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The Effect of Carboxymethyl Cellulose on the Stability of Emulsions Stabilized by Whey Proteins under Digestion *in vitro* and *in vivo*

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Abstract*—In vitro* gastro-duodenal digestion model was used to investigate the changes of emulsions under digestion conditions. Oil in water emulsions stabilized by whey proteins (2%) and stabilized by whey proteins (2%) with addition of carboxymethyl cellulose (0.75%) as gelling agent of continuous phase were prepared at pH7. Both emulsions were destabilized under gastric conditions; however the protective role of carboxymethyl cellulose was indicated by recording delay of fat digestibility of this emulsion. In the presence of carboxymethyl cellulose whey proteins on the interfacial surface of droplets were more resistant to gastric degradation causing limited hydrolysis of fat due to the poor acceptability of lipids for the enzymes. Studies of emulsions using *in vivo* model supported results from *in vitro* studies. Lower content of triglycerides in blood serum and higher amount of fecal fat of rats were determined when rats were fed by diet containing emulsion made with whey proteins and carboxymethyl cellulose.

***Keywords*—**Digestibility, emulsions, lipids, rats.

I. Introduction

CONTROLLED fat release in the gastrointestinal tract and delayed digestibility of lipids has been a subject of interest in recent years. It is well known that in the case of emulsions oil in water the properties of interfacial layer are very important for emulsions behavior during digestion process. A number of studies have been published about various aspects of developing emulsions with specific structural and physicochemical characteristics of interfacial layers that surround lipid droplets and are resistant to enzymatic degradation so that it is possible to reduce lipid bioavailability [1], [2].

Whey proteins (WP) are often used as emulsifier in the production of emulsions therefore their properties on interfacial layer during technological processes has been extensively investigated. It is important to note that hydrolysis of whey proteins on the interfacial layer of oil droplets was the objective of comprehensive studies as well [3]-[5]. These studies revealed that under simulated gastric conditions emulsions with WP on the interfacial layer were destabilized and oil droplets aggregated due to the hydrolysis of proteins by pepsin [6], [7]. However, it was determined that the proteolysis of WP depended on the unabsorbed proteins present in the emulsion [8]. The presence of hydrocolloids in emulsions was shown to have effect on the protein hydrolysis as well as on the physicochemical properties of emulsions during digestion [9].

Hydrocolloids are frequently used in emulsions as thickening or gelling agent in the continuous phase, some of them can act as emulsifying agent. So far, most attention has been paid towards characterization of emulsions stability at the presence of hydrocolloids together with proteins and their possible interactions at the interfacial layer of oil droplets [10]. Recently there have been considerable efforts to control digestibility of lipids through building the interfacial layers from proteins and polysaccharides by electrostatic deposition method (layer-by-layer) [11] or forming covalently cross-linked interfacial protein polysaccharide complexes based on the Maillard reaction [12].

Whilst results about emulsions stability under simulated gastric conditions are undoubtedly of some interest *in vitro-in vivo* correlations in digestion models are extremely important [13], [14]. However very limited information is available in this area. Some knowledge is gained from the experiments with lipid-based drugs. Dahan et al. [15] reported that a trend similar to that obtained in the *in vitro* lipolysis model was observed in *in vivo* experiments for lipophilic drug samples. Fatouros et al. [13] showed that the *in vitro* solubilization data correlated well with the *in vivo* data for lipid-based drug samples.

In earlier work Brandon et al. [16] suggested that the *in vitro* digestion models developed for food and soil could be partially validated by comparing the bioaccessibility with human *in vivo* bioavailability data or with animal data. Mun et al. [17] presented results of *in vitro* study about slower digestion of lipids coated by layer of chitosan. However, studies using mouse model showed no difference between body weight and fecal fat content of mince fed chitosan coated or uncoated lipid droplets [18]. Authors suggest several possible reasons for the nonconformity between *in vitro* and *in vivo* studies. One of them is that the time of food passage through the animals digestive tract is long enough therefore the possible decrease of digestion rate can be not recorded

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because the overall amount of digested lipids can be the same [19].

The aim of this work was first to investigate the influence of carboxymethyl cellulose on the stability of WP stabilized emulsions under simulated gastric conditions. Emulsions containing WP and carboxymethyl cellulose (CMC) were investigated under production and storage conditions by Girard, et al. [20]. From these studies it is known that under certain conditions CMC can increase stability of emulsions by increasing viscosity or gelling the aqueous phase. In our studies we used this method of emulsion stabilization and investigated the changes occurring in emulsions stabilized with either WP or mixture of WP and CMC using *in vitro* digestion models by simulating stomach and small intestine conditions. Secondly, bioavailability of fat from these emulsions was investigated using *in vivo* model.

II. Materials and Methods

A. Materials

Whey protein isolate (WP) (Lacprodan DI-9213) containing 88.5% of protein, 6.0% of moisture, 4% of ash and 0.1% of lactose was obtained from Arla Foods Ingredients Amba, Denmark. Carboxymethyl cellulose (CMC) from Carl ROTH GmbH Co, Germany, with purity ≥ 99.5 % and viscosity (2%, 25°C) 1000–3000mPa s was used. Rapeseed oil was purchased from the local supermarket. Pepsin A (601U/mg, P7000) and Pancreatin (4xUSP, P1750) were obtained from Sigma-Aldrich Chemie GmbH, UK, Bile salt (B8381) – from Sigma-Aldrich Chemie GmbH, New Zealand.

B. Preparation of Emulsions

Emulsion o/w stabilized by WP and CMC was prepared by simultaneous adsorption principle. Appropriate amounts of CMC and WP solutions were mixed for 5min at 24000rpm (Ultra Turrax (IKA Werke, Staufen, Germany)), the pH was adjusted to 7 (using 0.1 N NaOH) and mixture was stored for 1 hour. After 1 hour WP and CMC mixture was homogenized for 5min at 24000rpm with rapeseed oil. Emulsion contained 40% rapeseed oil, 2% WP, 0.75% CMC. Control emulsion was made without CMC and contained 40% oil, 2% whey protein.

C. Simulated Digestion Fluids Preparation (SDF)

Emulsions were passed through an *in vitro* digestion model that simulated the composition (pH, minerals and enzymes) of stomach and small intestine juices. Simulated gastric fluid (SGF) containing 2g NaCl, 6.9g pepsin (601U/mg), 7ml conc. HCl, diluted to 1L and pH adjusted to 1.2 using 1.0M HCl was prepared by modified method Sarkar and others [6]. Proteolytic activity of SGF was ~36.6U/ml. Simulated duodenal fluid (SDF) containing 0.039g pancreatin, 0.31g bile salt, 8ml H_2O, 5ml 0.9% NaCl, 2.37ml 0.15M HCl, 0.5ml 2M HCl, 11.5ml 0.15M Na_2HCO_3, diluted to 1L and pH adjusted to 1.2 using 1.0M HCl was prepared. Proteolytic activity of SDF was ~ 17U/ml, lipolytic activity~ 800U/ml.

D. In vitro Digestion of Emulsions

The *in vitro* emulsion digestion assay was performed in two steps with modification according to Almaas and others [21]. In brief, both digestion steps were performed for 240min each at 37^0C with continuous shaking at 100rev/min. In the first step each stock emulsion was mixed with SGF at ratio 4:1 w/w. The pH was adjusted to 5, 3 and 2 respectively after 10, 20 and 30 min using 2M HCl. In the second step each stock emulsion and SGF mixture was mixed with SDF at ratio 4:1 w/w. pH was raised up to 7 using 4M NaOH. Samples were withdrawn before, during and at the end of the digestion.

E. Measurements of Emulsions Stability under Digestion

Particles size distribution: oil droplets size was determined by means of a laser diffraction spectrometer Mastersizer 2000 (Malvern Instruments Ltd, UK). Measurements were performed in 24h after emulsions were made.

Degradation of the whey proteins: the content of β-lactoglobulin (β-LG), α-lactoalbumin (α-LA) and bovine serum albumin (BSA) in emulsions was examined by a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique [22] using the Bio-Rad Mini-PROTEANw 3 Cell system (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK).

Hydrolysis of lipids: the time-dependent lipolysis of lipids in emulsions was examined according to the following processes: extraction method of Folchs [23], solid phase extraction to get two phases: triglyceride (TG) and free fatty acids (FFA) [24]. Composition and quantitative analyses of FFA were carried out using GC-MS (Agilent 6890 DC, Agilent technology, Wilmington, DE, USA and Autospec Ultima MS, Micromass Ltd. Manchester, UK). Data were analysed using MassLynx v 4.0 (Waters, Miniford, MA, USA) and NIST 08 library (Gaithersburg, MD, USA).

F. In vivo Model

Adult male *Wistar* rats were maintained at controlled environment, individually housed in hanging stainless steel wire cages on a normal 12 hour light/12 hour dark cycle. Rats were divided into 3 groups, the number of rats was 7 per group. The rats were allowed a 1-week acclimation period prior to initiation of dietary treatment: control (usual diet); usual diet + emulsion without CMC (Diet-1); and usual diet + emulsion stabilized by WP and CMC (Diet-2). Rats were given free access to food and water, 2g of emulsion was given too once a day. Blood samples were collected after 1st and 2nd week of experiment and triglycerides in serum were determined using a Roche Integra 400 plus analyzer. Roche Diagnostics (Indianapolis, USA) reagents kits were used. Total fat content were determined in collected frozen fecal samples using Soxlet Extraction Unit (R 106 S, Behr Labor Technik, Germany). Fatty acids profile (saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA)) was determined by gas chromatography using a flame ionization detector (Shimadzu GC - 17A, using a BPX - 70, 120 m column, Japan).

G.Statistical Evaluation

The significant differences among means were analyzed using one-way ANOVA and *t* test (ANOVA for Excel vers. 3.1). Level of significance was set for $p < 0.05$.

III.Results

A.Stability of Emulsions under Digestion in vitro

Emulsions stabilized by WP alone (control) and emulsions stabilized by simultaneous addition of WP and CMC mixture at pH 7 were made and analyzed. It is known that WP–CMC interaction does not take place at a pH close to neutral. Therefore in emulsions stabilized by mixture of WP and CMC, the polysaccharide molecules remain unabsorbed on the oil droplet and form gel in the continuous phase of the emulsion with oil droplets locked in the network holes which prevented them from creaming.

In the first step of the experiment changes in droplet sizes during digestion process were measured. It was expected that emulsions of different mean droplet sizes can be obtained due to the different effect of enzymes on the interfacial layer of emulsions stabilized by WP alone and mixture of WP and CMC and that can facilitate the changes of fat release from the emulsions.

According to Armand and co-workers the rate of lipid hydrolysis under gastric conditions can be influenced by lipid droplet size. It was shown by *in vitro* experiments that decrease in the mean lipid droplet size caused the increase of rate of lipid hydrolysis [25]. On the other hand *in vivo* studies showed that lower initial fat droplet size facilitated fat digestion by gastric lipase in the stomach and duodenal lipolysis, however the overall fat assimilation in healthy subjects was not affected by differences in initial droplet size because of efficient fat digestion by pancreatic lipase in the small intestine. [26].

Fig. 1 illustrates the evolution of droplet size distribution during emulsions incubation with simulated gastric and duodenal fluids.

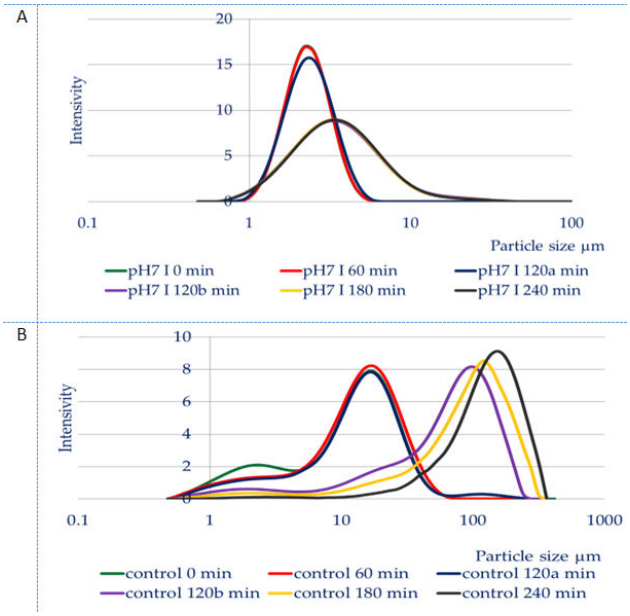


Fig. 1 Droplets size distribution in emulsions during incubation with SGF and SDF A emulsions stabilized by WP B emulsions stabilized by WP and CMC

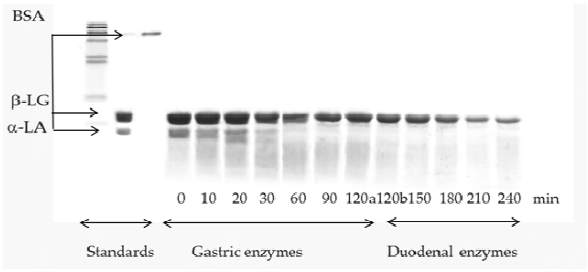
Comparing the mean droplet size of emulsions stabilized by WP alone and by WP with CMC it can be seen that initially (before digestion process) smaller droplets (about 4 µm) were recorded in emulsions stabilised by WP and CMC. So, it can be predicted that the fat release from these emulsions would be faster.

Analyzing data presenting changes of droplets distribution in emulsions under digestion conditions (Fig. 1), it can be concluded that addition of CMC markedly affected emulsions stability under digestion conditions. In emulsions which were made with WP and CMC (Fig. 1 A) only minor changes were recorded under gastric conditions. After addition of SDF more intensive increase in droplets size distribution was noted. However these changes in droplet size were observed immediately after addition of SDF into the emulsion and they were at the same level during all incubation time. Somewhat different results can be seen in the droplet size distribution of control emulsion (Fig. 1 B). A rapid increase in droplet size (above 10µm) was obtained within the first minutes of the gastric stage. Then formation of wide range of oil droplets size under duodenal conditions was determined. These results conform to the results published by Nik and co-workers that considerable aggregation and coalescence can occur during simulated small intestinal digestion of an emulsion stabilized by WP [2].

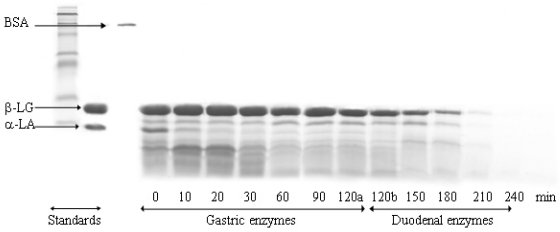
Despite the initial prediction that smaller droplets of emulsions stabilized by WP and CMC would be better available for enzymes to attach because of large surface area only slight disruption of the oil droplets started in the gastric stage of digestion. It can be that WP layer on the droplets was badly available for the pepsin from SGF due to the limited flow of liquid through the dense network of CMC gel in the

continuous phase of the emulsion. Destabilization of this emulsion because of the coalescence of oil droplets after addition of SDF could be explained by possible destructive action of bile salt on the CMC gel. Increased polydispersity of control emulsion during digestion process could be explained in terms of coalescence of oil droplets due to the hydrolysis of whey proteins adsorbed on the surface of droplets by SGF enzymes. The droplets coalescence since hydrolyzed WP cannot cover the entire surface area. These processes were followed by further destabilization of the system during incubation with SDF causing the transfer of polydispersed emulsion structure to the coarse emulsions structure.

Initially formulated hypothesis about the protective influence of CMC on the degradation of WP layer on oil droplets in emulsions was proved by the results of time study on the protein profile by SDS-PAGE of WP from emulsions degraded by SGF and SDF. Differences in the degradation of major WPs in emulsions made with addition of CMC as well as control emulsion (without CMC addition) are presented in Fig. 2. In both emulsions BSA was fully degraded at the very beginning of gastric digestion phase. The resistance of α–LA to the pepsin was slightly more expressed and survived after 20 min of emulsion incubation with SGF in the case of emulsion made with CMC (Fig. 2 (a)). In the control emulsion α–LA was degraded at the beginning of emulsion incubation with SGF (Fig. 2 (b)).



(a)



(b)

Fig. 2 SDS-PAGE protein profiles of emulsions during incubation with SGF and SDF: (a) – emulsions made with addition of WP and CMC; (b) control emulsion (no CMC added)

β–LG was resistant to gastric degradation, the protein pattern did not change much during 120min incubation with SGF for both emulsions. Addition of duodenal fluid resulted in its degradation after 180min of incubation in the case of control emulsion. The gradual reduction in β–LG was recorded in the emulsion made with CMC, but it was still β–LG remaining at the end of duodenal stage of digestion.

The time study on free fatty acids content variation in emulsions under duodenal conditions was performed. Fats were extracted, converted into methyl esters and analyzed by GC-MS. Results are presented in Fig. 3. In both emulsions free fatty acids content increased under digestion conditions. However at the end of duodenal stage of digestion it was recorded that in control emulsion FFA content was – 4.14% and in emulsion in which CMC was added, FFA content was 1.87%.

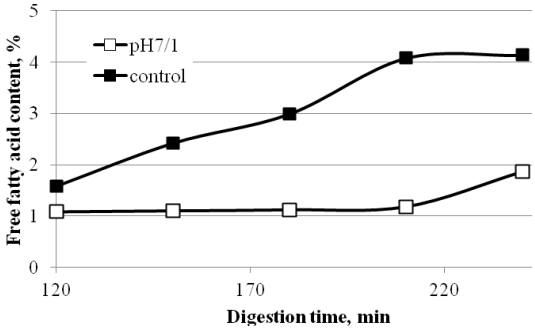


Fig. 3 Free fatty acids content variation during *in vitro* digestion

Results from this part of the experiment revealed that stabilization of emulsions by WP and CMC slowered degradation of emulsions under digestion conditions in comparison with emulsions stabilized by WP alone. The protective role of CMC network formation in the continuous phase of emulsion was shown by oil droplets size analysis and limited hydrolysis of fat in these emulsions as well as slower degradation of WP on the interfacial layer of the droplets.

The protective effect of polysaccharides concerning whey proteins on the interfacial layer of oil droplets was also reported for the systems where layer-by-layer coatings contained WP on an inner layer and polysaccharide on an outer layer. In these systems delay lipid digestability was determined and authors suggested that coating formed by the polysaccharides around lipid droplets restricted the access of the lipase to the emulsified triglycerides thereby retarding the lipolysis reaction [11]. In this study system containing WP on the interfacial layer of oil droplets and CMC in the continuous phase showed similar properties. It can be hypothesized that CMC gel network formed in the continuous phase of WP stabilized emulsion o/w reduced the rate of lipid digestion due to the suspended movement of gastric fluids in the emulsion and poor acceptability of lipids for the enzymes.

B. Fat Absorption in Rat Model

It was very important to identify whether the findings from *in vitro* model experiment about slower digestibility of fats in

the emulsions stabilized by WP and CMC can be supported by results about fat digestibility in emulsions under *in vivo* conditions. For that reason rat model was used in the second step of this study.

In the experiment one group of rats was fed by usual diet and other two groups were fed with usual diet and emulsions. Blood samples and fecal samples were collected after one week and two weeks of experiment.

Biochemical assay of blood samples showed a significant difference in triglycerides content, affected by the diet type after the first week and later after the second week of feeding (Table I). After 1st feeding period the difference between triglycerides content in blood of rats fed by Diet-1 (emulsion without CMC) and Diet-2 (emulsion with CMC) was almost 2-folds. After 2nd week of feeding amount of triglycerides in blood of rats fed by Diet-2 remained lower in comparison with blood of those fed by Diet-1 although not so remarkable (0.4mmol/L – Diet-1 and 0.3mmol/L – Diet-2).

Measurements of fat content in fecals of rats fed by different diets demonstrated that fat content in rats fecals after 1 week feeding by Diet-1 was lower than fecal fat content determined for fats fed by Diet-2; 4.15 and 5.47 %, respectively. The same tendency was observed after second week of feeding only the difference was less expressed (5.59% for Diet-1 and 5.77 % for Diet-2).

TABLE I
TRIGLYCERIDES CONTENT IN RATS BLOOD SERUM AND FECAL FAT CONTENT

Feeding	Triglycerides content in serum, mmol/L		Fecal fat content, %	
	1st week	2nd week	1st week	2nd week
Usual Diet	1.4±0.25	1.4±0.25	3.80±0.65	3.80±0.65
Diet-1	0.88±0.09	0.4±0.12	4.15±0.17	5.59±0.19
Diet-2	0.45±0.09	0.3±0.04	5.47±0.30	5.77± 0.16

The lower content of triglycerides in blood serum and higher amount of fecal fat of rats can be count as indicator of slower digestibility of fat by rat's organism. The lipolytic products of fat digestion are fatty acids, diglycerides and monoglycerides. Therefore one more possibility to judge about fat digestion is to analyze fatty acids composition in rats fecal. In our experiment rats feed contained 12.5g of fat from usual diet and 4g of fat from emulsion per 100g of diet. The composition and fatty acid source of the feed is shown in (Fig. 4 (a)). Comparison of fatty acid composition of usual diet and emulsion indicated higher amount of SFA and PUFA in usual rat's feed and higher amount of MUFA in emulsion. Finally saturated fatty acids comprise 21% from usual diet and 7% from emulsion, and unsaturated fatty acids 79% and 95%, respectively.

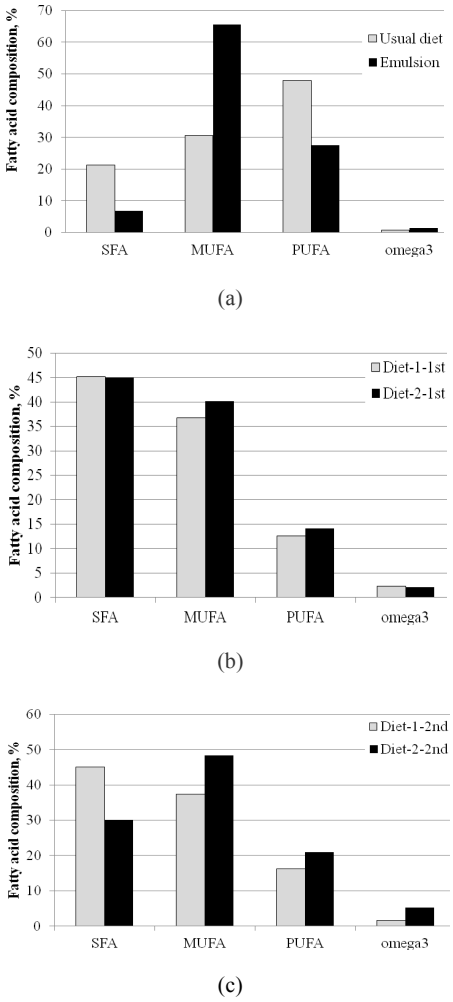


Fig. 4 Fatty acid composition of rats feed (a) and fecal samples after 1st week (b) and 2nd week (c) of feeding

Data about fatty acid composition of rats fecal fed by different diets are presented in Fig. 4 (b) and (c). It can be seen that fecal fatty acid excretion profile differed within diet type. In fecal fat of rats, fed by Diet-1 a higher rate of SFA was observed; it was 45% for Diet-1 and 30.1% for Diet-2. This difference was caused mainly by the more than 2-fold higher content of the decanoic acid (10:0) (from 0.6 to 0.2%); pentadecanoic acid (15:0) (from 0.8 to 0.5%); hexadecanoic acid (16:0) (from 24.3 to 16.3%); heptadecanoic acid (17:0) (from 1.3 to 0.1%); octadecanoic acid (18:0) (from 10.9 to 4.6%); eicosanoic acid (20:0) (from 0.7 to 0.3%).

According to the FFA composition in usual diet and emulsion the ratio of saturated and unsaturated FA was 3.7 for usual diet and 13.7 for emulsions. After the 1st week of feeding with usual diet and both emulsions, the ratio of saturated and unsaturated FA was in the range of 1.1-1.2. The relatively higher amount of saturated FA in rats fecal can be caused by different absorption of FA which depends on the amount of double bonds in the structure of FA. Saturated fatty acids are absorbed more slowly from the intestine than are unsaturated

fatty acids [27]. For better understanding of the lipolytic products absorption more comprehensive experiments *in vivo* are needed, because the quantity of feed and time of feeding are important factors to consider in animal studies.

IV. Conclusion

The results demonstrate that CMC added into WP stabilized o/w emulsion affected its stability under simulated gastric conditions. Emulsions which were stabilized by forming CMC gel around oil droplets covered by WP were significantly more stable under gastric and duodenal conditions in comparison with emulsions which were stabilized by WP alone. The protective effect of CMC concerning whey proteins on the interfacial layer of oil droplets caused the slower degradation of WP. As a result the droplets coalescence was delayed and limited hydrolysis of fat was determined due to the poor acceptability of lipids for the enzymes. Using *in vivo* model it was also shown that feeding of rats by emulsion stabilized with WP and CMC resulted in lower content of triglycerides in blood serum and higher amount of fecal fat compared with those fed by usual diet or emulsion stabilized only with WP.

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