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Bioaccessibility and stability of β-carotene encapsulated in plant-based emulsions: Impact of emulsifier type and tannic acid

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Abstract

The effect of two plant-based emulsifiers (quillaja saponin, QS and gum arabic, GA) and a polyphenol (tannic acid) on the formation, stability, digestibility, and β-carotene (BC) bioaccessibility of flaxseed oil-in-water emulsions was investigated. The gastrointestinal behavior of the emulsions was studied using a simulated gastrointestinal tract (GIT) consisting of mouth, stomach, and small intestine regions. In the absence of tannic acid, the initial extent of lipid digestion depended strongly on emulsifier type, with 45% and 76% of the free fatty acids being released after 5-min digestion for QS- and GA-emulsions, respectively. Even so, the lipid droplets were completely digested in both emulsions after 2 h incubation in the small intestine phase. Tannic acid addition (0.01% and 0.1% w/w) slowed down lipid digestion, but did not impact the final extent. The droplets in the QS-emulsions containing 0.1% tannic acid were highly flocculated in the stomach phase. Molecular docking simulations indicated that the interactions between tannic acid and the saponins were mainly driven by hydrogen bonding and hydrophobic interactions. Moreover, they showed that the interactions between tannic acid and QS were stronger at pH 2.5 than at pH 7.0, which would account for the extensive droplet flocculation observed under acidic conditions in the stomach. Emulsifier type and tannic acid addition had no significant influence on BC bioaccessibility. The GA-emulsions exhibited better stability than the QS-emulsions when stored at elevated temperatures (55 ºC for 7 days). Tannic acid addition effectively inhibited temperature-induced BC degradation. These results may facilitate the design of more efficacious nutraceutical-loaded functional foods and beverages.

Keywords: fortified emulsion; tannic acid; beta-carotene; lipid digestion; storage stability

1. Introduction

Many consumers are interested in incorporating more plant-based foods into their diets because their environmental impact, sustainability, healthiness, and ethics is perceived to be better than animal- or petroleum-based alternatives.[1] To satisfy this consumer demand, many food manufacturers are identifying, isolating, purifying, and characterizing the functional performance of plant-based ingredients derived from a wide range of sources. In particular, there has been considerable interest in their application as emulsifiers in various food and beverage products, such as creamers, sauces, soups, salad dressings, desserts, snacks, and dips.[2, 3] Numerous classes of plant-based emulsifiers have already been employed to form and stabilize oil-in-water emulsions including biosurfactants (saponins), polysaccharides (gum arabic and beet pectin), proteins (soy, pea, legume, and corn proteins), and phospholipids (soy and sunflower lecithin).[2]

The formation, stability, and functional performance of food emulsions depend strongly on the nature of the emulsifier used. For example, oil droplets coated by globular plant-based proteins (such as those found in soybeans or peas) are highly susceptible to aggregation at pH values around their isoelectric point, at high salt levels, and above their thermal denaturation temperature.[4-6] For this reason, there has been interest in identifying alternative plant-based emulsifiers that are less sensitive to environmental stresses. Quillaja saponin (QS) is a natural small molecule surfactant isolated from the Molina tree, which can form relatively stable oil-in-water emulsions at relatively low emulsifier levels.[7] Gum arabic (GA) is an amphiphilic polysaccharide that is widely used to stabilize oil-in-water emulsions, particularly those used in the beverage industry.[8, 9] Previous studies have shown that emulsions prepared using QS or GA are much less sensitive to environmental stresses than those stabilized by proteins[5, 7, 9]. Consequently, they are particularly suitable for forming plant-based emulsions that can be used for a wide variety of applications in foods and beverages.

In this study, β-carotene (BC) was utilized as a model hydrophobic bioactive

substance because of its provitamin A activity, as well as other nutraceutical effects.[10] This carotenoid is, however, extremely susceptible to chemical degradation because it contains numerous unsaturated groups, particularly when it is exposed to pro-oxidants, elevated temperatures, and light.[11] Consequently, some researchers have examined adding natural antioxidants to inhibit the degradation of β-carotene encapsulated in emulsions, such as tea polyphenols.[12] Tannic acid (TA) is also a good natural antioxidant, which can scavenge free radicals and inhibit lipid oxidation.[13] Additionally, tannic acid possesses antibacterial, antiviral, antimutagenic, and anticancer properties, which makes it suitable as a potential nutraceutical agent in foods.[13] Tannic acid has already been approved for use in foods by the US Food and Drug Administration (FDA), and has a long history of application in the food industry, such as in baked goods, beverages, desserts, and candy.[14] It is mainly used to improve the flavor and texture of products, and the dosage level that is considered safe depends on the nature of the food. For instance, FDA recommends that the maximum level of TA that can be used in baked goods, alcoholic beverages, and frozen dairy desserts is 0.01%, 0.015% and 0.04%, respectively.[15] It should be noted, however, that some studies have reported that TA can form complexes with components within the human gastrointestinal tract (such as metal ions, alkaloids, proteins, and polysaccharides) and therefore may interfere with digestion and absorption of nutrients.[13]

In the current study, we selected QS and GA as plant-based emulsifiers to produce emulsion-based delivery systems for β-carotene (BC) because of the good pH stability of emulsions stabilized by them.[5] The effect of plant-based emulsifier type and tannic acid addition on the gastrointestinal fate, carotenoid bioaccessibility, and storage stability of BC-loaded emulsions was investigated. Our study may provide valuable information that can be used to rationally design plant-based functional foods and beverages for improved human health.

2. Materials and Methods

2.1. Materials

Flaxseed oil was purchased from a grocery store and used without further purification (AAK Ltd., England, UK). Medium chain triglyceride (MCT) oil was purchased from SASOL (Miglyol® 812, Houston, TX). Gum arabic was provided by TIC Gums (Belcamp, MD). Quillaja saponin (Q-Naturale 200V) was donated by Ingredion Inc. (Westchester, IL). Tannic acid (from Chinese natural gall nuts), β-carotene (≥ 93%), and Nile red were purchased from Sigma-Aldrich Co. (St. Louis, MO). Double distilled water (Milli-Q) was used to prepare all solutions and emulsions.

2.2. Preparation of β-carotene emulsions

Initially crystalline β-carotene (2%, w/w) was dissolved in MCT oil by heating (140 °C, 30s) followed by continuous stirring until the sample appeared optically clear. MCT was used to dissolve the carotenoid because it is mainly comprised of medium chain saturated fatty acid chains that are not prone to oxidation during heating. An oil phase was then prepared by mixing BC-loaded MCT oil (10%, w/w) and flaxseed oil (90%, w/w) together. Flaxseed oil was used as a carrier oil because it is a good source of ω-3 fatty acids and should increase the bioaccessibility of the carotenoids in the gastrointestinal tract. Aqueous phases were prepared by dissolving the emulsifiers (0.75% QS or 5% GA) in 5 mM phosphate buffer (pH 7). Coarse BC-emulsions stabilized by QS or GA were prepared by mixing 5% (w/w) oil phase and 95% (w/w) aqueous phases using a high-speed blender for 2 min (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland). Then, the coarse emulsions formed were passed through a high-pressure homogenizer (Microfluidizer, M110Y, Microfluidics, Newton, MA) at an operational pressure of 12,000 psi for 3 cycles. The emulsions containing TA were prepared by mixing 5 % (w/w) oil-in-water emulsions, 1 % (w/w) TA solution, and buffer solution at different ratios to obtain final compositions of 0.01% or 0.1% TA and 2 % (w/w) oil. A BC-loaded emulsion without TA was used as a control.

2.3. Emulsion characterization

2.3.1. Storage stability

Immediately after preparation, the emulsions (8 mL) were placed in glass tubes and then sealed with plastic caps and Parafilm. The sealed emulsions were then maintained at 55 °C in the dark for 7 days. The β-carotene concentration in the BC-loaded emulsions was measured every day using a method reported previously with some minor modifications.[16] BC-emulsions were first diluted to an appropriate concentration with phosphate buffer, and then extracted with a hexane: iso-propanol (3:2, v/v) mixture. The absorbance at 450 nm was measured using a UV–visible spectrophotometer (Genesys 150, Thermo Scientific, Waltham, MA). The β-carotene concentration in the samples was calculated using a standard curve prepared using a series of samples of known β-carotene concentrations. The retention (R) of the β-carotene was expressed as $R(t) = 100 \times C(t)/C_0$, where C_0 is the initial carotenoid concentration and C is the carotenoid concentration after a particular storage time (t).

2.3.2. Particle size and ζ-potential measurements

The particle size distributions (PSDs) and mean particle diameters ($d_{4,3}$ and $d_{3,2}$) of the emulsions were determined using static light scattering (Mastersizer 2000, Malvern Instruments Ltd., Malvern, Worcestershire, UK). The electrical surface potential (ζ-potential) was measured using particle electrophoresis (Zetasizer Nano ZS, Malvern Instruments Ltd.). Samples were diluted with buffer solutions of the same pH as them to avoid multiple scattering effects and then stirred (1,200 rpm) to ensure that they were homogeneous. The refractive indices of the buffer solution and MCT-flaxseed oil mixture were taken to be 1.330 and 1.474, respectively.

2.3.3. Microstructure

A confocal scanning laser microscope with 60× objective lens (oil immersion) and 10× eyepiece (Nikon D-Eclipse C1 80i, Nikon, Melville, NY) was used to analyze the microstructure of the emulsions. The samples were stained with a 1 mg/mL Nile Red in ethanol solution to highlight the location of the oil phase.

2.4. Simulated gastrointestinal conditions

A three-stage simulated gastrointestinal tract (GIT) model (mouth, stomach, and intestine) was used to evaluate the gastrointestinal fate of the BC-loaded emulsions according to the previously reported standardized *in vitro* methods.[17, 18] The details of the compositions of the various simulated gastrointestinal fluids used are also included in these references.

***Mouth Phase*:** 15 mL of simulated saliva fluid (including 3 mg/mL mucin and various minerals) were mixed with 15 mL of BC-loaded emulsion (2 % (w/w) oil content). The resulting mixture was then adjusted to pH 6.8 and held in an incubator shaker (Innova Incubator Shaker, New Brunswick, USA) for 2 min at 37 °C to mimic agitation (100 rpm) in the mouth.

***Stomach Phase*:** 20 mL of the sample from the mouth phase was mixed with 20 mL of simulated gastric fluid (including pepsin, salts and acids) preheated to 37 °C. The resulting mixture was then adjusted to pH 2.5, and then the mixture was held in an incubator shaker (100 rpm) for 2 h at 37 °C to mimic the stomach environment.

***Intestine Phase*:** 30 mL of the sample from the stomach phase was adjusted to pH 7.0, followed by adding 1.5 mL of simulated intestinal fluid (including $CaCl_2$, NaCl and other minerals) and 3.5 mL of bile salt solution (0.1875 g of bile salts in 3.5 mL 5 mM pH 7 phosphate buffer) with constant stirring (560 rpm). The resulting mixture was then adjusted back to pH 7.00, and then 2.5 mL of lipase solution was added. An automatic titration unit with a stirring propeller (Metrohm, USA Inc.) was used to monitor the pH and maintain it at pH 7.0 by titrating 0.25 N NaOH solution into the reaction vessel for 2 h at 37 °C. The amount of free fatty acids released was calculated from the titration curves as described previously.[19]

2.5. Carotenoid Bioaccessibility

The bioaccessibility of the β-carotene was measured using a previously reported method with some modifications.[20] Briefly, after the small intestine stage was completed, the raw digesta was collected. Part of the sample was analyzed as is, whereas another part of the sample was centrifuged (12,000 rpm, 15 min) and the

clear supernatant obtained was taken to be the mixed micelle fraction. The β-carotene concentrations in the digesta and micelle fractions were then measured spectrophotometrically as described earlier (Section 2.3.2). The bioaccessibility (%) of the carotenoids was calculated using the following expression:

$$Bioaccessibility\ (\%) = \frac{C_{Micelle}}{C_{Digesta}} \times 100$$

Here, C_{Digesta} is the total β-carotene concentration in the small intestine phase after digestion, and C_{micelle} is the β-carotene concentration in the mixed micelle phase collected from the digesta after centrifugation.

2.6. Computational docking simulation

In order to explore the nature of the interactions between TA and the emulsifiers used, computational docking simulations were performed. The docking simulations utilized the CDOCKER Dock protocol of the Discovery Studio 3.1 program (BIOVIA, San Diego, CA) according to the method described by Dai et al.[21] The molecular structure of QS and TA were downloaded from PubChem database (https://pubchem.ncbi.nlm.nih.gov), using the MM2 force field for energy minimization in ChemBio 3D Ultra 18.0. To obtain a lowest energy minimized conformation, the TA and QS molecules were optimized using the small molecule module at pH 2.5 (the gastric pH value). The optimized structures of QS and TA were added to the CHARMm force field algorithm. The QS and TA were set as receptor and ligand, respectively. The docking parameters were set, as follows: heating steps were set to 2000 steps with a target temperature of 700 K; cooling steps were set to 5000 steps with a target temperature of 310 K (37 °C). The heating step was applied to the simulated molecules to put them into a disorganized (random) state, then the cooling step was applied to allow them to move into their preferred conformation (minimum free energy) under digestion conditions. The number of exhaustiveness was set to 10, and the pose cluster radius was set to 0.5. The pose with lowest CDOCKER energy (E_{CD}) and lowest CDOCKER interaction energy (IE_{CD}) was regarded as the optimized result. The interactions between QS and TA were

visualized using appropriate software (Discovery Studio 2019 Client). According to the same method, the docking between QS and TA was also performed at pH 7.0 (the small intestinal pH value).

2.7. Statistical analysis

All experiments were performed on at least two or three freshly prepared samples. The results are reported as mean and standard deviation values. All data were subjected to ANOVA and differences between means ($P < 0.05$) were identified by Tukeys test using a statistical software package (SPSS 25.0).

3. Results and Discussion

3.1. Effect of emulsifier type and TA on gastrointestinal fate of emulsions

The composition and structural properties of BC-loaded emulsions will be altered as they pass through the gastrointestinal tract due to the changes in environmental conditions they experience. To better understand the effects of emulsifier type and TA addition on the gastrointestinal fate of the BC-loaded emulsions, we measured changes in their particle size, ζ-potential and microstructures in the various stages of the simulated GIT.

3.1.1. Initial systems

In the absence of TA, the initial BC-loaded emulsions stabilized by QS and GA had monomodal particle size distributions (PSDs) with relatively small surface-weighted mean particle diameters: $d_{3,2} = 0.131 \pm 0.007$ and 0.330 ± 0.012 μm, respectively (Figs. 1A and 1B). The emulsions stabilized by QS contained smaller droplets than the ones stabilized by GA. This is most likely due to differences in the surface activities and adsorption kinetics of the two emulsifiers.[5] As reported in our previous study, QS had a higher surface activity and generated a stronger electrostatic repulsion than GA.[8, 22] QS is therefore highly effective at rapidly adsorbing to the lipid droplet surfaces, effectively covering their surfaces, and inhibiting their aggregation. TA addition (0.01% or 0.1%) had no significant influence on the mean particle size and PSDs of the emulsions stabilized by either QS or GA (Figs. 1 and 2). Additionally, the confocal microscopy images showed that emulsions stabilized by

both emulsifiers contained small lipid droplets that were uniformly spread throughout the samples, in both the absence and presence of TA (Fig. 3).

At neutral pH, the ζ-potential of the emulsions stabilized by QS and GA were around -63 and -32 mV, respectively, and did not depend on TA level (Fig. 4). These results suggested that TA is not a key factor affecting the physical stability of the emulsions under neutral pH conditions.

3.1.2. Mouth

The mean particle diameter and PSDs of the emulsions stabilized by QS changed when they were exposed to simulated mouth conditions. The mean particle diameter increased from 0.131 ± 0.007 to 0.270 ± 0.012 μm (Fig. 1A). The PSD was bimodal with a population of smaller particles (0.1 to 1 μm) and another of larger particles (1 to 10 μm) (Fig. 2 A-0% TA). The confocal microscopy images of the emulsions showed that the oil droplets were highly flocculated in the absence of TA (Fig. 3A). Interestingly, in the presence of TA, the mean particle diameter and PSDs of the emulsions did not change significantly compared to the initial emulsions (Figs. 1A and 2A). However, a small amount of droplet flocculation was observed in the confocal microscopy images (Fig. 3A). The ζ-potential of the emulsions changed from around -63 mV initially to around -26 mV after being exposed to the mouth environment. The changes of ζ-potential may be because some mineral ions in the simulated saliva accumulated around the surfaces of the lipids droplets, thereby causing electrostatic screening.[23] Other studies have reported that mucin can promote bridging or depletion flocculation of the emulsions.[24] The tendency for droplet flocculation to occur in the mouth may therefore have been due to alterations in electrostatic repulsion, bridging, or depletion effects. The ability of TA to reduce droplet flocculation in the mouth phase may have occurred for a variety of reasons, including its ability to bind to mucin or to bind to the droplet surfaces and alter the colloidal interactions between them.

For the emulsions stabilized by GA, the mean particle diameter and PSDs of the emulsions did not change appreciably after incubation in the mouth phase, in either

the absence or presence of TA (Figs. 1B and 2B). Moreover, the confocal microscopy images did not show any evidence of droplet aggregation in the simulated mouth environment (Fig. 3B). In addition, the ζ-potential of the emulsions only slightly decreased in both the absence and presence of TA. This result is consistent with our previous study that found that the ζ-potential decreased substantially for emulsions stabilized by QS but only slightly for emulsions stabilized by GA when exposed to simulated oral conditions.[5] Compared with the emulsions stabilized by QS, the emulsions stabilized by GA were, therefore, more resistant to simulated mouth environment.

3.1.3. Stomach

For the emulsions stabilized by QS, there were substantial changes in the mean particle diameter and PSD after exposure to simulated stomach conditions (Figs. 1A and 2A). The confocal microscopy images were in good agreement with the particle size measurements, as they showed that the emulsions contained a large number of highly aggregated oil droplets (Fig. 3A). The extent of droplet aggregation was greater in the emulsions containing the higher TA level. Previous studies suggest that droplet aggregation in the stomach could be due to changes in pH and ionic strength or due to depletion or bridging effects.[2, 25, 26] In the absence of TA, the depletion flocculation was likely caused by any non-absorbed polymers, while the bridging flocculation was caused by cross-linking between the oil droplets and mucin.[27] In the presence of TA, bridging flocculation could have been caused by both mucin- and/or TA-induced bridging. This is because TA itself is a crosslinker that can form insoluble complexes with proteins, polysaccharides, and metal ions.[28-30] Under acidic conditions, the full protonation of the hydroxyl groups on TA results in a strong hydrogen bonding potential.[31] Therefore, droplet flocculation could be caused by hydrogen bonding between TA and QS molecules adsorbed to the surfaces of the lipid droplets.

For the emulsions stabilized by GA, there were no obvious alterations in the mean particle diameter and PSD of the emulsions with or without TA after being

exposed to simulated stomach conditions (Figs. 1B and 2B). Moreover, the confocal microscopy images showed that the lipid droplets remained small and uniformly dispersed throughout the sample (Fig. 3B). These results suggest that the emulsions stabilized by GA had more tolerance to highly acidic gastric conditions. TA addition did not affect the stability of these emulsions in the stomach phase.

The ζ-potential measurements showed that the emulsions stabilized by both QS or GA had relatively low surface potentials in the highly acidic gastric conditions (Fig. 4A). The low negative charge observed in these emulsions may be due to protonation of the anionic groups on the QS and GA molecules under acidic conditions, the high ionic strength of the gastric fluids, and the presence of anionic mucin.[5] Again, the presence of the TA in the emulsions had little influence on their surface potentials under gastric conditions.

3.1.4. Small intestine

When foods are transferred from the stomach to the small intestine, the bases in the intestinal fluids neutralize the acids in the gastric fluids so that the pH returns to neutral.[17] In this study, we used a two-stage small intestine phase: (i) the pH was adjusted to pH 7 and the samples were analyzed; (ii) the digestive enzymes and bile salts were added and the samples were analyzed again after 2 hours. This procedure was carried out so that we could distinguish the impact of pH on the properties of the emulsions from that of the other digestive components.

For the emulsions stabilized by QS, when the sample was first adjusted to pH 7, the mean particle diameter of the emulsions without 0 or 0.01% TA decreased considerably from 5.20 to 0.52 μm and from 6.90 to 0.52 μm, respectively. Conversely, the emulsions containing 0.1% TA still had large mean particle diameters (14.37 μm), which suggested that high levels of tannic acid destabilized the system. The PSDs of the emulsions with 0 or 0.01% TA became bimodal after the systems were adjusted to pH 7, whereas the emulsions with 0.1% TA remained monomodal. The confocal microscopy images agreed with the particle size measurements. The images of the emulsions with low TA levels indicated that the flocs formed under

simulated stomach conditions were largely dispersed after incubation in small intestine conditions, while the flocculated droplets were not dispersed in the presence of 0.1% TA. These results indicated that the emulsions containing 0.1% TA were still highly flocculated when they entered the beginning of the small intestine phase, whereas the emulsions containing 0 or 0.01% TA were not. The precise physicochemical origin of this effect is currently unknown, but suggests that high levels of tannic acid may have altered the colloidal interactions between the oil droplets.

For the emulsions stabilized by GA, there were no obvious alterations in the particle size and PSDs of the emulsions with or without TA when the emulsions were adjusted to pH 7 after the stomach digestion (Figs. 1B and 2B). Moreover, the confocal microscopy images showed that these emulsions still contained small and uniform lipid droplets (Fig. 3B).

After adjusting to pH 7, there was an increase in the negative charge on the particles in all the emulsions, which can be attributed to an increase in negative charge on the adsorbed emulsifiers. For the systems stabilized by QS, the emulsions with 0.1% TA had a lower surface potential than those with 0 or 0.01% TA (Fig. 4A). For the systems stabilized by GA, the charge on the emulsions was not dependent on the TA level (Fig. 4B).

In the second stage of the small intestine phase, the digestive enzymes and bile salts were added, and then the samples were digested for two hours. At the end of small intestine phase, the mean particle diameter of all samples was below 0.45 μm (Fig. 1), but these samples contained a broad range of particles with different dimensions (Fig. 2). This is probably because the digesta contains a complex mixture of different colloidal substances, including undigested lipid droplets, digestive enzymes, micelles, vesicles, liquid crystals, calcium soaps, and other insoluble matter.[4] Furthermore, a variety of lipid-rich particles with different dimensions were also detected in the confocal microscopy images (Fig. 3). The ζ-potential measurements indicated that all the samples contained particles with a relatively high negative charge (Fig. 4), which was mainly caused by the ionization of anionic

groups, such as those on proteins, polysaccharides, digestive enzymes, free fatty acids, bile salts *etc*.

3.2. Molecular docking between emulsifier and TA

We attempt to explain the effects of TA on emulsion properties during digestion using computational molecular docking analysis. TA addition has little influence on the properties of GA-stabilized emulsions. Moreover, GA is a polysaccharide with a complex molecular structure that has not yet been fully elucidated.[32] Therefore, the molecular docking (CDOCKER) simulation was only used to predict and visualize the interactions between QS and TA at different pH values. Based on the CDOCKER protocol, the molecular arrangement that gives the lowest energy score is taken to be the most stable conformation. The arrangement that gave the lowest energy score (CDOCKER energy −118.78 and -168.25 kcal/mol; CDOCKER interaction energy −82.41 and -84.73 kcal/mol; at pH 2.5 and 7.0, respectively) was taken to represent the binding interaction of QS and TA (Table 1). The results of 3D docking clearly showed the binding of QS and TA was mainly driven by hydrogen bonds and hydrophobic interactions at both pH 2.5 and pH 7 (Fig. 5). Other researchers have also reported that hydrogen bonds and hydrophobic interactions are the major forces of the binding between TA and emulsifiers (such as proteins or polysaccharides).[33-35] As shown in Fig. 5, the binding between TA and QS at pH 7.0 has two hydrogen bonds (TA: H 153 and QS: O 95, 2.09 Å; TA: H 164 and QS: O 104, 2.17 Å) and eight hydrophobic interactions forces (eight Pi-Alkyls). At pH 2.5, the binding between TA and QS has four hydrogen bonds (TA: H 152 and QS: O 95, 1.94 Å; TA: H 160 and QS: O 29, 1.94 Å; TA: H 171 and QS: O 95, 2.79 Å; TA: H 173 and QS: O 29, 1.90 Å) and thirteen hydrophobic interactions (thirteen Pi-Alkyls). Analysis of the length of the hydrogen bonds and hydrophobic interactions was used to provide some analysis about their relative strengths at different pH values. The number of hydrogen bonds with a length below 2 units (*i.e.,* strong bonds), was three at pH 2.5 but zero at pH 7.0. Similarly, the number of hydrophobic interactions with a length below 5 (*i.e.,* strong bonds), was nine at pH 2.5 but three at pH 7.0. The 3D docking

images showed that the docking structure between TA and QS was tighter at pH 2.5 than at pH 7.0. Taken together, these results suggest that the interactions between TA and QS were more numerous and stronger at pH 2.5 than at pH 7.0. The instability of the emulsions observed under gastric conditions may therefore have been due to the stronger attractive interactions between the TA and QS under acidic conditions. In addition, some studies have reported that salts can increase the strength of hydrophobic interactions.[36, 37] This is probably the main reason why the QS-stabilized emulsion containing 0.1% TA was still flocculated when the emulsion entered the small intestine environment.

3.3. Effect of emulsifier type and TA addition on lipid digestion

The effect of emulsifier type and TA on lipid digestion was determined using a pH-stat method to monitor the FFA-release profile. In all samples, there was a rapid release of FFAs during the first few minutes followed by a slower release at longer times until a fairly constant final value was reached. The initial rate of lipid digestion depended on the type of emulsifier used and the level of TA present (Fig. 6). In particular, the QS-stabilized emulsions containing 0.1% TA had the slowest initial rate of lipid digestion. These results suggest that the initial rate of lipid digestion depended on the size of the particles in the emulsions at the beginning of the small intestine phase. Previous studies have shown that the rate of lipid digestion increases as the droplet size decreases because of the increase in lipid surface area exposed to the digestive enzymes.[38, 39]

To better compare the differences between the samples, we calculated the extent of FFAs released after 5 and 120 min of digestion (Fig. 6 C and D). In the absence of TA, the initial extent of lipid digestion from emulsions stabilized by QS was obviously lower than those of emulsions stabilized by GA. About 80% of the lipid droplets in the GA-stabilized emulsions was digested within the first 5 min (Fig. 6C). Additionally, the final extent of lipid digestion of the QS-stabilized emulsions was slightly lower than that of the GA-stabilized emulsions (Fig. 6D). This phenomenon is consistent with our previous studies.[5] The observed differences may have occurred for

a number of reasons: (i) the difference in the particle size between QS- and GA-stabilized emulsions (Fig. 3); (ii) QS can strongly adsorb to the surfaces of the lipid droplets, thereby hindering the adsorption of the lipase and/or bile salts; (iii) the interaction between cationic calcium and anionic saponins resulted in a decrease in the level of free calcium ions, which influenced their ability to precipitate long chain fatty acids from the lipid droplet surfaces.[5]

Interestingly, in the presence of TA, there were obvious differences in the initial extent of lipid digestion between the emulsions stabilized by different emulsifiers. For the emulsions stabilized by QS, a relatively low level of TA (0.01%) did not affect the initial extent of lipid digestion. However, a relatively high level of TA (0.1%) strongly influenced the initial extent of lipid digestion with only around 2.5% of lipid digestion occurring within the first 5 min. In this case, FFA release was slow during the first few minutes, increased steeply during the next few minutes, and then reached a level similar to that of the other QS-stabilized emulsions. This result suggests that a relatively high level of TA (0.1%) retards the initial stages of lipid digestion, but does not influence the final extent of lipid digestion.

Previous studies have shown that the size of the individual droplets, as well as their aggregation state, affects lipid digestion.[39, 40] Typically, the larger the individual droplets or the more flocculated they are, the slower the digestion rate. The confocal microscopy images of the emulsions containing 0.1% TA showed that the lipid droplets were highly flocculated when they first entered the small intestine (Fig. 4). As a result, one would expect that it would be more difficult for the lipase to access the surfaces of the lipid droplets in the interior of the flocs, thereby slowing their digestion. As lipid digestion proceeds, the flocs are broken down, and lipid digestion becomes faster and eventually all the lipid droplets are digested.

For the GA-stabilized emulsions, TA addition reduced the initial extent of lipid digestion, whereas the TA levels had no significant influence on the final extent of lipid digestion (Figs. 5 C and D). For these emulsions, all the droplets were relatively small and not strongly flocculated when they entered the small intestine (Figs 1-3), which suggests that droplet dimensions or aggregation state were not important in this

case. Another possible mechanism for the observed effects is that TA can chelate calcium ions in the gastrointestinal fluids.[41, 42] Calcium ions are critical for enhancing lipid digestion due to their ability to remove long chain fatty acids from the surfaces of lipid droplets though a precipitation mechanism, thus allowing the lipase to continue digesting the lipids inside the droplets.[4] If the fatty acids are not removed, then the lipase cannot readily reach the lipids. Therefore, the binding of TA to calcium ions could reduce the level of free calcium ions available to remove the long chain fatty acids from the droplet surfaces, thereby reducing the initial rate of lipid digestion. On the other hand, there was still enough free calcium present to allow the digestion reaction to go to completion, so that the final amount of FFAs released was similar.

3.4. Effect of emulsifier type and TA on carotenoid bioaccessibility

In previous studies, the bioaccessibility of carotenoids is usually measured at the end of the small intestine phase. In the current study, we measured changes in carotenoid bioaccessibility throughout the lipid digestion process to provide some additional insights into the mechanisms involved. In addition, the effect of emulsifier type and TA addition on carotenoid bioaccessibility was monitored. As shown in Figs. 7A and 7B, the BC bioaccessibility of all the samples increased to a maximum value after about 30 min of digestion, but then decreased, which is consistent with our previous study.[43] The origin of this effect can be related to the physicochemical mechanisms occurring during lipid digestion. Initially, the lipid droplets are digested by lipase, which leads to the formation of FFAs and the release of carotenoids. The free fatty acids and monoacylglycerols generated during lipid digestion interact with bile salts and phospholipids in the intestinal fluids, leading to the formation of mixed micelles. The carotenoid molecules released from the lipid droplets were the solubilized in the hydrophobic interiors of the mixed micelles. Consequently, there was initially an increase in the bioaccessibility of the carotenoids throughout the initial stages of small intestine digestion. Later, however, the anionic mixed micelles could react with the cationic calcium ions in the small intestine fluids,

which could cause them to aggregate and precipitate. This will result in a reduction of the level of carotenoids in the mixed micelle phase, thereby causing a decrease in BC bioaccessibility.

The final BC bioaccessibility of all the samples was around 45% (Fig. 7 C). Other researchers have reported that the bioaccessibility of BC depends on the nature of the carrier oil inside the lipid droplets, being around 70% when long-chain triglycerides (LCT) are used and around 10% when medium-chain triglycerides (MCT) are used [39, 44, 45] Corn oil was used as an LCT by these researchers, whereas we used a mixture of flaxseed oil and MCT (9:1, v/v) as a carrier oil for the BC. Therefore, the relatively low BC bioaccessibility observed in this study is probably due to two reasons: (i) the presence of the MCT reduced the carrying capacity of the mixed micelles; (ii) the level of polyunsaturated fatty acids (PUFAs) in flaxseed oil is higher than in corn oil. PUFAs tend to have highly bent structures and therefore form smaller hydrophobic domains inside the mixed micelles, which reduces their ability to solubilize the carotenoids. Overall, our results shown that emulsifier type (QS and GA) and TA addition (below 0.1%) did not influence the final bioaccessibility of the carotenoids.

3.5. Effect of emulsifier type and TA addition on the stability of BC emulsions

Finally, the effect of emulsifier type and TA addition on the physiochemical stability of the carotenoids was evaluated when the BC emulsions were stored at 55 ºC for 7 days. As shown in Fig. 8, the initial surface-weighted mean diameters ($d_{3,2}$) and volume-weighted diameters ($d_{4,3}$) of emulsions were 0.132 and 0.165 μm for QS-stabilized emulsions, and 0.340 and 0.387 μm for GA-stabilized emulsions, respectively. After storage at 55 ºC for 7 days, the $d_{3,2}$ of QS-stabilized emulsions increased slightly, but the $d_{4,3}$ values increased appreciably, suggesting that there was a small amount of droplet aggregation during storage. Interestingly, the final $d_{4,3}$ values of the emulsions containing TA were smaller than for the emulsions without TA, which suggests that the presence of the tannic acid may have helped stabilize them against aggregation. The $d_{3,2}$ and $d_{4,3}$ values of the GA-stabilized emulsions with

or without TA did not change during storage, indicating that they were highly stable. The confocal microscopy images showed that the QS-stabilized emulsions contain large individual oil droplets after storage, which suggests that some droplet coalescence occurred (Fig. 9B). The magnitude of the negative charge on the particles in the QS-stabilized emulsions slightly decreased after storage, but the charge on the GA-stabilized emulsions remained constant, with or without TA (Fig. 8C). Overall, these results show that the GA-stabilized emulsions were relatively stable during storage at elevated temperatures, but that the QS-stabilized emulsions exhibited some instability to droplet aggregation.

There was a decline in the BC content in all the emulsions during storage, which suggested that some chemical degradation of the carotenoids occurred (Fig. 8D). In the absence of TA, the rate of BC degradation was considerably faster in the QS-stabilized emulsions than the GA-stabilized ones. This may be because GA has larger molecular dimensions than QS and therefore forms a more protective interfacial barrier that restricts the interactions between the lipids and aqueous phase prooxidants.[46, 47] It should be noted, however, that the interfacial layers formed by large biopolymers (like GA) are often highly porous, and would therefore not form an effective barrier at the molecular level. The rate of BC degradation was considerably slower in the emulsions with TA than those without TA, and the yellow color of the emulsions without TA was less intense than those with TA (Figs. 8D and 9A). These results highlighted the strong anti-oxidant activity of TA, which could effectively inhibit carotenoid degradation.

4. Conclusions

In summary, the study showed that the gastrointestinal digestion and storage stability of BC-loaded emulsions depended on emulsifier type and TA concentration. The surface potential of the lipid droplets in different stages of the GIT mainly depended on emulsifier type and gastrointestinal environment. The aggregation stability of the lipid droplets in different stages of the GIT depended on TA concentration, especially for the QS-stabilized emulsions. These factors affected the

initial extent of lipid digestion. In the absence of TA, the initial extent of lipid digestion was significantly higher for GA-stabilized emulsions than for QS-stabilized ones, which was attributed to the ability of the QS to strongly adsorb to the lipid droplet surfaces and inhibit the attachment of the bile salts and/or lipase. Interestingly, addition of 0.1% TA to the QS-stabilized emulsions led to extensive droplet flocculation in the gastric fluids. This phenomenon was linked to the strong interactions between TA and QS via hydrogen bonds and hydrophobic interactions under acidic conditions, which was supported by molecular docking analysis. The QS-stabilized emulsions containing 0.1% TA were highly flocculated when they entered the small intestine, which reduced the ability of the lipase to access the surfaces of the lipid droplets in the interior of the flocs. Additionally, TA can interact with calcium ions, which may reduce their ability to promote lipid digestion by removing FFAs from the lipid droplet surfaces. The emulsifier type and TA concentration had no significant influence on BC bioaccessibility. The storage stability and temperature-induced BC degradation of BC-loaded emulsions depended on emulsifier type and TA concentration. The GA-stabilized emulsions had better resistance to a high temperature environment (55 ºC for 7 days) than QS-stabilized emulsions. TA addition effectively inhibited temperature-induced BC degradation. These results have important applications for the formation of functional foods and beverages that can control lipid digestion and protect oil-soluble bioactive components. Further studies are still needed to establish whether the results obtained using the in vitro methods are consistent with those obtained under more realistic GIT conditions, *e.g.,* using animal or human feeding studies.

Conflicts of interest

There are no potential conflicts between authors and others that bias our work.

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Figure captions

Fig. 1. The mean particle size diameter ($d_{3,2}$) of BC-loaded emulsions containing different amounts of TA (0, 0.01% and 0.1%) and different emulsifiers: (A) QS and (B) GA. Samples denoted with different capital case letters (A-D) were significantly different ($P < 0.05$) when compared between different GIT regions (same TA level). Samples denoted with lower case letters (a-c) were significantly different ($P < 0.05$) when compared between different TA levels (same GIT region).

Fig. 2. Particle size distribution of BC-loaded emulsions stabilized by different emulsifier (A: QS and B: GA) with containing different amounts of TA (control=0, 0.01% and 0.1%).

Fig. 3. Microstructure of BC-loaded emulsions stabilized by different emulsifiers (A: QS and B: GA) with containing different amounts of TA (0, 0.01% and 0.1%) after they were exposed to different regions of a simulated GIT. Scale bar is 50 μm.

Fig. 4. ζ-potential of BC-loaded emulsions containing different amounts of TA (0, 0.01% and 0.1%) and different emulsifiers: (A) QS and (B) GA. Samples denoted with different capital case letters (A-E) were significantly different ($P < 0.05$) when compared between different GIT regions (same TA level). Samples denoted with lower case letters (a-c) were significantly different ($P < 0.05$) when compared between different TA levels (same GIT region).

Fig. 5. 3D docking mode between QS and TA simulated by Discovery Studio and the docking size and distance.

Fig. 6. Total amount of FFA released from emulsion with different amounts of TA (0, 0.01% and 0.1%) and different emulsifiers: (A) QS and (B) GA. C: Initial extent of FFA released at initial 5 min. D: Final extent of FFA released after 2 h digestion. Samples denoted with different capital case letters (A-B) were significantly different ($P < 0.05$) when compared between different TA levels (same emulsifier). Samples

denoted with lower case letters (a-b) were significantly different ($P < 0.05$) when compared between different emulsifiers (same TA level).

Fig. 7. BC bioaccessibility from emulsions containing different amounts of TA (0, 0.01% and 0.1%) and different emulsifiers.

Fig. 8. Changes of particle size ($d_{3,2}$: A and $d_{4,3}$: B), ζ-potential (C) and BC retention (D) of BC-loaded emulsions containing different amounts of TA (0, 0.01% and 0.1%) and different emulsifiers during long-term storage at 55 ºC in dark. Samples denoted with different capital case letters (A-C) were significantly different ($P < 0.05$) when compared between different TA levels (same emulsifier and storage time). Samples denoted with lower case letters (a-c) were significantly different ($P < 0.05$) when compared between different emulsifiers and storage times (same TA level).

Fig. 9 Visual appearance (A) and microstructure (B) of BC-loaded emulsions produced using different amounts of TA (0, 0.01% and 0.1%) and different emulsifiers before and after 7 days of storage (55 ºC, in dark).

Fig. 1

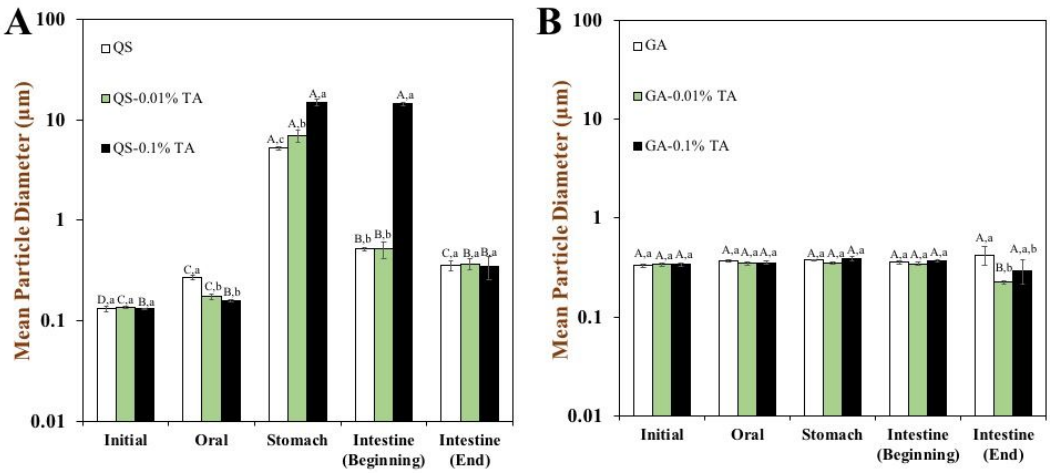

A
B
QS
QS-0.01% TA
QS-0.1% TA
GA
GA-0.01% TA
GA-0.1% TA
Mean Particle Diameter (μm)
100
10
1
0.1
0.01
Initial
Oral
Stomach
Intestine (Beginning)
Intestine (End)

Fig. 2

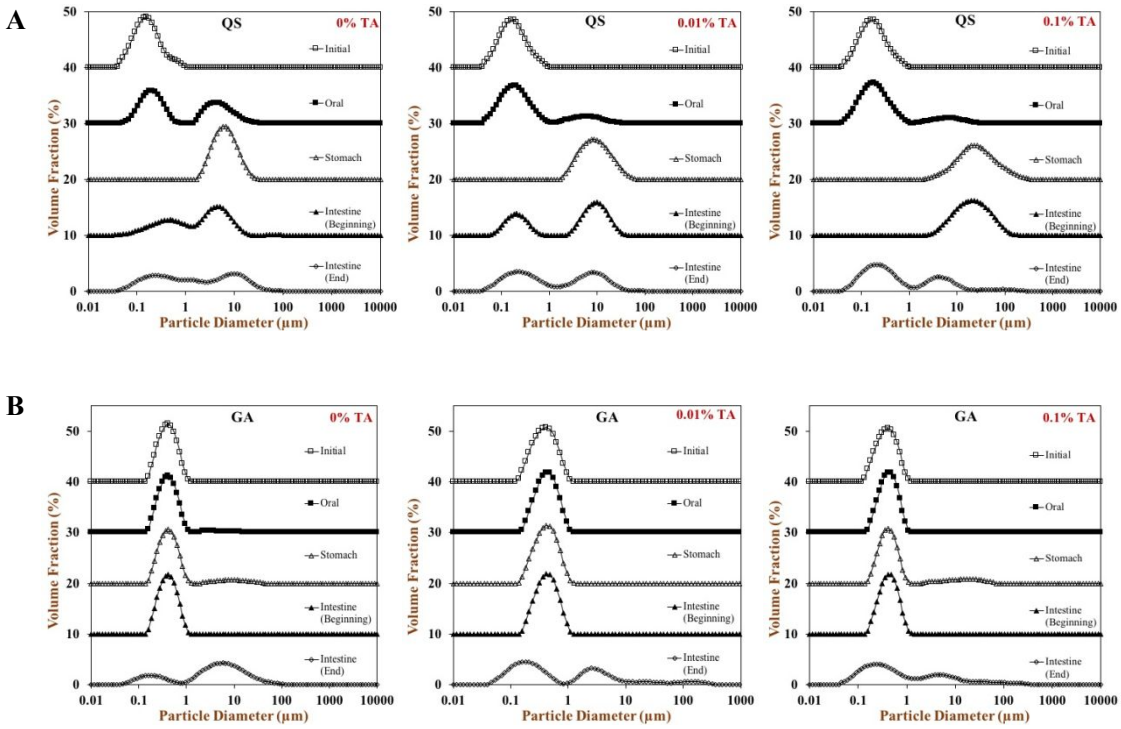

A
QS
0% TA
0.01% TA
0.1% TA
Initial
Oral
Stomach
Intestine (Beginning)
Intestine (End)
Volume Fraction (%)
Particle Diameter (μm)
B
GA
0% TA
0.01% TA
0.1% TA
Initial
Oral
Stomach
Intestine (Beginning)
Intestine (End)
Volume Fraction (%)
Particle Diameter (μm)

Fig. 3

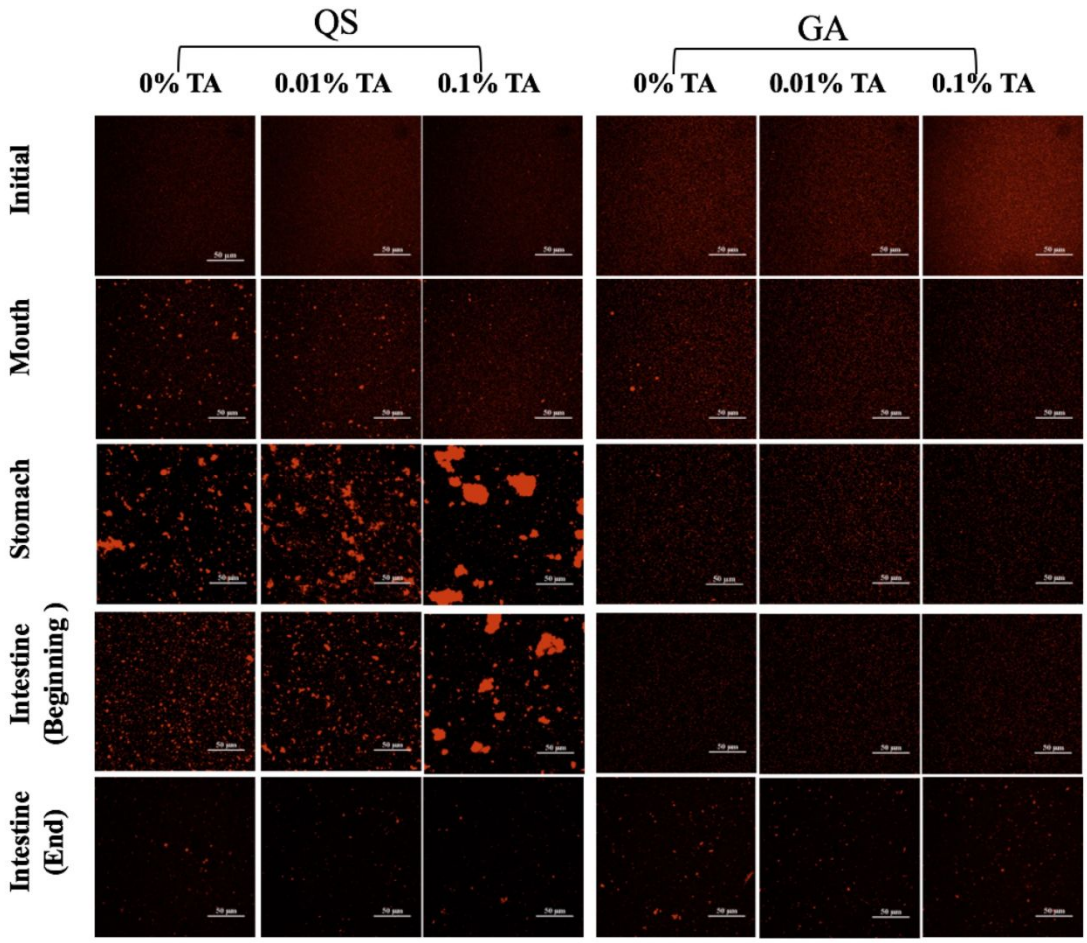

QS
GA
0% TA
0.01% TA
0.1% TA
0% TA
0.01% TA
0.1% TA
Initial
Mouth
Stomach
Intestine (Beginning)
Intestine (End)

Fig. 4

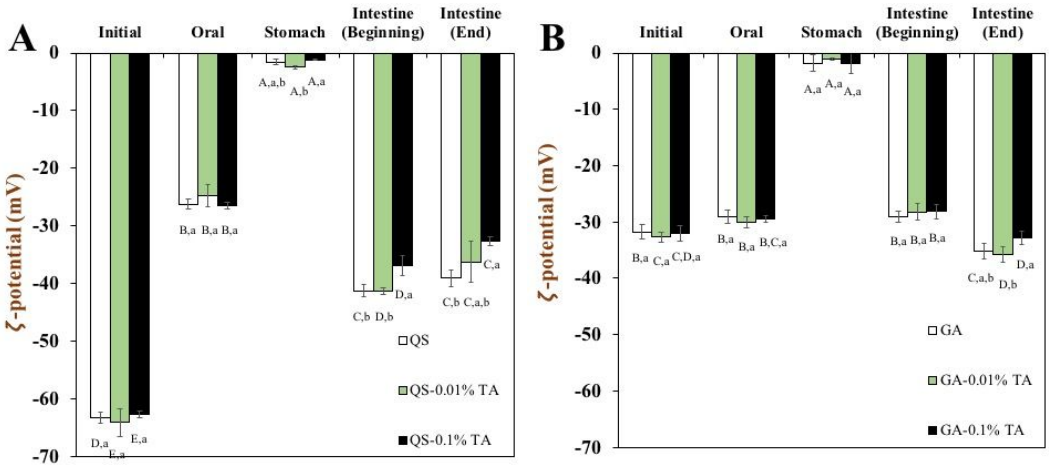

A
B
Initial
Oral
Stomach
Intestine (Beginning)
Intestine (End)
ζ-potential (mV)
0
-10
-20
-30
-40
-50
-60
-70
QS
QS-0.01% TA
QS-0.1% TA
GA
GA-0.01% TA
GA-0.1% TA

Fig. 5

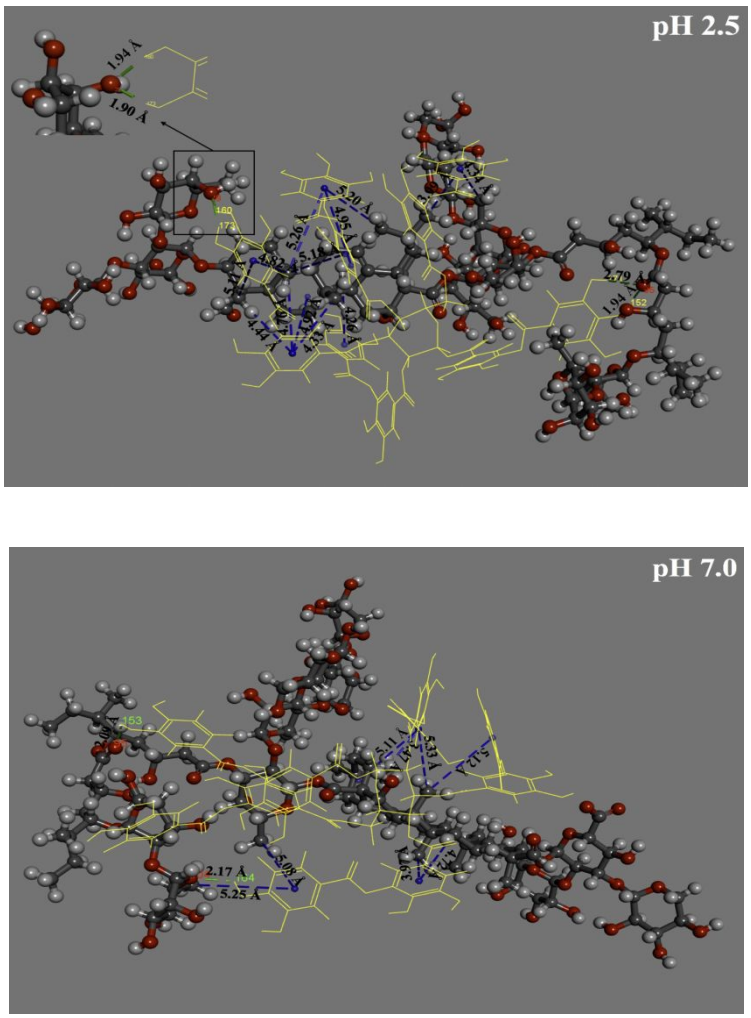

pH 2.5
1.94 Å
1.90 Å
5.20 Å
5.26 Å
5.14 Å
4.44 Å
4.33 Å
2.79 Å
1.94 Å
pH 7.0
5.11 Å
5.37 Å
5.12 Å
2.17 Å
5.25 Å

Fig. 6

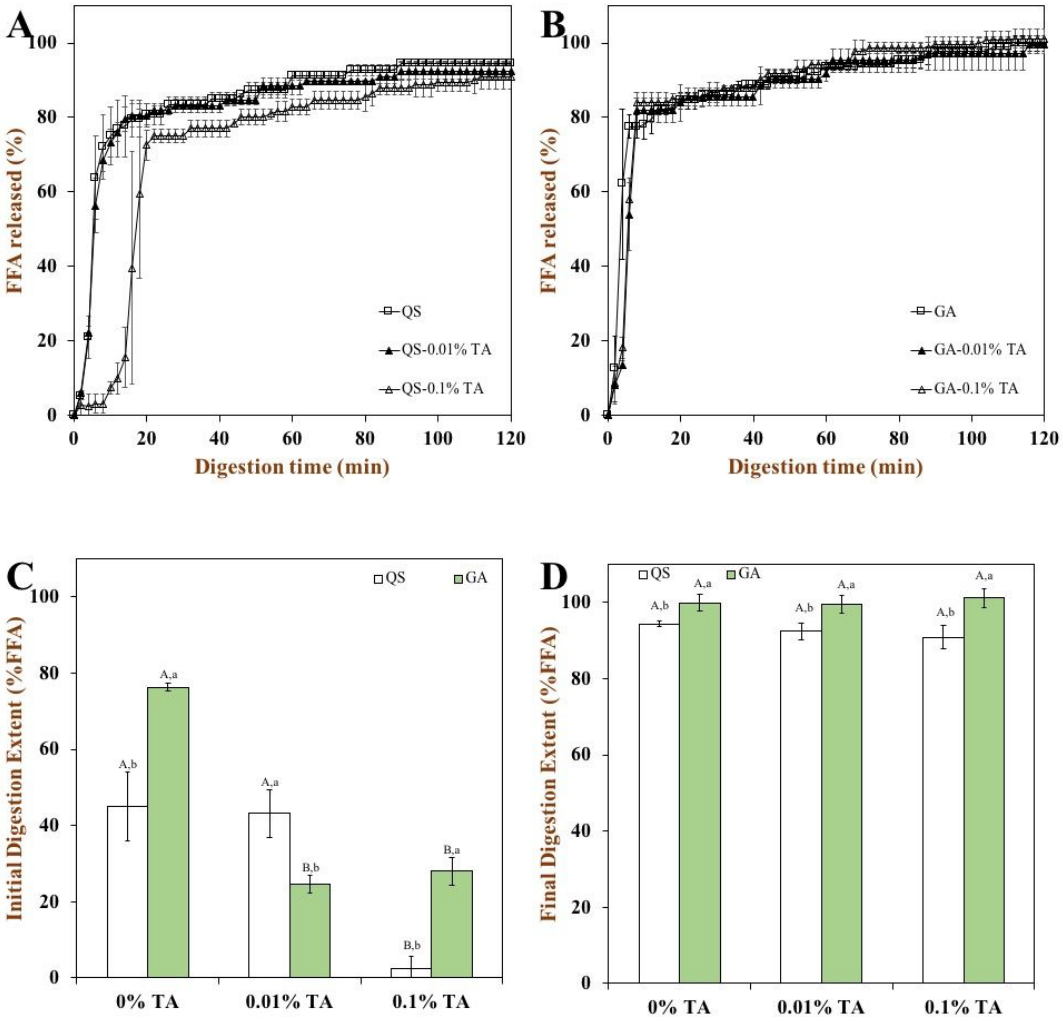

A
FFA released (%)
Digestion time (min)
QS
QS-0.01% TA
QS-0.1% TA
B
FFA released (%)
Digestion time (min)
GA
GA-0.01% TA
GA-0.1% TA
C
Initial Digestion Extent (%FFA)
QS
GA
A,b
A,a
A,a
B,b
B,b
B,a
0% TA
0.01% TA
0.1% TA
D
Final Digestion Extent (%FFA)
QS
GA
A,b
A,a
A,b
A,a
A,b
A,a
0% TA
0.01% TA
0.1% TA

Fig. 7

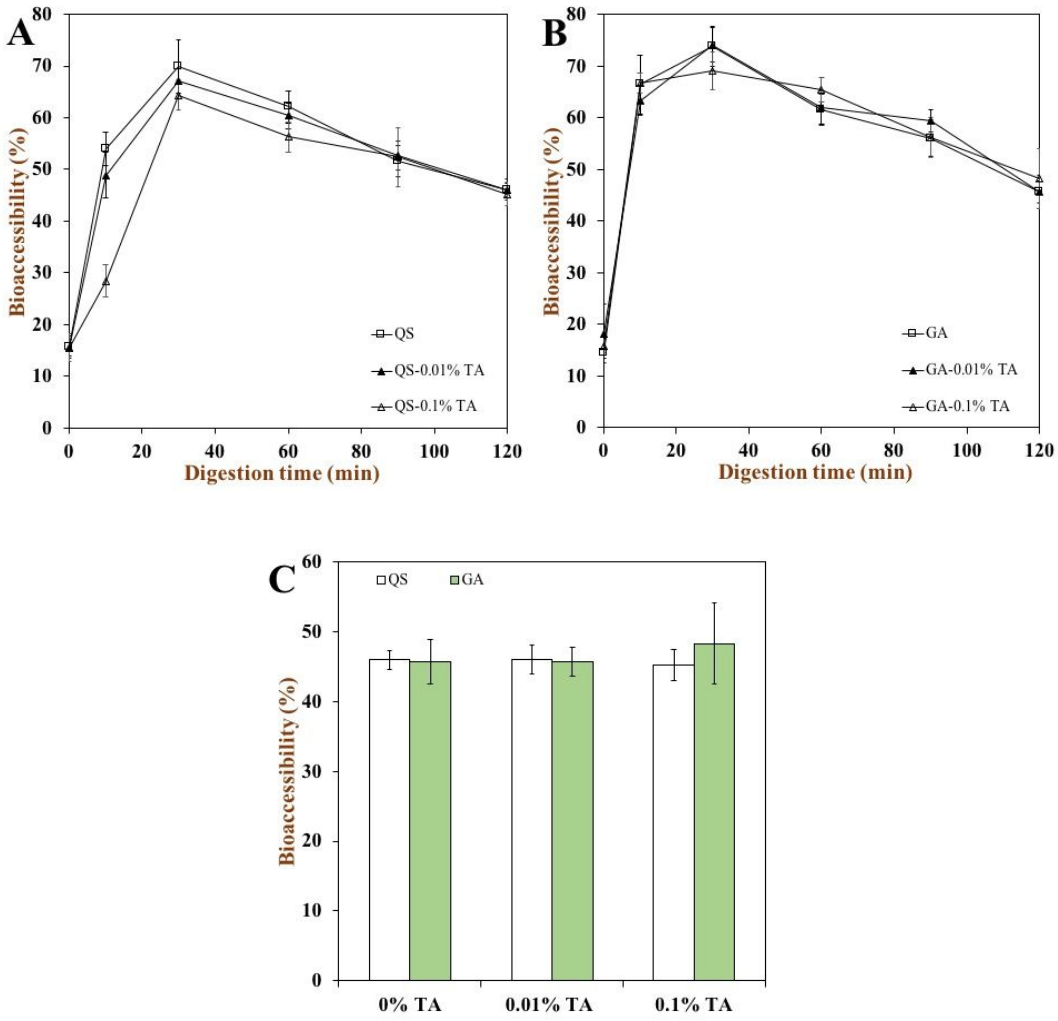

A
Bioaccessibility (%)
Digestion time (min)
QS
QS-0.01% TA
QS-0.1% TA
B
Bioaccessibility (%)
Digestion time (min)
GA
GA-0.01% TA
GA-0.1% TA
C
QS
GA
Bioaccessibility (%)
0% TA
0.01% TA
0.1% TA

Fig. 8

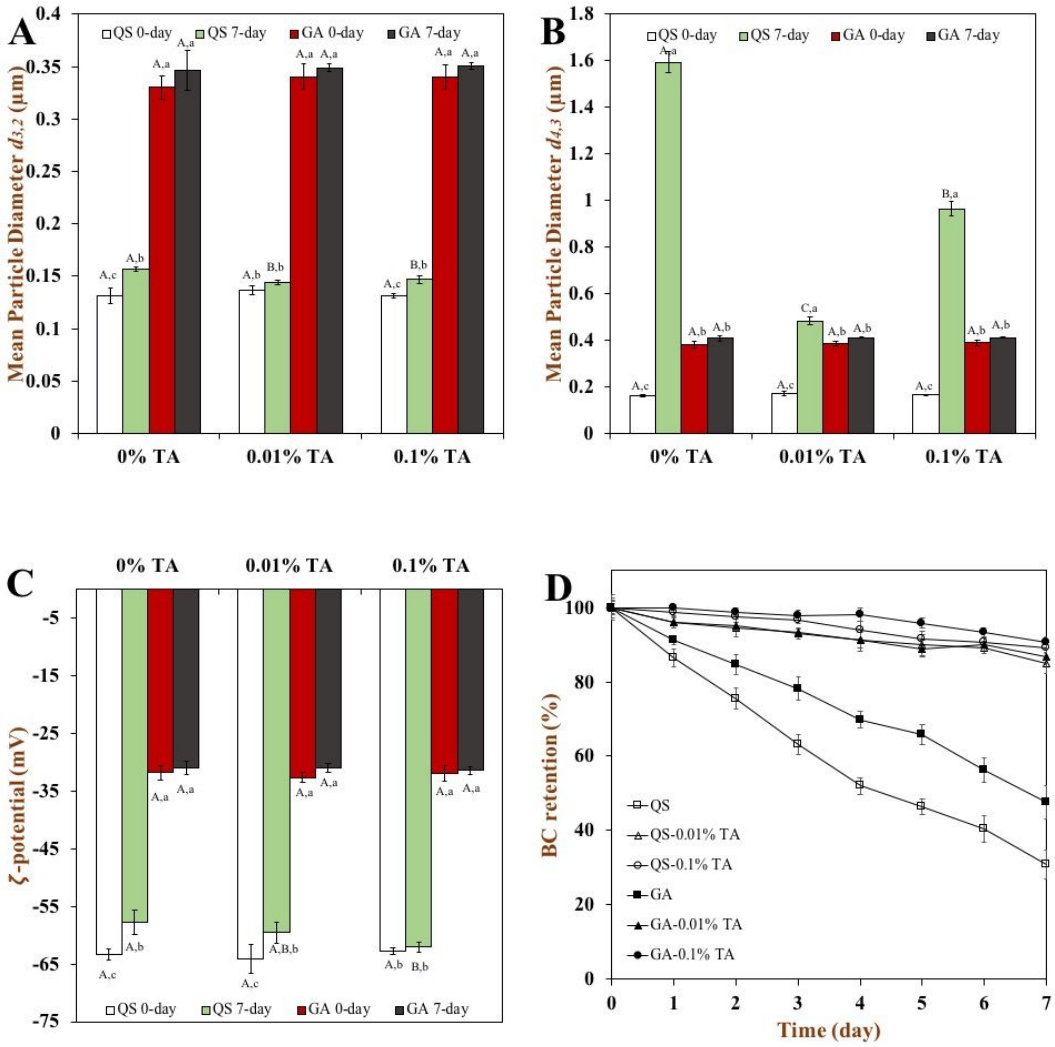

A
QS 0-day
QS 7-day
GA 0-day
GA 7-day
Mean Particle Diameter $d_{3,2}$ (μm)
0% TA
0.01% TA
0.1% TA
B
Mean Particle Diameter $d_{4,3}$ (μm)
C
ζ-potential (mV)
D
BC retention (%)
Time (day)
QS
QS-0.01% TA
QS-0.1% TA
GA
GA-0.01% TA
GA-0.1% TA

Fig. 9

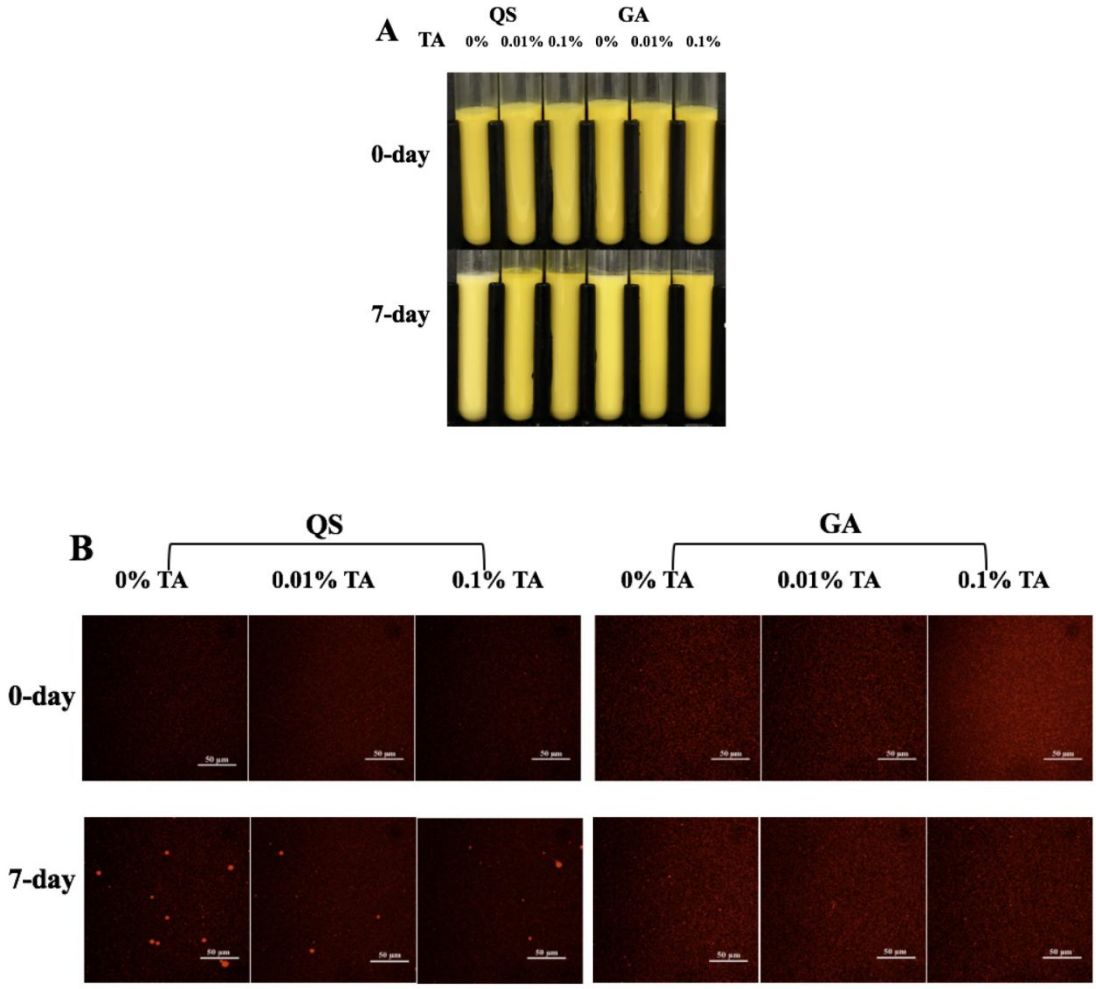
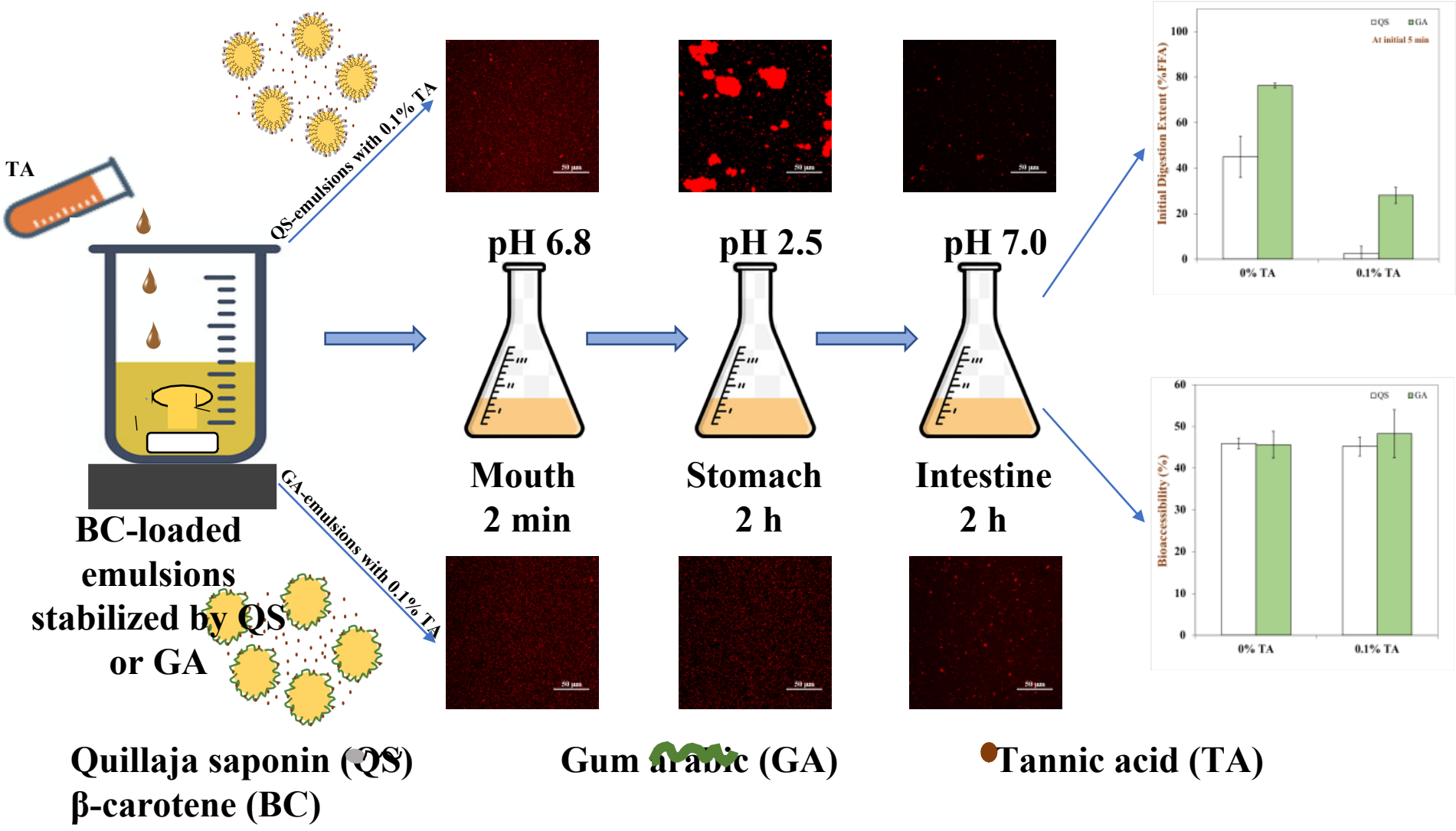

A
TA
QS
GA
0% 0.01% 0.1% 0% 0.01% 0.1%
0-day
7-day
B
QS
GA
0% TA
0.01% TA
0.1% TA
0% TA
0.01% TA
0.1% TA
0-day
7-day

Table 1. Parameters of CDOCKER protocol docking results

Pose number		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
pH 2.5	E_{CD} (kcal/mol)	**-118.78**	-118.17	-117.98	-117.43	-117.19	-116.99	-116.74	-116.56	-115.71	-115.53
	IE_{CD} (kcal/mol)	**-82.41**	-63.77	-55.28	-78.50	-62.85	-72.98	-72.30	-66.95	-67.76	-64.73
pH 7.0	E_{CD} (kcal/mol)	**-168.25**	-167.93	-161.74	-161.33	-158.50	-158.02	-156.08	-154.83	-153.06	-152.78
	IE_{CD} (kcal/mol)	**-84.73**	-77.47	-79.38	-63.75	-79.24	-80.52	-75.18	-62.81	-63.02	-61.29


TA
QS-emulsions with 0.1% TA
GA-emulsions with 0.1% TA
BC-loaded emulsions stabilized by QS or GA
pH 6.8
pH 2.5
pH 7.0
Mouth 2 min
Stomach 2 h
Intestine 2 h
Initial Digestion Extent (%FFA)
At initial 5 min
0% TA
0.1% TA
Bioaccessibility (%)
Quillaja saponin (QS)
β-carotene (BC)
Gum arabic (GA)
Tannic acid (TA)