Effect of homogenized and pasteurized versus native cow's milk on gastrointestinal symptoms, intestinal pressure and postprandial lipid metabolism

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Abstract

Some people experience gastrointestinal symptoms related to cow's milk consumption even if neither lactose intolerance nor cow's milk allergy can be diagnosed. To investigate whether milk homogenization could cause gastrointestinal problems, homogenized and pasteurized milk and native milk were served to eleven volunteers who reported such sensitivity in a random order together with an ingestible pressure measuring capsule. Postprandial lipemia did not differ between the two milk types, but significant differences were found in the postprandial plasma fatty acid composition. No significant difference was found in the amount of gastrointestinal symptoms or in the intestinal pressure after the consumption of native and processed milk. However, the obtained results on pressure in the large intestine ($P = 0.068$) as well as reported symptoms ($P = 0.103$) suggest that further studies in this area are needed with a bigger subject group and with longer exposure times to differently processed milk types.

1. Introduction

Fat is present in cow's milk as globules surrounded by the milk fat globule membrane (MFGM) composed of phospholipids, proteins, enzymes, cholesterol and glycoproteins (Mather, 2000). Commercial milk is generally homogenized for the purpose of physical stability.

Homogenization reduces the size of fat droplets from the average of 3 - 5 to 0.03 - 2 µm (Lopez, 2005; M. Michalski and Januel, 2006; Walstra, 1975). Breaking of the fat globules during homogenization creates new interface that cannot be entirely covered by the MFGM (Darling and Butcher, 1978). Thus, the new membrane contains also caseins and other surface active components that adsorb to the interface causing a fourfold increase in the protein content of the membrane (Houlihan, et al., 1992; Lee and Sherbon, 2002). No whey proteins are adsorbed unless homogenization is combined with heat treatment (Lee and Sherbon, 2002). If milk is first heated and then homogenized, the whey proteins denaturate and interact with the MFGM native proteins and micellar caseins. In addition the casein–whey complexes adsorb to the fat droplet interface (Houlihan, et al., 1992; Sharma and Dalgleish, 1993). When homogenization is done prior to heating the fat droplet interface is covered by semi-intact casein micelles or micellar fragments and the denaturized whey proteins are linked with MFGM proteins and adsorbed caseins via disulfide bonds. (Dalgleish and Banks, 1991; Lee and Sherbon, 2002). Various casein peptides and milk fat globule membrane proteins are reported to present either harmful (e.g. atherogenic) or beneficial bioactivity (e.g. hypotensive, anticarcinogenic), but there is no current knowledge of how homogenization influences these effects (M. C. Michalski, 2007).

In addition to the size of fat globules, processing of milk induces other changes to the milk composition. Whey proteins denaturate, aggregate and become insoluble during heating and form protein complexes with caseins. Especially, complexes between α-lactalbumin and κ-casein (Elfagm and Wheelock, 1978) as well as interactions between β-lactoglobulin and κ-casein (Dalgleish, 1990; Haque, et al., 1987; Jang and Swaisgood, 1990) have been reported. At the standard pasteurization temperature (72 °C) and time combination (15 seconds) the bioactive whey proteins retain most of their activity (Korhonen, et al., 1998). Pasteurization does not significantly destruct hormones, enzymes, growth factors, or proteins that bind to minerals, but heat treatments at higher temperatures will (Ebringer, et al., 2008). However, pasteurization lowers the concentration of water-soluble vitamins, especially vitamin C while the content of fat soluble vitamins and riboflavin remains intact (Ebringer, et al., 2008). No significant changes are seen in the antigenicity of milk proteins after standard pasteurization, but sterilization of milk may even increase allergic reactions (Kilshaw, et al., 1982). Due to the mechanical disintegration of casein micelles and milk fat globules, heat treatment and homogenization of milk might increase the ability of milk proteins to elicit allergic reaction in sensitized persons (Host and Samuelsson, 1988; Poulsen, et al., 1987).

Over the last years, the consumption of unpasteurized milk has increased in popularity in the Western countries despite the known risks associated with food-borne pathogens (Oliver, et al., 2009). Some people appear to experience milk-related gastrointestinal symptoms even when tested negative for lactose intolerance and milk allergy. It has been suggested that homogenization of milk may be involved in the induction of such gastrointestinal symptoms (Paajanen, et al., 2005). Native milk (NM) defenders and producers often advertise that consumption of NM has been associated with reductions in gastrointestinal symptoms but scientific evidence is currently lacking (Mummah, et al., 2014).

The few previously published studies on the topic have not confirmed possible gastrointestinal problems caused by cow's milk (Mummah, et al., 2014; Paajanen, et al., 2005; Pelto, et al., 2000). In those studies NM or homogenized milk was served to lactose intolerant or lactase persistence volunteers and gastrointestinal symptoms and immune responses were investigated. The SmartPill capsule, an ingestible probe measuring pH and pressure parameters, could provide an objective way to link differences in the gastrointestinal pressure with the gastrointestinal problems caused by certain food products. The SmartPill has been widely used by gastroenterologists for identifying functional digestive disorders (Willis, et al., 2011) but, to our knowledge, it has been previously used only in two nutrition related studies (Timm, et al., 2011; Willis, et al., 2011).

In this study, a double blind crossover trial with healthy volunteers who reported to be sensitive to processed milk was conducted with two separate meals (NM and HPM, homogenized and pasteurized milk) together with the use of SmartPill capsule. The aims of the study were to investigate 1) whether the two milk types induce different gastrointestinal problems as reported by the subjects 2) whether the two milk types induce different levels of low grade inflammation markers, differ in their postprandial intestinal transit times, intestinal pressure, glycemia, insulinemia or lipemia 3) whether the self-reported symptoms relate to changes in the intestinal pressure and also 4) to further study the applicability of the "smart pill system" to nutrition related trials.

2. Materials and Methods

2.1 *Milk*

NM and HPM were used as study milk samples. The milk samples were obtained from the Natural Resources Institute Finland, LUKE, research dairy farm and were processed and

packed at the LUKE Pilot Dairy plant in Jokioinen. Both milk types originated from the same herd of cows and same milk batches. Microbiological analysis was performed from every batch, and the milk was served at maximum two days after the milking. The HPM was first homogenized with two-stage homogenizer and then pasteurized. The pressure for homogenization was 160 bars and the pasteurization conditions were 72-73 °C for 15 seconds. The native milk was chilled and packed unprocessed. Both milk types were packed in a sealed tub the day before the test day and stored below 6 °C.

The milk fat globule size distribution of the milk samples was investigated once with Olympus BX60 BX-UCDB2 –microscope (Tokyo, Japan) combined with camera (Sony Power HAD 3CCD Color Video Camera DXC-950P, horizontal resolution 750TV lines, sensitivity 2000 lux, signal-to-noise ratio 58 dB; Tokyo, Japan). Imaging was done with Soft Imaging System analysis 3.0 program.

2.2 *Fatty Acid Analysis of Milk*

For fatty acid composition analysis, three replicates of each milk were analyzed and the lipids were extracted twice from each sample with chloroform-methanol (2:1) extraction (Folch, et al., 1957). After extraction, the lipids were fractionated into triacylglycerols and phospholipids by solid phase extraction using Sep-Pak Vac 6cc (500 mg) Silica Cartridges (Waters, Dublin, Ireland) (Hamilton and Comai, 1988). The fatty acid methyl esters (FAME) were prepared with a sodium methoxide method (Christie, 1982) for GC analysis.

The FAMEs were analyzed with Shimadzu GC-2010 gas chromatograph equipped with a flame ionization detector (Shimadzu Corporation, Kyoto, Japan). A wall-coated open tubular DB-23 column was used (60 m, i.d. 0.25 mm, liquid film 0.25 µm, Agilent technologies, J.W. Scientific, Santa Clara, CA, USA). Supelco 37 Component FAME Mix (Supelco, St. Louis, MO, USA), 68D (Nu-Chek-Prep, Elysian, MN, USA) and GLC-490 (Nu-Chek-Prep, Elysian, MN, USA) were used as external reference compounds.

2.3 *Determination of Lactose and Protein Concentration*

The lactose concentration of both milk samples was analyzed according to the NMKL method number 148, 1993. In short, weighted triplicate milk samples were diluted in ultrapure water and cleaned up with Sep-Pak C-18 classic (360 mg) Silica cartridges, (Waters, Dublin, Ireland). One milliliter of the milk sample was diluted to 3 mL of acetonitrile and the mixture was allowed to reach room temperature. The solution was filtered into a vial using 0.45 µm

polytetrafluoroethylene membrane filters. The analyses were carried out using an Agilent 1100 Series High Performance Liquid Chromatograph (HPLC) equipped with a refractive index detector (Agilent Technologies, Santa Clara, CA, USA). A Phenomenex AJO-4301 NH_2 (2.0 x 4.0 mm) pre-column was used with Phenomenex Luna NH_2 (3.0 x 150 mm, 5.0 µm particle size) column. Water–acetonitrile (1/4, v/v) was used as a mobile phase. L-(+)-lactic acid was used as an external standard (Sigma Aldrich, St. Louis, MO, USA).

The protein concentration was measured using Kjeldahl technique (AOAC, 1990). In short, the milk proteins were first digested with sulfuric acid followed by ammonia distillation and hydrochloric acid titration. The amount of protein in milk was calculated by multiplying the amount of nitrogen in the sample with a specific factor which is 6.38 for dairy.

2.4 *SmartPill Technology*

The SmartPill GI Monitoring System® (Given Imagine, Yoqneam, Israel) was used to monitor the changes in pH, pressure and temperature in the gastrointestinal tract. The system consists of a wireless, ingestible but non-digestible capsule (26 mm x 13 mm), a receiver for acquiring and storing the signals from the capsule and the MotiliGI software for displaying data on a computer.

Briefly, once the capsule is activated it starts to send pH, pressure and temperature data to the receiver. The SmartPill's pH sensor has an operating range of 1.0–9.0 ± 0.5 units, the pressure sensor has a range of 0 – 350 ± 5 mmHg and the temperature sensor has a range of 20 – 42 ± 1°C. The software uses the abrupt change from the acidic gastric pH to the alkaline duodenal pH to determine the gastric emptying time. SmartPill technology is also able to assess the transit times of small bowel, colon, and the whole gut.

2.5 *Clinical Trial*

Five healthy male and six healthy female volunteers (age 24 – 68 years; BMI 20.2 – 30.9 kg/m^2) were recruited to participate in a randomized cross-over clinical trial to consume both NM and HPM on two separate occasions separated by two to six weeks. This trial was limited to healthy subjects with normal liver and kidney functions, who reported stomach problems after drinking homogenized and pasteurized cow's milk but did not report those symptoms after having native cow's milk. The exclusion criteria were: history of cardiovascular disease, diabetes or any gastrointestinal (GI) conditions including history of gastric bezoar, suspected strictures, fistulas, GI obstruction, GI surgery within the past three months, dysphagia, Crohn's disease

or diverticulitis, implanted or portable electro-mechanical medical devices, regular medication, troubles with swallowing, celiac disease, regular smoker or participation in intervention two months prior to this study. Healthy subjects were recruited as diseases and medication may have impact on the digestive system which may give confounded results, and gastrointestinal conditions may increase the risk of the capsule to retain in the gastrointestinal tract. The trial was conducted according to the declaration of Helsinki. The ethics approval was obtained from the Ethics Committee, Hospital District of Southwest Finland. All subjects provided a written informed consent. The trial was registered prospectively to the U.S. National Institute of Health ClinicalTrials.gov registry (NCT02219126).

The subjects were on a non-dairy diet for five days prior to the study day and two days after the study day or until the capsule had exited the body. They were asked to keep a symptom diary until the capsule had exited the body.

In the morning of each study day, following an overnight fast, a catheter was inserted into an antecubital vein and a baseline sample was obtained. After that the study meal was served to the subjects. Each meal consisted of 4 dL of study milk and a SmartBar cereal bar. Nine participants swallowed the SmarPill capsule and two participants only ate the study meal because of a personal wish not to swallow the capsule. The milk samples were processed and packed one day prior to the study days and transported to the study site at temperature below 6 °C. The milk was served cold from paper cups covered with a lid and aluminum foil. Before serving the milk was shaken. The milk was drunk with a straw so that the mouth feel would be as similar as possible for both of the milk types. The SmartBar (Given Imagine, Israel) was provided to ensure the peristalsis of the gastrointestinal tract and to aid the movement of the capsule in the gut. Each SmartBar weighed 72 g and contained 2 g of fat, 50 g of carbohydrate and 2 g of fiber. The subjects were instructed to eat the SmartBar first and then swallow the capsule with the 4 dL of milk, all within a few minutes.

Blood samples were obtained from the subjects at 20, 40, 60, 90, 120, 180 and 240 min after ingestion of the capsule and milk for investigation of changes in the levels of blood glucose, insulin and triacylglycerols. The subjects were asked to restrain from eating or drinking for five hours after capsule ingestion to ensure that the capsule would exit the stomach simultaneously with the milk and before the next meal. The study subjects were offered a standardized salad lunch after five hours. Then the subjects were instructed to continue their non-dairy diet until

the capsule exited the body but otherwise eat and drink normally. To those two subjects, who did not swallow the capsule, the standardized lunch was offered after the last blood sampling.

The subjects were asked to wear the data receiver during the whole study time including nights until the capsule had exited the body with the stools, which took typically 1 to 3 days. The subjects were instructed to press the EVENT-button on the receiver every time they experienced stomach symptoms or when they ate, drank, went to bed, woke up or used toilet. The subjects were asked to write down in the diary the time and reason why they pressed the EVENT-button.

The subjects were instructed to use a provided disposable stool receiver on their toilet bowl and to observe their stool passage in order to confirm the exit of the capsule. After visual perception of the capsule, the subjects were instructed to return the data receiver. The subjects were required to avoid vigorous exercise and use of alcohol while the capsule was in the body. Also magnetic resonance imaging (MRI) was forbidden before the exit of the capsule for safety reasons.

2.6 *Plasma Insulin, Glucose, Cytokines, C-reactive Protein and Lipid Analysis*

Blood samples were collected to Li-heparin blood collection tubes (Vacuette®, Greiner Bio-One) and centrifuged at 2200 x g for 15 minutes for plasma separation. Plasma insulin was analyzed with electrochemiluminescence immunoassay. Plasma glucose was analyzed enzymatically with hexokinase assay. Plasma triacylglycerols were analyzed enzymatically with colorimetric method. All analyses were done with a Cobas 8000 (Roche Diagnostics, Basel. Switzerland).

The plasma samples from 0, 120 and 240 min postprandial time point were analyzed for indicators of low grade inflammation IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-10, TNFα and INFλ using Q-Plex™ High Sensitivity Human Cytokine Array (Quansys Biosciences, West Logan, Utah) as instructed by the kit provider. Samples were analyzed in duplicates. The test panel was measured by QuanSys-imaging system. High sensitive C-reactive protein was analyzed from 0 and 240 min postprandial time point samples, using CRP high sensitive ELISA-kit (IBL International EU59151, Hamburg, Germany) as instructed by kit provider.

For gas chromatographic analysis of the fatty acid composition of the plasma samples, the lipids were extracted with a modified Folch's chloroform-methanol extraction (Folch, et al.,

1957). Fractionation and methyl esters were prepared as explained earlier. The FAMEs were analyzed with gas chromatography as explained earlier.

2.7 *Lactose Malabsorption Genotyping*

In European Caucasian populations, the ability to digest lactose is associated with the SNP rs4988235 located in the *MCM6* gene (Enattah, et al., 2002). This SNP was genotyped from the blood samples by Sanger sequencing in order to investigate adult-type hypolactasia in our subjects. For genotyping, DNA was extracted from the blood samples using Qiagen's blood and tissue kit. Altogether 400bp around rs4988235 was amplified using the primer pair 5'-ACCCCCTTTTCAAAGACGAC and 5'-TGCTCATACGACCATGGAAT. Amplified DNA fragment was sequenced and individual genotypes were determined from the chromatograms.

2.8 *Interpretation of the SmartPill Data*

Data sets for the analysis were derived from the SmartPill raw pressure data. From the raw data, 45-minute moving averages (MA) were calculated for both the HPM and NM cases. Firstly the MA data was used to derive the normalized area under the curve (AUC) data using the trapezoidal rule to represent the overall pressure response for each subject. Secondly, the average difference of event pressure to the corresponding MA pressure data was produced. These derived data sets were acquired for the stomach, small intestine, large intestine and the whole digestive system.

2.9 *Statistical Analysis*

Statistical analyses were performed with SPSS 23.0 software (SPSS Inc, Chicago, IL, USA). Normal distribution of data was tested with Shapiro-Wilk test and logarithmic transformations for non-normally distributed data were performed when applicable. Depending on the normality of data, paired samples t-test or Wilcoxon matched-pairs signed ranks test was used to compare the measured responses. One-sample t-test was used to analyze differences in the event pressure. Statistical significance was indicated with $P < 0.05$. The relative changes of plasma cytokine levels in 120 min and 240 min as compared to those in 0 min of the same test subjects after each milk ingestion were calculated. These relative values were used in testing possible differences between treatments in 120 min and 240 min time points with Student's paired t-test.

3. Results

3.1 *Milk Composition*

No differences were observed in the fatty acid composition or contents of lactose or protein between NM and HPM milk samples. The major fatty acids in NM lipids were palmitic acid (16:0, 34.4 ± 0.1 %), oleic acid (18:1, 24.2 ± 0.1 %), stearic acid (18:0, 13.3± 0.0 %) and myristic acid (14:0, 11.3 ± 0.1 %). The content of lactose was 45.8 ± 1.7 g kg-1 and that of protein 34.8 ± 1.7 g kg-1. In HPM the major fatty acids were palmitic acid (34.8 ± 0.2 %), oleic acid (24.4 ± 0.1 %), stearic acid (13.5± 0.1 %), and myristic acid (11.3 ± 0.0 %). The content of lactose was 44.8 ± 0.5 g kg-1 and content of protein was 34.6 ± 0.6 g kg-1, respectively.

When analyzed by microscopy the milk fat globules of NM were bigger, approximately 5 µm in average diameter compared to milk fat globules from HPM, which were less than 1 µm in average diameter (Fig 1).

3.2 *SmartPill Data*

The SmartPill software generated reports with continuous measurements of pressure, pH and temperature (Fig. 2). The 45 minutes MA pressure measured by the SmartPill-capsule in the stomach, small intestine and the whole gut, calculated as AUC, did not differ between NM and HPM (stomach P = 0.963; small intestine P = 0.643; whole P = 0.53) respectively (data not shown). The AUC in the large intestine tended to be higher after the HPM compared NM but did not reach significant difference (P = 0.068). The intestinal transit times did not differ between the two milk types. The emptying or transit times for NM and HPM, respectively were: 4.6 ± 2 vs 5.4 ± 5 h for gastric, 4.7 ± 1 vs 4.1 ±1 h for small intestine and 21.0 ± 10 vs 26.4 ± 12 h for colon. The intestinal transit times did not differ between the two milk types. The pressure during adverse stomach symptom, indicated by EVENT button press, in the stomach, small intestine, large intestine or whole gut did not associate with the 45 minutes MA pressure in stomach, small intestine, large intestine or whole gut (stomach P = 0.428; small intestine P = 0.723; large intestine P = 0.292; whole P = 0.158).

3.3 *Symptom Diary*

HPM caused more reported adverse gastrointestinal symptoms in the whole gut compared to the NM. Events of adverse gastrointestinal symptom were reported 108 times after HPM. After NM adverse gastrointestinal symptoms were reported 55 times. Although more adverse

symptoms were reported after HPM the difference was not statistically significant due to the large variation (P = 0.103). Six subjects reported more adverse gastrointestinal symptoms after HPM, 3 subjects reported more adverse gastrointestinal symptoms after NM and 2 subjects reported equal amounts of gastrointestinal symptoms after both milk types. The most commonly reported symptom was flatulence, representing 55.6 % of all reported symptoms; other reported symptoms were abdominal pain/cramping 17.0 %; bowel movement 16.3 %; bloating 9.8 % and nausea 1.3 %. None of the subjects reported diarrhea. No gender difference was found between the gastrointestinal symptoms (P = 0.71).

3.4 *Plasma Insulin, Glucose, Cytokines, C-reactive Protein and Triacylglycerols*

We did not find any significant differences in plasma glucose and insulin concentrations between NM and HPM (data not shown). Despite the different lipid droplet size between the milk types, we did not find any significant differences in the blood TAG concentration between NM and HPM (Fig. 3).

All the cytokine levels were in ranges considered as normal for healthy adults. There was no statistically significant difference in the effects of the treatments on the inflammatory plasma markers (data not shown).

C-reactive protein levels were not affected by either of the milk types during the 4 h follow-up period (data not shown).

3.5 *Fatty Acid Composition of Plasma Lipids*

The most abundant FAs were oleic (18:1), palmitic (16:0), linoleic (18:2), stearic (18:0), myristic (14:0) and linolenic (18:3) acids (Fig. 4). At four hour time point we observed significantly more myristic (P = 0.021), palmitic (P = 0.047) and stearic acids (P = 0.028) after the HPM meal compared to NM meal. Also, the linoleic acid concentration tended to be higher after the HPM compared to NM at 4 h time point (P = 0.07). We did not observe any significant differences between the FA compositions of the two milk types at two hour time point.

3.6 *Lactose Malabsorption Genotypes*

When recruited, all eleven study participants reported adverse gastrointestinal symptoms after drinking HPM and that they were able to consume NM without symptoms. During the trial they were clinically tested for lactose malabsorption genotype and four out of eleven were carriers of the genotype C/C, four had C/T and three were carrying the T/T genotype. The C/C

genotype is associated with low lactase enzyme activity and the C/T and T/T are linked with lactase persistency (Jarvela, 2005). In the Finnish population the prevalence of C/C genotype is 17 % (Tolonen, et al., 2011). From the four C/C genotype carriers two reported more adverse symptoms after HPM, one reported more after NM and one reported equal amounts after both milk types.

Discussion

In this double blind crossover trial with healthy subjects, sensitive to cow's milk, one of our aims was to determine whether the two milk types induce different gastrointestinal problems reported by the study subjects.

The amount of reported GI symptoms by the study subjects did not show statistically significant difference ($P = 0.103$). Flatulence, abdominal pain, bloating and cramping were the most commonly reported symptoms after consumption of the milk samples as reported also by others (Mummah, et al., 2014; Paajanen, et al., 2003; Pelto, et al., 2000). Several studies have investigated GI symptoms and inflammation markers related to cow's milk consumption but like us, they found hardly any differences between the responses to NM and processed milk. Mummah et al. (2014) investigated in a crossover trial whether NM could reduce lactose intolerance symptoms relative to pasteurized milk in 16 subjects with self-reported lactose intolerance symptoms. Hydrogen breath tests results did not differ between the two milk types consumed by the subjects. Subjects did not observe any differences in the gastrointestinal symptom severity. Pasteurization does not affect the lactose content of milk and thus could explain why no differences were seen in the hydrogen breath test or symptoms severities between the two milk samples. Paajanen et al. (2003) conducted a randomized, double blind cross-over study with 44 subjects who self-reported better tolerance to NM than to HPM. During the two five-day study periods, separated by a nine day wash-out, the subjects experienced more symptoms during the second challenge period regardless of the milk sample type. No significant differences were found between the two study milks. Pelto et al. (2000) investigated the gastrointestinal symptoms and receptors CR1 and CR3 (which have been proven to be indicators of milk induced immunological changes) between homogenized and unhomogenized milk in three different groups of volunteers: lactose intolerant (N=6), milk hypersensitive (N=8) and healthy (N=6). They found no statistical differences in the symptoms or in the receptor expression between the groups. The lack of significant results might have

been influenced by small group sizes. None of these studies reported measurements of plasma triacylglycerol or FA composition nor intestinal pressure.

In addition to the gastrointestinal symptoms we investigated whether the intestinal transit times, glycemia, insulinemia and lipemia differed between the two milk types. We found no differences in the intestinal transit times. Also previous studies (Saad and Hasler, 2011; Sarosiek, et al., 2010; Timm, et al., 2011) have reported intestinal transit times in healthy subjects in ranges similar to ours. In the present study all of the measured inflammatory markers were in normal ranges for healthy subjects (Biancotto, et al., 2013; Kim, et al., 2011; Kokkonen, et al., 2010) and there was no statistically significant difference in the effects of the treatments on the inflammatory plasma markers.

Glycemia and insulinemia did not differ between the two milk types. Despite differing fat globule size in the ingested milk there were no significant differences in blood TAG concentration between the two milk types. However, we did find significantly more myristic, palmitic and stearic acids at the 4 h time point after ingestion of HPM compared to NM. Similarly, in an *in vitro* study by Garcia et al. (2014) they did not find any differences in the rate of digestion between native milk and homogenized milk when using simulated human gastro-duodenal digestion. However, they did reveal that the small native milk fat globules, present in both native and homogenized milk, were more rapidly hydrolyzed by gastric and pancreatic lipases than the large native milk fat globules due to an increased surface for lipase adsorption. Armand et al. (1999) investigated in a clinical trial the effect of fat globule size on gastro-duodenal fat digestion with two emulsions differing only in fat globule size. They found out that the smaller size fat globules were lipolysed faster than the larger ones even though the plasma TAG and chylomicron responses, given as iAUC, did not differ between the emulsions during the 7 h study period (Armand, et al., 1999). Ye et al. (2017) investigated in a human gastric simulator the effects of homogenization and heating of cow's milk on the formation and breakdown of protein clots. Their study revealed that native milk forms a firm clot which traps fat globules within the clot matrix. Homogenized and heated cow's milk forms loose and fragmented clot with crumbled structure. The great number of pores in the clot structure of heated and homogenized milk led to greater hydrolysis of proteins by pepsin and resulted in a faster release of fat globules to the digesta compared to native milk. This may lead to different rates of fat digestion in the stomach but also in the duodenum. Berton and colleagues (2009) as well as Ye et al. (2010) studied *in vitro* the action of human pancreatic lipase on native milk fat globules versus homogenized milk fat globules. They revealed that fat globules from

homogenized milk are more rapidly hydrolyzed by pancreatic lipase than fat globules of native milk. According to Berton et al. (2009) there is a lag phase in the hydrolysis of native milk fat globules due to the phospholipids at the MFGM which prevent the pancreatic lipase access to the interface. With homogenized milk there is no lag time and the interface is directly accessed by pancreatic lipase. Ye et al. (2010) also suggest that bile extract may alter the lipolysis by affecting the physicochemical interactions of fat globules during digestion. Vors and colleagues (2013) found the same phenomenon, as Berton et al. (2009) and Ye et al.(2010), when they served emulsified and non-emulsified milk and milk fat to healthy male volunteers and obese male volunteers. The emulsified fat resulted in a higher and sharper chylomicron triacylglycerol peak compared to non-emulsified fat in both healthy and obese men. They observed that during the first 300 minutes after ingestion of study fats, the iAUC of the emulsified fat was greater compared to non-emulsified fat in healthy men and it was significantly greater after emulsified fat compared to non-emulsified fat in obese men. According to Vors et al. (2013) and Bourlieu et al. (2015) homogenization increases the susceptibility of milk fat to luminal digestion and impacts the further metabolic fate of dietary fatty acids. On the other hand, smaller lipid droplets can result in slower gastric emptying even though they increase the rate of lipolysis. Our study supports previous findings where the postprandial lipemia is not affected by the perhaps faster lipolysis of smaller fat globules. However, our study is not directly comparable with the previous studies as many of them were *in vitro* –studies and as the SmartBar and especially its fiber, probably influences the absorption and digestion of the milk fat globules.

Thirdly, we wanted to investigate if the self-reported symptoms relate to changes in the intestinal pressure. The pressure measured by the SmartPill capsule in the stomach, small intestine and large intestine did not differ between the two milk types and we did not observe any differences between the moving average pressure area in stomach, small intestine or large intestine nor the pressure during adverse gastrointestinal symptoms. The fact that four of our nine subjects that swallowed the capsule turned out to have low lactase enzyme activity did not influence the results as two out the four C/C genotype study subject reported more adverse gastrointestinal symptoms after ingestion of HPM, one reported equal amounts after both milk types and one had no symptoms after ingestion of HPM. However, it cannot be ruled out, that the gastrointestinal symptoms might also be related to lactose malabsorption.

To our knowledge, our study is the first study investigating the relationship between reported gastrointestinal symptoms and intestinal pressure measured by the SmartPill capsule. Cassilly

et al. (2008) reported that the SmartPill capsule leaves the stomach together with the last pieces of the meal, and this indicates the completion of the gastric phase. It has been hypothesized that the body needs to reach a fasting state after a meal before it is possible for non-digestible solids, like SmartPill capsule, to empty from stomach (Cassilly, et al., 2008). If the second meal arrives before that, the SmartPill capsule stays in the stomach. This means that the capsule would not travel with the test meal in the GI tract and that the pressure recorded would not correlate with the adverse gastrointestinal symptoms caused by the test meal. In the present study 27 % of gastric emptying times (GET) were delayed suggesting that in those cases the capsule possibly did not travel with the test meal, and the capsule may not have been recording the "wanted" pressure. This could be one reason why the pressure at adverse gastrointestinal symptom did not correlate with the AM pressure.

Finally, we aimed to study the applicability of the "smart pill system" to nutrition-related trials. Previously, studies have been carried out for comparing gastrointestinal transit times (GITT) between the SmartPill capsule and conventional methods for measuring GITT (Camilleri, 2006; Cassilly, et al., 2008; Kuo, et al., 2008; Maqbool, et al., 2009; Rao, et al., 2009), or the system has been used to asses transit times in subjects with motility disorders (Kloetzer, et al., 2010; Rao, et al., 2009; Sarosiek, et al., 2010). To our knowledge only Timm et al. (2011) and Willis et al. (2011) have used the SmartPill capsule in dietary intervention studies before us. In a cross-over clinical trial by Timm et al. (2011) ten healthy subjects ate high fiber wheat bran cereals or low-fiber control cereals for 3 days before swallowing the SmartPill-capsule. The differences in intestinal transit times were compared between the cereals. The colonic transit time and whole gut transit time was significantly shorter after the wheat bran cereals compared to low-fiber control cereals. However, the GET was extremely delayed in 20 % of the subjects because the instructed 6 hours between the test meal and a following meal was not long enough for the capsule to exit the stomach. Willis and colleagues (2011) compared appetite and GET in 14 healthy subjects after consuming macronutrient- and fiber-matched solid and liquid meals with the same energy content. The SmartPill-capsule was used to define the GET. They found a negative association between hunger and GET and a longer GET after the solid meal compared to liquid meal. Like in the study of Timm et al., also Willis faced problems with delayed GET and of the measured GETs, 25 % were delayed and had to be excluded from the study results. In the present study of ours, 27 % of measured GETs were delayed which is consistent with the two other nutrition interventions. The large size of the SmartPill-capsule has been suggested to be the reason for the capsule to "hung up" in the stomach and to skew

438 the GET. Also fasted and fed states have different motility patterns in the stomach which may
439 have affected the migration of non-digestible object from stomach to small bowel (Camilleri,
440 2006; Cassilly, et al., 2008).

441 The SmartPill technology is relatively easy to use, sensitive and none of our participants had
442 trouble swallowing the capsule. Neither Timm (2011) nor Willis (2011) reported any
443 difficulties in swallowing the capsule. However, we did face problems with patchy and absent
444 data recording as reported also by Willis (2011). We had severe interruptions in recording in
445 17 % of our SmartPill data and in those cases only the whole gut transit time could be used.
446 Willis reported that 7 % of their data had absent data points and had to be left out from the
447 analysis. The data receiver needs to be kept within 30 cm from the body, basically tied to the
448 volunteer at all times. Our volunteers were instructed not to take shower if possible and sleep
449 with the receiver; still we got absent data points. Willis reported that they had absent data points
450 mainly during night time but we could not find a similar pattern from our data. Also the high
451 cost of the equipment and the capsules is a disadvantage.

452 **4. Conclusion**

453 Our results suggest that homogenization and pasteurization of cow's milk does not influence
454 the intestinal absorption of triacylglycerols from the milk fat globules but it influences the
455 balance between intestinal absorption and clearance rates of different FAs within the measured
456 four hours timeframe. This study supports earlier studies in which no significant difference was
457 found in the amount of gastrointestinal symptoms in sensitive individuals between native and
458 processed milk. However, the obtained results on pressure in the large intestine as well as of
459 reported symptoms (large intestine pressure $P = 0.068$; adverse events $P = 0.103$) suggest that,
460 although no significant differences were found, further studies in this area are needed with a
461 bigger subject group and with longer exposure times, such as several consecutive meals, to the
462 differently processed milk types. The SmartPill technology is not without faults, but with
463 careful study design it might be useful tool also in nutrition trials.

464

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624

Figure Captions

Fig.1 Microscopy images of fat globules in native milk (left) and homogenized and pasteurized milk (right) magnified by 400 times. The globule size distribution was investigated once during the trial. Heat treatment and homogenization were done with an industrial scale dairy plant according to manufacturer's instruction. Visual evaluation of each batch was done 7 days after homogenization. No cream separation was detected.

Fig.2 Graphs created by the SmartPill software from the data recorded by the capsule as it travels through the gastrointestinal tract. Pressure is marked as red bars, pH as a green line and temperature as a blue line. A) A typical, successful SmartPill data. Gastric emptying time is marked as vertical grey line, ileo-cecal junction is marked as a vertical light green line and body exit is marked with vertical purple line. B) A test data with technical failure. Several patchy, absent data points are recognized during data collection. The software was unable to determine the gastric emptying time and body exit. The graphs A and B are from the same subject but after different study visits

Fig.3 Plasma postprandial triacylglycerol (TAG) concentrations (deviation from baseline) after homogenized and pasteurized milk (HPM, dark grey line and dots) and native milk (NM, light grey line and squares). N=11, values are mean values with SD. No significant differences were observed between NM and HPM.

Fig.4 The major fatty acids of postprandial plasma at 2 and 4 hour time points after native milk (NM, black and light grey bars) and homogenized and pasteurized milk (HPM, striped and dark grey bars). N=11, values are mean with SD. Significant differences ($p < 0.05$) are marked with asterisk.

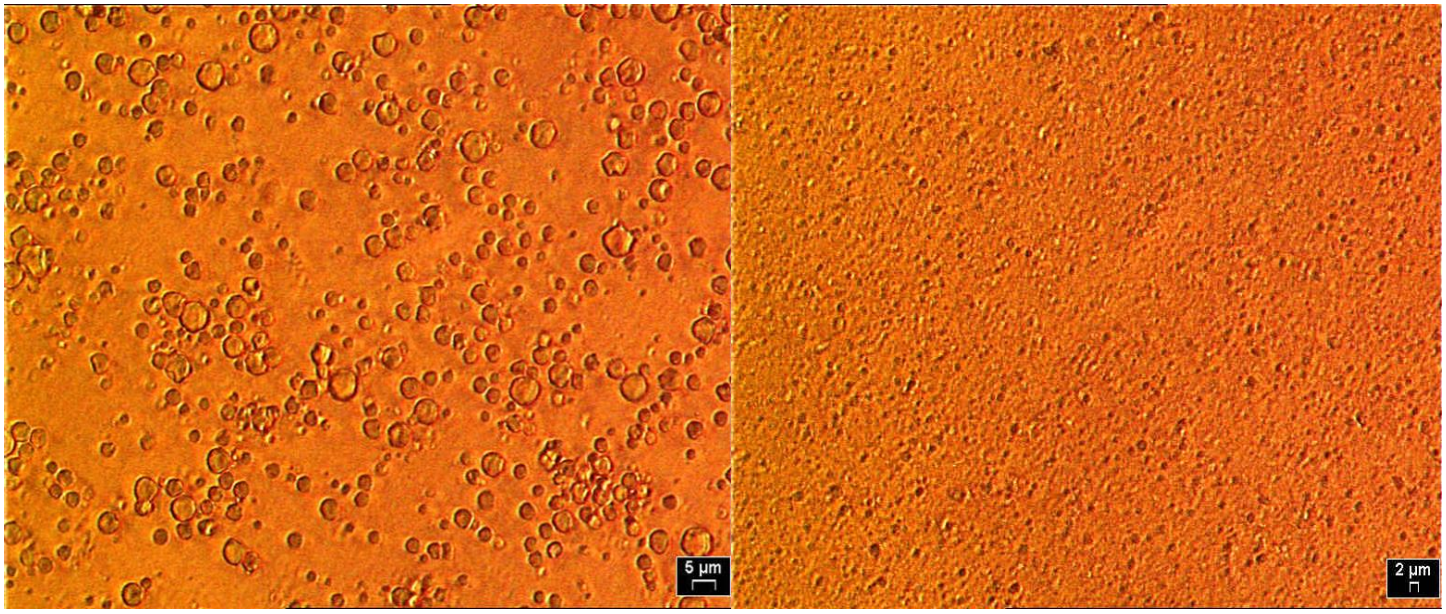

Figure 1

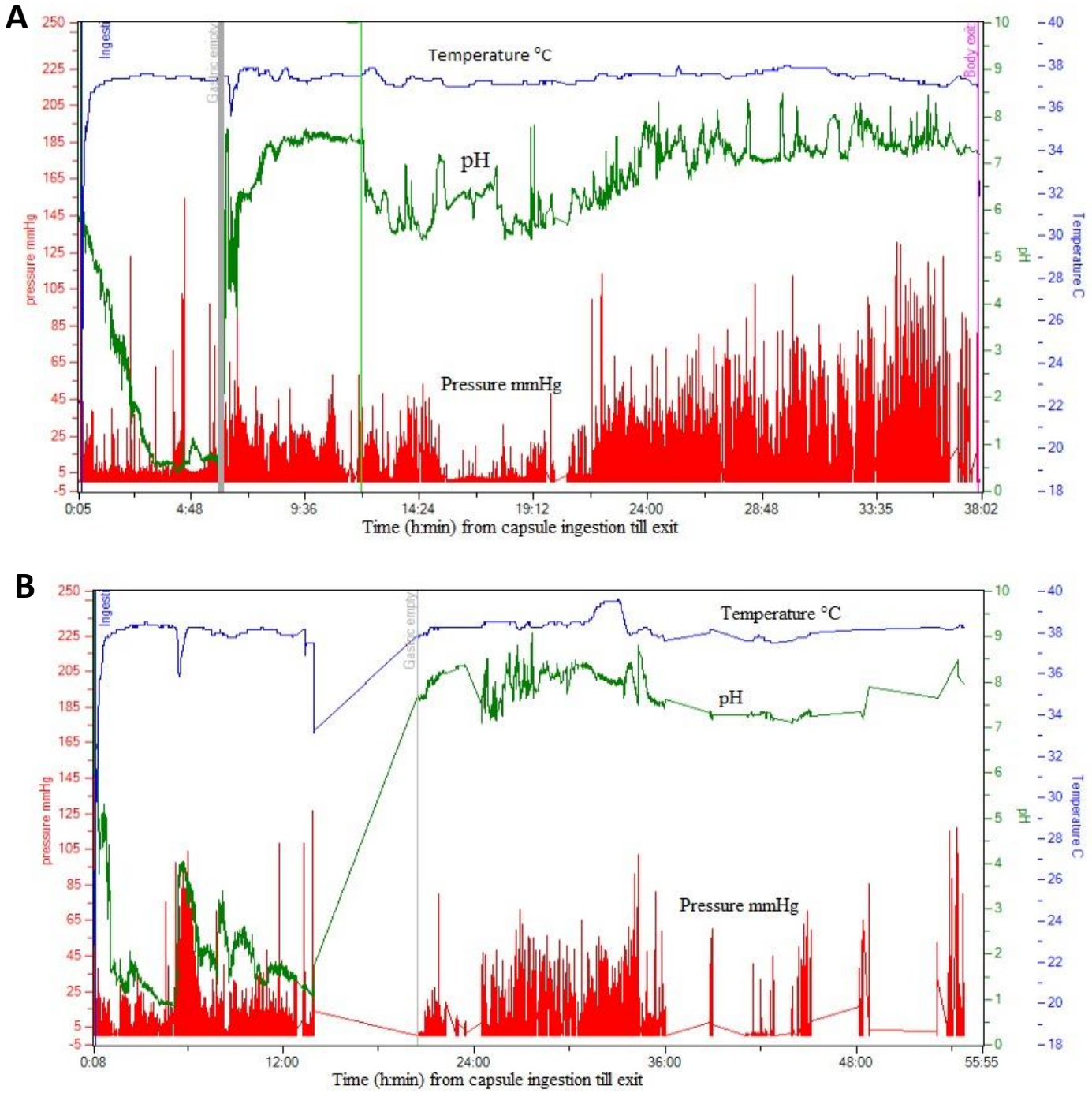
A
Temperature °C
pH
Pressure mmHg
pressure mmHg
pH
Temperature C
Time (h:min) from capsule ingestion till exit
B
Temperature °C
pH
Pressure mmHg
pressure mmHg
pH
Temperature C
Time (h:min) from capsule ingestion till exit

Figure 2

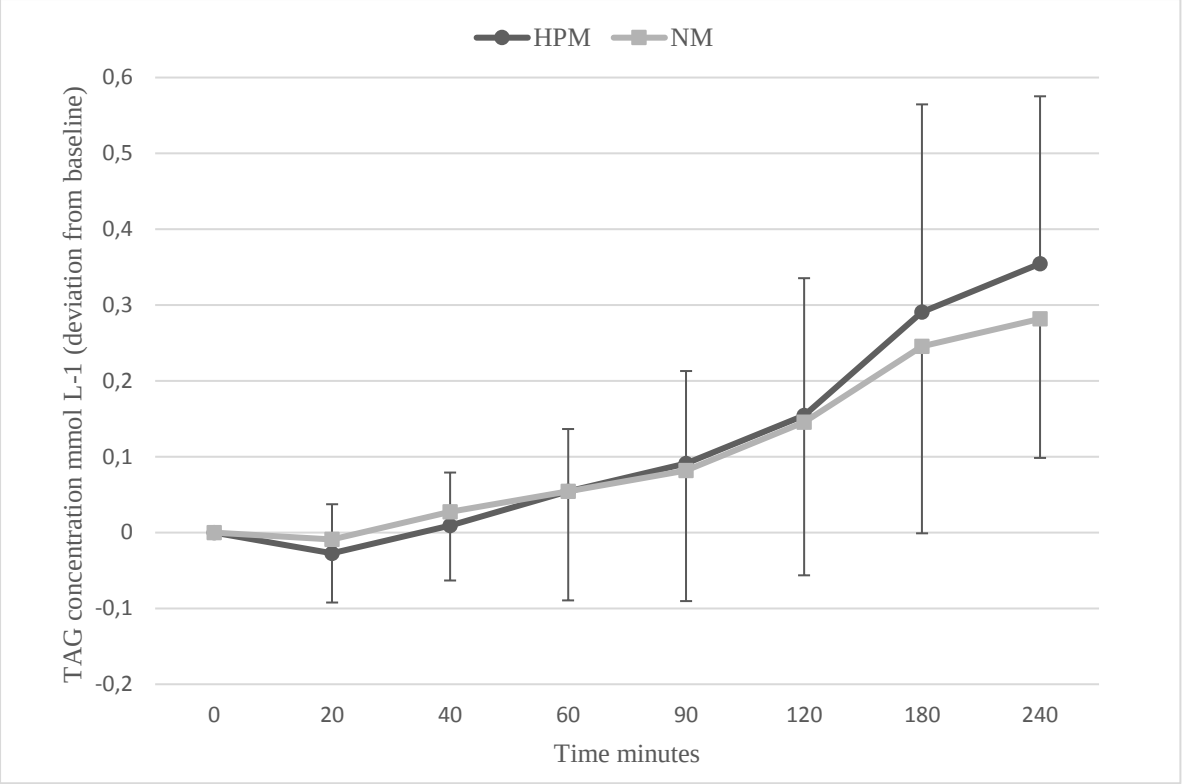
HPM
NM
TAG concentration mmol L-1 (deviation from baseline)
0,6
0,5
0,4
0,3
0,2
0,1
0
-0,1
-0,2
0
20
40
60
90
120
180
240
Time minutes

Figure 3

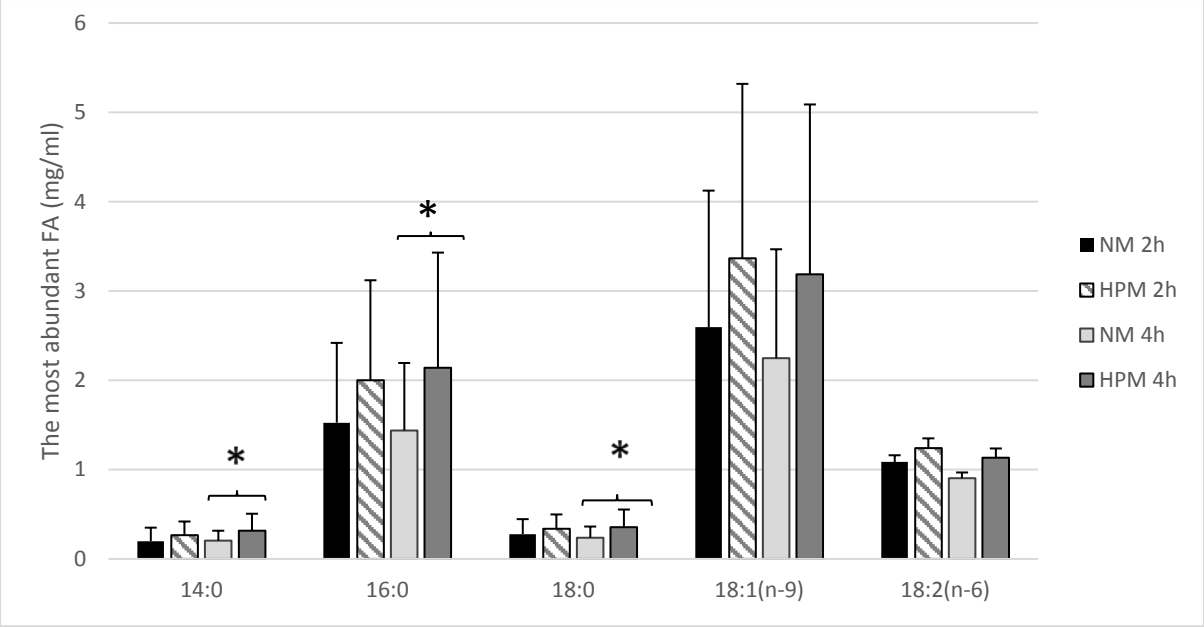
6
5
4
3
2
1
0
The most abundant FA (mg/ml)
*
*
*
14:0
16:0
18:0
18:1(n-9)
18:2(n-6)
NM 2h
HPM 2h
NM 4h
HPM 4h

Figure 4