Influence of emulsifier type on the *in vitro* digestion of fish oil-in-water emulsions in the presence of an anionic marine polysaccharide (fucoidan): caseinate, whey protein, lecithin, or Tween 80

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Abstract

The influence of an anionic marine polysaccharide (fucoidan) on the gastrointestinal fate of emulsified fish oils stabilized by different types of natural and synthetic emulsifier was examined: whey protein isolate (WPI); caseinate; lecithin; Tween 80. Oil-in-water emulsions in the absence and presence of fucoidan were passed through a simulated gastrointestinal tract (GIT) that included mouth, stomach, and small intestine phases. The change in droplet properties (particle size, charge, and organization) was measured throughout the GIT and the rate and extent of lipid digestion was measured in the small intestine phase. The presence of fucoidan increased the initial digestion rate of caseinate- and WPI-stabilized emulsions due to its ability to modulate lipid droplet aggregation. The fucoidan appeared to suppress isoelectric aggregation of the droplets, which increased the surface area of lipids available for the lipase molecules. On the other hand, the presence of fucoidan had little impact on the digestion of emulsions stabilized by lecithin or Tween 80, since it did not strongly impact the lipid droplet aggregation state. These results have important implications for the fabrication of functional foods and beverages that can control lipid digestion in the gastrointestinal tract.

Keywords

Emulsion; Digestion; Fucoidan; Polysaccharide; Emulsifier type

1. Introduction

Oil-in-water emulsions are an important element of numerous types of commonly consumed food products, including sauces, dressings, dips, desserts, ice cream, beverages, soups, and delivery systems (McClements, 2015). Traditionally, the major focus of research in this area was on understanding the physicochemical basis of their properties (such as appearance, texture, stability, and flavor), and using this information to design food emulsions with improved quality attributes. More recently there has been a focus on designing emulsions that can improve the nutritional aspects of foods (Mao & Miao, 2015; McClements & Xiao, 2012; Michalski, et al., 2013; Singh, Ye, & Ferrua, 2015). After an emulsion is ingested, its molecular composition and structural organization undergo a series of changes as it passes through the gastrointestinal tract (McClements, Decker, & Park, 2009; Singh, Ye, & Horne, 2009). The bioaccessibility of the emulsified oil and of any encapsulated hydrophobic bioactives is highly dependent on the gastrointestinal fate of the emulsion (Mao & Miao, 2015; Marze, 2015; Norton, Espinosa, Watson, Spyropoulos, & Norton, 2015; Qian, Decker, Xiao, & McClements, 2012). Consequently, food emulsions can be designed to increase the bioavailability of encapsulated substances, to control satiety, or to deliver components to specific regions of the gastrointestinal tract (Marze, 2015; McClements, et al., 2009).

Polysaccharides are commonly used in commercial food emulsions as stabilizers, texturizers, and health-promoting ingredients (Cui, 2005; Elleuch, et al., 2011; Stephen, Phillips, & Williams, 2006). Previous studies have reported many polysaccharides, such as pectin, chitosan, carrageenan, alginate, starch, and methyl cellulose, will impact the potential gastrointestinal fate of food emulsions (David-Birman, Mackie, & Lesmes,

2013; Espinal-Ruiz, Parada-Alfonso, Restrepo-Sanchez, Narvaez-Cuenca, & McClements, 2014; Li, et al., 2010; Mun, Kim, & McClements, 2015; Tokle, Lesmes, Decker, & McClements, 2012; Verrijssen, et al., 2014; Wooster, et al., 2014; Zhang, Zhang, Zhang, Decker, & McClements, 2015). The findings of these studies indicate that polysaccharides may impact lipid digestion through numerous physiochemical mechanisms: (i) they may adsorb to the surfaces of lipid droplets and form protective coatings that inhibit the ability of lipase to access the underlying lipid phase; (ii) they may alter the colloidal interactions between the lipid droplets, which alters their aggregation state, and therefore the surface area of lipids exposed to lipase; (iii) they may sequester components present in the gastrointestinal fluids (such as bile salts, fatty acids, phospholipids, calcium ions, or digestive enzymes) that normally play an important role in lipid digestion; (iv) they may alter the mass transport of digestive components due to their ability to increase the viscosity or form hydrogel networks. The ability and mechanisms of a given polysaccharide to impact lipid digestion relate to its molecular and physicochemical properties. Therefore, research is needed to identify the specific physicochemical mechanisms for a particular polysaccharide.

Fucoidan is marine polysaccharide consisting of a linear anionic backbone mainly composed of L-fucose and sulphate groups, which is typically isolated from brown algae and echinoderm (Ale, Mikkelsen, & Meyer, 2011; Wijesinghe & Jeon, 2012). Fucoidan has exhibited a number of biological activities that may lead to its application as a nutraceutical ingredient in functional foods, *e.g.*, antioxidant, anti-coagulant, anti-inflammatory, anti-tumor, and anti-viral activities (Vo & Kim, 2013). This anionic polysaccharide may also be utilized as a building block in the assembly of delivery

systems for bioactive agents, such as multilayer emulsions and biopolymer nanoparticles (Chang, Hu, & McClements, 2016; Chang & McClements, 2015; Huang, Chen, Lam, & Chen, 2014; Huang & Li, 2014; Lee & Lim, 2014). However, its effect on the digestion of emulsified lipids is currently unknown.

Food emulsions may be stabilized by a variety of different kinds of emulsifiers, including surfactants, phospholipids, proteins, and polysaccharides (Kralova & Sjoblom, 2009). Recent studies have shown that the nature of the emulsifier used to coat the lipid droplets may have a pronounced influence on their gastrointestinal fate (Hur, Decker, & McClements, 2009; Li & McClements, 2014; Nik, Wright, & Corredig, 2011; Qiu, Zhao, Decker, & McClements, 2015; Zhang, et al., 2015). These effects have been partly attributed to the influence of the emulsifier on the stability of the lipid droplets to aggregation within the GIT. If the droplets become flocculated or coalesced within the GIT, then the surface area of lipid exposed to the lipase is reduced, which slows down digestion. The rate of lipid digestion may also be reduced if the emulsifier molecules can prevent lipase from adsorbing to the droplet surfaces.

In the current study we compared the influence of three different natural emulsifiers (caseinate, whey protein isolate (WPI), and lecithin) and one synthetic emulsifier (Tween 80) on the rate and extent of lipid digestion in oil-in-water emulsions containing fucoidan. These emulsifiers were selected because they are commonly used within the food industry, and because they have different molecular characteristics (Kralova & Sjoblom, 2009; McClements, 2015): caseinate is a mixture of flexible proteins with an isoelectric point (pI) around pH 5; WPI is a mixture of globular proteins with a pI also around pH 5; lecithin is an amphiphilic phospholipid; and, Tween 80 is a non-ionic surfactant. We

hypothesized that the impact of fucoidan on emulsion digestion under simulated GIT conditions would depend on the nature of the emulsifiers used to coat the droplets, since fucoidan would be expected to interact differently with different emulsifiers.

The aim of the current study was therefore to examine the influence of fucoidan on the gastrointestinal fate of fish oil-in-water emulsions stabilized by different emulsifiers. A simulated static *in vitro* digestion model that includes the mouth, stomach, and small intestine phases was employed. A novel aspect of this study involved measuring the properties of the emulsions at the beginning and end of the small intestine phase to elucidate the aggregation state of the lipid droplets when lipase was added. The knowledge gained from this study should facilitate the rational design of functional foods that can control lipid digestion and improve the bioaccessibility of hydrophobic bioactives.

2. Experimental Methods

2.1. Materials

Fucoidan was isolated from the body wall of sea cucumber (*Acaudina Molpadioides*) according to a method described previously (Chang, et al., 2010). Briefly, the milled dried body wall of the sea cucumber was hydrolyzed by papain; the crude polysaccharide was precipitated with cetylpyridinium chloride; and then fucoidan was isolated from the crude polysaccharide fraction using ion exchange chromatography (Sephacrose Q Fast Flow, GE Healthcare, USA). The primary structure of fucoidan from *A. Molpadioides* has been clarified as $[\rightarrow 3\text{-}\alpha\text{-L-Fucp-1}\rightarrow 3\text{-}\alpha\text{-L-Fucp2,4(OSO}_3^-)\text{-1}\rightarrow 3\text{-}\alpha\text{-L-Fucp-1}\rightarrow 3\text{-}\alpha\text{-L-Fucp2(OSO}_3^-)\text{-1}\rightarrow]_n$ (Yu, et al., 2014; Yu, et al., 2013), and is presented schematically in Fig. S1 (supplementary material). The molecular weight of the fucoidan was determined

to be 1,321 kDa using high performance size exclusion chromatograph coupled with multiple angle laser light scattering in a previous study (Chang & McClements, 2015).

Fish oil (Ropufa 30 n-3 food oil) was obtained from DSM Nutritional Products Ltd. (Basel, Switzerland). The oil contained 101 mg of EPA/g of oil, 148 mg of DHA/g oil, and a total ω -3 PUFA of 312 mg/g of oil. Sodium caseinate was obtained from MP Biomedicals LLC. (Solon, OH), and was reported to contain 91.4 wt% protein and 5.0 wt% moisture. WPI was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA), and was reported to contain 92.7 wt% protein and 5.0 wt% moisture. Lecithin (Phospholipid 90G) was provided by Lipoid GmbH (Ludwigshafen, Germany), which was reported to have a phosphatidyl choline content of 96.6%. Mucin from porcine stomach (Type II, Product number M2378, Lot number SLBM5328V), pepsin from porcine gastric mucosa (250 units/mg, Product number P7000, Lot number SLBL6058V), porcine lipase (100–400 units/mg, Product number L3126, Lot number SLBH6427V), and porcine bile extract (Product number B8631, Lot number SLBM3452V) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used in this study were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Deionized water was used for the preparation of all solutions and emulsions.

2.2. Solution preparation

A stock buffer solution was prepared by dispersing 5 mM sodium phosphate monobasic and 0.02 wt% sodium azide (a non-food grade antimicrobial) in water and adjusting the pH to 7.0 by using sodium hydroxide. Caseinate, WPI, Tween 80, and lecithin solutions at 2 wt% were prepared by dispersing corresponding chemicals in the

stock buffer, and stirring for 3 h. The lecithin solution had to be further sonicated for 5 min at 40 kHz and 110 W using a ultrasonic bath (CPXH, Fisher Scientific, Pittsburgh, PA) to ensure proper dispersion as described previously (Ogawa, Decker, & McClements, 2003) with slight modification. Fucoidan solution at 1 wt% was prepared by dispersing fucoidan into the stock buffer and stirring overnight to ensure complete hydration.

Solutions for digestion experiments were prepared according to the methods reported in previous studies (Espinal-Ruiz, et al., 2014; Sarkar, Goh, & Singh, 2009). The simulated saliva fluid contained 1.594 g/L sodium chloride, 0.328 g/L ammonium nitrate, 0.636 g/L potassium phosphate, 0.202 g/L potassium chloride, 0.308 g/L potassium citrate, 0.021 g/L uric acid sodium salt, 0.198 g/L urea, 0.146 g/L lactic acid sodium salt, and 30 g/L mucin, and its pH was adjusted to 6.8. The simulated gastric fluid contained 2 g/L sodium chloride and 3.2 g/L pepsin, and pH was finally adjusted to 2.5 by using hydrochloric acid. The simulated intestinal stock salt solution contained 37.47 g/L calcium chloride dihydrate and 219.15 g/L sodium chloride. Bile salt solution (54 g/L) and lipase solution (24 g/L) were prepared by dispersing respective reagents in 5mM pH 7.0 phosphate buffer.

2.3. Emulsion preparation

Stock emulsions emulsified by caseinate, WPI, Tween 80, or lecithin (10 wt% fish oil, 1 wt% emulsifier) were prepared by homogenizing 10 wt% fish oil, 50 wt% emulsifier solution, and 40 wt% stock buffer using a high-speed blender (M133/1281-0, Biospec Products, Inc. ESGC, Switzerland) at 10,000 rpm for 2 min, followed by six passes at 12 kpsi through a high pressure homogenizer (Microfluidizer M-110L, Microfluidics, Newton, MA).

Emulsions containing 2 wt% oil droplets and 0.2 wt% fucoidan were prepared by diluting the stock emulsion with fucoidan solution and stock buffer solution, and stirring for 30 min. Similar emulsions containing no fucoidan were prepared by diluting the stock emulsion with only stock buffer solution. Previous studies have indicated that the effect of some polysaccharides (*e.g.*, pectin, chitosan, methyl cellulose) on emulsion digestion depends on polysaccharide concentration (Espinal-Ruiz, et al., 2014; Zhang, et al., 2015). In the current study, we therefore determined the impact of fucoidan on lipid digestion at both 0.2 and 0.5 wt%, however no appreciable difference in the results was found, and so only the data for the lower concentration is shown here for the sake of concision.

2.4. *In vitro* digestion model

To clarify the influence of fucoidan on emulsion digestion, the gastrointestinal fate of emulsions with and without fucoidan was studied and compared.

Emulsions were passed through a simulated *in vitro* digestion model that consisted of mouth, stomach, and intestine phases. The model was based on the one employed in previous studies (Espinal-Ruiz, et al., 2014) with slight modifications. Samples were taken out after each phase. However, an aliquot of sample was also drawn out of the small intestine phase before lipase digestion (*i.e.*, prior to the addition of lipase solution) to emphasize the influence of lipid droplet aggregation state. The temperature of the incubator and water bath used in the lipid digestion experiments was maintained at 37 °C unless otherwise indicated. Adjustment of pH was carried out using sodium hydroxide or hydrochloric acid solutions.

Initial system: 20 mL of 2 wt% oil-in-water emulsion was placed into a glass beaker in an incubator shaker.

Mouth phase: 20 mL of simulated saliva fluid was preheated to 37 °C and then mixed with the initial emulsion. After adjustment to pH 6.8, the mixture was placed in an incubated shaker for 10 min to mimic agitation in the mouth. This time is longer than a fluid product would normally spend within the mouth, but it was used to reduce variability amongst different samples.

Stomach phase: 20 mL of the sample from the mouth phase was mixed with 20 mL of simulated gastric fluid. After the pH was adjusted to 2.5, this mixture was held in an incubated shaker for 2 h to mimic stomach digestion.

Intestine phase 1 - before lipase digestion: 30 g of sample from the stomach phase was transferred to a 100 mL glass beaker that was placed in a water bath and adjusted to pH 7.00. Thereafter, 1.5 mL of simulated intestinal fluid and 3.5 mL of bile salt solution were successively added to the reaction vessel with constant stirring. After the pH was adjusted back to 7.00, 5 g sample was taken out for characterization.

Intestine phase 2 – after lipase digestion: 2.15 mL of lipase solution was added into the remainder of the sample from intestine phase 1 (approximately 30 g). The pH of the reaction system was then monitored and maintained at pH 7.0 for 2 h by titrating 0.1 M sodium hydroxide solution in the reaction chamber using an automatic titration unit (857 Titrand, Metrohm USA Inc., Riverview, FL). The percentage of free fatty acids (FFA) released was then calculated from the volume of sodium hydroxide used to neutralize the FFA using the following formula:

$$\% \text{FFA} = 100 \times \frac{V_{\text{NaOH}} \times m_{\text{NaOH}} \times M_{\text{Lipid}}}{W_{\text{Lipid}} \times 2}$$

Here, V_{NaOH} is the volume of sodium hydroxide required to neutralized the FFAs produced (in mL), m_{NaOH} is the molarity of the sodium hydroxide solution (0.1 M), W_{Lipid}

is the total weight of lipid initially present in the reaction vessel (0.129 g), and M_{Lipid} is the molecular weight of the fish oil (868 g/mol).

2.5. Characterization of emulsion samples

The ζ -potential of the samples was determined using a commercial instrument that combined dynamic light scattering and microelectrophoresis measurements (Nano-ZS, Malvern Instruments, Worcestershire, UK). Before analysis, samples were diluted to a droplet concentration of approximately 0.05 wt% using an appropriate buffer. Thus, the mouth phase samples were diluted using the simulated saliva fluid without mucin, the stomach phase samples were diluted using the simulated gastric fluid without pepsin, and the other samples were diluted using the stock phosphate buffer (pH 7.0).

The particle size distribution was determined using a static light scattering instrument (Mastersizer 2000, Malvern Instruments, Worcestershire, United Kingdom) after dilution in the appropriate buffer solution (as described for dynamic light scattering measurements). The mean particle size was reported as the volume-weighted mean diameter: d_{43} ($=\sum n_i d_i^4 / \sum n_i d_i^3$, where n_i is the number of particles with diameter d_i). The refractive indices of the aqueous phase and oil phase used in the light scattering calculations were 1.33 and 1.48 respectively.

The microstructure of samples was investigated using confocal scanning laser microscopy. A Nikon D-Eclipse C1 80i microscope (Nikon, Melville, NY) equipped with a 60 $\times$ oil immersion objective lens was used to capture the images. Before analysis, 2 mL samples were mixed with 0.1 mL Nile Red solution (1 mg/mL in ethanol) and 0.1 mL fluorescein isothiocyanate (FITC) solution (1 mg/mL in dimethyl sulfoxide) to respectively stain the oil phase and protein. The excitation and emission spectrum for

Nile Red were 543 nm and 650 nm, and for FITC were 488 nm and 515 nm. Images were recorded and analyzed by using the instrumental software (NIS-Elements, Nikon, Melville, NY).

2.6. Statistical Analysis

All experiments were performed at least three times, and average and standard deviations were calculated from these measurements. Student's t-test was performed utilizing SPSS v11.5 (SPSS Inc., Chicago, USA) to evaluate the difference between emulsion samples with and without fucoidan. Statistical significance was considered when $p < 0.05$.

3. Results and discussion

The particle size, microstructure, and electrical characteristics of the emulsions were measured at different stages within the simulated GIT to provide some information about the influence of fucoidan and emulsifier type on their gastrointestinal fate (Table 1, Figs. 1 to 3). In addition, the rate and extent of lipid digestion of the emulsions was measured during the small intestinal phase (Figs. 4 and 5). The results of these measurements are discussed in the following sections.

3.1 Initial system

In the absence of fucoidan, the initial emulsions stabilized by caseinate, WPI, lecithin, and Tween 80 all had fairly small mean particle diameters ($d_{43} = 283, 223, 358$ and 217 nm, respectively) and monomodal size distributions (Figs. 1 and 2). Confocal microscopy analysis showed that these emulsions all consisted of relatively small droplets that were evenly distributed throughout the sample, and rapidly moved around due to Brownian

motion (Fig. 2 and Fig. S2). These measurements indicated that the emulsion formulation and homogenization conditions used were appropriate for forming stable emulsions containing small droplets. All of the fucoidan-free emulsions were negatively charged, with the magnitude of their electrical charge depending on the nature of the emulsifier used (Fig. 3). The relatively high negative charge on the caseinate and WPI stabilized emulsions was expected since the pH of the initial system (pH 7.0) was well above the isoelectric points of the proteins ($pI \approx 5$). Lecithin, which mainly consisted of phosphatidyl choline, is a zwitterionic polar lipid that has previously been reported to give emulsion droplets a negative charge (Hu, Cao, Hu, & Feng, 2012; Uluata, McClements, & Decker, 2015). Tween 80 is a nonionic surfactant and so one might expect that lipid droplets coated by it would possess no charge. However, numerous studies have reported that lipid droplets stabilized by nonionic surfactants have a negative charge at neutral pH, which may be due to the presence of free fatty acid impurities in the surfactant or oil, or due to the preferential adsorption of OH^- (rather than H^+) from water to the droplet surfaces (Hsu & Nacu, 2003; Mun, Decker, & McClements, 2007).

The addition of fucoidan brought no significant difference in the particle size of caseinate, WPI, and Tween 80 stabilized emulsions measured by light scattering. However, flocs were observed in the microscopy images of the same emulsions, which suggests that the lipid droplets were held together by relatively weak attractive forces that were easily disrupted by the dilution and stirring conditions used in the light scattering measurements. This kind of behavior is characteristic of depletion flocculation, which is known to occur when relatively high concentrations of non-adsorbed polymer molecules are present in the aqueous phase surrounding the lipid droplets (Dickinson, Golding, &

Povey, 1997; McClements, 2015). Indeed, it has previously been reported that addition of 0.1 wt% fucoidan or higher can promote depletion flocculation in caseinate-stabilized emulsions at pH values above 6, where the droplets and biopolymer are both strongly negatively charged (Chang & McClements, 2015).

The emulsion stabilized by lecithin exhibited somewhat different behavior. The addition of fucoidan significantly increased the mean particle size of lecithin-stabilized emulsions (Table 1c), which can be attributed to the formation of irreversible aggregates in both of the particle size distribution (Fig. 1c) and the confocal microscopy images (Fig. 2). We hypothesize that the aggregates observed in these samples were a result of droplet coalescence. Droplet coalescence is often promoted when the emulsion droplets are brought into close proximity due to depletion flocculation (Klinkesorn, Sophanodora, Chinachoti, & McClements, 2004; Ye, Hemar, & Singh, 2004). Presumably, the interfacial coating formed by the lecithin molecules was less resistance to coalescence than that formed by the other emulsifiers.

The presence of fucoidan did not significantly influence the ζ -potential of any of the emulsions, which suggested that it did not adsorb to the surfaces of the lipid droplets under the conditions used. This would be expected since both the lipid droplets and fucoidan molecules were highly negatively charged at pH 7, and so there would be a strong electrostatic repulsion between them.

3.2 Mouth phase

After passing through the mouth phase, the mean particle size of all the fucoidan-free emulsions increased (Table 1), which can be attributed to the formation of a population of relatively large particles as seen in the particle size distributions (Fig. 1) and confocal

microscopy images (Fig. 2). Simulated saliva fluid mainly consists of inorganic salts and mucin, which is a heavily glycosylated and relatively large glycoprotein (0.5 to 20 MDa) (Bansil & Turner, 2006; Coles, Chang, & Zauscher, 2010; Strous & Dekker, 1992). The molecular weight of the mucin used in this study was determined to be around $2,981 \pm 21$ kDa by size exclusion chromatography coupled with multiple angle light scattering. It has been reported previously that mucin can promote depletion flocculation of lipid droplets stabilized by caseinate, Tween 80, or β -lactoglobulin under neutral pH (Chang & McClements, 2016; Ritzoulis, et al., 2012; Vingerhoeds, Blijdenstein, Zoet, & van Aken, 2005). Therefore, the droplet aggregation observed in the mouth phase can mainly be attributed to depletion flocculation induced by non-adsorbed mucin, or electrostatic screening caused by inorganic salts (Sarkar, et al., 2009).

The magnitude of the ζ -potential of the emulsions all decreased after 10 min exposure to the mouth phase, with the decrease being especially pronounced for the protein-coated droplets (Fig. 3). This change may have occurred to electrostatic screening effects associated with the presence of salt ions in the simulated saliva fluid (Singh & Sarkar, 2011), as well as the slight drop in pH from 7.0 to 6.8.

The addition of fucoidan to the emulsions caused no significant change in the particle size or ζ -potential of the emulsions (Table 1 and Fig. 3), which suggested that fucoidan only had a limited impact on the properties of the individual droplets in this state of the GIT. This effect can again be attributed to the fact that both the fucoidan and lipid droplets were negatively charged in the mouth phase, and therefore it was expected that the polysaccharide would not be electrostatically adsorbed onto the droplet surfaces. Having said this, droplet aggregation was still observed in the microscopic images of the

emulsions containing fucoidan in the mouth phase, which can be attributed to depletion flocculation due to the presence of both mucin and fucoidan.

3.3 Stomach phase

After 2 h exposure to the simulated stomach conditions, there was an appreciable increase in the particle size of the caseinate-, WPI-, and lecithin-stabilized emulsions (Table 1 and Fig. 1). The confocal microscopy images indicated that the lecithin- and protein-stabilized emulsions contained relatively large individual oil droplets, which suggests that coalescence had occurred in the stomach phase for these systems. This type of emulsion instability may occur for various reasons: (i) changes in pH and ionic strength may promote aggregation due to weakening of the electrostatic repulsion between the lipid droplets; (ii) hydrolysis of adsorbed proteins by pepsin may reduce droplet stability to coalescence; (iii) anionic mucin molecules may induce bridging flocculation of cationic protein-coated lipid droplets at acidic gastric pH (Singh & Sarkar, 2011; Singh, et al., 2009). In contrast, there was little change in the mean particle diameter, particle size distribution, or confocal images of the Tween 80-stabilized emulsions after exposure to the stomach phase (Table 1 and Figs. 1 to 2). These results suggest that Tween 80-stabilized emulsions were resistant to droplet aggregation under gastric conditions whereas lecithin- and protein-stabilized ones were not, which is in agreement with the findings of previous reports (van Aken, Bomhof, Zoet, Verbeek, & Oosterveld, 2011; Zhang, et al., 2015). This may have important consequences for designing emulsions that are able to induce satiety, since it has been shown that the rate of gastric emptying is less for gastric-stable emulsions (Marciani, et al., 2007; Steingoetter, et al., 2015).

A decrease in the magnitude of the negative ζ -potential was observed in all the fucoidan-free emulsions after the stomach phase (Fig. 3). The simulated gastric fluids have a relatively high ionic strength, which would result in a reduction in ζ -potential due to electrostatic screening (Israelachvili, 2011). It is interesting to note that the ζ -potential of the protein-coated emulsions was close to zero rather than highly positive, as would be expected at pH values appreciably below the isoelectric points of caseinate and WPI ($\approx$ pH 5). This effect can be attributed to the adsorption of anionic mucin molecules to the surfaces of the cationic protein-coated lipid droplets, which has been shown in recent studies (Chang & McClements, 2016). In addition, it may be due to partial or complete hydrolysis of the protein molecules adsorbed to the oil droplet surfaces, which may alter the interfacial composition and properties (Singh & Sarkar, 2011; Singh, et al., 2009).

The addition of fucoidan suppressed the decrease in ζ -potential of the protein-stabilized emulsions in the stomach phase (Fig. 3). Previous studies have shown that fucoidan will adsorb onto the surfaces of caseinate-stabilized emulsions at pH values below 6 (even though both the protein and polysaccharide are negatively charge), which was attributed to the electrostatic attraction of anionic sulfate groups on the fucoidan to cationic patches on the adsorbed caseinate molecules (Chang & McClements, 2015). As both fucoidan and mucin can adsorb onto the surfaces of protein-coated lipid droplets at gastric pH, then it is possible that competitive adsorption and displacement between those two polyelectrolytes might happen. Recently, it was reported that in the presence of a mixture of fucoidan and gum arabic, the fucoidan molecules preferentially adsorb to the surfaces of caseinate-coated lipid droplets, which was attributed to its higher charge density than gum arabic (Chang, et al., 2016). The highly charge density of fucoidan is

due to the presence of many sulfate groups along the polymer backbone, *i.e.*, an average of 0.75 sulfate groups per monosaccharide residue (Fig. S1) (Yu, et al., 2014; Yu, et al., 2013). On the other hand, mucin is considered to be a relatively weak polyelectrolyte, whose electrical characteristics are determined by ionizable groups in both the polypeptide backbone (*e.g.*, aspartic and glutamic acid residues, $pK_a \approx 4$) and the oligosaccharide side chains (*e.g.*, sialic acid residues, $pK_a \approx 2.6$; and, sulfate groups, $pK_a \approx 1$) (Cao, et al., 1999; Maleki, Lafitte, Kjoniksen, Thuresson, & Nystrom, 2008; Waigh, et al., 2002). We measured the ζ -potential of fucoidan and mucin (0.5 wt%) at pH 2.5, and found that the electric potential of fucoidan (-39.5 ± 1.5 mV) was much higher than that of mucin (-1.9 ± 0.2 mV). It therefore seems likely that fucoidan preferentially adsorbed onto the surfaces of lipid droplets in the presence to mucin. As a result, the protein-coated lipid droplets may have been coated by a highly charged fucoidan layer in these systems. Intriguingly, pectin, a weak electrolyte ($pK_a \approx 3.5$), is reported to have no significant impact on the ζ -potential of emulsions in the gastric phase (Zhang, et al., 2015). This may have occurred because the pectin molecules lost much of their negative charge under the highly acidic conditions of the gastric fluids. **Again it should be noted that the adsorbed proteins might have been partially hydrolyzed within the gastric phase due to the presence of pepsin, which may have altered the ability of the oil droplets to interact with the fucoidan molecules.**

The addition of fucoidan decreased the size of the aggregates formed within the gastric fluids for the caseinate- and WPI-stabilized emulsions (Table 1 and Fig. 1). Indeed, the particle size distribution plots indicated that there was a population of particles with dimensions similar to those present in the initial emulsions. Moreover, the

large coalesced droplets observed in the fucoidan-free emulsions were not observed in the emulsions containing fucoidan (Fig. 2). Droplet aggregation may have been inhibited in the presence of fucoidan because the highly anionic fucoidan molecules adsorbed to the surfaces of the protein-coated lipid droplets and increased the electrostatic and steric repulsion between them.

Fucoidan did not have an appreciable influence on the particle size or microstructure of the lecithin and Tween 80 emulsions compared to the fucoidan-free emulsions (Table 1), which may have been because this anionic polysaccharide did not adsorb to the surfaces of the anionic lipid droplets. Nevertheless, the presence of the fucoidan did increase the measured negative charge in these two emulsions after passing through the stomach phase (Fig. 3). This effect may have occurred because the anionic polysaccharide molecules contributed to the signal measured by the ζ -potential instrument.

3.4 Intestine phase 1 - before lipase digestion

In this series of experiments, the solution conditions were adjusted to simulate the small intestine, but lipase was not added. This procedure was carried out to provide some information about the aggregation state of the lipid droplets prior to lipid digestion. After adjusting the samples to pH 7.0 and adding bile salts and inorganic salts, the size of the particles in all the emulsions increased regardless of the presence or absence of fucoidan (Table 1 and Figs. 1 to 2). The size of the particles produced was particularly large in the emulsions containing caseinate-coated lipid droplets in the absence of fucoidan (Fig. 2). Particle aggregation may have been induced due to a number of physicochemical mechanisms. First, there was an increase in pH when the emulsions moved from the

stomach to the small intestine, which may have promoted aggregation in the protein-coated droplets as they moved through their isoelectric point. Second, the addition of inorganic salts may have promoted droplet aggregation by screening the electrostatic repulsion between the droplets (Israelachvili, 2011). Third, the bile salts may have adsorbed to the droplet surfaces and altered their interfacial compositions, thereby altering droplet-droplet interactions (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011; Wilde & Chu, 2011).

The presence of fucoidan in the emulsions had different effects depending on emulsifier type (Figs. 1 to 3). For lecithin-stabilized emulsions, the particle size distribution and microstructure did not appear to be strongly affected by the presence of fucoidan. This may have been because both the droplets and fucoidan were always negatively charged, and so there was little direct interaction between them. For Tween 80-stabilized emulsions, there appeared to be more small droplets remaining in the presence of fucoidan (Fig. 1), but extensive flocculation still occurred (Fig. 2). For protein-stabilized emulsions, the presence of fucoidan appeared to lead to the formation of fewer flocs, which contained less densely packed lipid droplets (Fig. 2). It is possible that the anionic fucoidan molecules adsorbed to the surfaces of the cationic protein-coated lipid droplets in the stomach phase, and therefore they prevented isoelectric flocculation when the pH was increased from 2.5 in the stomach phase to 7.0 in the small intestine phase.

3.5 Intestine phase 2 – lipase digestion

The influence of emulsifier type and fucoidan on the rate and extent of lipid digestion was examined by using a pH-stat method. The lipid digestion profiles of all the emulsions

456 followed a fairly similar pattern. A rapid increase in the release of FFAs was observed in
457 the initial period, followed by a more gradual increase at longer times, until a plateau was
458 reached (Fig. 4). To make a clearer comparisons, the initial rate of FFA release was
459 calculated by fitting a straight line to the first five minutes of digestion (Fig. 5a), and the
460 final extent of digestion was calculated at the end of 2 h incubation with lipase (Fig. 5b).

461 In the absence of fucoidan, the final extent of lipid digestion was greater than 85% in
462 the emulsions stabilized by different emulsifiers, suggesting an almost complete digestion
463 of the fish oil. Nevertheless, the initial digestion rate was appreciably influenced by
464 emulsifier type (Fig. 5a). The caseinate-coated lipid droplets were digested more slowly
465 than the other three emulsions, which could be attributed to the severe flocculation and
466 coalescence of the lipid droplets that occurred before the addition of lipase (Fig. 2).
467 Droplet aggregation resulted in a decrease in the surface area of the lipid droplets, and
468 thus reduced the access of lipase to the lipid substrate. The intermediate initial lipid
469 digestion rates for the WPI- and lecithin-coated droplets are consistent with the moderate
470 amount of droplet aggregation observed in these emulsions prior to lipase addition.
471 Somewhat surprisingly, the fastest rate of lipid digestion was observed for the Tween 80-
472 coated droplets, even though they appeared to form large dense aggregates at the
473 beginning of the small intestine phase (Fig. 2). It is possible that these aggregates
474 consisted of lipid droplets that were only weakly held together by attractive forces, and
475 were therefore easily disrupted during lipid digestion.

476 The addition of fucoidan to the emulsions had little impact on the rate or extent of
477 lipid digestion in the lecithin- and Tween 80-coated droplets (Fig. 5). This suggests that
478 this polysaccharide did not interfere with the lipid digestion process for these emulsions.

On the other hand, the addition of fucoidan to the emulsions containing caseinate-coated lipid droplets led to a significant increase in the initial digestion rate and final extent of digestion (Fig. 5). This increase could be attributed to ability of the fucoidan molecules to prevent extensive droplet aggregation in the GIT, therefore increasing the surface area of lipids available for the lipase to attach to. Fish oil naturally contains high levels of ω -3 fatty acids, which are beneficial to human health by improving cognitive function, and reducing the risk of coronary disease, inflammation and cancer (Laviano, Rianda, Molfinio, & Rossi Fanelli, 2013; Luchtman & Song, 2013; Nicholson, Khademi, & Moghadasian, 2013). Consequently, the presence of fucoidan may promote the potential health benefits of fish oils stabilized by this widely used dairy emulsifier.

Fucoidan also significantly increased the initial digestion rate of WPI, but the effect was much less than for caseinate (Figs. 5 and 6). Interestingly, the mean particle diameter of the WPI emulsion containing fucoidan (58 μ m) was higher than that of the fucoidan-free emulsion (42 μ m), and larger flocs were observed by confocal microscopy in the presence of fucoidan (Fig. 2). This suggests that the structure or stability of the aggregates may have been difference in the presence of fucoidan. Indeed, the confocal microscopy images suggests that the flocs formed in the presence of fucoidan have more open structures, which may have facilitated the penetration of lipase (Fig. 2). These results highlight the important role of the structural organization of emulsions in the GIT on their digestion.

After the incubation of the intestine phase, all the digested samples exhibited relatively high negative charges. These charges are likely to originate from the presence of various types of anionic particulate matter in the digesta (such as undigested protein aggregates,

undigested lipid droplets, micelles, or vesicles), which have negatively charged substances (such as peptides or free fatty acids) at their surfaces. Interestingly, the fucoidan had little impact on the overall charge measured in the small intestine phase, which may have been because its impact was dominated by all the other anionic species present.

The confocal microscopy images indicated that the large lipid-rich aggregates observed in the initial stages of the small intestine phase were largely disrupted or dissociated by the end (Fig. 2). This effect can be attributed to the conversion of the lipids into free fatty acids and monoacylglycerols by the lipase, and the conversion of the proteins into peptides and amino acids by the proteases. Nevertheless, some large particles were still observed in the caseinate-stabilized emulsions after lipid digestion (Fig. 2), which may be because the presence of large dense aggregates prevented complete lipid and/or protein digestion.

4. Conclusions

This study has shown that an anionic marine polysaccharide (fucoidan) influences the gastrointestinal fate of emulsified fish oils by an amount that depends on the type of emulsifier used to coat the droplets. The presence of fucoidan increased the initial digestion rate of caseinate- and WPI-stabilized emulsions due to its ability to modulate lipid droplet aggregation. On the other hand, the presence of fucoidan had little impact on the digestion of emulsions stabilized by lecithin or Tween 80. These results have important implication for the fabrication of functional foods and beverages that can control the lipid digestion.

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Fig. 1. Influence of fucoidan (FUC) on size distribution of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] during *in vitro* digestion. Solid and dash lines respectively stand for emulsions with and without fucoidan. The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase.

Fig. 2. Influence of fucoidan on microstructure of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] observed by confocal fluorescence microscopy. The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase. The scale bar indicates 20 μm . Images with higher resolution could be found in the supplementary data Fig. S2.

Fig. 3. Influence of fucoidan (FUC) on electrical characteristics (ζ -potential) of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] after expose to different phases of an simulated digestion model. The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase. Samples denoted with asterisks were significantly different ($p < 0.05$) from the corresponding fucoidan-free emulsions at the same digestion phase.

Fig. 4. Influence of fucoidan (FUC) on *in vitro* lipid hydrolysis of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80]. Free fatty acids (FFA) released from emulsions were measured using a pH-stat method.

Fig. 5. Influence of fucoidan (FUC) on the initial digestion rate and final digestion extent of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80]. Samples denoted with asterisks were significantly different ($p < 0.05$) from the corresponding emulsions without fucoidan.

Fig. S1 The structure of fucoidan from sea cucumber *Acaudina molpadioides*. The sulphate groups were highlighted in red.

Fig. S2 Representative microscopic images of emulsion samples in high resolution.

Fig. 1A

Fig. 1. Influence of fucoidan (FUC) on size distribution of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] during *in vitro* digestion. Solid and dash lines respectively stand for emulsions with and without fucoidan. **The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase.**

Fig. 1B

Fig. 1. Influence of fucoidan (FUC) on size distribution of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] during *in vitro* digestion. Solid and dash lines respectively stand for emulsions with and without fucoidan. The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase.

Fig. 1C

Fig. 1. Influence of fucoidan (FUC) on size distribution of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] during *in vitro* digestion. Solid and dash lines respectively stand for emulsions with and without fucoidan. **The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase.**

Fig. 1D

Fig. 1. Influence of fucoidan (FUC) on size distribution of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] during *in vitro* digestion. Solid and dash lines respectively stand for emulsions with and without fucoidan. **The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase.**

Fig. 2

Fig. 2. Influence of fucoidan on microstructure of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] observed by confocal fluorescence microscopy. The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase. The scale bar indicates 20 μm . Images with higher resolution could be found in the supplementary data Fig. S2.

Fig. 3A

Fig. 3. Influence of fucoidan (FUC) on electrical characteristics (ζ -potential) of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] after expose to different phases of an simulated digestion model. **The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase.** Samples denoted with asterisks were significantly different ($p < 0.05$) from the corresponding fucoidan-free emulsions at the same digestion phase.

Fig. 3B

Fig. 3. Influence of fucoidan (FUC) on electrical characteristics (ζ -potential) of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] after expose to different phases of an simulated digestion model. **The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase.** Samples denoted with asterisks were significantly different ($p < 0.05$) from the corresponding fucoidan-free emulsions at the same digestion phase.

Fig. 3C

Fig. 3. Influence of fucoidan (FUC) on electrical characteristics (ζ -potential) of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] after expose to different phases of an simulated digestion model. **The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase.** Samples denoted with asterisks were significantly different ($p < 0.05$) from the corresponding fucoidan-free emulsions at the same digestion phase.

Fig. 3D

Fig. 3. Influence of fucoidan (FUC) on electrical characteristics (ζ -potential) of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80] after expose to different phases of an simulated digestion model. **The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase.** Samples denoted with asterisks were significantly different ($p < 0.05$) from the corresponding fucoidan-free emulsions at the same digestion phase.

Fig. 4A

Fig. 4. Influence of fucoidan (FUC) on *in vitro* lipid hydrolysis of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80]. Free fatty acids (FFA) released from emulsions were measured using a pH-stat method.

Fig. 4B

Fig. 4. Influence of fucoidan (FUC) on *in vitro* lipid hydrolysis of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80]. Free fatty acids (FFA) released from emulsions were measured using a pH-stat method.

Fig. 4C

Fig. 4. Influence of fucoidan (FUC) on *in vitro* lipid hydrolysis of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80]. Free fatty acids (FFA) released from emulsions were measured using a pH-stat method.

Fig. 4D

Fig. 4. Influence of fucoidan (FUC) on *in vitro* lipid hydrolysis of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80]. Free fatty acids (FFA) released from emulsions were measured using a pH-stat method.

Fig. 5A

Fig. 5. Influence of fucoidan (FUC) on the initial digestion rate and final digestion extent of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80]. Samples denoted with asterisks were significantly different ($p < 0.05$) when compared with the corresponding emulsions without fucoidan.

Fig. 5B

Fig. 5. Influence of fucoidan (FUC) on the initial digestion rate and final digestion extent of emulsions stabilized by different emulsifiers [(A) caseinate; (B) WPI; (C) lecithin; (D) Tween 80]. Samples denoted with asterisks were significantly different ($p < 0.05$) from the corresponding emulsions without fucoidan.

Table. 1. Mean particle diameter (d43, in µm) of emulsions stabilized by different emulsifiers as they pass through different phases of an *in vitro* digestion model, in absence or presence of fucoidan (FUC). The time of emulsions staying in the mouth and stomach phase was 10 min and 2 h; intestine 1 and intestine 2 stood for status before and after 2 h lipase digestion in the intestine phase. Samples denoted with asterisks were significantly different ($p < 0.05$) from the corresponding fucoidan-free emulsions at the same digestion phase.

	Caseinate Emulsion		WPI Emulsion		Lecithin Emulsion		Tween 80 Emulsion	
	Without FUC	With FUC	Without FUC	With FUC	Without FUC	With FUC	Without FUC	With FUC
Initial	0.283±0.003	0.281±0.003	0.225±0.006	0.222±0.001	0.356±0.001	0.758±0.028*	0.217±0.120	0.216±0.010
Mouth	7.045±0.866	5.749±0.006	6.730±1.319	6.928±2.341	5.484±0.023	6.700±1.930	5.499±0.491	5.504±0.229
Stomach	40.298±3.264	10.051±1.904*	23.614±3.424	8.620±2.893*	8.385±0.954	10.602±1.414	5.477±0.581	4.635±0.544
Intestine 1	107.983±13.845	29.863±0.244*	42.084±4.991	58.342±18.799	98.377±25.046	79.323±5.506	59.917±8.838	51.459±4.583
Intestine 2	17.322±4.738	15.710±9.852	22.436±12.015	12.434±1.212	29.428±4.207	8.590±2.253*	11.948±5.505	13.958±2.666