca(II)-alginate based beads. The aim of the present work was to evaluate the effects of the two by-product extracts and different formulations, by including additives in the alginate matrix, over *in vitro* gastrointestinal digestion-fermentation on the release of antioxidant capacity and the production of SCFAs as modulation markers of the gut microbiota activity.

2. Materials and Methods

2.1. Extract and bead preparation.

Beet (*Beta vulgaris* var conditiva) (1 kg) was purchased in a local market. Stems and leaves were separated from root; they represented 51 ± 2% in fresh weight of the total beet. Then, each by-product was separately washed, scalded and homogenized (as a puree) in a blender (model HR 1372, Philips). Each puree (stem and leaf) was mixed with water (1:1.5, meaning 15g of puree:22.5mL of water) for 5 min at 20 °C by magnetic stirring (1500 rpm) and then centrifuged at 6,000 rpm during 30 min, finally obtaining two separate extracts. Seven formulations were prepared according to Aguirre-Calvo[23]. The inclusion of co-materials such as sugars and hydrocolloids provides additional advantages in Ca(II)-alginate systems. Table S1 of the Supplementary file includes a detailed description of each excipient as well as their concentrations. Briefly, the following formulations were prepared: alginate (A); alginate-sucrose (AS); alginate-sucrose-guar gum (ASGG); alginate-sucrose-arabic gum (ASAG); alginate-sucrose-high methoxy pectin (ASH); alginate-sucrose-low methoxy pectin (ASL); alginate-sucrose-dextran (ASD). Beads were generated by the dropping method[21]: the sodium alginate solutions prepared for stem or leaf extracts were dripped into a calcium chloride solution (2.5% w/v, 0.1 M buffer acetate pH 5.5 with or without 20% w/v sucrose, depending on the system). The extrusion speed of the peristaltic pump (model 7518–00, Cole Parmer,

Masterflex, USA) was set at 20 rpm, with a 6 cm distance between the 0.45 mm tip and the surface of the calcium chloride solution. The formed beads were maintained to harden for 5 min in the gelling bath. Then, they were washed out two times, using distilled water, and kept until further use in a conventional fridge at 5±1 °C.

2.2. In vitro Gastrointestinal Digestion.

The activities for the enzymes were previously determined with several methods to establish the concentration to be used in the digestion protocol. Alpha-amylase from Bacillus (Sigma-Aldrich Ref.:A6380) the enzymatic assay method was EC 3.2.1.1[24]; for pepsin from porcine gastric mucosa (Sigma-Aldrich Re.: P700) the enzymatic activity was determined with the method EC 3.4.23.1,[24] for pancreatin from porcine pancreas (Sigma-Aldrich Re.: P7545) the trypsin activity method EC 3.4.21.4[24] was used and for bile salts (Sigma-Aldrich Re.: B8631) a quantitative determination of Total Bile Acids-IVD-(Spinreact Re.:1001030) was performed.

All samples (extracts and beads) were subjected to an *in vitro* digestion process following the protocol by Minekus et al.[24] and Perez Burillo et al.[25,26]. Briefly, *in vitro* digestion is composed of three phases that mimic gastrointestinal conditions. The oral phase was performed by mixing 5.0 g of sample with 5.0 mL of Simulated Salivary Fluid (SSF), with α-amylase (150 U/mL) and 25.0 µL of $CaCl_2$ then incubated at 37 °C for 2 min. Followed by the gastric phase, 10.0 mL of Simulated Gastric Fluid (SGF) with pepsin (4000 U/mL) and 5.0 µL of $CaCl_2$ were added to the oral phase, the pH was lowered to 3.0 by adding 1 N HCl, and then samples were incubated at 37 °C for 120 min. Finally, for the intestinal phase, 20.0 mL of Simulated Gastric Fluid (SGF) with pancreatin (26.74 mg/mL), bile salts (20 mM) and 40.0 µL of $CaCl_2$ were added to the gastric phase; the pH was raised to 7.0 with 1 N NaOH, after which it was incubated at

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37 °C for 120 min. The enzymatic reactions were halted by immersing the tubes in iced water. The samples were then centrifuged at 6000 rpm for 10 min at 4 °C and the supernatants were then labeled as *digested fractions* (*d*). After *in vitro* digestion, the solid residue (fraction not available for absorption) that is left after removing the supernatant plus 10% of the digestion supernatant were used as sample for fermentation procedure (*sfp*).

2.3. In vitro Fermentation

The *in vitro* fermentation was performed according to the protocol by Perez-Burillo et al[25]. Fermentation final solution was prepared with 10 mL fermentation medium (peptone water 15g/L), 0.5 mL reductive solution (51.5 mM cysteine, 80 mM sodium sulfide, and 0.04 M NaOH) and 13.12 µL of 0.01 %w/v of resarzurin. For the inoculum, feces were collected from healthy donors (n = 3 -two male, one female-, mean body mass index = 21.3, mean age = 34.2 years, not taking antibiotics). Briefly, *sfp* was weight (500 mg) and 7.5 mL of fermentation final solution and 2.0 mL of inoculum (32% feces in 100 mM phosphate buffer pH 7.0) were added. The anaerobic atmosphere was produced by bubbling nitrogen through the mix; then, it was incubated at 37 °C for 20 h under oscillation. Immediately afterward, the samples were immersed in ice to stop the microbial activity and centrifuged at 500 rpm for 20 min. The supernatant was collected as a soluble fraction potentially absorbed after fermentation and stored at -80 °C, then labeled as *fermented fractions* (*f*).

After *in vitro* gastrointestinal digestion and *in vitro* fermentation, the two fractions (*d* and *f*) were used to determine the antioxidant capacity in the different stages: digestion supernatant (fraction available for absorption at the small intestine) and fermentation supernatant (fraction available for absorption at the large intestine).

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2.4. Antioxidant capacity methods

The supernatants stored from both processes were analyzed to evaluate the global antioxidant response of the beads under *in vitro* digestion-fermentation. Five different methods were used to determine the antioxidant capacity: the gallic acid equivalents antioxidant capacity referred to Folin ($GEAC_{FOLIN}$)[25]; the Trolox equivalents antioxidant capacity referred to reducing capacity against ABTS or AAPH radicals ($TEAC_{FRAP}$, $TEAC_{ABTS}$, $TEAC_{AAPH}$, respectively)[25-26]; and the Catechin equivalents antioxidant capacity against OH radicals or referred to reducing capacity method ($CEAC_{OH}$, $CEAC_{RED}$, respectively)[26]. Controls have been made to assure that the contributions of betacyanins from the samples did not interfere with the readings for $TEAC_{FRAP}$, $TEAC_{AAPH}$, $CEAC_{OH}$, and $CEAC_{RED}$.

2.5. SCFAs analysis

As a measure of the gut microbiota functionality, the production of SCFAs was assessed following the procedure described by Aguirre-Calvo et al.[23] and Delgado-Andrade et al.[27]. This analysis was carried out on Accela 600 HPLC (Thermo Scientific) determining acetic, propionic, and butyric acids. A 0.1 M phosphate buffer pH 2.8:acetonitrile (99:1, v/v) delivered at a 1.25 mL/min flow rate was used as a mobile phase. An Aquasil C18 reverse phase (Thermo Scientific) (150 × 4.6 mm, 5 μm) column was used, with a total run-time of 30 min. Detection was made at 210 nm with a UV-VIS PDA. Briefly, an aliquot of 1.0 mL of the supernatant after the fermentation process was centrifuged, filtered (0.22 μm nylon filter) and analyzed by means of a HPLC system. Standard solutions for the acids were quantified with concentrations ranging from 10,000 to 5 ppm. Finally, the results were expressed as mmol of SCFA per g of beads.

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2.6. Statistical analyses

Data are expressed as mean values of triplicates (n = 3) ± standard deviation (SD). One-way ANOVA with Tukey's post-test were performed by using Prism 6.01 (GraphPad Software Inc., San Diego, CA, USA) to determine significant differences among mean values on all the measured parameters ($p < 0.05$).

3. Results

Leaf and stem extracts were successfully encapsulated in Ca(II)-alginate beads containing several excipients. The advantage of including co-materials such as sugars and hydrocolloids in Ca(II)-alginate beads was already demonstrated producing benefits by increasing the entrapment efficiency and stability of the encapsulated compounds [23,28-32]. The loading efficiencies values ranged between 16-35 % and 28-60 % for betacyanin and total phenolic compounds, respectively, depending on the extract and formulation, as previously observed[28,29].

Since there is no single chemical assay that can accurately evaluate the contribution of bioactive compounds nor evaluate the antioxidant potential in them due to the diversity of oxidation processes involved in the human body[26,33], several analyses involving the GAR method were performed for the digested and fermented fractions of Ca(II)-alginate based beads.

Total phenolic compounds or TP ($GEAC_{FOLIN}$) were assessed for both the digested and fermented fractions, as shown in Fig 1, in order to consider the antioxidants potentially absorbed in both small and large intestines, respectively. The $GEAC_{FOLIN}$ of the unprotected extracts, as well as the contribution of plain Ca(II)-alginate beads (without extracts), were included for comparison purposes.

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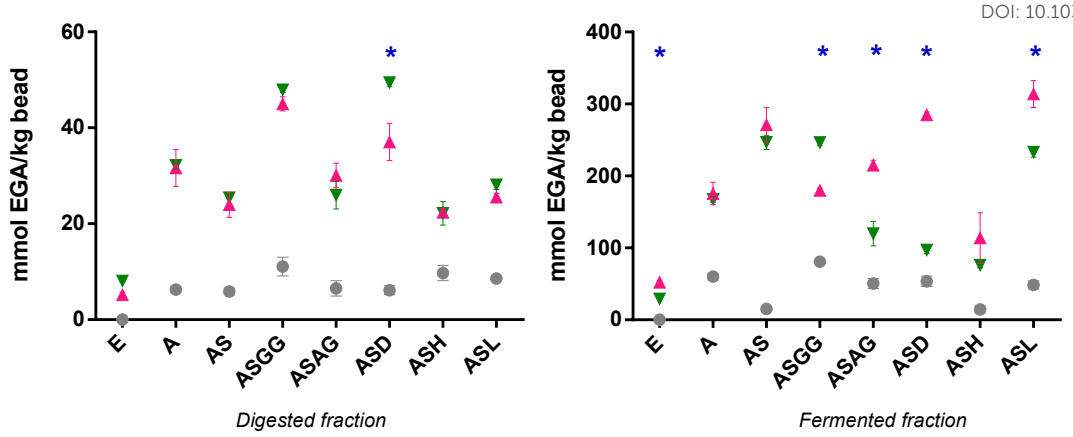

Fig 1. Total phenolic compounds $GEAC_{FOLIN}$ (mmol Equivalent of gallic acid/g beads) values from digested and fermented fractions of Ca(II)-alginate based beads with leaf (green inverted triangles) and stem (pink triangles) extracts and without extracts (grey circles, adapted from Aguirre-Calvo et al.[23]). The values corresponding to the non-encapsulated extracts (E) were included. A: alginate; S: sucrose; GG: guar gum; AG: arabic gum; D: dextran; H: high methoxyl pectin; L: low methoxyl pectin. Mean ± standard deviations values are reported. Asterisk (*) indicates significant differences between extracts for samples of the same formulation ($p < 0.05$).

Even though some phenolic compounds from the non-encapsulated extracts reached both digested and the fermented fractions, these concentrations are much lower than those that succeed by any of the bead's formulations. Among the digested beads (Fig 1A), both encapsulated extracts showed no significant difference between them; only dextran (ASD) addition produced a significant difference between extracts, being the formulation with the leaf extract higher than that with stem extract. Moreover, the levels of total phenolic compounds of the fermented fraction were up to ten times higher than those of the digested fraction (Fig 1). As a general trend, stem extract produced higher levels of phenolic compounds upon fermentation. Some excipients exerted a positive effect, but only for a specific extract (stem), such as sucrose plus arabic gum, dextran, and low methoxyl pectin, showing a higher content of TP with respect to Ca(II)-alginate plain beads (A).

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As mentioned earlier, due to the diversity of oxidation processes, the antioxidant activity must be analyzed through several methods to understand the real antioxidant potential of a food or extract. Then, the ferric reducing capacity and the capacity against two radicals were performed (Fig 2) for both digested and fermented fractions. The encapsulated extracts produced much higher $TEAC_{FRAP}$ values than the non-encapsulated ones, obtaining values between two and four times higher for the digested beads, and even a much higher response after fermentation. Significative differences among extracts were obtained for both digested and fermented beads. However, it is not possible to assure which extract produced higher levels of antioxidant activity based on the reduction of Fe^{3+} to Fe^{2+}, and there is no clear trend regarding which excipients enhance the activity, considering both digested and fermented groups. The addition of sucrose (AS) and guar gum (ASGG) produced the higher $TEAC_{FRAP}$ values for both extracts for the digested fraction (Fig 2A), in line with the $GEAC_{FOLIN}$ values (Fig 1A). Among the fermented ones, the inclusion of sucrose (AS) and arabic gum (ASAG) produced the best behavior for both extracts (Fig 2B).

It is worth noting that not all the excipients on the beads exerted protection of the antioxidants of the extracts against $ABTS^{*+}$, as observed for ASGG and ASD with stem extract, and ASAG for both extracts, for the digested fraction (Fig 2C). Regarding the fermented fraction (Fig 2D), only ASH with stem extract showed no significant differences with respect to the extract, revealing an overall positive effect by Ca(II)-alginate encapsulation with excipients. Both digested extracts behave very similarly for the digested fraction, even though the activity was significantly higher in ASGG and ASD for leaf extract (Fig 2C). Leaf extract showed an overall higher antioxidant activity against $ABTS^{*+}$ for fermented systems, except for ASAG systems (Fig 2D). This trend was the opposite to that observed against another radical (AAPH) for the fermented

fraction, reaching AS, ASAG, and ASL the highest activities for stem extract (Fig 2F). Among the digested fractions, leaf extract showed higher antioxidant values than stem (Fig 2E). The presence of sucrose, guar gum and dextran improved the antioxidant capacity of the beads with respect to A beads (Fig 2E). Finally, the overall antioxidant capacity (expressed as mmol of Trolox/kg of beads) against AAPH was higher than for ABTS, obtaining protection of the extracts by its encapsulation, which even was boosted by the presence of some excipients.

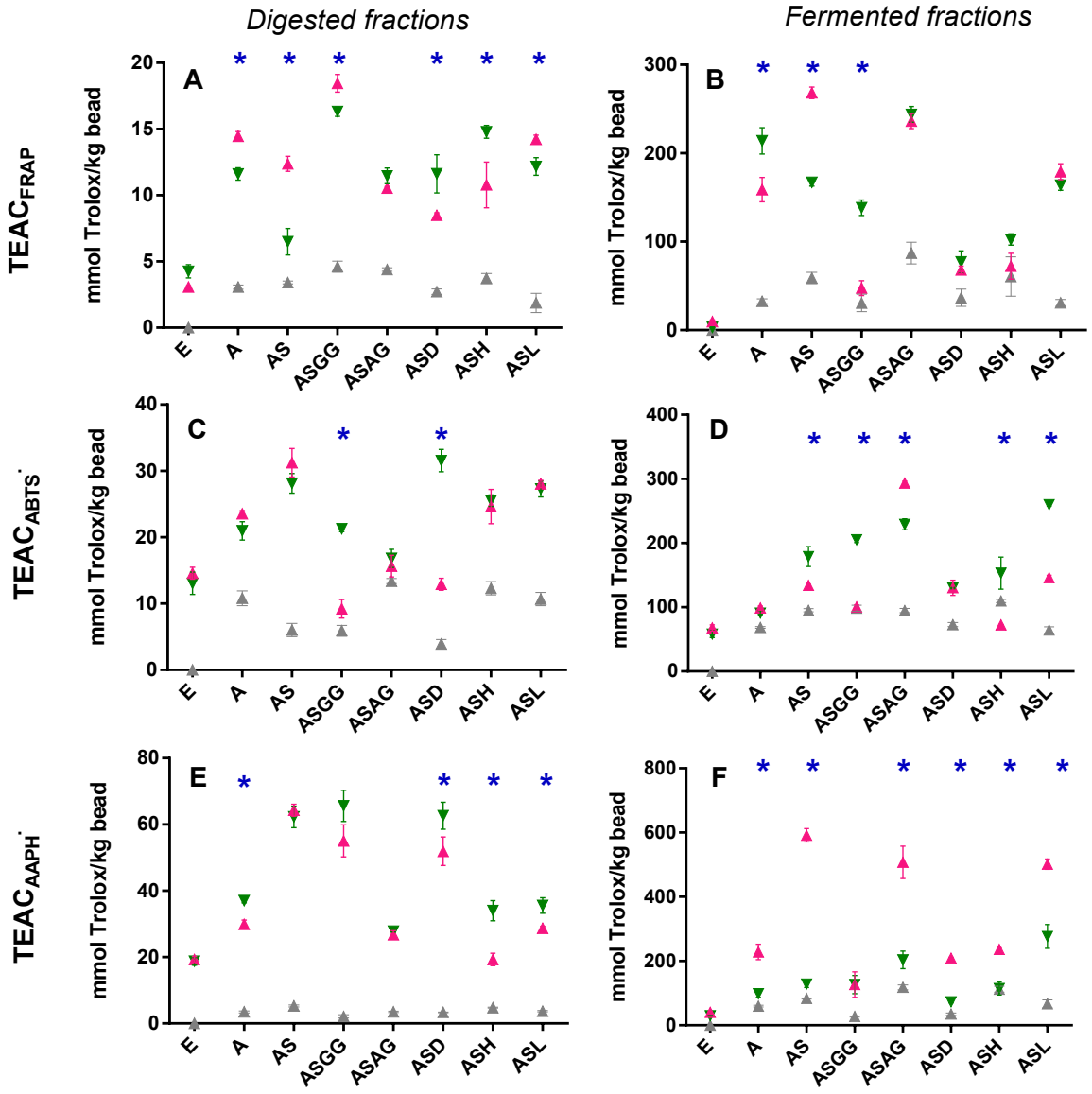

Fig 2. Antioxidant capacity from digested (A, C, E) and fermented (B, D, F) fractions for Ca(II)-alginate beads with leaf (green inverted triangles) and stem (pink triangles) extracts containing

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different excipients. A-B show $TEAC_{FRAP}$, C-D $TEAC_{ABTS}$, E-F $TEAC_{AAPH}$. The values for beads without extracts (grey circles, adapted from Aguirre-Calvo et al.[23]) were included for comparative purposes. A: alginate; S: sucrose; GG: guar gum; AG: arabic gum; D: dextran; H: high methoxyl pectin; L: low methoxyl pectin. Mean ± standard deviations values are reported. Asterisk (*) indicates significant differences between extracts for samples of the same formulation ($p < 0.05$).

The capacity against ferric ions at physiological pH ($CEAC_{RED}$ method) of digested and fermented fractions was measured as the global reduction capacity[25]. The activity of the non-encapsulated leaf extract was enhanced by its encapsulation (Fig 3A). Furthermore, the inclusion of excipients such as guar gum and both types of pectin produced higher protection of the bioactive compounds in the digested fraction with respect to the beads with alginate and sucrose or plain beads (Fig 3A). On the other hand, stem extract did not show a great improvement by encapsulation for the digested fractions, with the only exception of high methoxyl pectin (Fig 3A). However, fermented samples showed a huge boost on the activity, especially for stem extract, even though most of the included excipients did not improve the activities (with once again the exception of guar gum for leaf extract) (Fig 3B). In both digestion and fermentation, the encapsulation of the extract allowed higher protection of the encapsulated compounds with respect to the extract that was not encapsulated.

After fermentation, several compounds are produced and released, thus the antioxidant activities measured result from different molecules than the parent compound introduced into the *in vitro* system. In a previous work of our group,[28] a good correlation between antioxidant activity measured by ABTS and phenolic compounds content encapsulated in Ca(II)-alginate beads ($R^2 = 0.846$) has been obtained, however no such correlation was found with betacyanins. In the present work, some correlations with $GEAC_{FOLIN}$ were obtained: for beads containing stem extract correlates with $TEAC_{AAPH}$ and $TEAC_{FRAP}$ for digested samples, and $TEAC_{AAPH}$ and $TEAC_{ABTS}$ and $CEAC_{RED}$ for

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fermented samples. Instead, for beads containing leaf extract only correlates with $TEAC_{AAPH}$ for digested samples, showing no correlations with fermented ones. The correlations are shown as Supplementary Figures (S2-S4) in the Supplementary File.

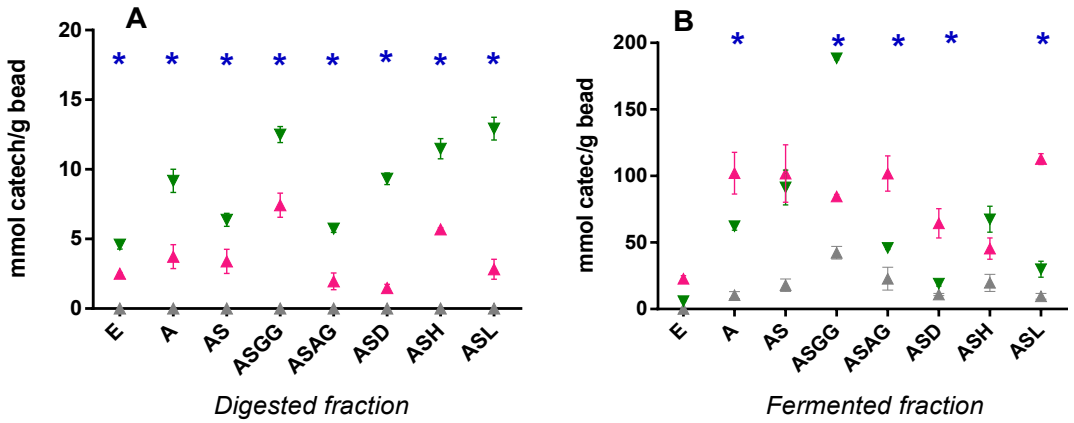

Fig 3. Antioxidant capacity ($CEAC_{RED}$) from digested and fermented fractions of Ca(II)-alginate beads with leaf (green inverted triangles) and stem (pink triangles) extracts containing different excipients. The values for beads without extracts (grey circles, adapted from Aguirre-Calvo et al.[23]) were included for comparative purposes. A: alginate; S: sucrose; GG: guar gum; AG: arabic gum; D: dextran; H: high methoxyl pectin; L: low methoxyl pectin. Mean ± standard deviations values are reported. Asterisk (*) indicates significant differences between extracts for samples of the same formulation ($p < 0.05$).

Non-digestible carbohydrates consumed are usually fermented by colonic microbiota resulting in the production of SCFA (acetate, propionate, and butyrate), usually endorsed with various health beneficial properties[34,35]. Fig. 4 depicts the total SCFAs, acetate, propionate and butyrate released after fermentation of Ca(II)-alginate-based beads containing extracts of leaf and stem. The high content of SCFAs (Fig 4) evidences the fermentative activity, as by-products of the colonic microbiota metabolism. Some studies suggest that polyphenols and their metabolites could selectively stimulate some microorganisms' metabolic pathways, like SCFAs production[36,37]. The differences between the controls and the prebiotic action exerted by the excipients have been discussed previously[21] and will not be further analyzed. Beads containing leaf extract

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produced a higher response for total SCFAs in comparison to stem beads, except for AS system. Particularly, among the leaf beads, the addition of guar gum showed a significant increase in the production of SCFAs (especially propionate and butyrate), which correlates with the studies regarding its role and help in metabolism through the SCFAs increase[38]. Regarding the response of both propionate and butyrate, the stem extract was higher in the production of these compounds, except in the case of guar gum, as previously commented, which greatly favored the leaf extract.

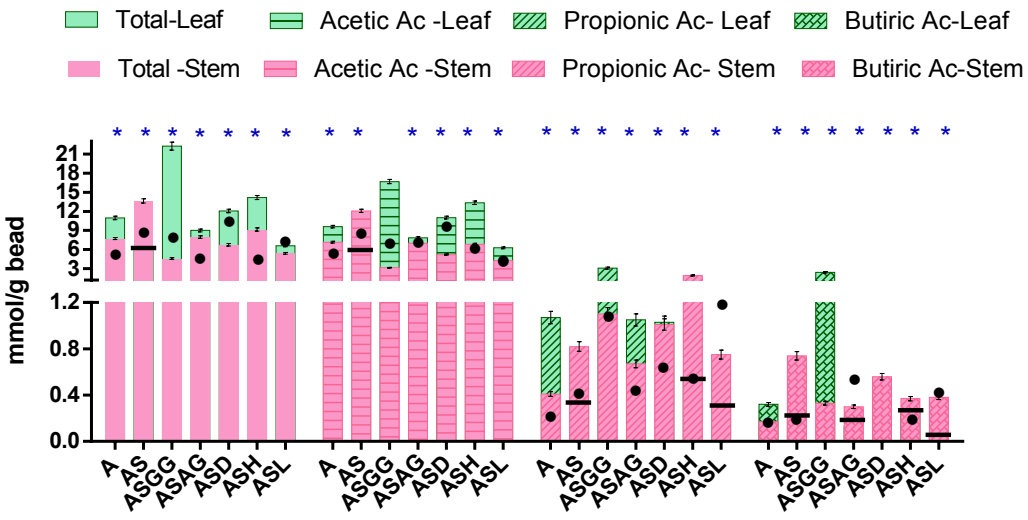

Fig 4. Release of SCFAs (mmol per g of beads) for systems of Ca(II)-alginate based beads with leaf and stem extracts containing excipients after fermentation. The hidden values behind each column are indicated with lines. The values for beads without extracts (dots were adapted from Aguirre-Calvo et al.[23]) were included as points for comparative purposes. A: alginate; S: sucrose; GG: guar gum; AG: arabic gum; D: dextran; H: high methoxyl pectin; L: low methoxyl pectin. Mean ± standard deviations values are reported. Asterisk (*) indicates significant differences between extracts for samples of the same formulation ($p < 0.05$).

4. Discussion

Table 1 summarizes the global antioxidant response (GAR) by integrating the response of both the digested and fermented phases, obtained for each of the systems analyzed for both encapsulated extracts. Also in this table, the $CEAC_{(OH)}$ values were

added but only for the fermented phase (as shown in Supplementary Fig. S1), since the digested one did not show any antioxidant capacity against hydroxyl radicals.

In general terms, Ca(II)-alginate beads had more antioxidant capacity in the different phases (digested and fermented) than the extracts without encapsulation; this allows them to be a suitable vehicle, reaching duodenum or colon fermentation despite the severe conditions of the gastrointestinal tract. Different studies have provided compelling evidence that the ingestion of beet and derivatives offers beneficial physiological effects that may translate to improved clinical outcomes for several pathologies[39-41]. The knowledge of absorption and metabolism of the antioxidant compounds is critical to assure an adequate bioavailability of the antioxidants. Betalain availability is high, being betacyanins less bioavailable than betaxanthins[42]. However, intestinal bacteria are actively involved in betalains metabolism, interfering with their absorption and bioavailability[43]. Some phenolic compounds such as flavonoids also can be substrates for intestinal bacteria, especially if they are in their glycosylated form[44]. Also, some flavonoids are able to enter the red blood cells exhorting cellular antioxidant activity. It was demonstrated by Angelino and co-workers[45] that the liver received an unchanged flavonoid (apigenin-8-C-glucoside-2-O-xyloside) which was returned to the gut by enterohepatic recirculation for reabsorption at ileum. More studies are needed to correlate the *in vitro* bioaccesibility with *in vivo* bioaccesibility to finally understand the full capacity of these systems.

Some of the beads with excipients (sugars and/or biopolymers) presented a better overall response (GAR) than the controls that only had alginate. According to the information obtained from Table 1 for the leaf beads, the highlighted systems are those that have arabic gum, guar gum, and low methoxyl pectin. It has been proved that the addition of sugars and can not only optimize the encapsulation efficiency and control the

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release of bioactive compounds, but also compensate for the deficiencies that alginate control presents[28,29,42]. Thus, these functional ingredients obtained from a beet by-product generated not only antioxidant compounds but also the formation of SCFAs, which exert *per se* more beneficial effects. Then, it is important to analyze altogether the information of both Table 1 and Fig 4, considering the overall effects given by each formulation, and keeping in mind that the fermentative phase is the one that contributes more to the antioxidant capacity. The compounds released after the fermentation are different from the parent compound introduced into the *in vitro* system, so the antioxidant capacity is relative to the new compounds, not the parent ones (see Fig. S2-S4 in the Supplementary File for a complete analysis of correlations).

The high total SCFAs (Fig 4) evidenced the fermentative activity since they are by-products of the colonic microbiota metabolism. Various health beneficial properties were already reported for acetate, propionate, and butyrate, such as balance redox equivalent production in the anaerobic environment of the gut[47] and lowering the intestinal pH, which hinders pathogens and enhances the nutrient absorption[35]. Ca(II)-alginate beads produced mainly acetic acid, which is typically the most abundant SCFA in the colon, since it is generated by most enteric bacteria as a result of carbohydrates fermentation and makes up more than half of the total SCFAs detected in feces[48]. A significant increase for leaf beads was observed in the total SCFAs content with the addition of biopolymers and sucrose, with respect to plain Ca(II)-alginate beads. Sucrose and guar gum produced an increase in acetic acid, and propionic acid was increased in all cases, disclosing an enormous potential to employ these beads as ingredients in functional foods. Then, it is important to analyze the particular effect in the inclusion of different co-materials during synthesis, especially dietary fibers, which may help increase

beneficial fermentative products in the distal regions of the colon and improve colonic health[22].

Several factors are involved in the digestion and fermentation processes, affecting both the way substrates are digested and fermentation products are formed, such as food composition (particularly amount of the fermentable substrate) and its physical form, interactions between different groups of bacteria, and availability of inorganic electron acceptors[49]. Besides, it is known that the abundance of specific bacterial taxa varies upon DF supplementation, explaining the changes in SCFAs profiles. *Bacteroidaceae-Ruminococcaceae* and *Prevotellaceae-Ruminococcaceae* dominated microbiota produced more butyrate (up to 96%) or propionate (up to 40%), respectively[48]. Then, less fermentable fibers will impact the butyrate production[50], which was increased by the excipients except for dextran and guar gum, revealing that among the non-digestible carbohydrates these two are more fermentable than the others[50]. Instead, arabic gum will increase propionate but not acetate[51] and low methyl pectin will increase both[50], since they induce growth and/or activity of specific beneficial populations. The result of total SCFAs is higher than the values obtained for a well-known prebiotic such as inulin[52] for all the systems, revealing the huge ability of Ca(II)-alginate beads to improve gut microbiota metabolism. Thus, considering also acetate, propionate and butyrate released after fermentation, it can be highlighted that the addition of the excipients exerts a positive effect throughout the entire stage of *in vitro* conditions.

On the other hand, the overall difference for leaf and stem extracts could be related to the types of compounds present in each extract, as well as to the particularities that they impose in the microstructure of the Ca(II)-alginate beads. The leaf and the stem contain phenolic compounds and betacyanins, although not in the same proportion. In previous works[28,29] it has been studied that the amount of betacyanin in the stem extract

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is significantly higher than in the leaf (around 7 times); in contrast, the content of phenolic compounds is around 2.5 times higher in the leaf extract than in the stem extract.

Finally, an additional aspect should be considered. As previously demonstrated, the presence of natural extracts and excipients prompts important structural changes in the alginate network, affecting key parameters that define the encapsulation performance in most of the industrial and environmental applications[28,29]. During gelation, alginate chains associate in dimers (at scale: ~ 1 nm), which then self-associate forming a rod-like structure (at scale: ~ 10 nm) with different degrees of interconnection (at scale: ~ 100 nm), giving the network gel. The presence of leaf extract produced changes at all the scale levels, provoking a higher interconnected network of more dense rods. Stem extract also showed similar but slighter changes[28]. Besides, the presence of sugars and hydrocolloids affected the alginate dimers size and density, the size and compactness of the rods as well as their interconnectivity[28,29], even though these changes are strongly overlaid by the presence of each extract. In a recent study[23], the microstructural analysis of Ca(II)-alginate by SAXS allowed studying these three scales under digestion conditions. The size and density of the alginate dimers in beads including excipients increasingly resemble the alginate plain system in each successive step of the digestion, which could be due to the loss of each excipient (especially sucrose). The rods compactness and size slightly incremented along with salivary and gastric fluids for all the systems. It is worth noting that the salivary phase is too short to induce a significant structural change and the acidic conditions of the gatric fluid, far from dissolving the system, are expected to produce a reinforcement of the network by the protonation of non-crosslinked sites. By the contrary, through the intestinal fluid an important decrease for both parameters was observed revealing a partial loss of the structure (in average for the systems with excipients, size of rods changes from ~7.3 nm to ~4.8 nm and their compactness decreases

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from a fractal density of ~2.7 to ~2.2). These changes are also related to the increase in the interconnectivity of the Ca(II)-alginate network at larger scales. Then, from the microstructural point of view, all beads showed a similar and advantageous behavior: they slightly change in oral and gastric fluids, and they partially dissolve their structure in intestinal fluid where the absorption takes place, protecting the extract throughout the entire stage of *in vitro* conditions.

Then, the behavior obtained in the present work for each formulation is a consequence of the composition and the microstructural association between components, which will affect the availability of the compounds along with digestion and fermentation. In this way, the specific compounds could exert diverse antioxidant behaviors, leading to different general responses in relation to the nature of the interactions between biopolymers/extracts in Ca (II)-alginate beads in the gastrointestinal environment.

Table 1. Global antioxidant response (GAR) for Ca(II)-alginate based beads containing leaf and stem extracts.

		Total^{GAR}	**GEAC$_{FOLIN}$**	**TEAC$_{FRAP}$**	**TEAC$_{ABTS}$**	**TEAC$_{AAPH}$**	**CEAC$_{RED}$**	**CEAC$_{OH}$**
LEAF	extract	37 ± 1^{j}	7 ± 1^{h}	71 ± 7^{f}	48 ± 2^{h}	11 ± 1^{f}	0.20 ± 0.01^{f}
	A	200 ± 5^{g}	226 ± 15^{bc}	112 ± 5^{ef}	135 ± 12^{g}	71 ± 4^{d}	5.05 ± 0.09^{jcd}
	AS	272 ± 10^{cd}	174 ± 5^{d}	207 ± 17^{bc}	191 ± 14^{e}	98 ± 14^{c}	3.6 ± 0.3^{d}
	ASGG	294 ± 5^{bc}	155 ± 9de	227 ± 5^{b}	192 ± 33^{e}	201 ± 2^{a}	8.0 ± 0.4^{ab}
	ASAG	146 ± 20^{h}	255 ± 9^{ab}	246 ± 10^{b}	232 ± 28^{d}	52 ± 2^{e}	7.7 ± 0.7^{ab}
	ASD	147 ± 6^{h}	89 ± 14^{fg}	161 ± 3^{cde}	135 ± 7^{g}	28 ± 2^{f}	2.5 ± 0.7^{e}
	ASH	98 ± 6^{i}	117 ± 7^{ef}	179 ± 26^{cd}	149 ± 23^{fg}	79 ± 11^{cd}	5.1 ± 0.9^{cd}
	ASL	261 ± 8^{cde}	176 ± 6^{d}	287 ± 4^{a}	312 ± 39^{c}	43 ± 7^{e}	9.2 ± 0.8^{a}
STEM	extract	57 ± 2^{j}	13 ± 2^{h}	82 ± 6^{f}	60 ± 3^{h}	25 ± 2^{f}	0.2 ± 0.1^{f}
	A	207 ± 19^{fg}	173 ± 14^{d}	122 ± 4^{e}	258 ± 25^{d}	106 ± 17^{bc}	6 ± 1^{bc}
	AS	295 ± 27^{bc}	281 ± 7^{a}	165 ± 4^{cd}	656 ± 22^{a}	105 ± 22^{bc}	3.6 ± 0.5^{d}

	ASGG	225 ± 4 ^{efg}	66 ± 9 ^{g}	110 ± 4 ^{ef}	182 ± 45 ^{ef}	92 ± 2 ^{c}	2.4 ± 0.8 ^{e}
	ASAG	245 ± 10 ^{def}	247 ± 9 ^{ab}	309 ± 4 ^{a}	534 ± 52 ^{b}	104 ± 14 ^{bc}	2.0 ± 0.9 ^{e}
	ASD	322 ± 8 ^{ab}	77 ± 4 ^{g}	143 ± 13 ^{de}	261 ± 6 ^{d}	66 ± 11 ^{d}	3.7 ± 0.4 ^{d}
	ASH	136 ± 36 ^{hi}	83 ± 16 ^{fg}	97 ± 3 ^{ef}	256 ± 7 ^{d}	51 ± 8 ^{e}	6.3 ± 0.7 ^{bc}
	ASL	339 ± 19 ^{a}	193 ± 9 ^{cd}	174 ± 4 ^{c}	530 ± 17 ^{b}	116 ± 5 ^{b}	6.1 ± 0.3 ^{jbc}

$GEAC_{FOLIN}$ are expressed in mmol gallic acid/ kg beads or L; $TEAC_{(FRAP,\ ABTS\ and\ APPH)}$ are expressed in mmol Trolox/kg beads or L and $CEAC_{(RED)}$ are expressed in **mmol** catechin/kg bead or L s and $CEAC_{(OH)}$ **mol** catechin/kg beads or L, respectively. Different lowercase letters on the columns (a-h) indicates significant differences between systems for the GAR determination ($p < 0.05$). A: alginate; S: sucrose; GG: guar gum; AG: arabic gum; D: dextran; H: high methoxyl pectin; L: low methoxyl pectin. Mean ± standard deviations values are reported.

5. Conclusions

The effects of *in vitro* simulated digestion-fermentation on the total phenolic content, antioxidant capacity and SCFAs production of Ca(II)-alginate beads were studied. The fermented fraction had up to 10-times higher antioxidant capacity compared to the digested fraction, revealing that both the encapsulated compounds and the sugars and biopolymers were responsible to enhance microbiota production. In each antioxidant capacity method, there were systems that improve the protection of the extract. Sucrose, arabic and guar gums, and low methoxyl pectin were the best excipients that exhibit protection in most of the antioxidant capacity for any of the fractions (digested or fermented). Moreover, among the used hydrocolloids, guar gum achieves two main goals: increased functional properties (total phenolic content and antioxidant capacity) in digested-fermented fractions and improved the generation of SCFAs by the gut microbiota. Furthermore, the addition of guar gum together with sucrose can lead to Ca(II)-alginate beads with improved properties for preservation of the beads and protection of encapsulated compound, being a promising ingredient or even as functional food.

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Conflict of interest

The authors declare the absence of conflict of interest.

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