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Influence of encapsulation of emulsified lipids with chitosan on their in vivo digestibility

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Abstract

The influence of interfacial composition on the in vivo digestibility of emulsified and encapsulated lipids was investigated. An electrostatic layer-by-layer deposition technique was used to prepare soybean oil-in-water emulsions that contained lipid droplets coated by lecithin or by lecithin–chitosan. Thirty six 4-week-old male mice were divided into four groups and fed treatment diets for 4 weeks; atherogenic diets supplemented with (A) non-emulsified fat, without chitosan (control), (B) non-emulsified fat, with chitosan, (C) emulsified fat, without chitosan, or (D) emulsified fat encapsulated by chitosan. There were no differences in body weights, food intake, major organ weights, or fecal fat contents between all treatment groups, where total fat absorption was >90%. The results suggest that encapsulation of lipids by chitosan does not inhibit their in vivo digestibility, even though previous studies indicate that chitosan does inhibit their in vitro digestibility. Consequently, it should be possible to use chitosan to microencapsulate lipids and lipid-soluble components without compromising their bioavailability, although further human studies are needed to confirm this.

Keywords: Emulsions; Encapsulation; Chitosan; Cholesterol; Bioavailability; Digestibility of lipids

1. Introduction

Dietary fats are usually broken down into oil-in-water emulsions in the mouth, stomach and/or small intestine due to the presence of various surface-active and stabilizing components present in the food or secreted by the body (Van Aken, 2004). In humans, digestion of dietary fat begins in the stomach and final absorption of the lipid digestion products occurs in the small intestine (Armand et al., 1992, 1994, 1999; Brockman, 2000; Mu & Hoy, 2004). The substrate for digestion of dietary fats is therefore usually fat droplets embedded in an aqueous medium, with the surfaces of the droplets being coated by a complex layer of surface-active material that may come from a variety of different sources (Mun, Decker, Park, Weiss, & McClements, 2006).

Fat digestion in the small intestine is due to the hydrolysis of the lipids by pancreatic lipase and also to the hydrolysis by gastric lipase in the stomach. The ability of pancreatic lipase to adsorb to the fat droplet surfaces is increased by the presence of bile salts that are released from the gall bladder or directly from the liver, and by the presence of co-lipase that is released from the pancreas (Brockman, 2000; Chapus, Rovery, Sarda, & Verger, 1988; Labourdenne, Brass, Ivanova, Cagna, & Verger, 1997). Therefore, it has been proposed that the composition, structure and physicochemical properties of the interfacial layer surrounding the fat droplets should play an important role in determining the extent of enzyme binding to the emulsion surface and therefore the extent of lipolysis (Wickham, Garrood, Leney, Wilson, & Fillery-Travis, 1998).

In a previous study, we altered the characteristics of the interfaces surrounding emulsified lipids using an electrostatic layer-by-layer (LbL) deposition method. The thick-

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ness and electrical charge of the interfacial layer was varied by adsorbing a positively charged polysaccharide (chitosan) onto the surfaces of negatively charged oil droplets (coated by lecithin). In an in vitro study, we found that the extent of lipid digestion by pancreatic lipase was much less in an emulsion containing lecithin–chitosan coated droplets than in an emulsion containing lecithin coated droplets (Faldt, Bergenstahl, & Claesson, 1993; Mun et al., 2006; Peniche, Aruelles-Monal, Peniche, & Acosta, 2003). This difference was attributed to the fact that the chitosan layer was positively charged and relatively thick, and should therefore prevent the lipase from coming into close contact with the lipid substrate inside the fat droplets (Mun et al., 2006). The purpose of the current study was to use an in vivo model to examine the influence of interfacial characteristics on the digestion of fat droplets coated either by lecithin or by lecithin–chitosan. We used the same electrostatic layer-by-layer deposition method used in a previous study to create these interfacial layers with different compositions, structures and charges (Ogawa, Decker, & McClements, 2003a, 2004). The lecithin coated droplets are surrounded by a relatively thin negatively charged layer, whereas the lecithin–chitosan coated droplets are surrounded by a relatively thick positively charged layer.

2. Materials and methods

2.1. Materials

Powdered lecithin was obtained from ADM-Lecithin (Decatur, IL). Powdered cellulose and chitosan (medium molecular weight; 75–85% deacetylation; viscosity of 1 wt% solution in 1 wt% acetic acid, viscosity = 200–800 cps) were purchased from the Sigma–Aldrich Chemical Company (St. Louis, MO). Maltodextrin (designated M 150) was donated from Grain Processing Corporation (Muscatine, IA). Acetic acid and diethyl ether (anhydrous) were purchased from Fisher Scientific (Chicago, IL). Soybean oil was purchased from a local grocery store and used without further purification. Basal diet was purchased from Harlan Teklad (Madison, WI). Tritridecanoin (Glyceryl tridecanoate) was purchased from Nu-Chek Prep (Elysian, MN).

2.2. Diets

The basal diet was purchased from Harlan-Teklad (TD 04488, Madison, WI). To satisfy the minimal lipid requirement as well as aid absorption of fat-soluble vitamins, soybean oil (1% of diet) was provided in the basal mix. The additional fats (15.5% of diet) were prepared as described below and combined with basal mix. Treatments were: (A) non-emulsified fat, without chitosan (control), (B) non-emulsified fat, with chitosan, (C) emulsified fat, without chitosan, or (D) emulsified fat encapsulated by chitosan (Table 1).

All treatment diets contained the same amounts of soybean oil (14%), lecithin (1.4%), tritridecanoin (0.1%), maltodextrin, chitosan (or cellulose), and acetic acid in the final diets. Tritridecanoin was added as a marker for fat absorption rate during digestion.

The method used to prepare the emulsified soybean sample was described previously (Klinkesorn, Sophanodora, Chinachoti, Decker, & McClements, 2005a). An oil-in-water (O/W) emulsion, 'the primary emulsion', was prepared by homogenizing 14 wt% soybean oil and 0.1% tritridecanoin with 85 wt% lecithin solution (1.76 wt% lecithin dispersed in acetate buffer, pH 3) in a high-speed blender for 2 min (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) followed by three passes through a two stage high-pressure valve homogenizer: 2500 psi first stage; 500 psi second stage (LAB 1000, APV-Gaulin, Wilmington, MA). This primary emulsion was diluted with aqueous chitosan solution (0.4 wt% chitosan in acetate buffer) to form a secondary emulsion. Any flocs formed in the secondary emulsion were disrupted by passing it once through a two stage high-pressure valve homogenizer: 1500 psi first stage; 500 psi second stage. The electrical charge on the droplets changed from negative (~−42.5 mV) for the primary emulsion to positive (~+55.9 mV) for the secondary emulsion, which indicated that the cationic chitosan molecules adsorbed to the surface of the anionic lecithin-coated emulsion droplets. The primary and secondary emulsions were mixed with maltodextrin solutions, and freeze dried before mixing with basal diet. Treatment diets were refrigerated until use.

Table 1
Diet composition

Basal ingredients	g/kg
Casein	220.0
L-Cystine	3.3
Sucrose	93.8
Soybean oil	10.0
Cholesterol	10.0
Cholic acid	5.0
Cellulose	50.0
Mineral mix AIN-93G(TD94046)	52.5
Calcium phosphate[a]	8.5
Magnesium oxide[a]	1.2
Vitamin mix AIN-93VX(TD94047)	11.5
Choline bitartrate	4.5
TBHQ	0.03
Soybean oil	140.0
Lecithine	14.0
Tritridecanoin	1.0
Maltodextrin	325.67
Cellulose or chitosan	50.0

[a] Since chitosan has been reported to interfere with mineral absorption (Braier & Jishi, 2000), additional calcium and magnesium have been added. Treatment A: control. Diet for treatment B contains chitosan. Diet for Treatment C contained emulsified soybean oil, lecithin, and tritridecanoin but no chitosan. Diet for Treatment D contained emulsified soybean oil, lecithin, and tritridecanoin with chitosan as encapsulating agent.

2.3. In vivo testing

Weanling (3-wk-old) male ICR mice from Charles River Laboratories (Wilmington, MA) were housed in wire-bottomed individual cages in a windowless room on a 12-h light/dark cycle in strict accordance with guidelines established by the Institutional Animal Care and Use Committee at the University of Massachusetts, Amherst. After 1-week adaptation period, animals were randomly divided into four groups and fed diets indicated in Table 1 for 4 weeks. Water and food were provided ad libitum, provided freshly three times a week. Body weights were measured weekly and food intake was monitored throughout the experiment. Mouse feces collected for 24 h after 2 week feeding were analyzed for their fat content using a solvent extraction method (see below). After the feeding periods, the mice were sacrificed by CO_2 asphyxiation. Blood was collected by cardiac puncture and sera were analyzed for total cholesterol and triglyceride using commercial kits (Cholesterol kit, TR-13421, and triglyceride kit, TR-22421, both from Equal Diagnostic, Exton, PA). Organs (liver, kidneys, lung, heart, spleen and adipose tissue) were weighed. Gut contents from the stomach, the small and the large intestines, and the colon were collected to be used for particle size analysis (see below).

2.4. Analysis for fat content from feces

Feces were dried at 63 °C for 3 h using a Drying Vacuum Oven (Macalaster Bicknell, Catalog No. 3600; Cambridge, MA) then extracted with diethyl ether using a Soxtec system extraction unit (HT-1043, FOSS, Prairie, MN). The difference in weight before and after solvent extraction was used to determine fat content.

2.5. Particle size measurements

The particle size distribution was determined in diets and feces using laser light scattering (Mastersizer X, Malvern Instruments Ltd., Malvern, UK). Before measurements, samples were dispersed in water and dense particulate materials (such as cellulose) were removed by centrifugation at 6500 rpm for 10 min. The supernatants were diluted with distilled water before measuring particle sizes. The instrument finds the particle size distribution that gives the best fit between the experimental measurements and predictions made using light scattering theory (Mie theory). A refractive index ratio of 1.08 was used by the instrument to calculate the particle size distributions (Aoki, Decker, & McClements, 2005). Each individual particle size measurement was determined from the average of three readings.

2.6. Optical microscopy

The upper small intestine (the duodenum and part of jejunum) contents were dispersed in water and a drop of dye solution (Oil Red O dissolved in corn oil) was added. The microstructure of selected emulsions was determined using optical microscopy (Nikon microscope Eclipse E400, Nikon Corporation, Japan). The images were acquired using a CCD camera (CCD-300-RC, DAGE-MTI, Michigan City, IN) connected to a Digital Image Processing system (Micro Video Instruments Inc., Avon, MA).

2.7. Analysis for tritridecanoin content from livers

Hepatic lipids were extracted as described (Folch, Lees, & Sloane-Stanley, 1957). Fatty acid methyl esters were prepared by reaction with 4% HCl in methanol for 20 min at 60 °C and identified by comparison with standards (Sigma Chemical Co., St. Louis, MO) by gas chromatography equipped with a flame ionization detector (Shimadzu GC-17A; Tokyo, Japan). A Supelcowax-10 fused silica capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness, Supelco Inc., Bellefonte, PA) was used and oven temperature was programmed from 100 to 200 °C, increased 20 °C per min, held for 40 min, increased 10 °C per min to 230 °C, and held for 20 min. Both injector and detector were set at 250 °C.

2.8. Statistics

Data in all the tables and figures were analyzed by Ducan's Multiple Range Test and one-way ANOVA Procedure of the SAS Institute. Differences were considered significant at a p value of <0.05.

3. Results

Body weights and food intake were not significantly different between all treatment groups (Table 2). There was no difference in tissue weights (liver, kidneys, lung, heart, spleen, adipose tissues, and small and large intestines, Fig. 1). These findings indicate the treatment diets caused no adverse effects.

There was no difference between the measured total fecal fat contents between all treatment groups (Fig. 2). To ensure the efficiency of fat extraction from encapsulated particles, test diets were tested for fat content as well. Close to 100% of the non-encapsulated fat samples (Diets A to C) could be extracted. On the other hand, we found that only 61% of encapsulated fat (Diet D) could be extracted, which was probably because chitosan formed a physical barrier around the individual fat droplets and/or because it promoted extensive droplet flocculation thereby limiting the ability of the organic solvent to penetrate into sample and solubilize the lipids. It should be noted that the total fecal fat contents were measured on samples that had passed through the entire mouse digestive tract. During passage through digestive tract, the fecal fats may have been no longer encapsulated, then the extraction efficiency would not have been a problem. If we assume that the fat extrac-

Table 2
Effect of emulsified or encapsulated lipids on food intake and body weights from mice fed for 4 weeks[a]

Week	Treatment				SEM
	A	B	C	D	
Body weight (g)					
0	17.97	17.60	17.94	17.99	*0.42*
1	24.66	23.91	25.06	24.22	*0.67*
2	27.70	27.49	27.78	26.62	*0.81*
3	29.38	29.51	29.21	27.74	*0.93*
4	30.38	30.15	30.43	28.66	*0.99*
Food intake (g/mouse)					
1	30.77	30.90	31.86	31.07	*0.78*
2	32.00	33.77	33.02	31.12	*1.00*
3	30.89	32.88	31.38	30.04	*0.95*
4	29.56	29.15	29.97	29.86	*1.23*
Total	123.24	126.69	126.23	122.10	*3.44*

[a] Treatments were A: control, B: chitosan, C: emulsified fat (soybean oil, lecithin, and tritridecanoin) without chitosan, and D: emulsified fat (soybean oil, lecithin, and tritridecanoin) with chitosan as encapsulating agent. Numbers are means of nine animals.

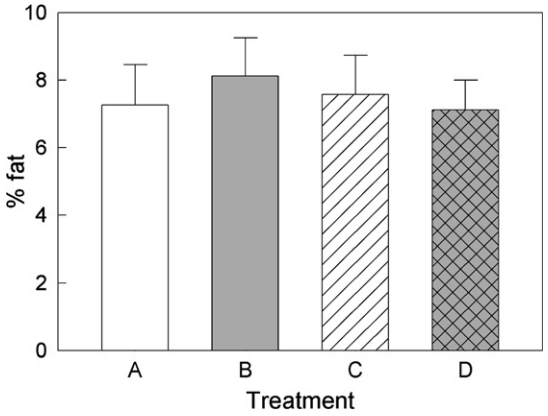

Fig. 2. Fecal fat content. Animals were fed one of the following diets: (A) non-emulsified fat, without chitosan (control), (B) non-emulsified fat, with chitosan, (C) emulsified fat, without chitosan, or (D) emulsified fat encapsulated by chitosan. Numbers are mean ± S.E. $n = 9$.

tion was indeed incomplete, the previously determined extraction efficiency could be used to calculate a fecal fat content. Based on this calculation, the fecal fat content from animals fed treatment diet D would in fact be significantly higher (11.3%) than other groups (7.3–8.1%). However, at present we do not have strong evidence that encapsulated fat particles remain intact in the intestines. In addition, when total fat absorbed was calculated based on food intake and fecal fat content (allowing for the lower extraction efficiency), animals fed treatment diet D had 96.0% fat absorption compared to 96.2–96.6% in the other three treatments. In addition, we also determined the extent of fat digestion and absorption by measuring hepatic tridecanoic acid levels (a marker for fat absorption). No significant differences in hepatic tridecanoic acid levels were found (% tridecanoic acid; Diet A, 0.68 ± 0.19; Diet B, 0.61 ± 0.14; Diet C, 0.42 ± 0.15; Diet D, 0.85 ± 0.44), suggesting that the lipids were equally digested and absorbed in all diet treatments. These results suggest that the difference in remaining fecal fat by chitosan encapsulation is negligible compared to the total absorbed fat. Thus we conclude that the overall fat absorption was not affected by encapsulating fat with chitosan.

Laser diffraction measurements were used in an attempt to provide some information about the changes in the size of the fat droplets during the in vivo digestion process. The particle size distributions of the particles present in the test diets and feces are shown in Table 3. Over 95% of the particles were less than 10 μm in the primary and secondary emulsions used to prepare the diets

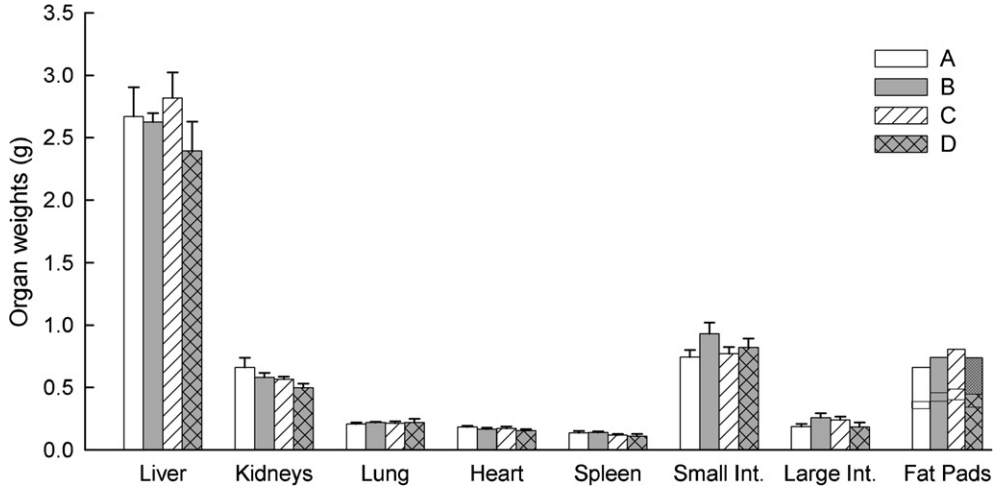

Fig. 1. Comparison of organ weights (liver, kidneys, lung, heart, spleen, small intestine, large intestine, and adipose tissue). Animals were fed one of the following diets for 4 weeks: (A) non-emulsified fat, without chitosan (control), (B) non-emulsified fat, with chitosan, (C) emulsified fat, without chitosan, or (D) emulsified fat encapsulated by chitosan. For adipose tissue, stacked bars indicate epididymal, retroperitoneal and mesenteric fat pads from bottom to top. Numbers are mean ± S.E. $n = 9$.

Table 3
Particle size distributions in test diets and feces[a]

Particle diameter (μm)	Test diet (%)			
	A	B	C	D
<1	64.6 ± 27.0	65.1 ± 28.9	60.0 ± 24.2	48.1 ± 33.1
1–10	11.0 ± 5.8	14.8 ± 11.1	12.5 ± 5.5	10.4 ± 9.1
10–100	4.7 ± 5.4	4.5 ± 5.8	3.8 ± 4.7	2.7 ± 3.7
>100	19.7 ± 27.94	15.6 ± 27.9	23.7 ± 25.4	38.8 ± 39.4
	Fecal samples (%)			
	A	B	C	D
<1	34.0 ± 8.1	37.6 ± 12.4	31.1 ± 1.8	33.4 ± 7.4
1–10	23.9 ± 1.8	26.5 ± 3.2	24.1 ± 0.3	33.1 ± 5.4
10–100	35.8 ± 7.2	34.6 ± 13.8	42.5 ± 1.3	25.0 ± 3.2
>100	6.4 ± 2.6	1.5 ± 1.8	2.4 ± 0.2	8.6 ± 9.5

[a] Treatments were A: control, B: chitosan, C: emulsified fat (soybean oil, lecithin, and tritridecanoin) without chitosan, and D: emulsified fat (soybean oil, lecithin, and tritridecanoin) with chitosan as encapsulating agent. Numbers are means of three samples.

($d_{32} = 0.50$ and 0.49 μm for primary and secondary emulsion, respectively). In all diets, about 50–60% of the particles were less than 10 μm in diameter and the remaining percent would be other particulate materials. There was no significant difference in the particle size distribution of the four kinds of test meal. Since the oil was initially in the form of bulk fat in groups A and B, but as small oil droplets in groups C and D, the fact that there was no difference in particle sizes suggests that the particles measured may not only be oil droplets, but also possibly cellulose, undigested protein aggregates, etc. In the feces, the proportion of particles <1 μm and particles larger than 100 μm decreased, whereas the proportion of particle with diameter in the range 1–10 μm and 10–100 μm increased compared to particle size distribution of diets. The diet D containing droplets initially surrounded by chitosan had a similar particle size distribution as the other three treatment diets, which again suggests that the particles observed may either not be oil droplets and/or that encapsulation of the lipid droplets by chitosan had little affect on lipid digestion.

To investigate any difference in the microstructure of small intestine contents from animals that consumed four different test meals, the microstructures were determined by optical microscopy (Fig. 3). To discriminate the lipid droplets from the other components observed in the microstructure of intestine content, lipid soluble dye solution (Oil Red O) was also placed on the microscope slide to stain the lipid phase. The overall microstructure was not different among the four different intestine contents (Fig. 3).

Finally, there was no difference in serum triglyceride levels. However, additional chitosan in the diet reduced serum cholesterol level significantly compared to control. This was not observed when chitosan was used for encapsulation (Fig. 4).

4. Discussion

Use of microencapsulation technology for biologically valuable lipid and/or lipid soluble components has drawn considerable attention in the food industry. Chitosan has been proposed as a particularly effective ingredient for encapsulating fats because it is one of the few food-grade biopolymers that has a positive charge across a wide pH range. Lipids encapsulated by chitosan have been shown to have improved stability to thermal processing, lipid oxidation, freeze-thaw cycling, and high ionic strengths than non-encapsulated lipids (Aoki et al., 2005; Ogawa, Decker, & McClements, 2003b; Ogawa, Decker, & McClements, 2004; Klinkesorn et al., 2005a, Klinkesorn, Sophanodora, Chinachoti, Decker, & McClements, 2005b). However, the bioavailability of the encapsulated lipids is still unclear, since a recent in vitro study in our laboratory showed that encapsulation of lipids by chitosan reduced the extent of lipid digestion by pancreatic lipase (Mun et al., 2006). In this report, we found that the fat absorption was not affected by the relatively small levels of free chitosan used (Diet B) or when the emulsified lipids were initially encapsulated by chitosan (Diet D). This is also supported by the lack of changes in intestine contents and/or intestine weights, which may have been altered if there was any need for adjustment for fat absorption. Since there was no difference in fat absorption, it was not surprising to observe no difference in body weights, food intake and major organ weights. These results suggest that encapsulation of emulsified lipids by chitosan does not impact their subsequent digestibility, which means that chitosan encapsulation could be useful as a tool to delivery bioactive lipids and lipid soluble compounds without affecting fat absorption. However, further human studies are needed to confirm this.

The reason that we observed a decrease in lipid digestion upon chitosan encapsulation of fat droplets in our previous in vitro study (Mun et al., 2006), but not in our current in vivo study, suggests that the in vitro digestion model used previously did not adequately simulate the natural mouse digestion process. There may be additional enzymes, dilution steps, mechanical stresses or surface active components that help disrupt the lecithin–chitosan layers surrounding the fat droplets during animal digestion that were not included in the fairly simple in vitro digestion model used in our study where samples were simply adjusted from pH 3 to 7 and then pancreatic and bile extracts were added.

Previously, chitosan supplements have been reported to reduce cholesterol in both animals and humans, although doses used for this effect are significantly higher (2–15% of diet) than those used in our experiment (0.5%) (Ausar et al., 2003; Chiang, Yao, & Chen, 2000; Ebihara & Schneeman, 1989; Gallaher, Munion, Hesslink, Wise, & Gallaher, 2000; Gallaher et al., 2002; Han, Kimura, & Kuda, 1999; Lehoux & Groundin, 1993; Sugano et al., 1980). It was interesting though that the supplement of chitosan sig-

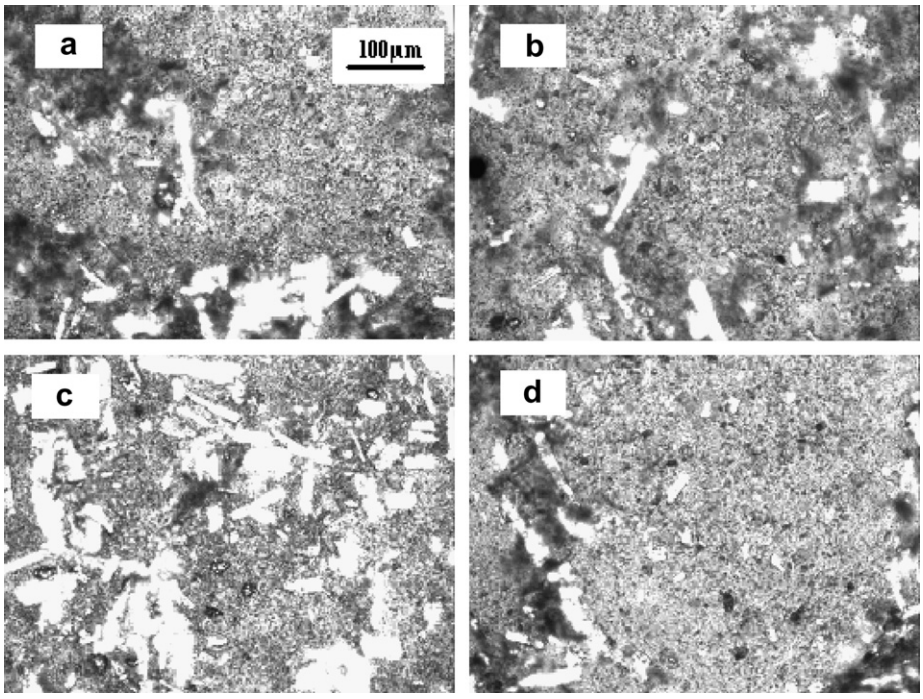

Fig. 3. Photomicrographs of duodenal contents. Animals were fed one of the following diets: (a) non-emulsified fat, without chitosan (control), (b) non-emulsified fat, with chitosan, (c) emulsified fat, without chitosan, or (d) emulsified fat encapsulated by chitosan.

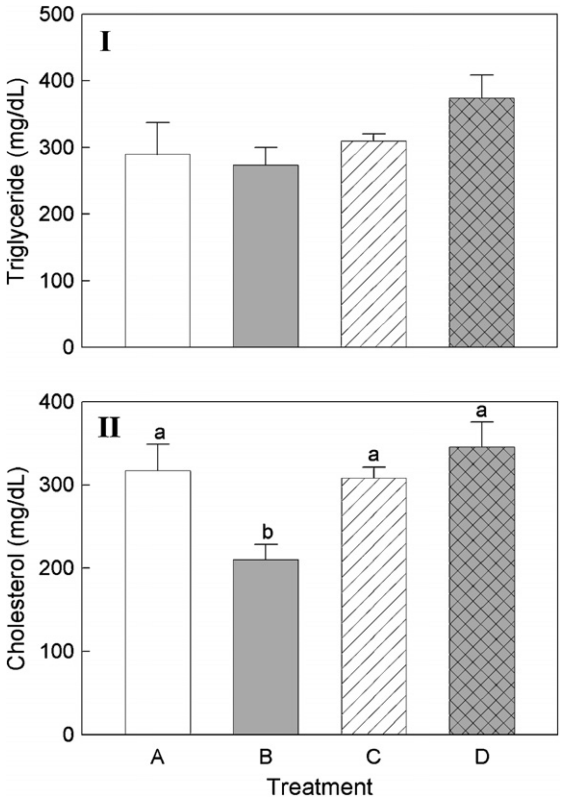

Fig. 4. Serum triglyceride (I) and cholesterol (II) concentrations. Animals were fed one of the following diets for 4 weeks: (A) non-emulsified fat, without chitosan (control), (B) non-emulsified fat, with chitosan, (C) emulsified fat, without chitosan, or (D) emulsified fat encapsulated by chitosan. Numbers are mean ± S.E. $n = 6–8$. Means with different letters are significantly different at $p < 0.05$.

nificantly reduced serum cholesterol levels (Treatment B), but when chitosan was provided as encapsulation there was no difference in serum cholesterol levels (Treatment D). This supports the direct interaction between free chitosan and bile or cholesterol to reduce intestinal absorption as previously suggested (Ebihara & Schneeman, 1989; Gallaher et al., 2000).

In summary, our results suggest that lipid digestibility was not inhibited by emulsification of fat nor by encapsulation of fat droplets with chitosan in vivo. This encapsulating technology can be a very useful tool to provide biologically active lipid and lipid soluble components, such as fish oil. This method can protect stability when applied in a number of food systems without compromised fat bioavailability. The taste as well as processing stabilities of these molecules still needs to be determined.

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