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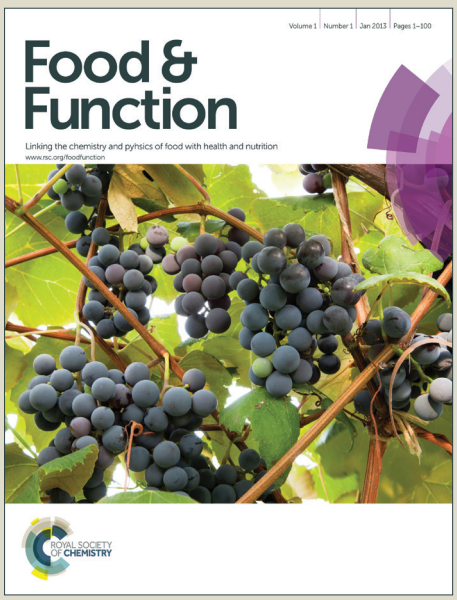

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Impact of dietary fibers [methyl cellulose, chitosan, and pectin] on digestion of lipids under simulated gastrointestinal conditions

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Abstract

A simulated *in vitro* digestion model was used to elucidate the impact of dietary fibers on the digestion rate of emulsified lipids. The influence of polysaccharide type (chitosan (cationic), methyl cellulose (non-ionic), and pectin (anionic)) and initial concentration (0.4 to 3.6% (w/w)) was examined. 2% (w/w) corn oil-in-water emulsions stabilized by 0.2% (w/w) Tween-80 were prepared, mixed with polysaccharide, and then subjected to an *in vitro* digestion model (37 °C): initial (pH 7.0); oral (pH 6.8; 10 min); gastric (pH 2.5; 120 min); and, intestinal (pH 7.0; 120 min) phases. The impact of polysaccharides on lipid digestion, ζ-potential, particle size, viscosity, and stability was determined. The rate and extent of lipid digestion decreased with increasing pectin, methyl cellulose, and chitosan concentrations. The free fatty acids released after 120 min of lipase digestion were 46, 63, and 81% (w/w) for methyl cellulose, pectin, and chitosan, respectively (3.6% (w/w) initial polysaccharide), indicating that methyl cellulose had the highest capacity to inhibit lipid digestion, followed by pectin, and then chitosan. The impact of the polysaccharides on lipid digestion was attributed to their ability to induce droplet flocculation, and/or due to their interactions with molecular species involved in lipid hydrolysis, such as bile salts, fatty acids, and calcium. These results have important implications for understanding the influence of dietary fibers on lipid digestion. The control of lipid digestibility within the gastrointestinal tract might be important for the development of reduced-calorie emulsion-based functional food products.

Keywords: Pectin, chitosan, methyl cellulose, emulsion, lipid digestion, gastrointestinal tract, flocculation.

1. Introduction

Diets rich in fat have been associated with high incidences of obesity and elevated risks of coronary heart disease, diabetes, and certain forms of cancer[1, 2]. A potential strategy for combating these chronic diseases is to reduce the total level of fat present in food products[3-5]. However, the development of fat-reduced products is challenging because fats have a major impact on the physicochemical, sensory, and nutritional properties of foods[6, 7]. For instance, fat contributes to the desirable texture of dairy products[8], the mouthfeel and texture of bakery products[9], and the creamy texture, milky appearance, desirable flavor, and satiating effects of emulsion-based products, such as sauces, spreads, dressings, and dips[10]. Foods with reduced fat levels must therefore be carefully formulated to ensure that they maintain their desirable physicochemical, sensory, and nutritional properties (*e.g.* appearance, flavor, texture, shelf life, and satiety effects), otherwise they will not be acceptable to consumers[6].

Rather than simply reducing the total amount of fat present within foods, it may also be possible to improve their healthfulness using other strategies associated with controlling fat digestion. For example, if the rate and extent of lipid digestion within the small intestine can be decreased then the post-prandial spike in blood lipid levels that normally occurs after ingestion of a fatty food can be reduced[11]. In addition, retarded lipid digestion may also increase the feelings of satiety and satiation, which may lead to lower total calorie consumption[12-14]. Dietary fibers are known to have an impact on the behavior of lipids within the gastrointestinal tract and can therefore be used to modulate the response of humans to ingested lipids[12, 15-17]. Dietary fibers may influence lipid digestion through a variety of mechanisms[16]: (i) they may bind to species that play a critical role in digestion, such as bile salts, phospholipids, enzymes or calcium[18]; (ii) they may increase the viscosity of the intestinal phase, and thereby alter mass transport processes[19, 20]; (iii) they may form protective coatings around lipid droplets thereby inhibiting lipase access[14, 21, 22]; (iv) they may promote lipid droplet aggregation thereby changing the amount of lipid surface exposed to lipase[23, 24]; (v) they may inactive digestive enzymes[25-27]; (vi) they may alter the microbial population within the large intestine[28]. The ability of dietary fibers to impact lipid digestion through these and other mechanisms ultimately depends on their molecular and

78 physicochemical properties[29]. At present, there is a relatively poor understanding of the
79 relationship between dietary fiber structure and their impact on the lipid digestion process.

80 In the present study, we used a simulated gastrointestinal tract (GIT) model to study
81 the influence of anionic (pectin), cationic (chitosan), and non-ionic (methyl cellulose)
82 polysaccharides on the potential gastrointestinal fate of emulsified lipid droplets. These
83 three polysaccharides were selected because of their different charge characteristics, and
84 because they can be used as functional ingredients in food and beverage products. Pectin
85 consists of a group of natural polymers with similar chemical features: linear regions of
86 α(1-4) linked galacturonic acid units separated by branched regions of neutral sugars[30-32].
87 A fraction of the galacturonic acid groups are esterified with methyl groups, which means
88 that the overall molecular charge depends on the ratio of esterified to non-esterified groups,
89 as well as the pH relative to the pK_a of the acid groups ($pK_a \approx 3.5$). Thus, pectin molecules
90 tend to be negatively charged at high pH, but lose their charge at pH values appreciably
91 below pH 3.5. Chitosan is one of the few cationic biopolymers available for application in
92 food products [30-32]. It primarily consists of a linear chain of β(1-4) linked acetyl-
93 glucosamine (uncharged) and glucosamine ($pK_a \approx 6.5$) units. The electrical characteristics
94 of chitosan therefore depend on the ratio of acetylated to non-acetylated groups, as well as
95 the pH relative to the pK_a of the amino groups. Chitosan therefore tends to be positively
96 charged at low pH values, but loses its charge when the pH is raised above 6.5. Methyl
97 cellulose consists of a linear backbone of β(1-4) linked glucose units, with methyl groups
98 attached to a fraction of the glucose units, and is therefore neutral[30-32]. We hypothesized
99 that these three polysaccharides would have different effects on lipid digestion due to their
100 different molecular and physicochemical characteristics. In particular, we focused on their
101 influence on the rheology of gastrointestinal fluids, the aggregation stability of lipid
102 droplets in different stages of the GIT, the rate and extent of lipid digestion, and their
103 interactions with other charged molecular species involved in lipid digestion.

104 The aim of the study was to obtain a better understanding of the role of dietary fiber
105 characteristics on the gastrointestinal fate of ingested lipids. The knowledge gained from
106 this study might be useful for the fabrication of healthier functional food products designed
107 to promote health and wellness[5, 33].

2. Materials and methods

2.1. Chemicals

Corn oil was purchased from a commercial food supplier (Mazola, ACH Food Companies Inc., Memphis, TN) and stored at 4 °C until use. The manufacturer reported that the corn oil contained approximately 14, 29, and 57% (w/w) of saturated, monounsaturated, and polyunsaturated fatty acids, respectively. Tween 80 (Sigma-Aldrich Chemical Company, St Louis, MO) was used as a model food-grade non-ionic surfactant to prepare the oil-in-water emulsions used in this study. Powdered methyl cellulose (M0262, 41 kDa molecular weight, 27.5-31.5% methylation, viscosity of 2% (w/w) aqueous solution, η=400 cps), and powdered chitosan (448877, medium molecular weight (190-310 kDa), 75-85% deacetylation, viscosity of 1% (w/w) solution in 1% (w/w) acetic acid, η=200-800 cps) were purchased from Sigma-Aldrich Chemical Company (St Louis, MO). Commercial powdered high methoxyl pectin (Genu Pectin (Citrus), USP/100) was kindly donated by CP Kelco (Lille Skensved, Denmark) and was used without further purification. The composition of this material as provided by the manufacturer was 6.9% moisture, 89.0% galacturonic acid, and 8.6% methoxyl groups, which corresponds to a degree of esterification of approximately 62%. The average molecular weight was reported by the manufacturer as 200 kDa. Fat soluble fluorescent dye Nile Red (N3013), lipase from porcine pancreas (Type II, L3126, triacylglycerol hydrolase E.C. 3.1.1.3), bile extract (porcine, B8631), mucin from porcine stomach (Type II, M2378, bound sialic acid ≤ 1.2%), and pepsin A from porcine gastric mucosa (P7000, endopeptidase E.C. 3.4.23.1, activity ≥ 250 units/mg solid) were purchased from Sigma-Aldrich Chemical Company (St Louis, MO). The supplier has reported that lipase activity is 100-400 units/mg protein (using olive oil) and 30-90 units/mg protein (using triacetin) for 30 min incubation (one unit of lipase activity was defined as the amount of enzyme required for the release of 1 μeq of fatty acid from either triacetin (pH 7.4) or olive oil (pH 7.7) in 1 h at 37 °C). The composition of the bile extract has been reported as 49% (w/w) total bile salt (BS), containing 10-15% glycodeoxycholic acid, 3-9% taurodeoxycholic acid, 0.5-7% deoxycholic acid, 1-5% hydrodeoxycholic acid, and 0.5-2% cholic acid; 5% (w/w) phosphatidyl choline (PC); Ca^{2+} ≤ 0.06% (w/w); critical micelle concentration of bile extract 0.07 ± 0.04 mM; and mole

ratio of BS to PC being around 15:1. All other chemicals were purchased from Sigma-Aldrich Chemical Company (St Louis, MO). Double distilled water was used to make all solutions.

2.2. Solution and emulsion preparation

2.2.1. Polysaccharide stock solutions preparation

Pectin, chitosan, and methyl cellulose stock solutions (4% w/w) were prepared by dispersing 10 g of powdered pectin, chitosan, or methyl cellulose into 240 g of 5 mM phosphate buffer (pH 7) for pectin and methyl cellulose, and 5 mM acetate buffer (pH 4) for chitosan. The solutions were then stirred at 800 rpm for 12 h (overnight) at room temperature to ensure complete dispersion and dissolution. Pectin, chitosan, and methyl cellulose stock solutions were finally adjusted to pH 7 using 1 N sodium hydroxide and hydrochloric acid solutions, and then equilibrated for 10 min before use.

2.2.2. Stock emulsion preparation

A stock emulsion was prepared by mixing 20% (w/w) corn oil and 80% (w/w) buffered emulsifier solution (5 mM phosphate buffer pH 7.0, containing 2.5% (w/w) Tween 80) together for 5 min using a bio-homogenizer (Speed 2, Model MW140/2009-5, Biospec Products Inc., ESGC, Switzerland). The coarse emulsion obtained was then passed 5 times through a high-pressure homogenizer (Microfluidizer M-110L processor, Microfluidics Inc., Newton, MA) operating at 11,000 psi (75.8 MPa).

2.2.3. Polysaccharide-emulsion mixture preparation

Polysaccharide-emulsion mixtures were prepared by mixing the stock emulsion (containing 20% (w/w) corn oil and 2% (w/w) Tween 80) with buffered stock solutions of 4% (w/w) chitosan (cationic), methyl cellulose (non-ionic), or pectin (anionic), to obtain systems of varying composition: 2% (w/w) corn oil, 0.2% (w/w) Tween 80, and 0.2-3.6% (w/w) polysaccharide (corresponding to mass ratio polysaccharide to corn oil ranging from 0.1 to 1.8). The emulsion-polysaccharide mixtures were then stirred with a high-speed stirrer (Fisher Steadfast Stirrer, Model SL 1200, Fisher Scientific, Pittsburgh, PA) at 1000 rpm and stored overnight at room temperature. Analysis of the emulsion-polysaccharide

mixtures was then carried out before (initial) and after subjection to the different stages of the *in vitro* digestion model.

2.3. Static *in vitro* digestion model

Each emulsion sample (initial phase) was passed through a simulated static *in vitro* digestion model that consisted of oral (section 2.3.1), gastric (section 2.3.2), and intestinal (section 2.3.3) phases. Measurements of emulsion microstructure and stability, particle size distribution, particle charge, and viscosity were performed after each phase (section 2.4). The standardized static *in vitro* digestion model used in this study was a modification of those described previously[13, 34].

2.3.1. Oral phase

Simulated saliva fluid (SSF, pH 6.8) containing 3% (w/w) mucin was prepared according to the composition shown in **Table 1**. The SSF composition was based on those reported in previous studies[35]. Each emulsion (initial phase) was mixed with SSF (ratio 1:1 w/w) and the resulting mixture containing 1% (w/w) corn oil and 0.1-1.8% (w/w) pectin was used for characterization after the incubation period. The oral phase model consisted of a conical flask containing emulsion-SSF mixture incubated at 37 °C with continuous shaking at 100 rpm for 10 min in a temperature controlled air incubator (Excella E24 Incubator Shaker, New Brunswick Scientific, NJ, USA) to mimic the conditions in the mouth. The resulting oral phase (bolus) was used in the gastric phase (section 2.3.2).

2.3.2. Gastric phase

Simulated gastric fluid (SGF) was prepared by adding 2 g NaCl, 7 mL concentrated HCl (37% w/w), and 3.2 g pepsin A (from porcine gastric mucose, 250 units/mg) to a flask and then diluting with double distilled water to a volume of 1 L, and finally adjusting to pH 1.2 using 1 M HCl. Samples taken from the oral phase (bolus) were mixed with SGF (ratio 1:1 w/w) so that the final mixture contained 12 mM NaCl, 0.16% (w/w) pepsin A (corresponding to an enzymatic activity of 400 units/mL), 0.5% (w/w) corn oil, and 0.05-0.9% (w/w) pectin. This mixture was then adjusted to pH 2.5 using 1 M NaOH and incubated at 37 °C with continuous shaking at 100 rpm for 2 h (this time represents the half

194 emptying of a moderately nutritious and semi-solid meal[23]). Since lipase activity is
195 markedly lower in the gastric compartment compared to that in the duodenal tract, the
196 addition of gastric lipase in this phase can be omitted[24]. Samples were taken for
197 characterization at the end of the incubation period (gastric phase). The resulting gastric
198 phase (chyme) was used in the intestinal phase (section 2.3.3).

199 **2.3.3. Intestinal phase**

200 Samples obtained from the gastric phase (20 mL chyme containing 0.5% (w/w) corn
201 oil and 0.05-0.9% (w/w) pectin) were incubated for 2 h at 37 °C in a simulated small
202 intestine fluid (SIF) containing 2.5 mL pancreatic lipase (24 mg/mL), 3.5 mL bile extract
203 solution (54 mg/mL), and 1.5 mL salt solution containing 0.25 M $CaCl_2$ and 3 M NaCl, to
204 obtain a final composition of the intestinal fluid in the reaction vessel of 0.36% (w/w) corn
205 oil, 0.05-0.65% (w/w) pectin, 2 mg/mL pancreatic lipase (corresponding to an enzymatic
206 activity of 550 units/mL), 7 mg/mL bile extract, 15 mM $CaCl_2$, and 150 mM NaCl. The
207 free fatty acids (FFA) released were monitored by determining the amount of 0.1 M NaOH
208 needed to maintain a constant pH 7.0 within the reaction vessel using an automatic titration
209 unit (pH stat titrator, 835 Titrando, Metrohm USA, Inc.). All additives were dissolved in
210 phosphate buffer solution (5 mM, pH 7.0) before use. Lipase addition and initialization of
211 the titration program were carried out only after the addition of all pre-dissolved ingredients
212 and balancing the pH to 7.0. Samples were taken for physicochemical and structural
213 characterization at the end of the digestion period (intestinal phase). The volume of 0.1 M
214 NaOH added to the emulsion was recorded over time and then was used to calculate the
215 concentration of FFA generated by lipolysis. The amount of FFA (% w/w) released was
216 calculated using the following equation:

217

218 $$\text{FFA (\%w/w)} = 100 \times \left(\frac{V_{NaOH}(L) \times C_{NaOH}(M) \times MW_{Lipid}(g/mol)}{2 \times w_{Lipid}(g)} \right) \quad (1)$$

219

220 Where, C_{NaOH} is the concentration of the sodium hydroxide (0.1 M), MW_{Lipid} is the
221 average molecular weight of corn oil (872 g/mol), W_{Lipid} is the initial weight of corn oil in
222 the intestinal phase (0.1 g), and V_{NaOH} is the volume of NaOH (L) titrated into the reaction

vessel to neutralize the FFA released, assuming that all triacylglycerols (TAG) are hydrolyzed in two molecules of FFA and one molecule of monoacylglycerol (MAG). Titration blanks were performed by inactivating lipase in boiling water for 15 min prior to initialization of the titration program.

2.4. Emulsion characterization

2.4.1. Gravitational separation

Ten milliliters of samples were transferred into glass test tube, sealed with a plastic cap, and then stored at room temperature for 24 h. Digital photographs of the samples were taken after storage to record their stability to phase separation and gravitational separation.

2.4.2. Emulsion microstructure

The microstructure of the emulsions was characterized by confocal microscopy. An optical microscopy (C1 Digital Eclipse, Nikon, Tokyo, Japan) with a 60x objective lens was used to capture images of the emulsions. Emulsions were gently stirred to form a homogeneous mixture without introducing air bubbles. A small aliquot of the emulsions (6 μL) was then transferred to a glass microscope slide and covered with a glass cover slip. The cover slip was fixed to the slide using nail polish to avoid evaporation. A small amount of immersion oil (Type A, Nikon, Melville, NY) was placed on the top of cover slip. Emulsions samples were stained with fat soluble fluorescent dye Nile Red (0.1% (w/w) dissolved in 100% (w/w) ethanol) to visualize the location of the oil phase. All confocal images were taken using an excitation (543 nm) argon laser and emitted light was collected between 555-620 nm, and then characterized using the instrument software (EZ CS1 version 3.8, Niko, Melville, NY).

2.4.3. Apparent viscosity measurements

The apparent viscosity of samples was measured using a dynamic shear rheometer (Kinexus Rotational Rheometer, Malvern Instruments Ltd., Worcestershire, UK). A cup and bob geometry consisting of a rotating inner cylinder (diameter 25 mm) and a static outer cylinder (diameter 27.5 mm) was used. The samples were loaded into the rheometer measurement cell and allowed to equilibrate at 37 °C for 5 min before the beginning all

experiments. Samples underwent a constant shear treatment (10 s^{-1} for 10 min) prior to analysis to standardize the shear rate of each sample. The apparent viscosity (η) was then obtained from measurements with a shear rate of 10 s^{-1}.

2.4.4. Particle size distribution measurements

The emulsions were diluted to a droplet concentration of approximately 0.005% (w/w) using buffer solution at the appropriate pH prior to analysis to avoid multiple scatterings effects. The particle size distribution of emulsions was then measured using a static light scattering instrument (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). A refractive index ratio of 1.47 (corn oil) was used in the calculations of the particle size distribution. Background corrections and system alignment were performed prior to each measurement when the measurement cell was filled with the appropriate buffer solution. Particle sizes were reported as particle size distribution profiles (volume fraction (%) vs. particle diameter (μm)) for a mass ratio polysaccharide:corn oil of 1.8.

2.4.5. Particle electrical charge measurements

The electrical charge (ζ-potential) of emulsions was determined using a particle electrophoresis instrument (Zetasizer NanoSeries, Malvern Instruments Ltd., Worcestershire, UK). The emulsions were diluted to a droplet concentration of approximately 0.005% (w/w) using buffer solution at the appropriate pH prior to analysis. Diluted emulsions were injected into the measurement chamber, equilibrated for 120 s and then the ζ-potential was determined by measuring the direction and velocity that the droplets moved in the applied electric field. Each ζ-potential measurement was calculated from the average of 20 continuous readings made per sample. To determine the effect of pH on the ζ-potential of the polysaccharides (0.5% w/w), a titration between pH 2-8 was performed with an automatic titration unit (Multi Purpose Titrator MPT-2, Malvern Instruments Ltd., Worcestershire, UK) and 0.25 M NaOH. The ζ-potential was recorded at each pH after 60 s equilibrium.

2.5. Data analysis

All measurements were performed at least three times using freshly prepared samples. Averages and standard deviations were calculated from these triplet measurements.

3. Results and discussion

3.1. Electrical properties of dietary fibers

Initially, we measured the ζ-potential *versus* pH profiles of the three polysaccharides used in this study to characterize their electrical properties (**Fig. 1**). The ζ-potential of the chitosan went from highly positive at pH 2 to close to zero at pH 8, which can be attributed to the presence of cationic amino groups ($-NH_3^+ \leftrightarrows -NH_2 + H^+$) with a pK_a value around pH 6.5 along the polymer backbone[36]. The ζ-potential of the methyl cellulose was close to zero across the entire pH range due to the fact that it is a neutral polymer with no charged groups. The ζ-potential of the pectin went from close to zero at pH 2 to highly negative at pH 8, which can be attributed to the presence of anionic carboxyl groups ($-COOH \leftrightarrows -COO^- + H^+$) with a pK_a value around pH 3.5[37]. Visual observations of the samples indicated that they remained transparent across the entire pH range studied, suggesting that self-association, precipitation, and sedimentation did not occur.

3.2. Influence of dietary fibers on physicochemical properties of lipid droplets in simulated gastrointestinal tract (GIT)

In this series of experiments, we examined the influence of the three polysaccharides on the physicochemical and structural properties of lipid droplets as they passed through a simulated GIT. Different types and amounts of dietary fiber were mixed with stock emulsions, and then the properties of the resulting mixtures were characterized as they were passed through the simulated mouth, stomach, and small intestine stages. The particle size distribution, microstructure, charge, and stability of the samples were measured after each stage of the GIT model.

3.2.1. Initial Samples

The particle size distribution measured by static light scattering (SLS) indicated that all of the initial emulsions contained relatively small droplets (d_{32} < 250 nm), with a monomodal distribution that had a peak around 310 nm (**Fig. 2a**). On the other hand, the confocal microscopy images suggested that there were very large flocs present in the emulsions containing methyl cellulose and pectin, and some small flocs in the emulsions containing chitosan (**Fig. 3a**). The fact that droplet flocculation was not evident in the light scattering data, but was in the microscopy images, can be attributed to the fact that the emulsions were highly diluted prior to SLS measurements, which will breakdown any weakly flocculated droplets[38].

One would not expect an electrostatic attraction between anionic or neutral polymers and oil droplets stabilized by a non-ionic surfactant. We therefore attribute the extensive droplet flocculation observed in the emulsions containing methyl cellulose or pectin to a depletion effect[39], *i.e.,* the generation of an osmotic attraction between the droplets due to the exclusion of non-adsorbed polymers from the droplet surfaces[38, 40]. Conversely, the small amount of flocculation observed in the emulsions containing chitosan may be attributed to either a depletion or bridging effect[39]. Measurement of the ζ-potential of the Tween 80-stabilized oil droplets indicated that they had a slight negative charge (-6 mV) at neutral pH, which may have been due to the presence of anionic impurities (such as fatty acids) in the oil or surfactant, or due to preferential adsorption of hydroxyl ions (rather than hydronium ions) from water by the lipid droplet surfaces[41]. Thus, there may have been a weak electrostatic attraction between the anionic lipid droplets and cationic chitosan molecules initially leading to some bridging flocculation[42]. In addition, any non-adsorbed chitosan molecules may have promoted depletion flocculation[42, 43]. However, the fact that much less flocculation occurred within the sample containing chitosan suggests that neither depletion nor bridging effects were particularly strong[39]. Bridging flocculation may have been limited due to the relatively weak electrostatic interactions at this pH[42], whereas depletion flocculation may have been limited because of the relatively low molecular weight of the chitosan used[36, 43, 44].

Measurements of the creaming stability of the initial emulsions in the presence of the different polysaccharides also supported the observation that flocculation occurred in some of the samples (**Fig. 4a**). In the absence of dietary fiber, the emulsions appeared homogenous after storage and could therefore be considered to be stable to creaming. The initial emulsions containing chitosan were stable to creaming at all dietary fiber concentrations studied, which suggests that extensive droplet flocculation did not occur. The stability of the chitosan emulsions could be attributed to a number of factors: (a) a weak electrostatic attraction due to the low droplet charge; (b) a weak depletion attraction due to the low molecular weight of the chitosan molecules; (c) an increase in aqueous phase viscosity (**Fig. 5a**). The emulsions containing methyl cellulose and pectin were stable to creaming at low levels (0.4%), but highly susceptible to creaming at higher levels (**Fig. 4a**). At low levels of these polysaccharides, the depletion attraction is not strong enough to overcome the steric and/or electrostatic repulsion between the oil droplets and therefore flocculation does not occur. At higher polysaccharide levels, the depletion attraction is strong enough to promote flocculation and therefore rapid creaming occurs because of the resulting increase in particle size[38]. The height of the cream layer increases at high polysaccharide levels because of the formation of a three-dimensional network of strongly aggregated droplets that inhibits their movement[35]. Viscosity measurements of the samples containing high levels of the polysaccharides indicated that they were relatively viscous, and could therefore inhibit particle movement[45] (**Fig. 5a**).

Measurements of the electrical charge in the emulsion-polysaccharide systems showed that there was little change in the ζ-potential when methyl cellulose or chitosan was added, but that there was an appreciable increase in the negative charge when pectin was added (**Fig. 6a**). These results suggest that methyl cellulose and chitosan did not strongly interact with the lipid droplets, which can be attributed to the relatively low charge of the droplets and polysaccharides at this pH. The large increase in negative charge that occurred when pectin was added can probably be attributed to the fact that the micro-electrophoresis instrument measured the electrical characteristics of the pectin molecules rather than those of the lipid droplets[46].

3.2.2. Oral Phase

The emulsion samples were then subjected to a simulated oral phase, and their physicochemical and structural properties were measured. The particle size distribution measured by SLS indicated that the majority of lipid droplets in all of the emulsions remained relatively small, but that there was a population of highly aggregated lipid droplets (**Fig. 2b**). The confocal microscopy images confirmed that large flocs were present in all of the emulsions containing polysaccharides, but that there were also some smaller flocs in the control emulsion containing no dietary fiber (**Fig. 3b**). Visual observations indicated that all the emulsions were highly unstable to gravitational separation: after storage they all had a thin white layer of fat droplets at the top and a watery serum layer at the bottom (**Fig. 4b**). These results suggest that the conditions in the oral phase promoted extensive droplet flocculation in all of the emulsions. In the control emulsion, droplet aggregation can be attributed to depletion flocculation induced by the presence of the mucin molecules in the simulated oral fluids[47]. In the other emulsions, droplet flocculation may have been a result of depletion and bridging flocculation caused by the mucin and dietary fiber molecules[38, 47]. The presence of mucin would have increased the osmotic attraction between the fat droplets due to the presence of non-adsorbed polysaccharides in the aqueous phase. In addition, there may have been some electrostatic attraction between anionic groups on the mucin and cationic groups on chitosan in the emulsions containing this dietary fiber[48].

Similar to the initial samples (Section 3.2.1), measurements of the electrical charge characteristics of the emulsion-polysaccharide systems showed that there was little change in the ζ-potential when methyl cellulose or chitosan was added, but that there was a large increase in negative charge when pectin was added (**Fig. 6b**). Again, these results suggest that methyl cellulose and chitosan did not strongly interact with the lipid droplets under oral conditions, which can be attributed to the relatively low charge of the lipid droplets (-12 mV) and these two polysaccharides (**Fig. 1**) at this pH. The large increase in negative charge that was observed when pectin was added to the emulsions can again be attributed to the fact that the micro-electrophoresis instrument was more sensitive to the pectin molecules than the lipid droplets[49].

Shear viscosity measurements indicated that all of the samples containing polysaccharides were relatively viscous after exposure to oral conditions (**Fig. 5b**, η>1 Pa s). The increase in viscosity in the presence of the polysaccharides depended on dietary fiber type: methyl cellulose > chitosan > pectin. These differences can be attributed to differences in the molecular characteristics of the dietary fibers, such as molecular weight, conformation, and self-association. In general, the apparent viscosity of a polymer solution increases with increasing molecular weight, decreasing branching, and increasing self-association[50].

3.2.3. Gastric Phase

After passage through the oral phase, the samples were subjected to a simulated gastric phase, and again changes in their physicochemical and structural properties were measured. Both the light scattering and confocal microscopy measurements indicated that extensive droplet aggregation occurred in all of the systems (**Fig. 2c and Fig. 3c**). The irregular shape of the particles observed in the confocal microscopy images suggested that the droplets were flocculated, rather than coalesced under gastric conditions. Visual observations indicated that all the control and chitosan emulsions were relatively stable to gravitational separation: after storage they had a fairly uniform cloudy appearance throughout (**Fig. 4c**). On the other hand, the emulsions containing methyl cellulose or pectin had white sediments at the bottom of the test tubes after exposure to the gastric phase (**Fig. 4c**). The amount of sediment present in these samples increased as the polysaccharide concentration increased. These results suggest that the flocs formed by these two polysaccharides in the simulated gastric fluids were large enough and dense enough to rapidly sediment. On the other hand, the flocs formed in the control and chitosan emulsions did not appear to be strongly susceptible to gravitational separation, perhaps because of their smaller size or lower density contrast[38]. Pectin molecules may also have self-associated and sedimented due to the reduction in their negative charge under highly acidic gastric conditions.

Electrical charge measurements of the emulsion-polysaccharide systems under gastric conditions showed that there was little change in ζ-potential when methyl cellulose or pectin was added, but that there was a large increase in positive charge when chitosan

was added (**Fig. 6c**). These results suggest that methyl cellulose and pectin did not strongly interact with the lipid droplets through electrostatic interactions under gastric conditions, which can be attributed to the relatively low charge of the fat droplets (-1 mV) and these polysaccharides (**Fig. 1**) at pH 3. The large increase in positive charge that occurred when chitosan was added to the emulsions can be attributed to the fact that the chitosan molecules became strongly cationic under acidic conditions (**Fig. 1**). The measured positive charge may therefore have been indicative of interactions between the lipid droplets and chitosan[36], or due to the fact that the micro-electrophoresis instrument was more sensitive to the chitosan molecules than the lipid droplets.

The viscosity of all the emulsions was relatively low under simulated gastric conditions, which can be attributed to the fact that the samples were diluted at each stage of the gastrointestinal tract model so the polymer concentration would be relatively low, *i.e.,* below the polymer overlap region[21].

3.2.4. Intestinal Phase

After passage through the gastric phase, the samples were subjected to a simulated small intestine phase, and changes in their physicochemical and structural properties were again measured. Light scattering and confocal microscopy measurements suggested that extensive droplet aggregation occurred in all of the systems, but that there were distinct differences between their microstructures (**Figs. 2d and 3d**). The fat phase was fairly evenly distributed throughout the sample in the control emulsion containing no polysaccharide (**Fig. 3d**) and many small particles were detected by SLS (**Fig. 2d**). Presumably, the majority of these particles were "mixed micelles" formed by the lipid digestion process[23, 24, 34]. Mixed micelles consist of small (<10 nm) micelle-like structures, as well as much larger (50 – 5000 nm) liposome-like structures[51]. They consist of phospholipids and bile salts from the intestinal fluids, as well as free fatty acids and monoacylglycerols resulting from digestion of the triacylglycerols[52, 53]. The mixed emulsions containing chitosan contained some irregular shaped particles, but these were appreciably smaller than those observed in the mixed emulsions containing either pectin or methyl cellulose **(Fig. 3d**). The particles in these systems were probably a mixture of undigested fat droplets and mixed micelles. Visual observations indicated that the control

emulsions and the emulsions containing chitosan had a relatively uniform yellowish brown appearance (**Fig. 4d**). The emulsions containing methyl cellulose or pectin also had a yellowish brown color but there was evidence of some sediment at the bottom of the test tubes after exposure to the intestinal phase (**Fig. 4d**). This is difficult to see in the digitable photographs since both the sediment and liquid above were optically opaque, but it could clearly be observed by eye. The brownish yellow color can be attributed to the presence of bile salts, since the stock solution of these digestive components had a dark brown color.

Electrical charge measurements indicated that the control emulsions had a relatively high negative charge (-35 mV) under simulated intestinal conditions (**Fig. 6d**), which can be attributed to the presence of anionic substances at the particle surfaces, such as free fatty acids, phospholipids, and bile salts. The ζ-potential changed appreciably with increasing polysaccharide concentration, with the direction of the change depending on initial polysaccharide type. The particles became more positive when chitosan was added, more negative when pectin was added, and changed little when methyl cellulose was added (**Fig. 6d**). These results suggest that methyl cellulose did not strongly interact with the fat droplets through electrostatic interactions under intestinal conditions, which can be attributed to its neutral charge characteristics (**Fig. 1**) at pH 7. On the other hand, the increase in positive charge on the particles when chitosan was added to the emulsions may have been due to the fact that cationic chitosan molecules interacted with the anionic lipid particles, and other anionic species such as mixed micelles containing bile salts and free fatty acids. The increase in negative charged when increasing amounts of pectin were added may have been due to binding of pectin to the negative lipid particles, but this is unlikely due to strong electrostatic repulsion between them[21, 25]. Instead, the micro-electrophoresis instrument may have been more sensitive to the pectin molecules than the lipid particles. The viscosity of all the emulsions was relatively low under simulated intestinal conditions (**Fig. 5d**), which can be attributed to the progressive dilution that occurs after passage through each stage of the gastrointestinal model[23, 24, 34].

Finally, we examined the influence of polysaccharide type and concentration on the rate and extent of lipid digestion using a pH stat method (**Fig. 7**). In the absence of polysaccharide, the emulsions were rapidly and completely digested. Indeed, the fat phase

was almost fully digested within the first 5 minutes of incubation. In the presence of polysaccharides, there was a decrease in both the rate and extent of lipid digestion, with the amount depending on the polysaccharide. There was a slight decrease in the total amount of fatty acids produced after 2 hours of digestion with increasing chitosan concentration, but a much more appreciable decrease with increasing pectin or methyl cellulose concentration (**Fig. 8**).

3.3. Potential Mechanisms

Overall, this study has shown that different polysaccharides have different effects on the rate and extent of lipid digestion. In particular, our results suggest that both pectin and methyl cellulose were able to appreciably inhibit lipid digestion. In this section, we examine some potential physicochemical mechanisms that may account for the observed influence of polysaccharides on lipid digestion.

Rheology: The type and amount of polysaccharides present in the initial systems influenced the rheological properties of the fluids in the various stages of the simulated gastrointestinal tract. Changes in the rheology of the gastrointestinal fluids may impact the rate and extent of lipid digestion through a number of mechanisms. At the molecular level, an increase in the micro-viscosity of a sample will slow down the movement of any molecular species involved in the lipid digestion process, *e.g.,* bile salts and lipase towards the droplet surfaces, or free fatty acids and monacylglycerols away from the droplet surfaces. Consequently, dietary fibers could decrease the rate and extent of lipid digestion due to their ability to slow down molecular diffusion. However, it should be stressed that polysaccharides may cause a large increase in the macro-viscosity of a sample, but have little effect on the micro-viscosity since small molecules can easily diffuse through the large pores in polymer networks. An increase in the macro-viscosity associated with the presence of dietary fibers may influence the intimate mixing of the samples with the digestive components, which could also inhibit the ability of lipase to get to the lipid droplet surfaces. In the small intestinal phase, the increase in apparent viscosity due to the presence of the different polysaccharides was relatively modest (**Fig. 5**d), and therefore we do not believe that this mechanism played a major role in influencing lipid digestion.

Flocculation: The presence of polysaccharides within the gastrointestinal fluids may have promoted flocculation of the lipid droplets due to bridging, depletion, or other mechanisms. The ability of lipase to interact with the lipid droplet surfaces and digest the encapsulated triglycerides may be reduced if the droplets are trapped within large flocs (**Fig. 9**). One would expect that the inhibition of lipid digestion would increase as the floc size increased, and as the packing of droplets and polymers within the flocs increased, since these factors would reduce the ability of lipase molecules to rapidly diffuse through the entire flocs. Based on our confocal microscopy and other measurements, this mechanism appears to be important in accounting for the observed inhibition of lipid digestion, since the emulsions containing methyl cellulose and pectin were highly flocculated (**Fig. 3**) and also had reduced digestion rates (**Fig. 8**).

Electrostatic interactions: One would expect cationic chitosan molecules to interact with various anionic species involved in the lipid digestion process, such as lipid droplets, bile salts, phospholipids, free fatty acids, and mixed micelles. These interactions may either inhibit or promote lipid digestion depending on their nature. For example, chitosan may bind free fatty acids produced during triglyceride lipolysis and remove them from the lipid droplet surfaces, thereby allowing the lipase to continue acting on the non-digested triglycerides. On the other hand, if chitosan forms a protective layer around the lipid droplet surfaces, then it may inhibit digestion by preventing the lipase from reaching the non-digested triglycerides within the droplets. One would also expect anionic pectin molecules to interact with any cationic species involved in the lipid digestion process. For example, anionic pectin may strongly bind cationic calcium ions and prevent them from precipitating long-chain fatty acids at the lipid droplet surfaces. As a result, lipid digestion may be inhibited because the formation of a layer of free fatty acids around the lipid droplets can prevent lipase from reaching the non-digested triglycerides. Electrostatic interactions may therefore also play an important role in the ability of certain polysaccharides to inhibit lipid digestion. In future studies, it would be useful to carry out a more detailed study of the interactions of dietary fibers with specific digestive components so as to better understand the potential importance of these interactions.

4. Conclusions

The objective of this work was to study the impact of three polysaccharides (chitosan, methyl cellulose, and pectin) on the physicochemical characteristics and microstructure of emulsified lipids during passage through a simulated gastrointestinal tract. Pectin and methyl cellulose promoted depletion flocculation when present at sufficiently high concentrations, whereas chitosan promoted bridging flocculation under acidic pH conditions. Pectin and methyl cellulose reduced the rate and extent of lipid digestion appreciably, whereas chitosan caused a slight decrease. Our results suggest that droplet flocculation may have restricted the access of lipase to the lipid droplet surfaces, thereby reducing hydrolysis of the emulsified lipids (**Fig. 9**). In addition, electrostatic interactions of polysaccharides with oppositely charged species involved in lipid digestion may also impact digestion. This information may be used for designing functional foods that give healthier lipid profiles and thereby promote health and wellness.

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References

1 R. M. van Dam and J. C. Seidell, *European Journal of Clinical Nutrition*, 2007, **61**, S75-S99.
2 G. A. Bray, S. Paeratakul and B. M. Popkin, *Physiology & Behavior*, 2004, **83**, 549-555.
3 B.-c. Wu, B. Degner and D. J. McClements, *Food Chemistry*, 2013, **141**, 3393-3401.
4 Y. Mao and D. Julian McClements, *Journal of Food Science*, 2012, **77**, E144-E152.
5 C. Chung, B. Degner and D. J. McClements, *Food Hydrocolloids*, 2013, **32**, 263-270.
6 I. Heertje, *Food Structure*, 2014, **1**, 3-23.
7 S. S. Narine and A. G. Marangoni, *Food Research International*, 1999, **32**, 227-248.
8 D. Rousseau, *Food Research International*, 2000, **33**, 3-14.
9 B. S. Ghotra, S. D. Dyal and S. S. Narine, *Food Research International*, 2002, **35**, 1015-1048.
10 S. Ghosh and D. Rousseau, *Current Opinion in Colloid & Interface Science*, 2011, **16**, 421-431.
11 G. Michas, R. Micha and A. Zampelas, *Atherosclerosis*, 2014, **234**, 320-328.
12 D. Kritchevsky, *Annual Review of Nutrition*, 1988, **8**, 301-328.
13 Y. Li, M. Hu and D. J. McClements, *Food Chemistry*, 2011, **126**, 498-505.
14 Y. Li and D. J. McClements, *Food Hydrocolloids*, 2014, **35**, 367-374.
15 M. Galisteo, J. Duarte and A. Zarzuelo, *The Journal of Nutritional Biochemistry*, 2008, **19**, 71-84.
16 P. Gunness and M. J. Gidley, *Food & Function*, 2010, **1**, 149-155.
17 J. L. Slavin, *Nutrition*, 2005, **21**, 411-418.
18 G. Dongowski, *Food Chemistry*, 2007, **104**, 390-397.
19 H. Fabek, S. Messerschmidt, V. Brulport and H. D. Goff, *Food Hydrocolloids*, 2014, **35**, 718-726.
20 M. Kristensen and M. G. Jensen, *Appetite*, 2011, **56**, 65-70.
21 O. K. Simo, Y. Mao, T. Tokle, E. A. Decker and D. J. McClements, *Food Research International*, 2012, **48**, 337-345.
22 T. Tokle, U. Lesmes and D. J. McClements, *Journal of Agricultural and Food Chemistry*, 2010, **58**, 9825-9832.
23 S. J. Hur, B. O. Lim, E. A. Decker and D. J. McClements, *Food Chemistry*, 2011, **125**, 1-12.
24 D. J. McClements and Y. Li, *Food & Function*, 2010, **1**, 32-59.
25 M. Beysseriat, E. A. Decker and D. J. McClements, *Food Hydrocolloids*, 2006, **20**, 800-809.
26 I. A. Brownlee, *Food Hydrocolloids*, 2011, **25**, 238-250.
27 M. Espinal-Ruiz, F. Parada-Alfonso, L.-P. Restrepo-Sánchez and C.-E. Narváez-Cuenca, *Bioactive Carbohydrates and Dietary Fibre*, 2014, **4**, 27-38.
28 M. Castillo, S. M. Martín-Orúe, M. Anguita, J. F. Pérez and J. Gasa, *Livestock Science*, 2007, **109**, 149-152.
29 D. Mudgil and S. Barak, *International Journal of Biological Macromolecules*, 2013, **61**, 1-6.
30 S. W. Cui, *Food Carbohydrates: Chemistry, Physical Properties and Applications*, Taylor and Francis, Boca Raton, FL, 2005.
31 A. Imeson, *Food Stabilizers, Thickeners and Gelling Agents*, John Wiley & Sons, Chichester, UK, 2010.
32 P. A. Williams, in *Handbook of Industrial Water Soluble Polymers*, ed. P. A. Williams, Blackwell Publ., Oxford, UK, 2007, pp. 73-97.
33 R. S. Khan, J. Grigor, R. Winger and A. Win, *Trends in Food Science & Technology*, 2013, **30**, 27-37.
34 M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carriere, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le

Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D. J. McClements, O. Menard, I. Recio, C. N. Santos, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies and A. Brodkorb, *Food & function*, 2014, **5**, 1113-1124.

35 Y. Mao and D. J. McClements, *Food & Function*, 2012, **3**, 1025-1034.

36 F. Yuan, Y. Gao, E. A. Decker and D. J. McClements, *Journal of Food Engineering*, 2013, **114**, 1-7.

37 O. G. Jones, U. Lesmes, P. Dubin and D. J. McClements, *Food Hydrocolloids*, 2010, **24**, 374-383.

38 D. J. McClements, *Food Hydrocolloids*, 2000, **14**, 173-177.

39 K. Furusawa, M. Ueda and T. Nashima, *Colloids and Surfaces, A: Physicochemical and Engineering Aspects*, 1999, **153**, 575-581.

40 P. Jenkins and M. Snowden, *Advances in Colloid and Interface Science*, 1996, **68**, 57-96.

41 C. V. Nikiforidis and V. Kiosseoglou, *Food Hydrocolloids*, 2011, **25**, 1063-1068.

42 S. Biggs, M. Habgood, G. J. Jameson and Y.-d. Yan, *Chemical Engineering Journal*, 2000, **80**, 13-22.

43 F. Renault, B. Sancey, P. M. Badot and G. Crini, *European Polymer Journal*, 2009, **45**, 1337-1348.

44 Y. Li and D. J. McClements, *Food Hydrocolloids*, 2013, **33**, 368-375.

45 T. Kawakatsu, G. Trägårdh and C. Trägårdh, *Journal of Food Engineering*, 2001, **50**, 247-254.

46 R.-Y. Tsai, P.-W. Chen, T.-Y. Kuo, C.-M. Lin, D.-M. Wang, T.-Y. Hsien and H.-J. Hsieh, *Carbohydrate Polymers*, 2014, **101**, 752-759.

47 M. H. Vingerhoeds, T. B. J. Blijdenstein, F. D. Zoet and G. A. van Aken, *Food Hydrocolloids*, 2005, **19**, 915-922.

48 O. Svensson, K. Thuresson and T. Arnebrant, *Journal of Colloid and Interface Science*, 2008, **325**, 346-350.

49 M. Tholstrup Sejersen, T. Salomonsen, R. Ipsen, R. Clark, C. Rolin and S. Balling Engelsen, *International Dairy Journal*, 2007, **17**, 302-307.

50 D. E. Dunstan, E. Chai, M. Lee and D. V. Boger, *Food Hydrocolloids*, 1995, **9**, 225-228.

51 D. J. McClements and Y. Li, *Advances in Colloid and Interface Science*, 2010, **159**, 213-228.

52 M. Almgren, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 2000, **1508**, 146-163.

53 Y. Yang and D. J. McClements, *Journal of Colloid and Interface Science*, 2013, **405**, 312-321.

Figure Captions

Figure 1. Influence of pH on the electrical charge (ζ-potential) of diluted 0.5% (w/w) chitosan (●), methyl cellulose (○), and pectin (■) solutions.

Figure 2. Influence of chitosan (●), methyl cellulose (○), and pectin (■) (mass ratio polysaccharide:corn oil of 1.8) on the particle size distribution of emulsions under simulated gastrointestinal conditions consisting of an initial (a), oral (b), gastric (c), and intestinal (d) phases.

Figure 3. Influence of chitosan, methyl cellulose, and pectin (mass ratio polysaccharide:corn oil of 1.8) on the microstructure of emulsions observed by confocal fluorescence microscopy under simulated gastrointestinal conditions consisting of an initial, oral, gastric, and intestinal phases.

Figure 4. Influence of the concentration (mass ratio polysaccharide (P):corn oil (CO)) of chitosan, methyl cellulose, and pectin on creaming stability of emulsions under simulated gastrointestinal conditions consisting of an initial, oral, gastric, and intestinal phases.

Figure 5. Influence of the concentration of chitosan (●), methyl cellulose (○), and pectin (■) on the apparent viscosity of emulsions under simulated gastrointestinal conditions consisting of an initial (a), oral (b), gastric (c), and intestinal (d) phases.

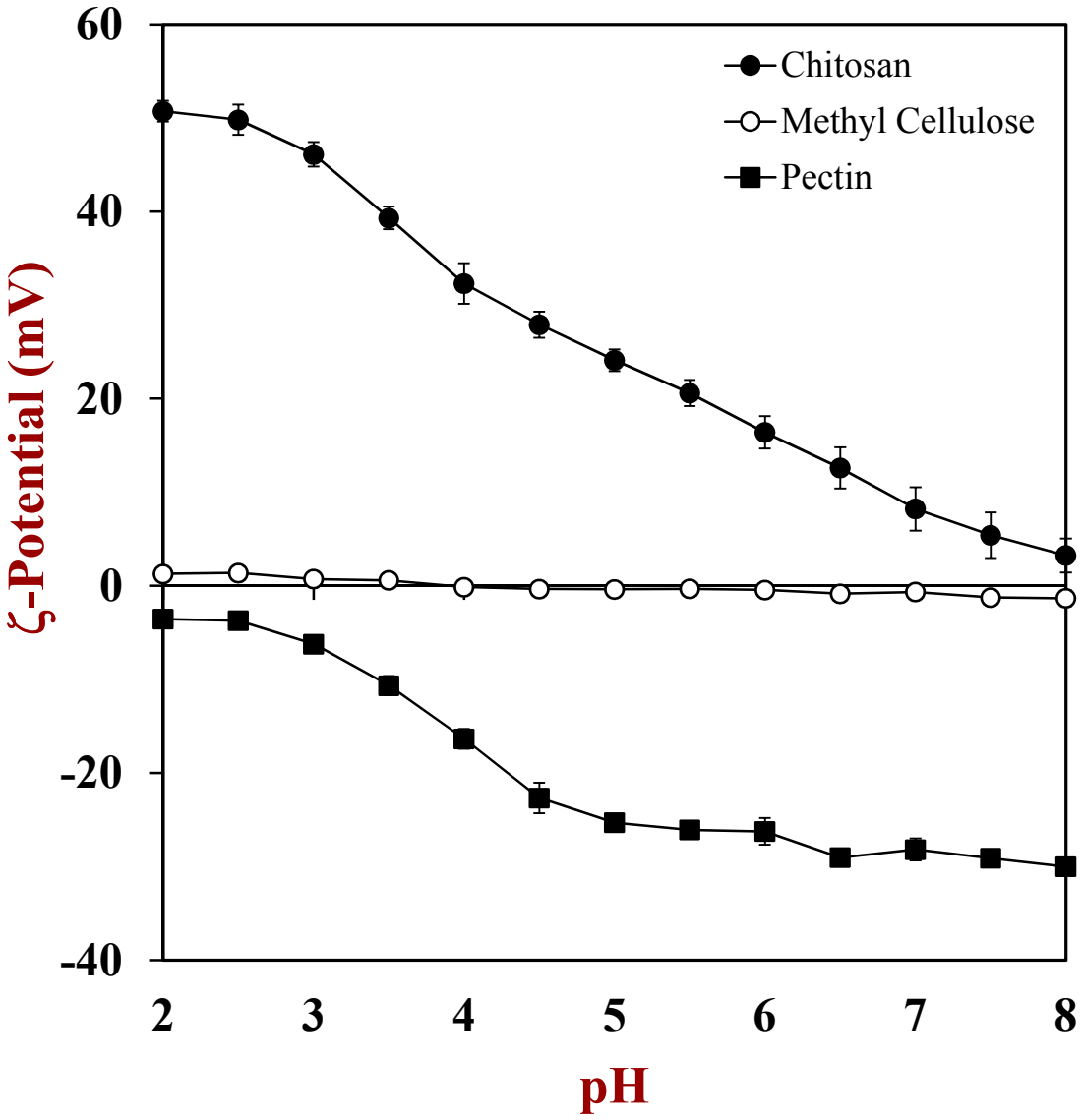
Figure 6. Influence of the concentration of chitosan (●), methyl cellulose (○), and pectin (■) on the electrical charge (ζ-potential) of emulsions under simulated gastrointestinal conditions consisting of an initial (a), oral (b), gastric (c), and intestinal (d) phases.

Figure 7. Influence of the concentration of chitosan (a), methyl cellulose (b), and pectin (c) on *in vitro* hydrolysis (percentage of free fatty acids (FFA) released by pH stat method) of lipid droplets (0.5 % w/w) under simulated gastrointestinal conditions. The concentrations

of polysaccharides in the intestinal phase were 0.00 (●), 0.07 (○), 0.15 (▲), 0.44 (△), and 0.65 % (w/w) (■).

Figure 8. Influence of the concentration of chitosan (●), methyl cellulose (○), and pectin (■) on free fatty acids (FFA) released after 2 hours of digestion (intestinal phase).

Figure 9. Schematic representation of inhibition of lipid droplets digestion rate by polysaccharides. Lipid droplets of o/w Tween 80 stabilized emulsion (a), digestion of lipid droplets by lipase (b), polysaccharides may lead a decrease on the digestion rate of lipid droplets by embedding them into their structure (c).



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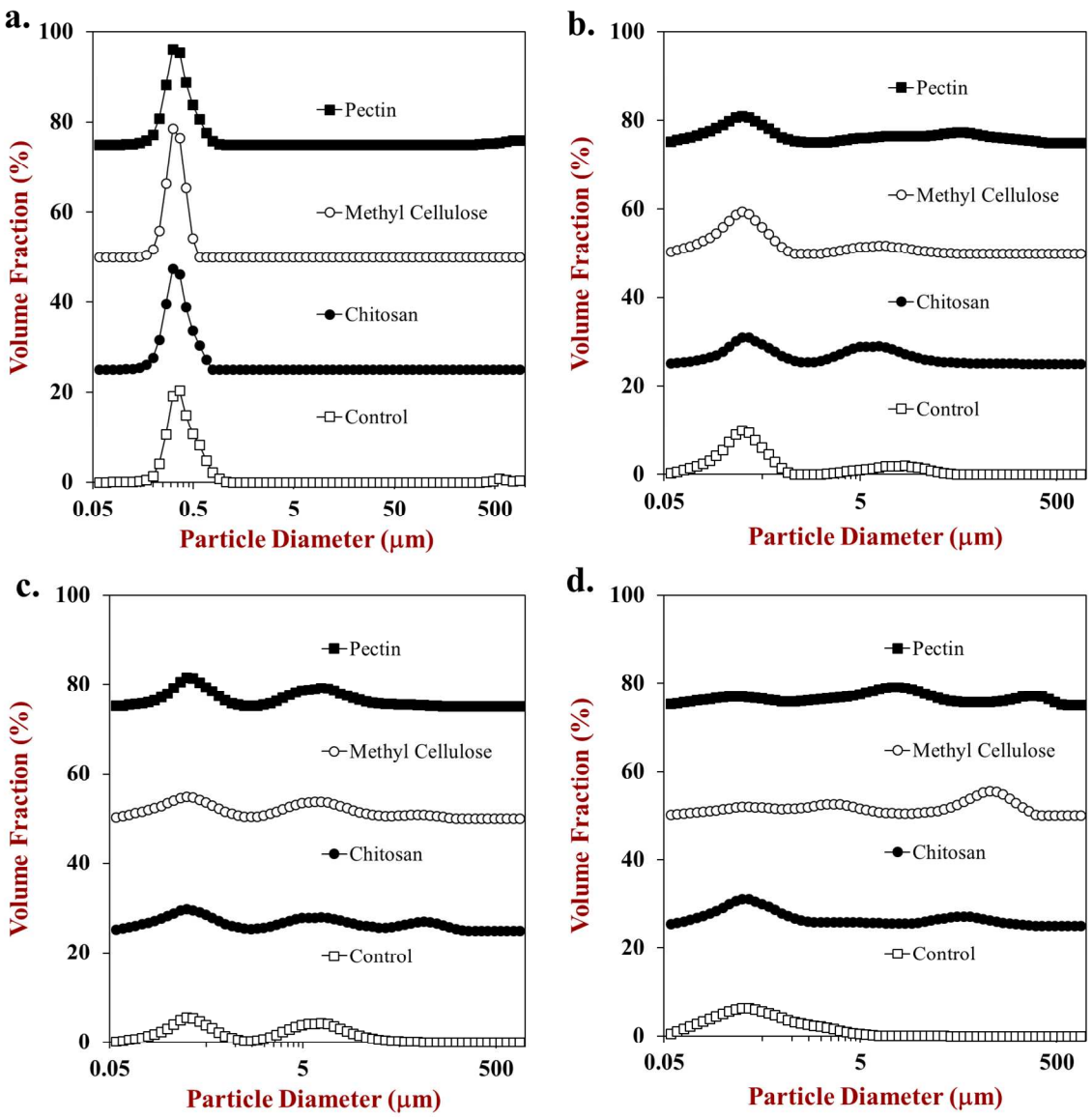

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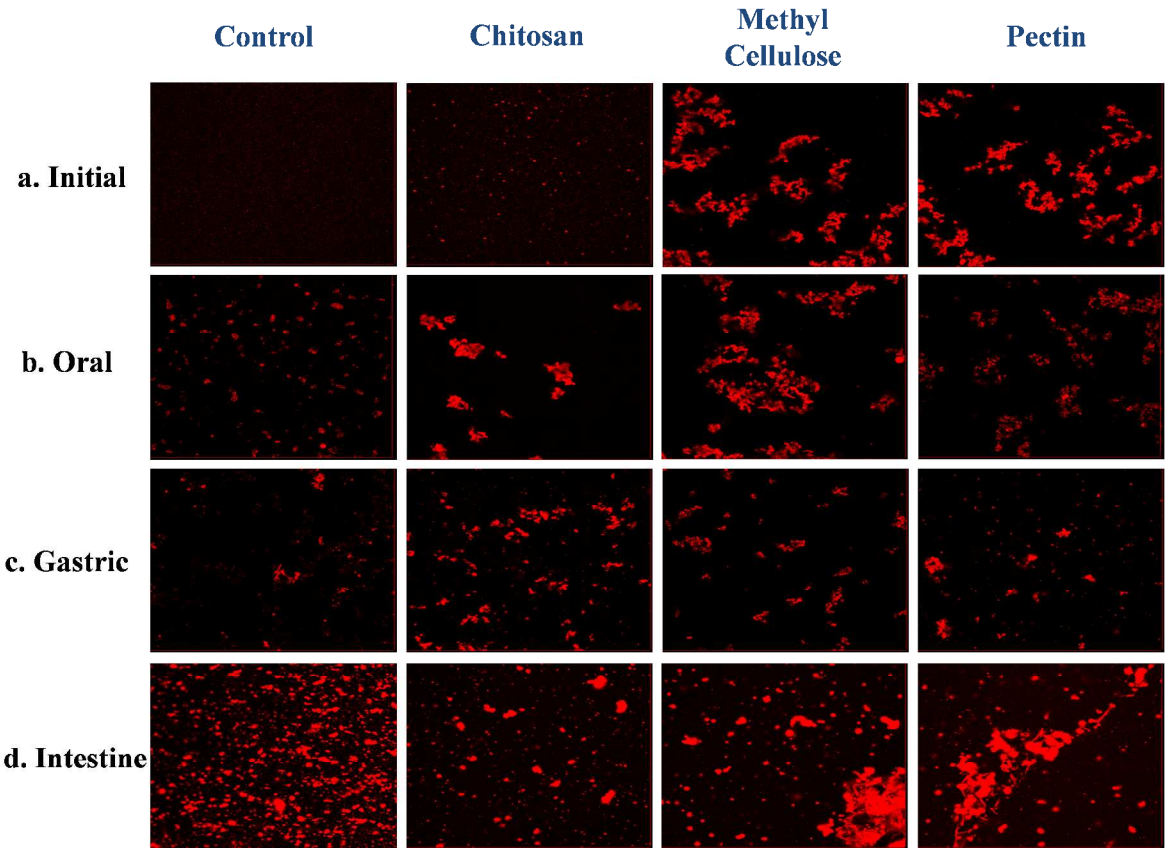

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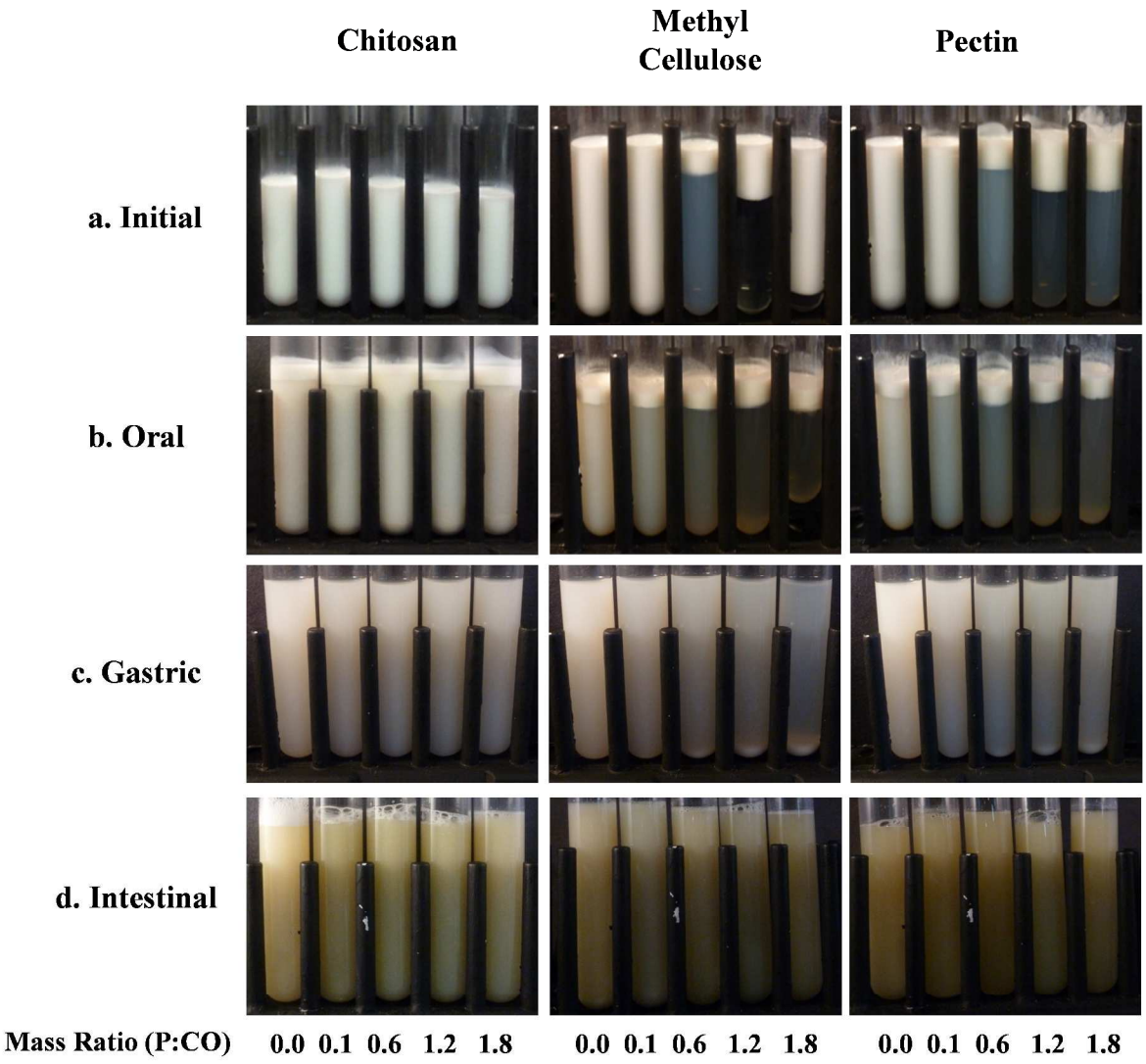

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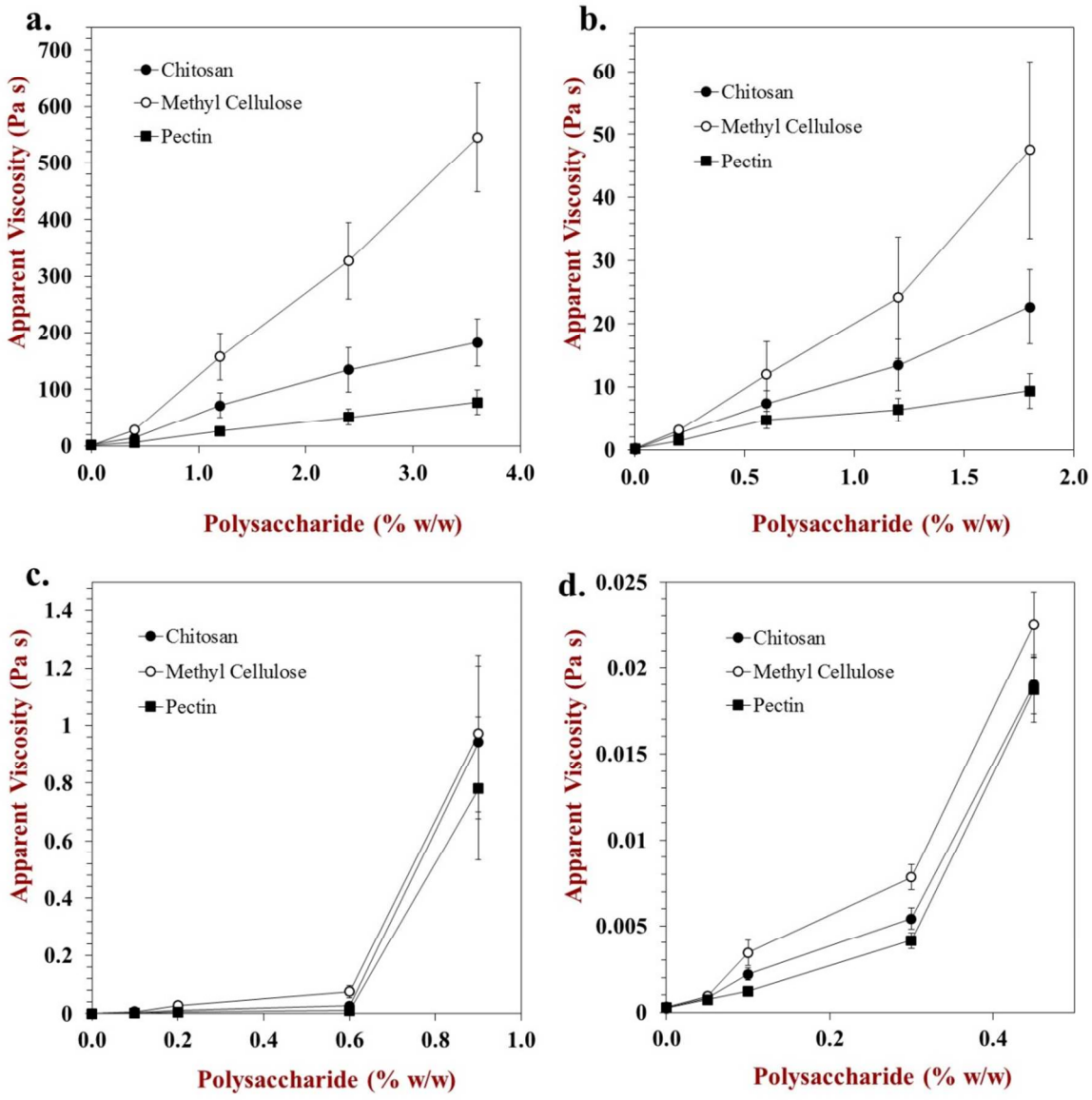

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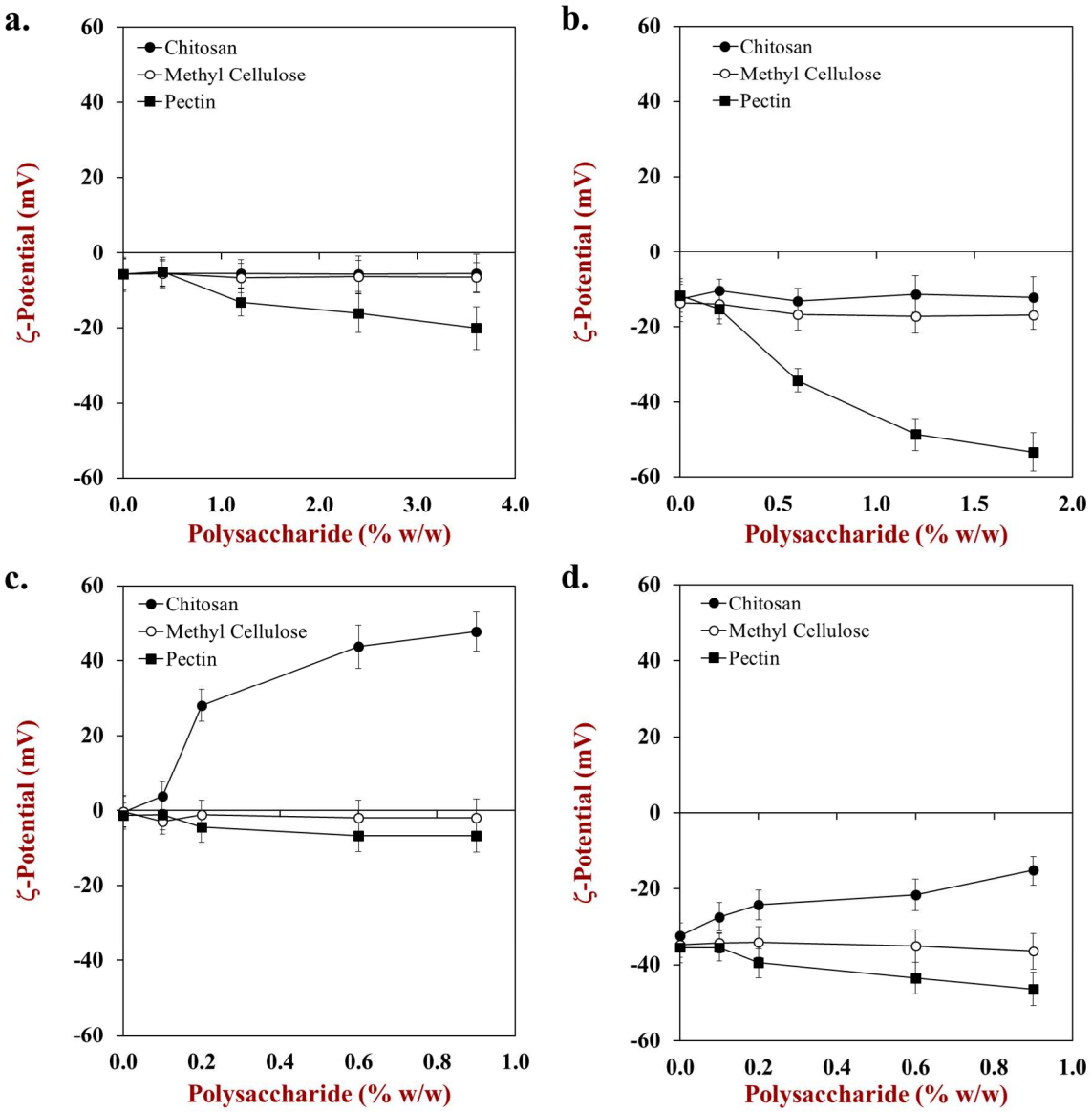

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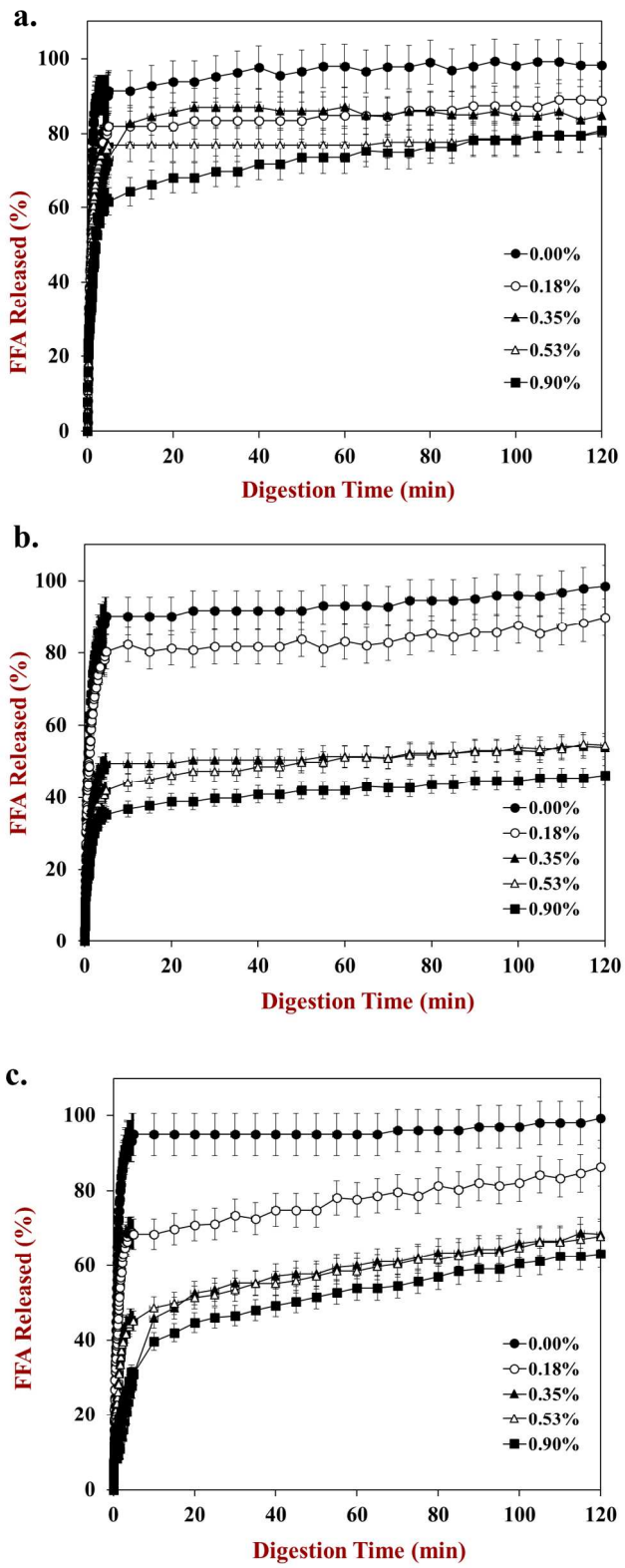

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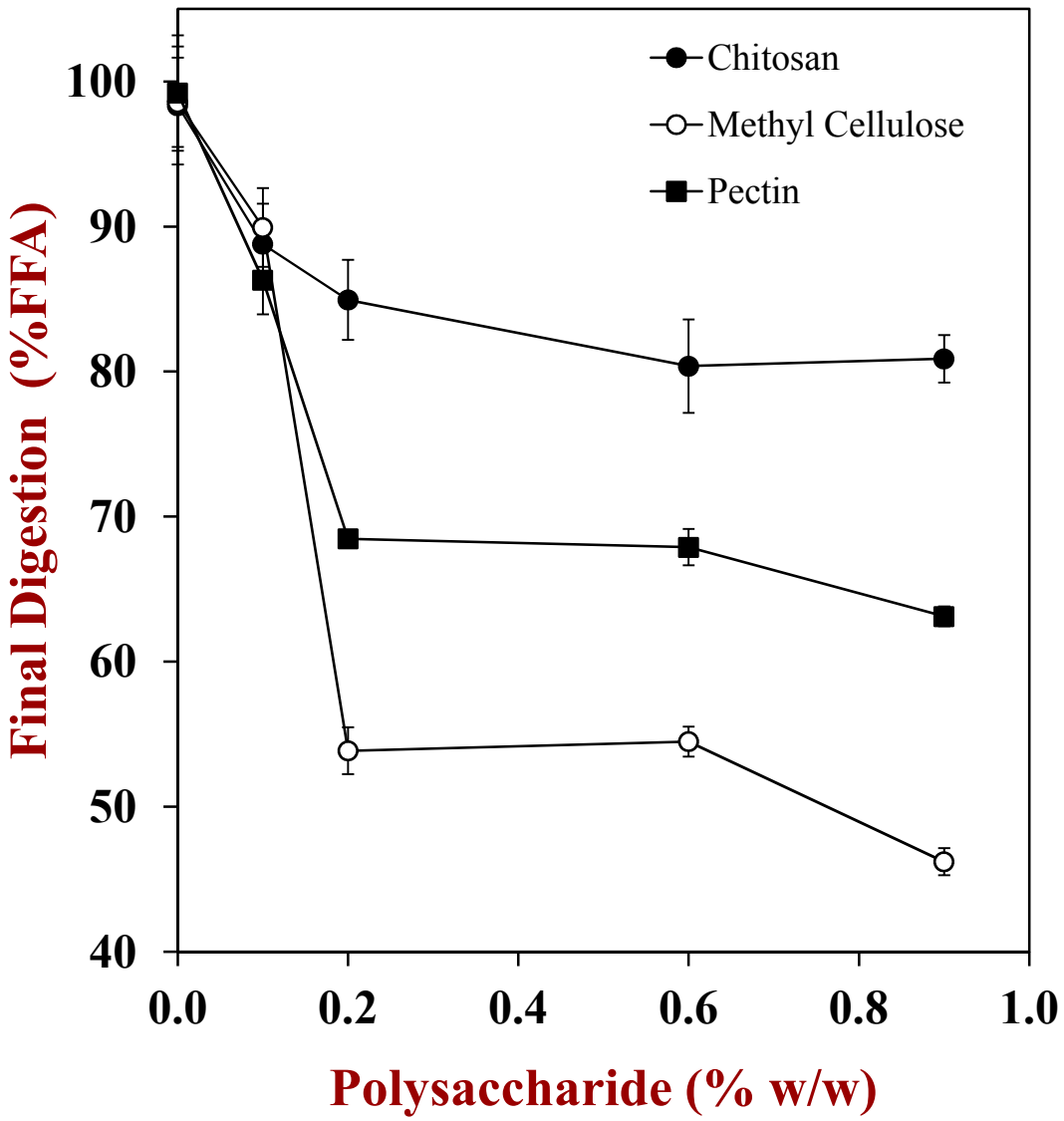

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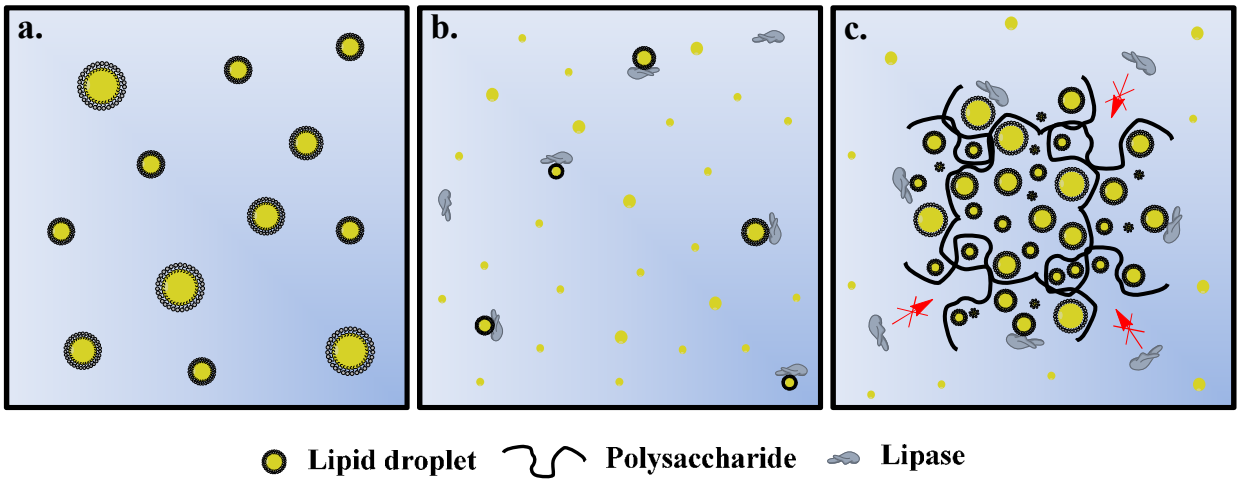

17

18 **Figure 9.**

Tables

Table 1. Chemical composition of simulated saliva fluid (SSF) used to simulate oral conditions.

Compound	Chemical formula	Concentration (g/L)[1]
Sodium chloride	NaCl	1.594
Ammonium nitrate	NH_4NO_3	0.328
Potassium dihydrogen phosphate	KH_2PO_4	0.636
Potassium chloride	KCl	0.202
Potassium citrate	$K_3C_6H_5O_7 \cdot H_2O$	0.308
Uric acid sodium salt	$C_5H_3N_4O_3Na$	0.021
Urea	H_2NCONH_2	0.198
Lactic acid sodium salt	$C_3H_5O_3Na$	0.146
Porcine gastric mucin (Type II)	----	30

[1]The SSF was prepared in double distilled water and then pH 6.8 was adjusted.