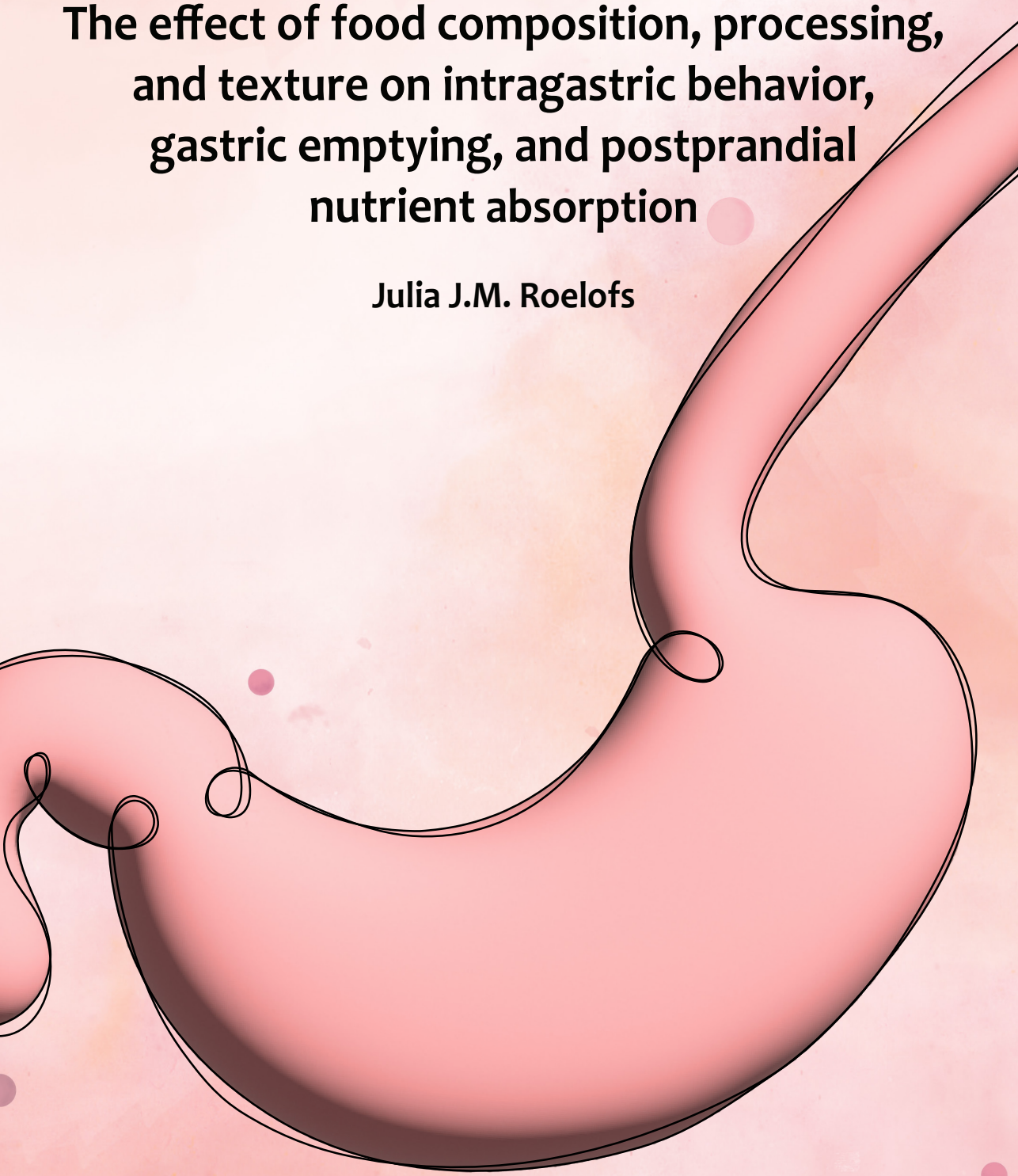
The effect of food composition, processing, and texture on intragastric behavior, gastric emptying, and postprandial nutrient absorption

Julia J.M. Roelofs



PROPOSITIONS

1. *In vitro* work is quintessential before doing *in vivo* digestion studies.
   (this thesis)
2. Individual variation is ignored in digestion research.
   (this thesis)
3. During a PhD, acquiring knowledge is secondary to developing skills.
4. All manuscript require statistical audits before submission.
5. Banning social media will benefit humanity.
6. The protein transition, although presented as a global challenge, is a problem of high-income countries.

Propositions belonging to the thesis entitled:
The effect of food composition, processing, and texture on intragastric behavior, gastric emptying and postprandial nutrient absorption

Julia J.M. Roelofs
Wageningen, 28 May 2024

The effect of food composition, processing, and texture on intragastric behavior, gastric emptying, and postprandial nutrient absorption

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Julia J.M. Roelofs

Thesis

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Prof. Dr C. Kroeze,

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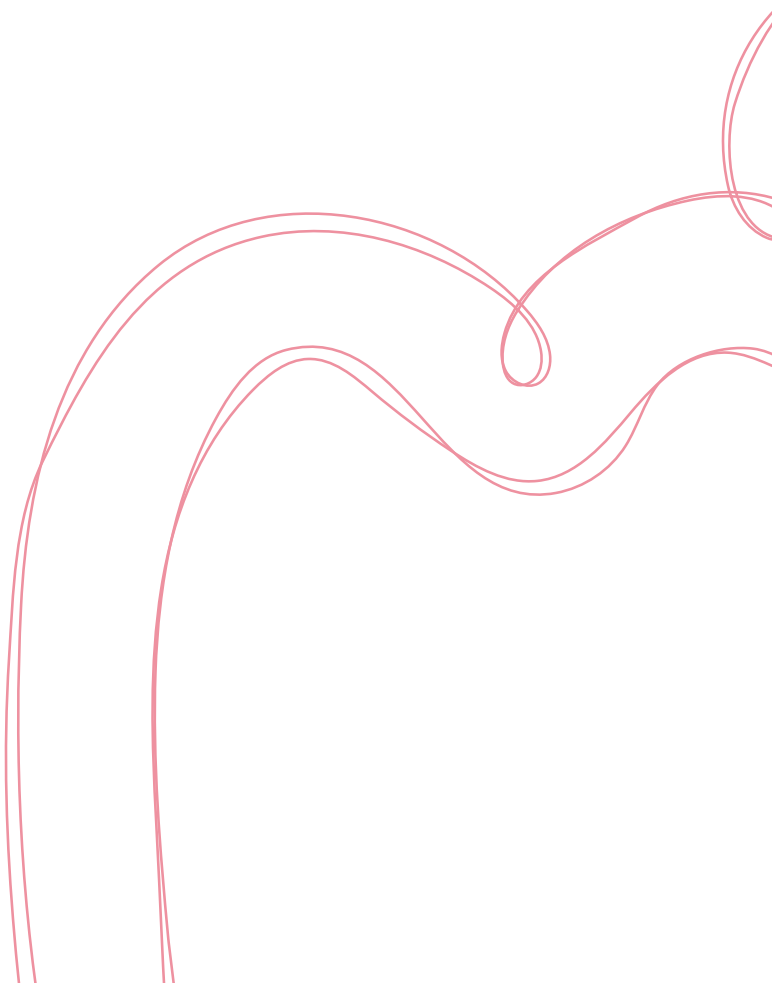
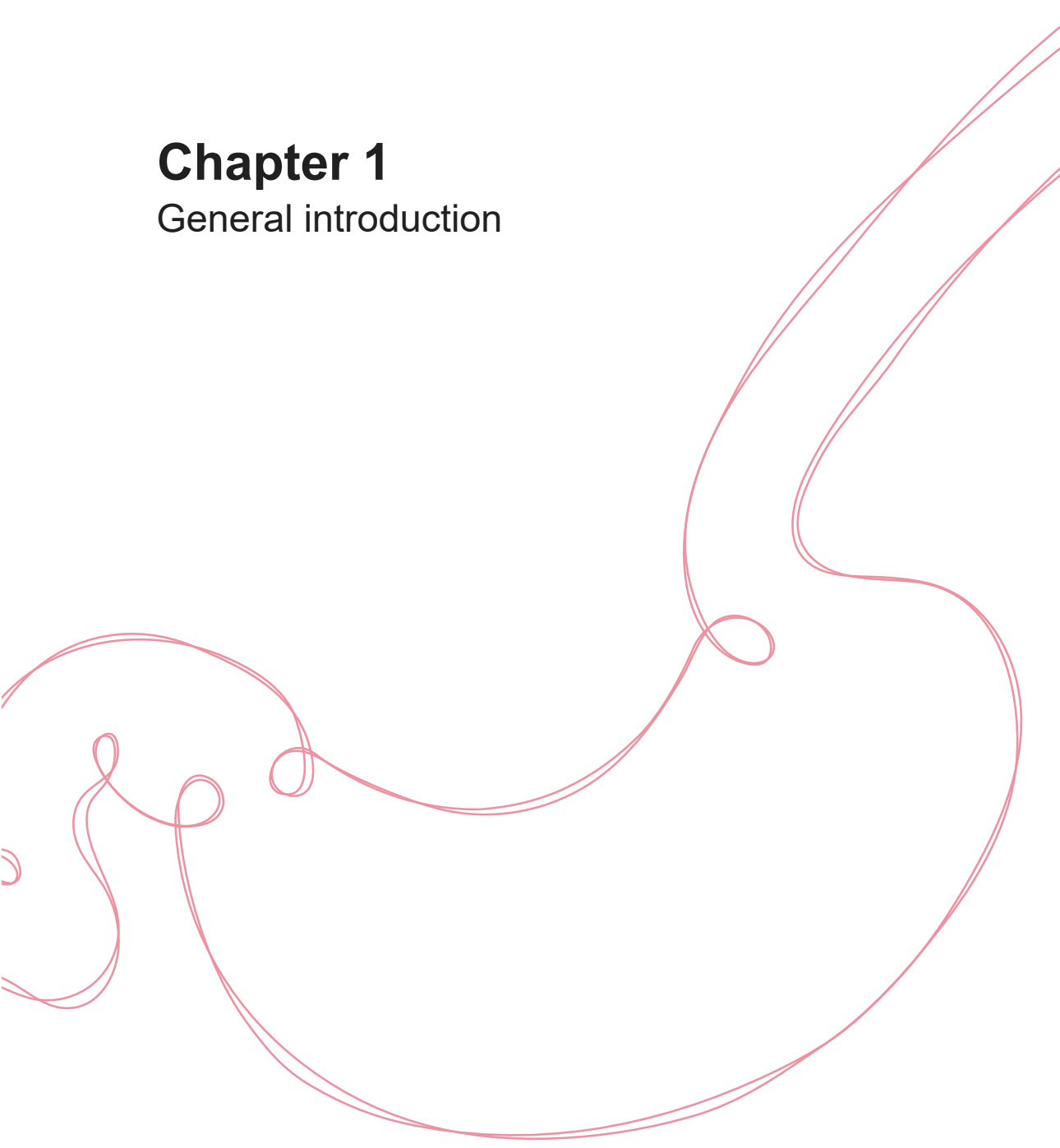
Contents



Chapter 1
General introduction



To maintain normal body composition, function, and health throughout your life, adequate food of sufficient quality is essential (WHO, 2007). Food contains three essential macronutrients: carbohydrates, fats, and proteins. Proteins provide essential amino acids, which cannot be (adequately) synthesized by our body. Amino acids are essential for the synthesis of many physiological important molecules, such as hormones, enzymes, and DNA, and regulate key metabolic processes that are vital to health, growth, development, reproduction and homeostasis (Wu, 2009).

Before our body can utilize the nutrients we consume, they need to be digested. The digestion is influenced by many factors, including their composition, how they are processed, the texture of the food, and our biological variety. These factors can either enhance or decrease the digestibility of foods. Thus, simply ingesting sufficient amounts of the nutrients we require, does not always result in sufficient absorption. It matters from which source the food comes, how it is made, in what form it is ingested, and many more factors. Not all foods are digested and absorbed to their full extent. By studying gastric digestion and post-prandial response, and thereby gaining an understanding of these processes, one might be able to optimize the digestibility and use it to its advantage. For example, with the goal of improving health, maximum utilization of nutrients or influencing the rate at which food is digested and absorbed. Especially for those who may experience difficulty ingesting sufficient nutrients, such as elderly, critically ill or athletes, this might be beneficial (Coelho-Júnior et al., 2018; Liao et al., 2017; Sieber, 2019).

Another reason is the growing interest in more sustainable food sources. The demand for proteins from more sustainable sources than animal protein is rapidly growing. However, the digestibility of plant-based proteins is generally lower compared to animal-based proteins (Berrazaga et al., 2019; Pasiakos et al., 2015). The lower digestibility of proteins from plants is explained by the intact cell wall that hinders direct contact between intracellular macronutrients and the digestive enzymes. This slows down or even completely prevents the access of proteases to the cell contents and limits intracellular protein hydrolysis. Thus, the digestibility of

plant-based proteins depends on the fraction of broken cells generated during their processing (Zahir et al., 2018). Moreover, plant-based proteins are known for their lower solubility compared to animal-based proteins, which influences digestibility (Rivera del Rio et al., 2020). Plants also contain anti-nutritional factors. These are compounds that reduce nutrient utilization and/or food intake of plants or plant products used as human foods which can be removed or inactivated by processing (Thakur et al., 2019). This lower digestibility of plant-based proteins results in suboptimal utilization of the nutrients and results in waste. Improving digestion of these proteins may thus be beneficial, such that the protein can be absorbed to its full extent (Fardet et al., 2019; Mahe et al., 1996; van Vliet et al., 2015). Thus, nutrient intake does not always reflect nutrient absorption, while absorption of sufficient nutrients is important for health. This highlights the need for further research.

The overall aim of this thesis is to obtain a better understanding of how food composition, processing, and texture affect intragastric behavior (gastric coagulation and layering), gastric emptying, and postprandial nutrient absorption. The introduction continues with an overview of gastric digestion and subsequent nutrient absorption. Next, the influence of food properties and individual variability on digestion will be discussed. After which a short insight on how gastric digestion can be measured will follow. The introduction ends with the aim and outline of this thesis.

1.1. Digestion

Digestion is the breakdown of food into particles that can be absorbed by the body. Digestion consists of a series of mechanical, physiological, and biochemical processing steps leading to the breakdown of food structures that eventually allows for absorption and utilization of nutrients (Mackie, 2019). This process starts with the oral phase, where mastication and secretion of saliva leads to the formation of a food bolus that can safely be swallowed, and continues in the gastrointestinal tract (Koç et al., 2013; Witt & Stokes, 2015).

1.1.1. Gastric digestion

After the oral phase, the bolus will travel through the esophagus to the stomach, where the gastric phase takes place. The stomach is a muscular organ that contracts (peristalsis) and is responsible for storing, mixing, and physically breaking down the bolus (Bornhorst & Singh, 2014; Chew, 2004). Gastric digestion is a crucial step in the absorption of energy and nutrients from the ingested food. Gastric fluids, secreted by the secretory cells within the stomach mucosa, play a critical role in gastric digestion by aiding in the breakdown of the ingested food. These gastric fluids are a complex combination of water, hydrochloric acid, enzymes, intrinsic factor, salts (Na^+, K^+, and Cl^-), and mucus (Chew, 2004; Martinsen et al., 2019).

Especially for protein-rich foods, gastric digestion is an important phase. Gastric fluid contains pepsinogen, which is activated to pepsin under the acidic conditions in the stomach (Chew, 2004; Heda et al., 2019; Wilson & Stevenson, 2019). These acidic conditions, with a pH between 1.4-2.0 in a fasting state, are maintained by the constant secretion of hydrochloric acid and are essential for the proper functioning of pepsin (Heda et al., 2019). Pepsin initiates the breakdown of proteins into smaller peptides via pepsin and gastric acid.

In addition to inducing these first steps of digestion, gastric conditions can induce several intragastric behaviors. Intragastric behavior can be described as changes to the food bolus in response to the gastric conditions and include gastric sieving, gastric layering, and coagulation (**Figure 1**). Gastric sieving is a phenomenon where one component of the stomach contents empties faster compared to another component (Marciani et al., 2012). It can happen when the ingested food consists of a (semi-)solid component and a liquid component. In this situation, the liquid content of the stomach empties quickly, leaving behind the (semi-)solid fraction (Hinder & Kelly, 1977; Marciani et al., 2012). Camps et al. (2017) showed that this sieving can also occur when only liquids are ingested. If a liquid meal, followed by a glass of water does not mix in the stomach, a nutrient rich layer can remain separate in the stomach. The watery, nutrient low layer will then empty from the stomach first. However, this gastric layering not only happens when water is ingested together with

a meal but can also happen when emulsions destabilize in the stomach. Emulsions are often stabilized by proteins. Under gastric condition, these will undergo pepsin-mediated protein digestion, causing the emulsion to destabilize. This results in the formation of multiple layers in the stomach, that differ in nutrient content. Another intragastric behavior is coagulation. Coagulation is a change in the structure of protein from liquid to a more solid form resulting in the formation of curds. Coagulation happens in reaction to heat, acids or enzymes. Especially caseins are prone to coagulation, while whey proteins remain soluble (Huppertz & Chia, 2021). Although gastric digestion is frequently studied, visualization of the stomach content is not frequently done. Especially for intragastric behavior, *in vivo* data is scarce, and these behaviors have rarely been quantified before.

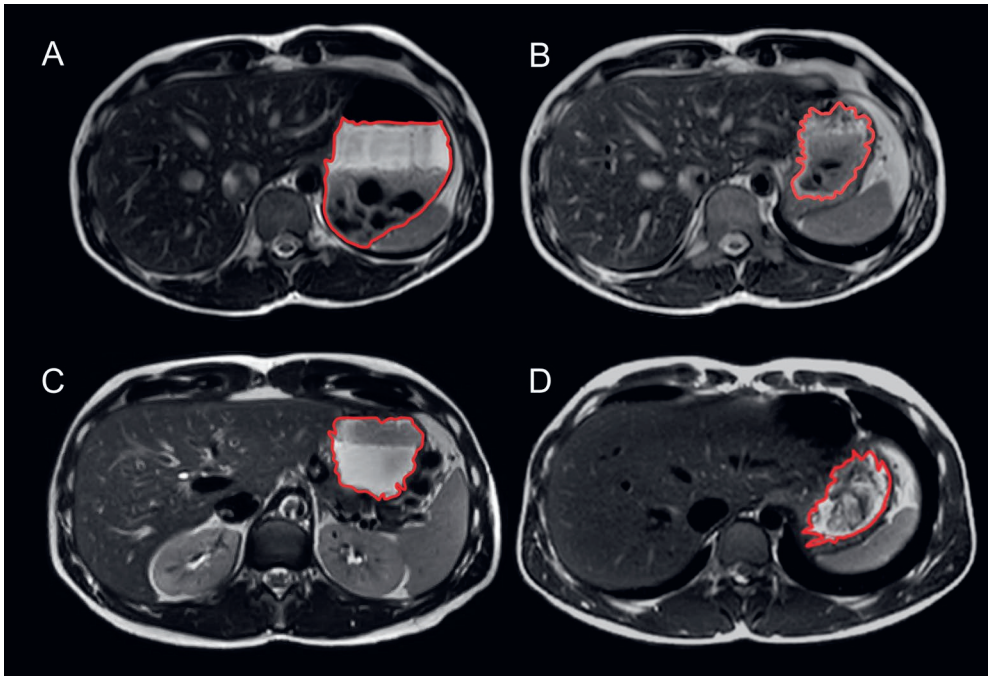


Figure 1. Examples of the intragastric behavior. A: Gastric content at t = 10 after consumption of a semi-solid together with water, where the bright top layer corresponds to the water that is ingested. B: The same participant at t = 90, where the bright top layer is now minimal, while there is still quite some semi-solid content left, indicating that gastric sieving takes place. C: Gastric content showing phase separation. D: Gastric coagulation after consumption of milk.

Moreover, the stomach's muscular contractions, known as peristalsis, further contribute to the mechanical breakdown of food, facilitating better access to digestive enzymes (Bornhorst & Singh, 2014; Chew, 2004). This combination of chemical and mechanical processes in the stomach ensures that food particles are adequately broken down into a form that can be effectively absorbed in the intestines.

1.1.2. Gastric emptying

After gastric digestion, the partially digested chyme is gradually released into the duodenum through a controlled process called gastric emptying. The dynamics of gastric emptying are influenced by physiological feedback mechanisms, involving hormonal and neuro-systemic responses (Hellström et al., 2006). The emptying is also affected by the chemical characteristics of the food; predominantly macronutrient content and energy density, and physical characteristics, such as the viscosity (Camps et al., 2016; Mackie, 2019; Marciani et al., 2001; Roy et al., 2022). The food matrix plays an important role in digestibility because of its influence on the kinetics of transit and hydrolysis of macronutrients. For example, liquids empty faster from the stomach compared to semi-solid foods with equal nutrient density (Camps et al., 2016; Clegg & Shafat, 2014; Mackie et al., 2013; Zhu et al., 2013). The pylorus can only pass particles smaller than 0.5 – 2 mm, thus solid foods need to be sufficiently broken down before they can be passed to the intestine (Kelly, 1980; Marciani et al., 2001; Meyer, 1980).

Moreover, it is known that the intragastric behaviors discussed above can affect gastric emptying. For example, when caseins coagulate, proteins and fat globules are captured in the curd. Since the pylorus cannot pass these curds, they need to be broken down first. This results in the initial emptying of the liquid fraction and slowing down of the more solid fraction that contains the caseins and fat (Huppertz & Chia, 2021). The same happens with gastric sieving and gastric layering, where one fraction, often the nutrient poor layer, empties out first. Again, these effects have not yet been extensively studied *in vivo* yet.

1.1.3. Small intestine

When entering the duodenum, a mixture of enzymes, bile and bicarbonate are added to the chyme. The enzymes further digest the food, after which the majority of the nutrients are absorbed (Joye, 2019; Mackie, 2019). Once transported inside the intestinal cells, the nutrients are used for energy or synthesis of compounds. When they are not used by the cell itself, they are transported into the blood stream (Joye, 2019).

Gastric emptying determines the delivery of the food bolus to the duodenum and can therefore affect (the rate of) intestinal digestion and absorption. In addition, the hormones (CCK, GLP-1, PPY) that are secreted in response to the nutrients present can slow down gastric emptying (Moran et al., 2021). This highlights the influence of intragastric behavior on digestion, where the fractions of the food bolus that enter the small intestine is affected. Nutrient absorption kinetics are frequently studied, but combining the assessment of gastric digestion and absorption kinetics is unique.

1.2. Food properties

Gastric digestion and nutrient absorption are influenced by many factors, this includes the composition of the food, how they are processed and their texture. The digestion of an individual isolated nutrient is different compared to the same nutrient imbedded in a food matrix. The different compounds in foods interact physically and chemically with each other and thereby influence accessibility, stability and digestibility (Aguilera, 2019). For example, texture influence the rate of eating/need for oral mastication, the accessibility to enzymes, but also gastric emptying and thus absorption. The presence of certain compounds or structures can hinder or enhance digestion. An example is the presence of minerals in dairy. Caseins in milk form casein micelles with calcium, phosphate, and magnesium. Changing the mineral composition is shown to alter the coagulating properties *in vitro*, and might therefore alter the (rate of) digestion (Huppertz & Chia, 2021).

An example where structures can hinder digestion is the intact cell wall in plants that hinders direct contact between intracellular macronutrients and their digestive

enzymes. By processing, these cells can be broken down, thereby allowing access to the intracellular macronutrients (Zahir et al., 2018). Moreover, processing can also remove or inactivate the anti-nutritional factors found in plants, which are compounds that reduce nutrient utilization (Thakur et al., 2019). This shows that processing of food can be important for digestion and optimal utilization of nutrients. Food processing, such as gelling, enzymatic hydrolysis and heating, alters the chemical and physical characteristics and can significantly alter digestion under gastric conditions (Joye, 2019; Loveday, 2022). Heat treatment is a frequently used processing method that is known to impact digestion, especially that of proteins. Depending on the type of protein, the temperature and time of the heating, the digestibility of the protein may either improve or decrease. Proteins either lose their tightly folded structure, which results in higher accessibility of the peptide chain for enzymes, or aggregate, which impairs digestion. Moreover, the pH, ionic strength and food matrix will also influence the effect of heat treatment on protein digestibility (Joye, 2019). For example, Ye et al. (2019) showed that heat treatment of milk affected the coagulation of protein, and the formation and structure of the coagulates. Subsequently, this change can alter the rate of protein hydrolysis, gastric emptying, and absorption. In addition to coagulating properties, heating can also affect gastric emulsion stability (Ye et al., 2020). Particularly whey proteins are susceptible to heat treatment. Denatured whey proteins are able to form complexes with caseins (Anema et al., 2004; Fox et al., 2015; Guyomarc'h et al., 2009; Mulet-Cabero et al., 2019). In turn, these whey-casein complexes increase emulsion stability (Raikos, 2010). Using processing to change food properties can be used to our advantage in developing certain products, such as infant formula where emulsion stability is important.

Although the effects of processing are quite well studied in animal-based proteins, there is more research needed on the effects of processing on digestion and absorption of plant-based proteins. The aforementioned effects of processing on digestibility show the potential that processing might also have in plant-based proteins. With the shift towards a more plant-based diet, isolated plant-based proteins are consumed more often. The isolation of plant-based proteins often

includes a thermal denaturation step, which may either improve or decrease their digestibility. *In vitro* work on pea protein showed that heating disrupts the structure, thereby increasing the number of smaller better digestible particles. Conversely, these heat-induced aggregates are up to 50% less digestible compared to before the heat treatment (Mulet-Cabero et al., 2019; Rivera del Rio et al., 2020). However, the effect of this is not yet studied *in vivo*.

The above highlights the many factors involved factors that can improve or decrease the digestibility and should be considered.

1.3. Biological variety

Not only the chemical and physical characteristics of food influence digestion, but the morphology and physiology of the gastrointestinal tract is influenced by personal factors such as age, sex, and BMI (Stillhart et al., 2020). This variability is present in gastric juice secretion, gastric emptying, transit times, and nutrient absorption. For example, fasted gastric secretion rate was found to be positively correlated with body weight, being male, and age (Baron, 1969; Goldschmiedt et al., 1991; Hassan & Hobsley, 1971; Kekki et al., 1982; Vakiland & Mulekar, 1965; Whitfield & Hobsley, 1987). In turn, fasted gastric volume content is known to influence digestion and intragastric behaviors (Camps et al., 2021).

Moreover, gastric emptying is known to be longer in woman. However, post-menopausal women tend to have similar gastric emptying as men (Stillhart et al., 2020). Studies on age-related effects on gastric emptying are conflicting, but a review of Stillhart et al. (2023) indicates that healthy aging might be related to slightly slower gastric emptying of both liquid and solid meals. Studies on BMI are inconsistent, with some studies showing no difference between obese people and controls, some showing a slower or faster gastric emptying (Stillhart et al., 2020).

In addition to the factors mentioned above, many others can influence the digestive tract, including body size, diseases, pregnancy, diet, and ethnicity (Stillhart et al., 2020).

1.4. Methods

The digestion of food products is predominantly studied with *in vitro* digestion models. However, digestion consists of many complex processes, making it challenging to study *in vitro*. These models are based on *in vivo* data, but they cannot account for all the complex processes, factors such as the mixing of the food in the stomach or the individual variability (Muttakin et al., 2019). Therefore, *in vivo* research is needed to understand to what extent *in vitro* digestion models represent *in vivo* digestion.

There are several techniques to measure gastric emptying, both indirect and direct. Indirect measurements include C-isotope breath analysis, direct measurements include scintigraphy and MRI. Magnetic Resonance Imaging (MRI) is a non-invasive and direct approach to measure gastric emptying. The advantage over scintigraphy is that MRI does not expose participants to radiation. It uses magnetization in combination with radiofrequency (RF) pulsus to obtain RF pulses from nuclei of interest. Generally, these nuclei of interest are water protons (^{1}H). The magnetic field lines up these water protons in the body and exposes them to a specific RF pulse which will lead to excitation of these protons. When these protons relax again, they emit an RF signal which can be measured with the coil. This signal varies based on the tissue properties, which allows us to visualize and differentiate between the different tissues of the body (Berger, 2002).

The anatomical detail provided by MRI allows accurate visualization of the stomach and its contents, and thus gastric emptying (de Zwart & de Roos, 2010). With these images, gastric content volume can be calculated based on manual delineation of every slice (**Figure 2**). In addition, MRI allows for differentiation between different food fractions, which can be useful if a meal consists of multiple components. In addition to the assessment of gastric content volume, MRI can capture intragastric behavior, such as phase separation and coagulation (Smeets et al., 2021). Although these intragastric behaviors have been visualized before, it has hardly been quantified before.

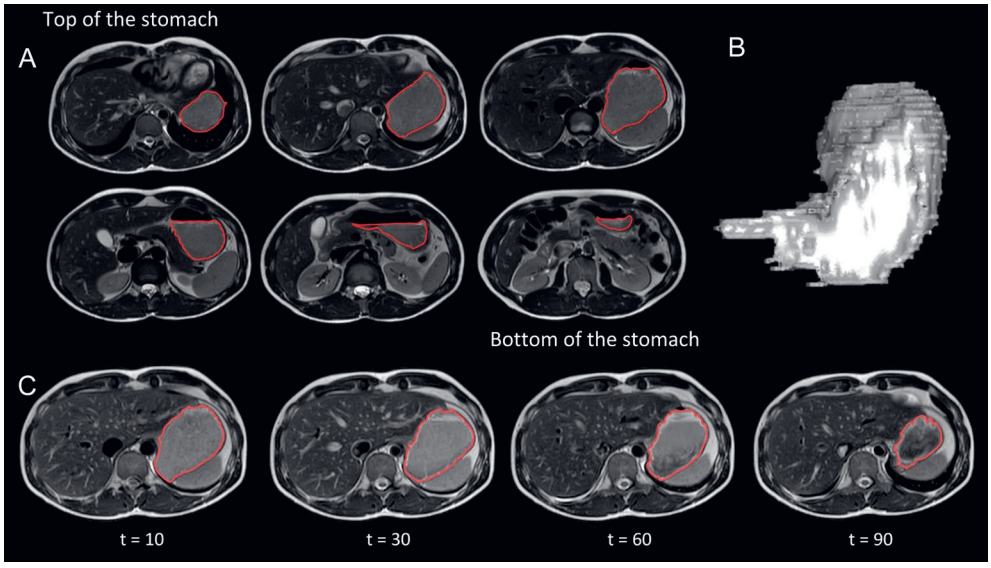


Figure 2. A) Cross-sectional slices from top to bottom at one timepoint in which the stomach is delineated in red. B) 3D rendering of all delineated slices, showing total gastric content. C) Time series of gastric volume over time.

1.5. Aim and outline of thesis

The above highlights the complexity of the digestion of food and the many factors that are involved. Having a thorough understanding of these processes can contribute to improving digestibility with the goal of improving health, maximum utilization of nutrients or influence the rate at which food is digested and absorbed.

Although there is a substantial amount of *in vitro* data on gastric digestion and intragastric behavior, *in vivo* data is still relatively scarce. This highlights the need to not only further exploration *in vivo*, but also to combine *in vitro* and *in vivo* digestion models to validate the representation of the model. Moreover, combining the measurements of *in vivo* gastric digestion with absorption kinetics will give new, and more complete insights in digestion.

The overall aim of this thesis is to obtain a better understanding of how food composition, processing, and texture affect intragastric behavior (gastric coagulation

and layering), gastric emptying, and postprandial nutrient absorption. This was studied using abdominal MRI in combination with blood sampling to assess postprandial absorption kinetics. In addition, side-by-side *in vitro* experiments were performed (**Figure 3**). Specific questions addressed in this thesis are:

- Does casein mineralization affect gastric coagulation, and how does this affect gastric emptying?
- What is the effect of minimal processing and alteration of the fat-globule interface in infant-formula on emulsion stability, gastric emptying, and the postprandial plasma metabolome compared to a control IF (CF)?
- What is the effect of heat treatment and food texture on intragastric behavior, gastric emptying, and amino acid absorption kinetics of pea protein?
- What are the intra- and interindividual variation in fasted gastric content and to what extent is this variation associated with age, sex, weight, and height?

Chapter 2 explores the effect of casein mineralization in milk on gastric coagulation and the subsequent effects on gastric emptying and amino acid absorption kinetics. **Chapter 3** explorers the potential of minimal processing and altering the fat-globule interface to change the emulsion stability. Thereby aiming at earlier phase separation in the stomach and consequently a phased release of nutrients in the intestine as observed with breastfeeding. This was measured with gastric MRI and post-prandial fat absorption kinetics. Although there is ample information about the digestion of animal-based protein, *in vivo* data on plant-based proteins is often still lacking. Pea protein is one of the more promising plant-based proteins due to its wide availability, low cost, and relatively balanced amino acid profile. **Chapter 4** therefore studies the effect of heat treatment and food texture on intragastric behavior, gastric emptying, and nutrient absorption by comparing an unheated and a heated drink with a heated semi-solid. As large variations in fasted gastric content were observed within the trials, data of 24 studies measuring gastric volume with MRI were pooled to assess this variation in **Chapter 5**. Intra- and interindividual differences were analyzed, including the influence of age, sex, weight, and height. In **Chapter 6** the overall results and their implications are discussed and suggestions for future research are given.

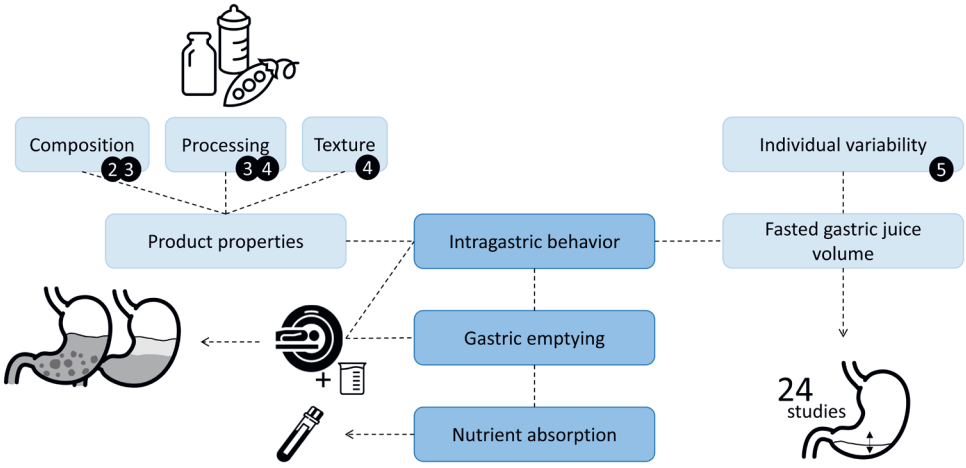


Figure 3. Schematic overview of this thesis. The numbers indicate the different chapters.

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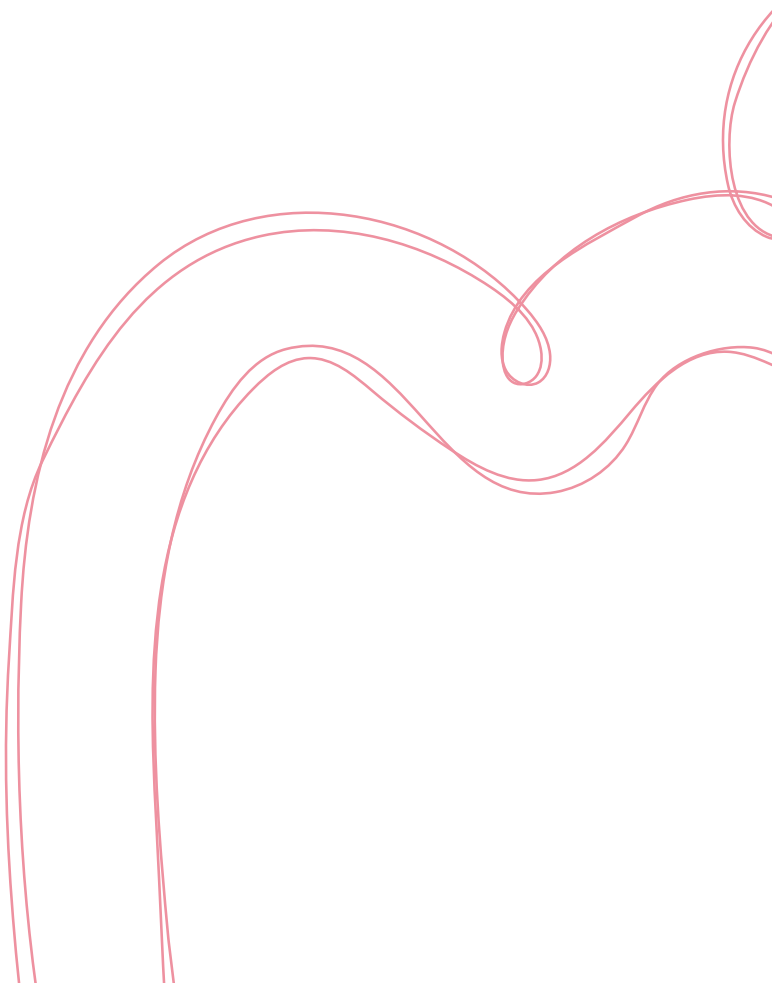

Chapter 2

Gastric coagulation and postprandial amino acid absorption of milk is affected by mineral composition: a randomized crossover trial

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ABSTRACT

Background: *In vitro* studies suggest that casein coagulation of milk is influenced by its mineral composition, and may therefore affect the dynamics of protein digestion, gastric emptying, and appearance of amino acids (AA) in the blood, but this remains to be confirmed *in vivo*.

Objective: This study aimed to compare gastrointestinal digestion between two milks with the same total calcium content but different casein mineralization (CM).

Design: Fifteen males (age 30.9±13.8 y, BMI 22.5±2.2 kg/m2) participated in this randomized cross-over study with two treatments. Participants underwent gastric magnetic resonance imaging (MRI) scans at baseline and every 10 min up to 90 min after consumption of 600 ml milk with low or high CM. Blood samples were taken at baseline and up to 5 hours postprandially. Primary outcomes were postprandial plasma AA concentrations and gastric emptying rate. Secondary outcomes were postprandial glucose and insulin levels, gastric coagulation as estimated by image texture metrics, and appetite ratings.

Results: Gastric content volume over time was similar for both treatments. However, gastric content image analysis suggested that the liquid fraction emptied quicker in the high CM milk, while the coagulum emptied slower. Relative to high CM, low CM showed earlier appearance of AAs that are more dominant in casein, such as proline (MD 4.18 µmol/L, 95%CI [2.38-5.98], p<0.001), while there was no difference in appearance of AAs that are more dominant in whey protein, such as leucine. The image texture metrics homogeneity and busyness differed significantly between treatments (MD 0.007, 95%CI [0.001, 0.012], p=0.022; MD 0.005, 95%CI [0.001, 0.010], p=0.012) likely because of a reduced coagulation in the low CM milk.

Conclusions: Mineral composition of milk can influence postprandial serum AA kinetics, likely due to differences in coagulation dynamics.

Keywords: milk, digestion, casein, micellar calcium, gastric emptying, amino acids, glucose, insulin

1. INTRODUCTION

Protein is an essential macronutrient for many functions in the human body (Coelho-Junior et al., 2018; Sullivan et al., 1999). Consuming sufficient protein can be a challenge. Therefore, optimal digestion and absorption of the consumed protein support bioavailability of amino acids (AA) (Fardet et al., 2019; Mahé et al., 1996). A common source of protein is bovine milk (van Rossum, 2018), which generally contains about 3.5% protein, of which caseins represent around 80% and whey proteins around 20% (Haug et al., 2007). While whey protein remains soluble, casein coagulates in the stomach when casein micelles are destabilized by pepsin proteolysis (Boirie et al., 1997). This leads to the formation of curds containing protein and fat globules that impact overall digestion kinetics, such as casein digestion and absorption (Ye et al., 2019).

Previous studies, predominantly *in vitro*, suggest that casein coagulation is affected by several factors, including processing-induced protein modifications, overall product composition, including mineral composition, and variations in gastric acidification and protease secretion (Corredig & Salvatore, 2016; Horstman & Huppertz, 2022; Huppertz & Chia, 2021; Mulet-Cabero et al., 2020). It is important to fully understand the effect of processing-induced protein modifications of milk on coagulation since coagulation could influence the rate at which protein empties from the stomach and thereby affect protein digestion and absorption kinetics (Roy et al., 2022). Gastric emptying (GE) is the rate-limiting step in the delivery of nutrients to the small intestine for further breakdown and absorption (Kong & Singh, 2008). Gastric emptying is mainly influenced by energy density and the viscosity or structure of the gastric chyme (Camps et al., 2016). However, compared to whey proteins, the coagulated casein fraction of milk empties later, because the stomach only empties particles sized below 1–2 mm (Elashoff et al., 1982; Kong & Singh, 2008). Accordingly, whey proteins, which remain soluble, have a higher GE rate than caseins (Aguilera, 2019; Sakata et al., 2022). Processing of milk alters its functional properties including casein and its coagulation (Dalgleish & Corredig, 2012), which could influence GE and related digestion kinetics. This is supported by recent *in vivo* work showing that the processing of casein and the resulting alterations in the

product matrix can have a strong effect on postprandial AA responses. For instance, cross-linked sodium caseinate was more rapidly digested than micellar casein and calcium caseinate and upon the ingestion of dairy products containing 25 g protein, and a higher increase in EAA concentrations in blood was observed after consumption of yoghurt, compared to milk and cheese (Horstman et al., 2021; Trommelen et al., 2020). Thus, the degree of casein coagulation in the stomach could affect the dynamics of gastric protein digestion, stomach emptying, and subsequent intestinal digestion and absorption of AA.

Both the physical (e.g., compactness, hardness and elasticity, size of fat globules) and the chemical parameters (e.g., protein/lipid ratio, P/Ca ratio) can influence the milk matrix and could therefore affect the bioavailability of AA (Fardet et al., 2019). *In vitro* studies indicate that casein coagulation is affected by mineral composition(Yang et al., 2023) since caseins form casein micelles with calcium, phosphate, and magnesium (Farrell et al., 2006). Partial decalcification of casein micelles results in looser-formed gastric clots and greater proteolysis (Zhang et al., 2023). Casein mineralization (CM) also affected the coagulation of milk proteins in a model infant formula (Huppertz & Lambers, 2020). The effect of CM on coagulation was followed up by a study on the coagulating behavior of bovine casein micelles under infant, adult, and elderly conditions where gastric coagula became looser and the formation of free amino groups and small peptides increased with an increasing level of decalcification(Wang et al., 2023). Knowing whether and how proteins coagulate is relevant for applications in infant formula and possibly for milk tolerance, since soft curds may be linked to less GI symptoms (van Eijnatten et al., 2023). Human milk has a more open coagulum, while cow milk has a more dense coagulum, which is likely more difficult to digest (Roy et al., 2020). Mineralization is a factor of interest since a low mineralization would result in a softer, less dense coagulum which is potentially easier to digest. In infants this may be linked to reduced digestive complications such as cramping or abdominal pain (Hill, 1931; Van de Heijning et al., 2014). This remains to be shown in clinical studies.

However, thus far, only *in vitro* or indirect (by measuring AA kinetics) *in vivo* studies have been done, in which other product differences than only mineralization were studied. MRI could be a helpful tool in assessing casein coagulation. Currently, the main use of MRI in gastric research is measuring GE (Smeets et al., 2020; Spiller & Marciani, 2019). But MRI can also be used to visualize intragastric processes, such as changes from liquid to solid state, gastric sieving and phase separation (Camps et al., 2017; Marciani et al., 2012), which is an advantage over ultrasound or tracer-methodology. Since gastric protein coagulation involves a change from a liquid to a semi-solid state, MRI could potentially be used to quantify the degree of coagulation. So far, gastric coagulation has only been visually assessed using MRI (Coletta et al., 2016), however image texture analysis may provide a more objective and accurate quantification (Smeets et al., 2020).

This study aimed to compare gastrointestinal digestion (coagulation, GE, and postprandial AA dynamics) between skimmed bovine milks with the same total calcium content but a different degree of CM. We hypothesized that gastric protein coagulation would be more prominent in milk with higher CM and consequently delay gastric protein emptying, serum AA appearance, and related glycemic responses.

2. PARTICIPANTS AND METHODS

2.1. Design

This study was a randomized, single-blinded, crossover study with two treatments. Washout was at least one week, and sessions were a maximum two months apart. Primary outcomes were postprandial plasma amino-acid concentrations and gastric emptying rate. Secondary outcomes were postprandial glucose and insulin levels, gastric coagulation, and other product instabilities if visible and appetite ratings obtained after each MRI measurement. The results are presented in the order of (I) coagulation, (II) gastric emptying, (III) AAs, (IV) insulin / glucose and (V) appetite ratings.

2.2. Participants

Healthy males, aged 18-55 years, BMI 18.5-25.0 kg/m^2, were recruited from the Wageningen area from December 2020 to March 2021. Because there may be sex differences in gastrointestinal function (Lajterer et al., 2022; Soldin & Mattison, 2009), males were chosen as the study group. Exclusion criteria were bovine milk allergy, lactose intolerance (either self-reported or diagnosed), gastric disorders or regular gastric complaints, such as heartburn, use of proton pump inhibitors or other medication which alters the normal functioning of the stomach, recreational use of drugs, within one month prior to the pre-study screenings day, alcohol consumption of more than 14 standard units/week, being vegan, smoking, or having a contraindication to MRI. Because there are sex differences in gastrointestinal function (Lajterer et al., 2022; Soldin & Mattison, 2009), males were chosen as the study group. The sample size was calculated for the first primary outcome, the postprandial AA profile, based on the expected difference in peak serum AA concentration. We estimated the peak difference to be 0.4 pmol/ml, with an SD of 0.3 pmol/ml based on an earlier study performed with differently treated dairy products that are comparable to these study products (Horstman et al., 2021). The power was set to 0.9. This resulted in an estimated sample size of n=14. However, to account for a possible smaller difference, it was decided to include 15 participants. Fifteen males (age: 30.9±13.8 years; BMI: 22.5±2.2 kg/m^2) completed this study. See the flow diagram in **Supplementary figure 1**. The study was conducted according to the principles of the Declaration of Helsinki (October 2013) and registered with the Dutch Trial Registry under number NL8959 accessible through https://trialsearch.who.int/Trial2.aspx?TrialID=NL8959. All participants provided written informed consent.

2.3. Study procedures

On the day before each test session, participants consumed a standardized 500-kcal rice dinner, which they could finish or eat less if they preferred. There were no restrictions on the beverage consumed with the meal. After this, participants fasted for at least 12 hours until the test session the following morning. Drinking water was allowed up to 1.5 hours before the session. Participants were instructed to refrain

from heavy sports starting from the day before the test session and to use the same mode of transportation on both test days. Participants arrived at the hospital Gelderse Vallei, Ede, The Netherlands, at 8 or 10 AM and were measured at the same time on both study days. **Figure 1** shows an overview of a test session. Upon arrival, an intravenous (IV) cannula was placed in an antecubital vein. Baseline measurements consisted of appetite ratings, an abdominal MRI scan, and a blood sample. Subsequently, participants were instructed to consume 600 ml of a test product at ~7 degrees within five minutes, but all finished between one and four minutes. Gastric MRI scans were performed at baseline and every ten minutes, starting at T = 10 min up to 90 minutes after the start of consumption. During the MRI session, participants verbally rated subjective appetite: hunger, thirst, fullness, desire to eat, and prospective consumption on a scale from 0 (not at all) to 100 (most imaginable) at each time point (Blundell et al., 2010; Hjermstad et al., 2011). Blood samples were drawn at baseline and at T = 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 270, and 300 min.

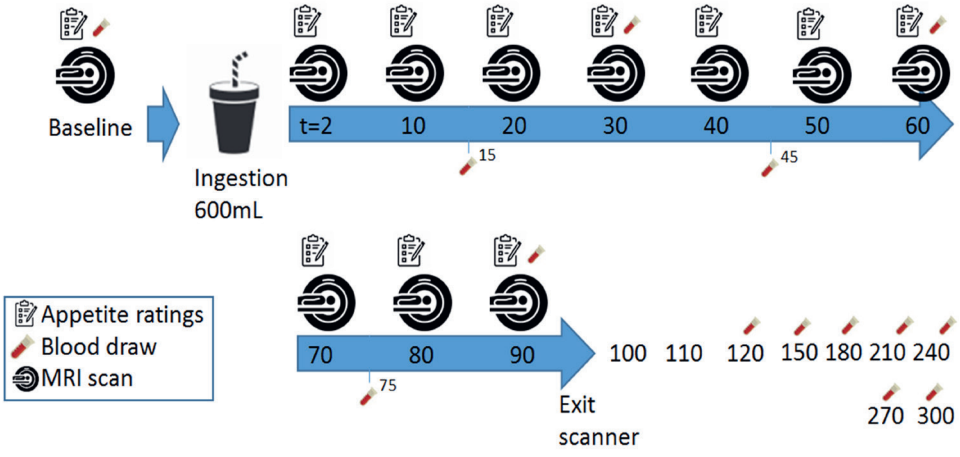

Figure 1. Overview of a test session.

The test products were two skimmed milks with different micellar mineral composition. The low CM product was pasteurized skimmed milk (FrieslandCampina, The Netherlands) with 20 mM added trisodium citrate, resulting

in a degree of CM of 4.3 mmol protein-associated Ca/10 g casein, determined as described previously (Huppertz & Timmer, 2020). Adding citrate alters the micellar calcium content and thereby the casein micelle integrity (Priyashantha et al., 2019). To maintain a similar buffering capacity in the high CM product, disodium hydrogen phosphate was added to skimmed milk at a level of 20 mM, resulting in a degree of CM of 8.9 mmol protein-associated Ca/10 g casein. Solutions were made at the same time in the afternoon preceding the test day and were slowly mixed with a magnetic stirrer at 4° C overnight to create an equilibrium of micellar and non-micellar calcium. The difference in gastric protein coagulation was determined by wet weight measurements of the coagulates during *in vitro* gastric digestion (**Supplementary figure 2**). The nutritional value of both test products per 600 ml prepared product (calculated using label information) was 831 kJ/195 kcal, 0 g fat, 27 g carbohydrate, 21 g protein and 762 mg calcium. Participants were randomly allocated by block randomization using randomizer.org to receive either the low CM or the high CM milk first. The milks were similar in appearance and taste and participants were blinded to the milk they received.

2.4. MRI

Participants were scanned in a supine position with the use of a Philips Ingenia Elition X 3.0T MRI scanner. A T_2-weighted ~20-s 2-D Turbo Spin Echo sequence (37 4-mm slices, 2 mm gap, 1 x 1 mm in-plane resolution, TR = 550 ms, TE = 80 ms, flip angle: 90 degrees) was used with breath-hold command on expiration to fixate the position of the diaphragm and the stomach. The software Medical Imaging Processing And Visualization (MIPAV, version 11.0.3) was used to do a bias field correction and manually delineate gastric content on every slice(McAuliffe et al., 2001). Volumes on each time point were calculated by multiplying the surface area of gastric content per slice with slice thickness, including gap distance, and summed over the total number of slices showing gastric content. To assess changes in gastric coagulation, image texture analysis of the stomach content was performed using the software LIFEx (version 7.2.0, Institut national de la santé et de la recherche médicale, France) (Nioche et al., 2018) as previously applied in human *in vivo* MRI research on casein coagulation (van Eijnatten et al., 2023). Homogeneity,

coarseness, contrast, and busyness were calculated. These image metrics provide information on the spatial patterns of voxel intensity (Thomas et al., 2019). The Gray-Level Co-occurrence Matrix (GLCM) method was used for homogeneity (degree of similarity between voxels) and Neighborhood Gray-level Difference Matrix (NGLDM) difference of grey-levels between one voxel and its 26 neighbors in 8 dimensions was used for contrast (local variations), coarseness (spatial rate of change in intensity) and busyness (spatial frequency of changes in intensity). The number of grey levels for texture metric calculation was set at 64, intensity rescaling at relative (ROI: min/max), and dimension processing at 2D. On each postprandial time point, texture metrics were calculated per slice for the stomach content. Subsequently, a weighted average texture metric was calculated based on the gastric content volume in each slice such that slices with little stomach content contributed less to the average than those with more stomach content. To quantify the (relative) volume of liquid and semi-solid stomach contents the number of lighter (more liquid), intermediate and darker (semi-solid) voxels was calculated by determining intensity thresholds with the use of Otsu's method (Otsu, 1979) in Matlab (version R2023a, multitresh function) an approach previously used on in vitro MRI images of milk digestion (Mayar et al., 2023). The number of intermediate and darker voxels were summed and interpreted as reflecting coagulation. This was done because visual inspection of the thresholding results showed that in these images a separation in two categories was not accurate. In the context of this study, changes in image texture metrics were interpreted as reflecting changes in the degree of coagulation. An example of stomachs with and without coagulation and their corresponding image texture measures can be found in **Supplementary figure 3**.

2.5. Blood collection and analysis

Blood samples (10 ml) were drawn from the IV cannula into sodium-fluoride, serum-, and lithium-heparin tubes. After collection, sodium-fluoride and lithium-heparin tubes were centrifuged at 1000 x *g* for 10 min at 22°C within 30 min, to obtain blood plasma. To obtain blood serum, serum tubes were first allowed to clot for 30 min before being centrifuged at the same conditions as the other tubes. Following

centrifugation, serum aliquots of 500 µl and 250 µl were pipetted in 5 ml tubes and stored at -80°C until they were analyzed in bulk.

Analysis and quantification of serum AA concentrations were done using liquid chromatography mass spectrometry (LC-MS) triple quad, with an internal standard and 13C reference mixture(Hermann et al., 2018). For determination of the glucose concentrations, plasma samples were processed using an Atellica CH Glucose Hexokinase_3 (GluH_3) assay kit and quantified using an Atellica CH analyzer (Siemens Healthineers, The Netherlands). The lower limit of detection was 0.2 mmol/l and intra-assay CVs were at most 4.5%. Serum insulin was processed and its concentrations were quantified using an enzymatic immunoassay kit (ELISA, Mercodia AB, Sweden) with a limit of detection of 0.008 mmol/l and intra assay CVs of at most 6.8%.

2.6. In vitro digestion

In vitro gastric digestion was performed using a semi-dynamic digestion model simulating adult gastric conditions as described previously for infant conditions (Lambers et al., 2023). In short, to mimic adult digestive conditions digestion units contained 6 ml simulated gastric fluid (SGF) pH 1.5, containing 30 mM HCl (Sigma-Aldrich) and 1000 U/mL pepsin (Sigma-Aldrich, P6887), at the start of the experiment to simulate the fasting state. 60 mL formula was added and SGF with a flow of 0.72 ml/min was added until sampling pH using a preprogrammed DAS-box scripts. Subsequently, protease inhibitors (Pepstatin A, 5 µM, Sigma-Aldrich) were added to stop the enzymatic reactions before analyses of coagulation behavior by image analyses and wet-weight measurements after filtration (2 mm mesh, representing the stomach pyloric filter cut-off).

2.7. Statistical analysis

To estimate gastric emptying half time (GE-t50), a commonly used summary measure, for each scan session, a curve was fitted according to an established linear-exponential model as developed based on earlier models of GE to the data of gastric volume over time using R statistical software(Camps et al., 2018; Elashoff et

al., 1982; Fruehauf et al., 2011; R development Core Team, 2017). This method works well for gastric content that increases due to gastric excretion in the early phase (lag phase) and afterward empties almost linearly. Further analyses were performed in SPSS (version 22, IBM, Armonk, USA). GE-t50 was compared between low and high CM milk with a paired t-test. The serum AA were categorized into three groups: branched-chain amino acids (BCAA), essential amino acids (EAA), and non-essential amino acids (NEAA) and their content was summed. Overall blood parameters, gastric volume, image texture metrics, and appetite ratings were tested using linear mixed models with treatment, time, and treatment*time as fixed factors and baseline values as covariate. An extra analysis on the first 90 min of the blood parameters was conducted since this is when differences in gastric coagulation were expected. Proline, an AA more dominant in casein, and leucine, an AA more dominant in a whey protein, were used as a showcase since the expected difference in AA kinetics would be driven by differing casein coagulation and not whey protein. After this, we estimated the contribution of casein and whey proteins by determining the casein/whey protein AA ratio 'Q' of serum AA from the concentrations of proline, phenylalanine, aspartic acid, asparagine, and alanine according to the method of Jacobs et al.(Jacobs et al., 2019) described for food products. The formula Q = (asparagine + alanine) / (proline + phenylalanine) was used. Asparagine and alanine are more dominant in whey protein and proline and phenylalanine are more dominant in casein. Normality was confirmed by quantile-quantile plots of the residuals, except for insulin, which was log-transformed to meet normality. Missing data was handled using a Maximum Likelihood estimation. The significance threshold was set at p = 0.05. Data are expressed as mean ± SD unless stated otherwise.

3. RESULTS

3.1. Coagulation metrics

Qualitatively, coagulation was visible on the MRI images for both high and low CM milk (**Supplementary figure 4**). The image texture metrics homogeneity and busyness were higher for high CM milk (MD 0.007, 95% CI [0.001, 0.012], p=0.022

and MD 0.005, 95% CI [0.001, 0.010], p=0.012, respectively). Coarseness (MD 0.001, 95% CI [-0.001, 0.002], p=0.512),) and contrast (MD -0.086, 95% CI [-0.203, 0.031], p=0.149) were not significantly different. There were no time*treatment interaction effects. **Figure 2** shows graphs of homogeneity busyness, coarseness, and contrast over time**.**

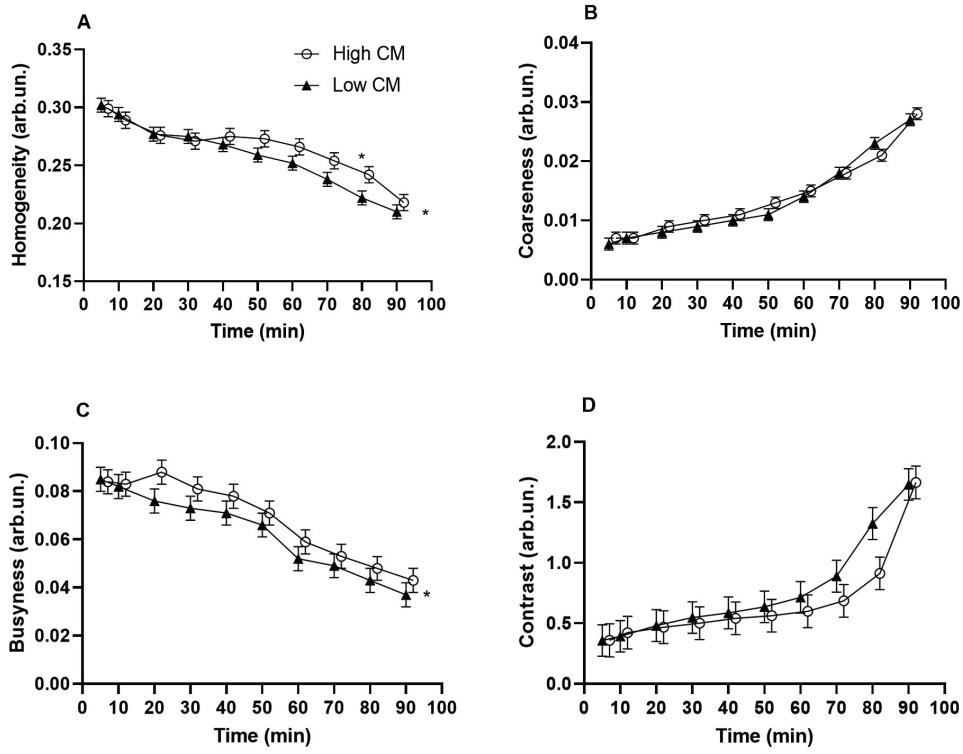

*Figure 2. Mean ± SEM of image texture metrics homogeneity (A), coarseness (B), busyness (C) and contrast (D) of gastric content as visible on MRI after low and high CM milk ingestion. *p < 0.05 placed above the value denotes a significant time point, at the right of the graph it denotes a significant treatment effect. A difference in image texture metrics may reflect a difference in casein coagulation.*

3.2. Gastric emptying

There was no significant difference in gastric volume over time between treatments (MD 3.8, 95% CI -8.2, 15.8, p = 0.53). This is in line with the GE-t50: low CM milk

45.6 ± 7.8 min and high CM milk 46.6 ± 8.7 min (MD 1.0, 95% CI [-1.9, 4.0], p = 0.46) (**Figure 3**). There was a higher proportion of liquid over time between treatments in the low CM condition (MD 2.2 %, 95% CI [0.30, 4.1], p = 0.023) (**Figure 4**). There were no significant timepoints driving the difference. **Supplementary figure 5** shows an example of a cross section through a stomach colored after applying the thresholding method.

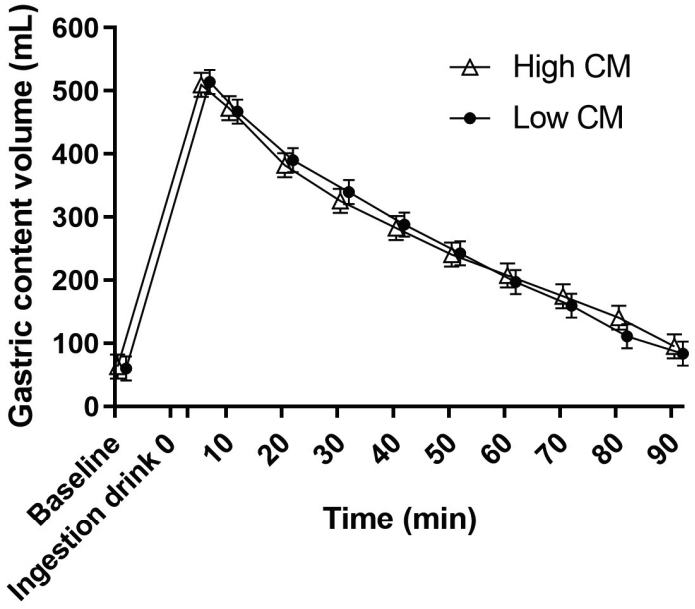

Figure 3. Mean ± SEM gastric content over time after ingestion of 600 ml of low and high CM milk. There were no significant differences between the two treatments.

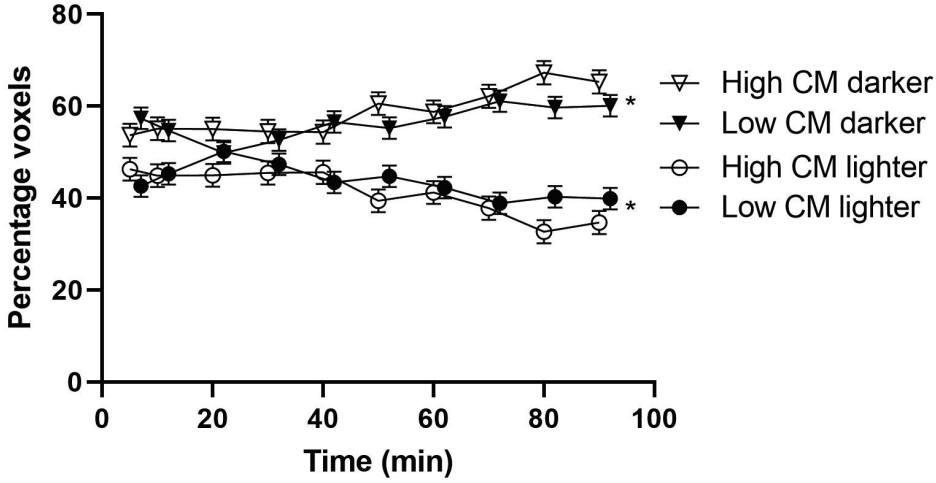

*Figure 4. Mean of percentage of two intensity categories of voxels of stomach content (lighter (more liquid) and darker (more solid)) after applying the thresholding method. *Denotes a significant difference between treatments (n=15).*

3.3. Amino acids

The total EAA postprandial response over time was similar for low and high CM milk (MD -10.5 µmol/L, 95% CI [-34.1, 13.0], p = 0.379, time p < 0.001, time * treatment p = 0.374) and total NEAA response over time was higher for the low CM milk with a trend for the interaction of time and treatment (MD -17.1 µmol/L, 95% CI [-29.6, -4.6], p = 0.008, time*treatment p = 0.095), driven by time point T = 60 min, p < 0.001 (**Figure 5**). The BCAA valine (MD -7.1, 95% CI [-14.1, -0.2], p = 0.045, time p < 0.001, time* treatment p = 0.135) driven by t = 60 min, p = 0.001) was significantly higher for the low CM milk. Figures of separate AAs can be found in **Supplementary figures 6, 7 and 8**.

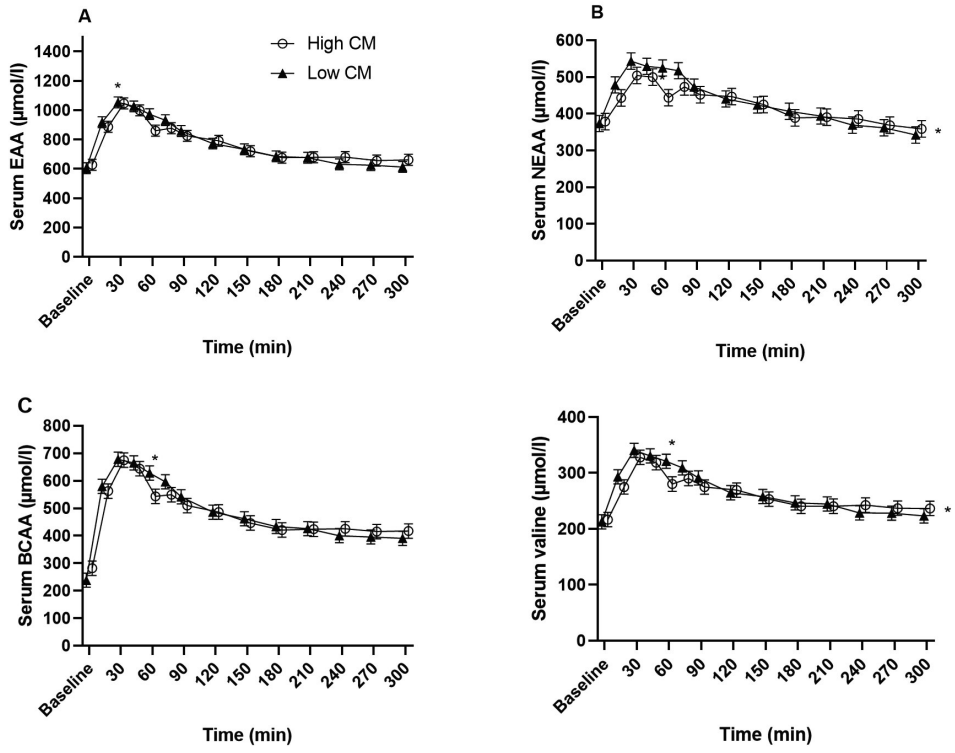

*Figure 5. Mean ± SEM of serum essential amino acid (A), non-essential amino acid (B), branch chained amino acids (C) and valine (D) concentrations after low and high CM milk ingestion. *p < 0.05 placed above the value denotes a significant time point, at the right of the graph it denotes a significant treatment effect.*

Analysis of the first 90 min, when the effect of a reduced casein coagulation is to be expected, showed higher total serum AA for low CM for both EAA (MD -46.8 µmol/L, 95% CI [-81.6 – -12.0], p = 0.009 and T = 60, p = 0.008) and NEAA (MD -42.4 µmol/L, 95% CI [-61.4 – 23.5], p < 0.001 and T = 60, p < 0.001).

Relative to high CM milk, low CM milk showed earlier appearance of AAs more dominant in casein, such as proline (MD 16.7 µmol/L, 95 % CI [9.5 - 24.0], treatment p < 0.001), while there was no difference in AA appearance of AAs more dominant in whey protein, such as leucine (MD 2.3 µmol/L, 95 % CI [-4.0 – 8.5], treatment p = 0.477). In line with these observations, analysis of the estimated serum casein/whey

protein AA ratio showed a difference between the treatments (MD 0.22, 95% CI [0.088-0.36], treatment p = 0.002, T = 30 min p = 0.015) (**Figure 6**).

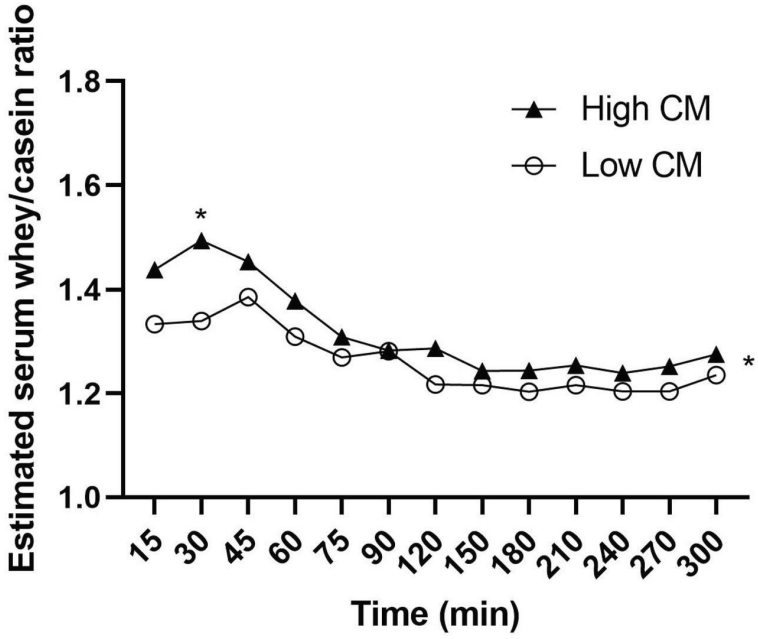

*Figure 6. Estimated serum whey protein/casein AA ratio over time after low and high CM milk ingestion. *p < 0.05 placed above the value denotes a significant time point, at the right of the graph it denotes a significant treatment effect.*

3.4. Glucose and insulin

Glucose over time did not differ between treatments (MD -0.047 mmol/L, 95% CI [-0.373, 0.278], p = 0.915) and there was no time*treatment interaction (p = 0.99). The insulin response over time was significantly lower for the high CM milk (MD 0.072 (mIU/L), 95% CI [0.019, 0.125], p = 0.008). Post-hoc t-tests showed that this is driven by time points T = 30 (MD 8.4, 95% CI [2.8, 14.0], p = 0.004) and T = 45 min (MD 6.9, 95% CI [1.3, 12.5], p = 0.016). There was no time*treatment interaction (p = 0.38). Graphs of both insulin and glucose can be found in **Figure 7**.

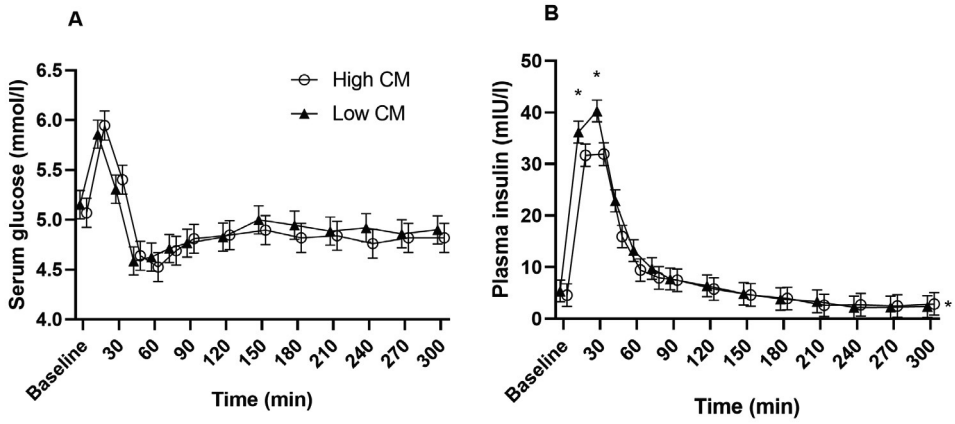

*Figure 7. Mean ± SEM plasma glucose (mmol/L) and serum insulin (mIU/L) concentrations over time after low and high CM milk ingestion. *p < 0.05 placed above the value denotes a significant time point, at the right of the graph it denotes a significant treatment effect.*

3.5. Appetite ratings

Hunger (MD 2.7, 95% CI [-0.418, 5.87], p = 0.089), fullness (MD 0.97, 95% CI [-2.06, 3.99}, p = 0.53), desire to eat (MD 0.26, 95% CI [-2.80, 3.32], p = 0.87), prospective consumption (MD -1.22, 95% CI [-3.99, 1.56], p = 0.388), thirst (MD -2.56, 95% CI [-5.96, 0.84], p = 0.139) did not differ between treatments (**Supplementary figure 9**). There were no interaction effects for any rating.

3.6. Time effects

There was a significant time effect for all mixed model analyses of AA, GE, glucose, and insulin, coagulation metrics and appetite ratings (all p < 0.001).

4. DISCUSSION

To the best of our knowledge this is the first *in vivo* study that directly evaluated the effect of milk mineral composition on gastric casein coagulation, GE, AA, and glycemic responses.

We used image texture metrics as an objective measure to quantify the degree of coagulation. Among the image texture metrics, homogeneity and busyness were higher for the high CM milk compared to the low CM milk. A difference in homogeneity and busyness implies a difference in coagulation between the high CM milk and the low CM milk. This is in line with previous *in vitro* work applying model IF formula and simulated infant digestion (Huppertz & Lambers, 2020) and our *in vitro* digestions of the test products under simulated adult conditions (**Supplementary figure 2**). Both *in vitro* studies showed a substantial difference in coagula between low and high CM samples, in line with an overall reduced casein coagulation and formation of a more open structured curd for the low CM milk. However, these measures should be further validated, since such image analyses to quantify the degree of structure formation have been used in other areas (Do et al., 2019; Gao et al., 2020), but are novel for characterizing gastric protein coagulation (Smeets et al., 2020; van Eijnatten et al., 2023). In the current study, homogeneity and busyness were both higher, which seems contradictive: a higher homogeneity may imply a lower degree of coagulation, while higher busyness may imply a higher degree of coagulation. What should be considered is that higher homogeneity could not only reflect a homogenous liquid, but also result from the presence of large coagulates. Another aspect to consider is that not only the size, but also the structure is an important characteristic of coagula. For instance, we know that some coagulates are firm and have a dense structure, and greater weight than less dense coagulates with approximately the same volume (Wang et al., 2018). Indeed, lower CM resulted in smaller and softer curd particles *in vitro*, likely because of higher concentration of non-micellar casein, which hinder enzymatic coagulation of casein micelles (Huppertz & Lambers, 2020). MRI is sensitive to water content, so could provide information on water contained in the coagulum and therefore its density. It should be noted that MRI image texture parameters are affected by the resolution of the input images and could detect differences in image intensity patterns that are not clearly distinguishable by viewing the MRI images. This needs further validation by concomitant analysis of MRI images and coagulates that differ in size and density. Other MRI techniques are being developed that can provide molecular-level information such as measurement of the magnetization transfer ratio

and relaxation rates (Deng et al., 2023; Deng et al., 2022; Mayar et al., 2023). These measurements require additional MRI spectra to be recorded but could be used in follow-up research to examine more subtle differences in protein coagulation *in vivo*.

The differences in coagulation between the two milks did not affect gastric emptying rate, as gastric volume curves over time were similar for both milks. This was expected, since the milk samples only differed in mineral composition. However, the threshold analysis showed that most of the liquid fraction emptied sooner than the semi-solid (coagulated) fraction. It is known from *in vitro* and animal *in vivo* models that complete breakdown and subsequent GE of the coagulated casein fraction can take longer than complete emptying of the liquid fraction (Boirie et al., 1997; Huppertz & Lambers, 2020; Mahé et al., 1996; Roy et al., 2022; Sakata et al., 2022; Ye et al., 2016). This is likely a consequence of the increased particle size exceeding the maximum size that can pass the pyloric filter (Kong & Singh, 2008) and may be physiologically important to provide a sustained release of amino acids to the neonate. Accordingly, whey proteins, which remain soluble, have a higher GE rate than caseins (Aguilera, 2019). Studies comparing whey protein and casein show a difference in overall GE as assessed with MRI and ultrasonography (Brun et al., 2012; Kuyumcu et al., 2015; Sakata et al., 2022). However, one study with preterm infants using whey protein- and casein-dominant formulas found similar GE (Thorkelsson et al., 1994). Likely, overall GE rates vary between studies likely as a consequence of the different formulations used.

In this paper, we measured the amino acids as a proxy to measure the effect of mineralization on casein coagulation *in vivo* in humans. The serum AAs that are more dominant in casein, such as proline, and estimated serum casein/whey protein AA ratio were significantly higher for the low CM milk, predominantly driven by time point T = 60 min. Gastric casein coagulation differences can likely explain the observed difference in overall postprandial AA profiles. Looking at the first 90 min, when the effect of differences in casein coagulation could be expected, both EAA and NEAA responses were significantly lower for high CM milk. This is in line with our hypothesis and is probably caused by the delay in emptying of the coagulated

casein fraction. Indeed, proline, an AA more dominant in casein AA was significantly different between the treatments, whereas leucine, an AA that is more dominant in whey protein, was not, further illustrating that micellar calcium only affects the coagulation and digestion of the casein fraction. This is strengthened by the findings of differences in the estimated serum whey protein/casein AA ratio. A reduced casein mineralization can also explain recent *in vivo* observations where overall digestion of mineral-depleted milk protein concentrate was faster than that of a regular CM milk (Chan et al., 2019).

No differences in glucose responses were observed, which is not surprising since carbohydrate (lactose) concentration was the same for both treatments and gastric emptying did not differ. The lower insulin levels observed in response to high CM milk are likely due to the difference in BCAAs and/or other insulinotropic AAs present in the milk. In this study the BCAA valine was significantly higher for the low CM milk. The BCAA have the potential to influence insulin responses (Fajans et al., 1969; Floyd et al., 1966; Sloun et al., 2020). There was no difference in glucose response. The insulin response was significantly lower for the high CM milk: driven by time points T = 30 and T = 45 min which were in line with differences in postprandial BCAA valine.

In conclusion, milks with different mineral compositions show different coagulating properties, as measured with higher serum AA in low CM milk, confirming *in vitro* results. Coagulation differences were further supported by MRI image analyses. Although the different coagulation properties did not influence overall GE the liquid fraction emptied quicker, while the coagulum persisted. This is in line with the difference in AA kinetics where the effects were predominantly driven by AAs more dominant in casein. The results suggest that the mineral composition of milk can influence gastric coagulation and protein digestion. This knowledge may help to determine the optimal processing of dairy products and their effect on digestion and health. Future studies should focus on improving measurements of the degree of coagulation and coagulum structure with MRI and examining the physiological relevance of the observed differences.

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Elise J.M. van Eijnatten: Investigation, Formal analysis, Writing – original draft. **Julia J.M. Roelofs**: Investigation, Writing – review & editing. **Guido Camps**: Writing – review & editing. **Thom Huppertz**: Resources, Writing – review & editing. **Tim T. Lambers**: Conceptualization, Resources, Writing – review & editing. **Paul A.M. Smeets**: Conceptualization, Writing – review & editing, primary responsibility for the final content. All authors read and approved the final manuscript. This study was funded by FrieslandCampina and TL and TH are employed by FrieslandCampina. All other authors declare no further conflict of interest. We thank Lisa van den Berg, Caya Lindner, Jinke Oosterhof, and Jesper Rietmeijer for assisting with data collection, Jacques Vervoort and Sebas Wesseling for their work on the AA analysis and Christophe Nioche for his support with the LIFEx software. The use of the 3T MRI was made possible by WUR Shared Research Facilities.

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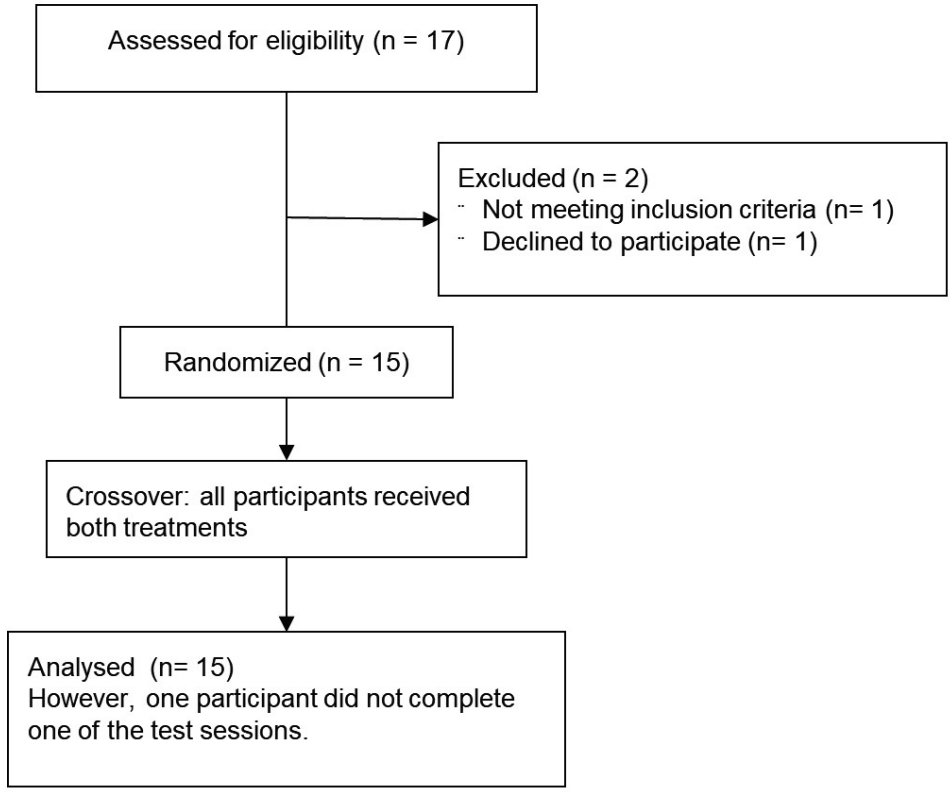
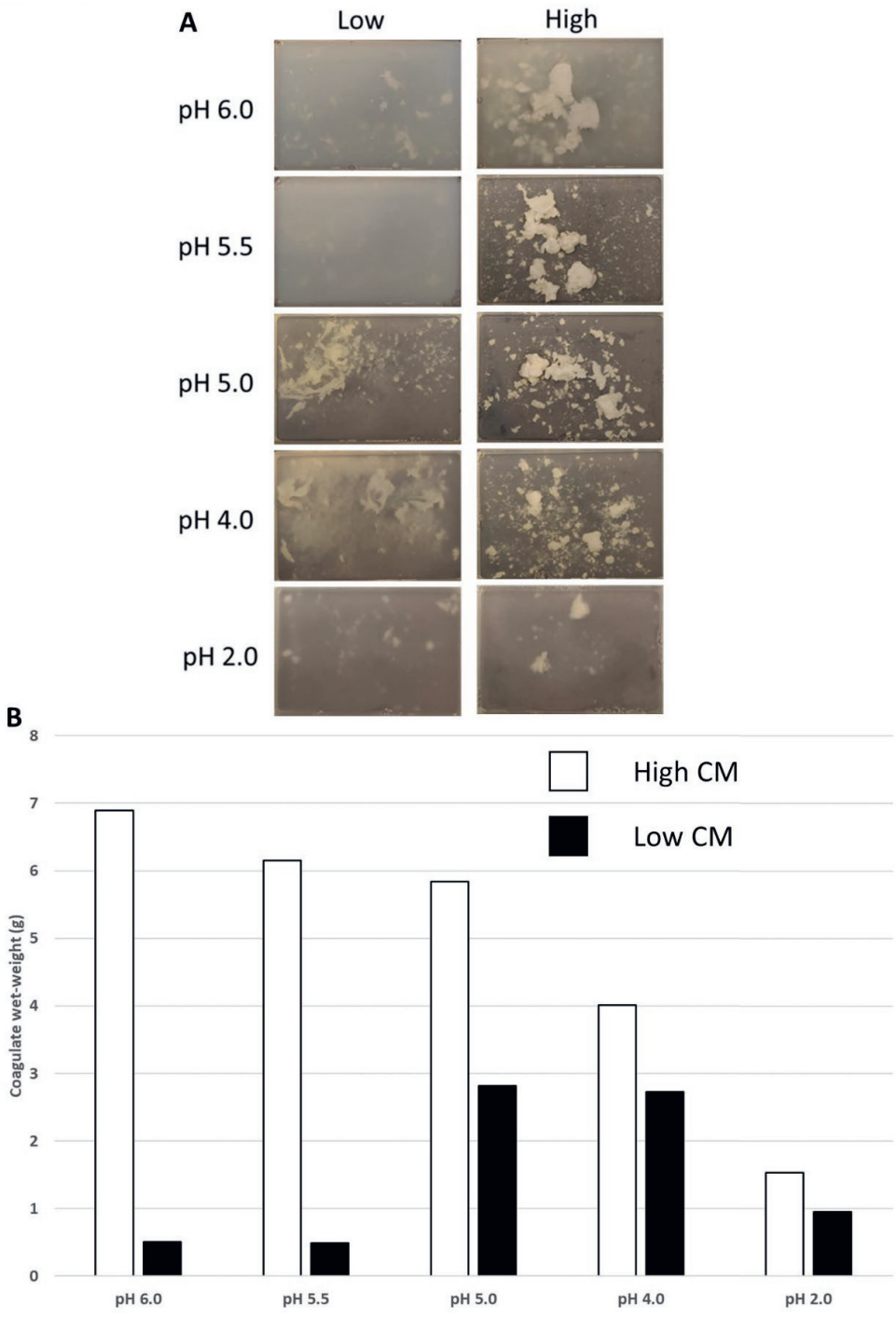
Zhang, J., Liu, D., Xie, Y., Yuan, J., Wang, K., Tao, X., Hemar, Y., Regenstein, J. M., Liu, X., & Zhou, P. (2023). Gastrointestinal digestibility of micellar casein dispersions: Effects of caprine vs bovine origin, and partial colloidal calcium depletion using in vitro digestion models for the adults and elderly. *Food Chemistry*, *416*, 135865. https://doi.org/https://doi.org/10.1016/j.foodchem.2023.135865

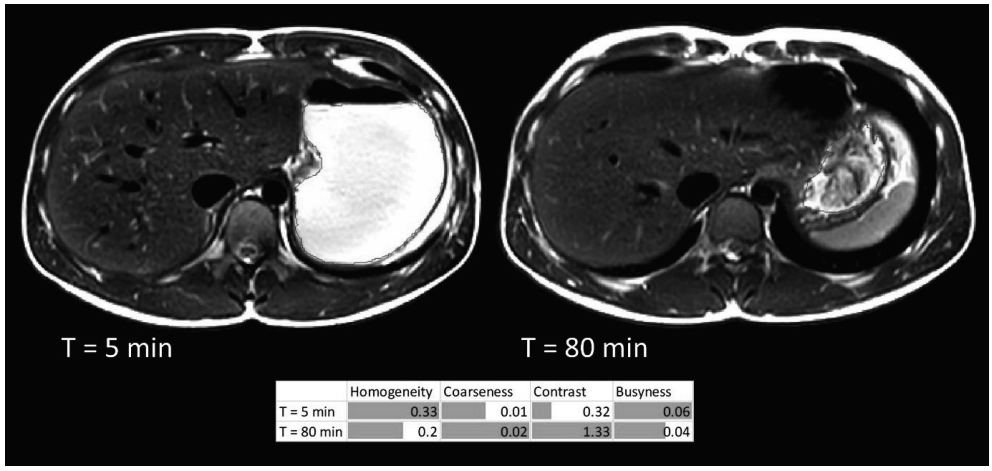
7. SUPPLEMENT



Supplementary figure 1. Study flow diagram.

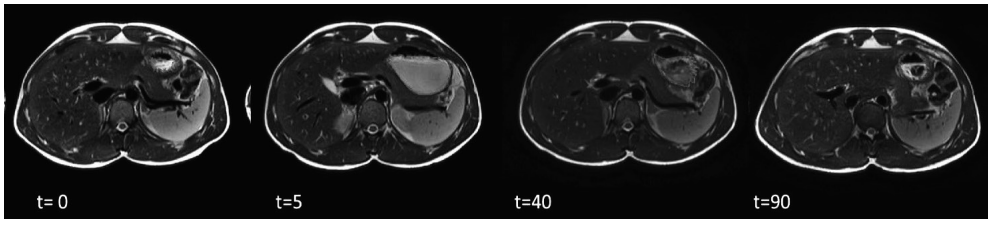


Supplementary figure 2. Coagulation in vitro.

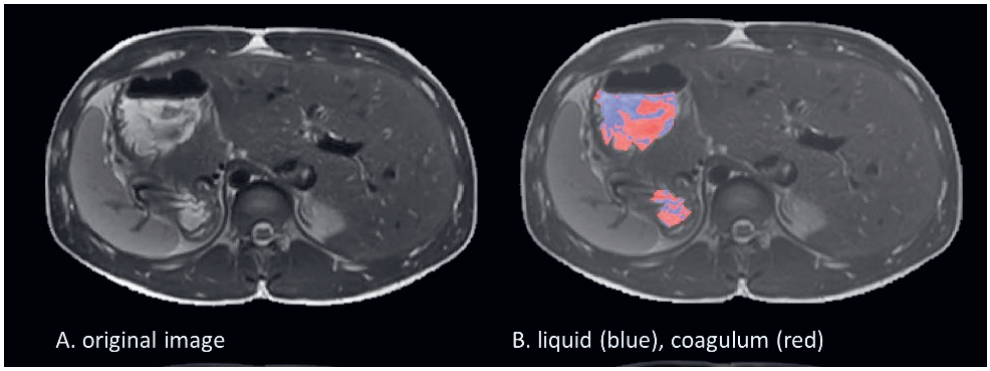


	Homogeneity	Coarseness	Contrast	Busyness
T = 5 min	0.33	0.01	0.32	0.06
T = 80 min	0.2	0.02	1.33	0.04

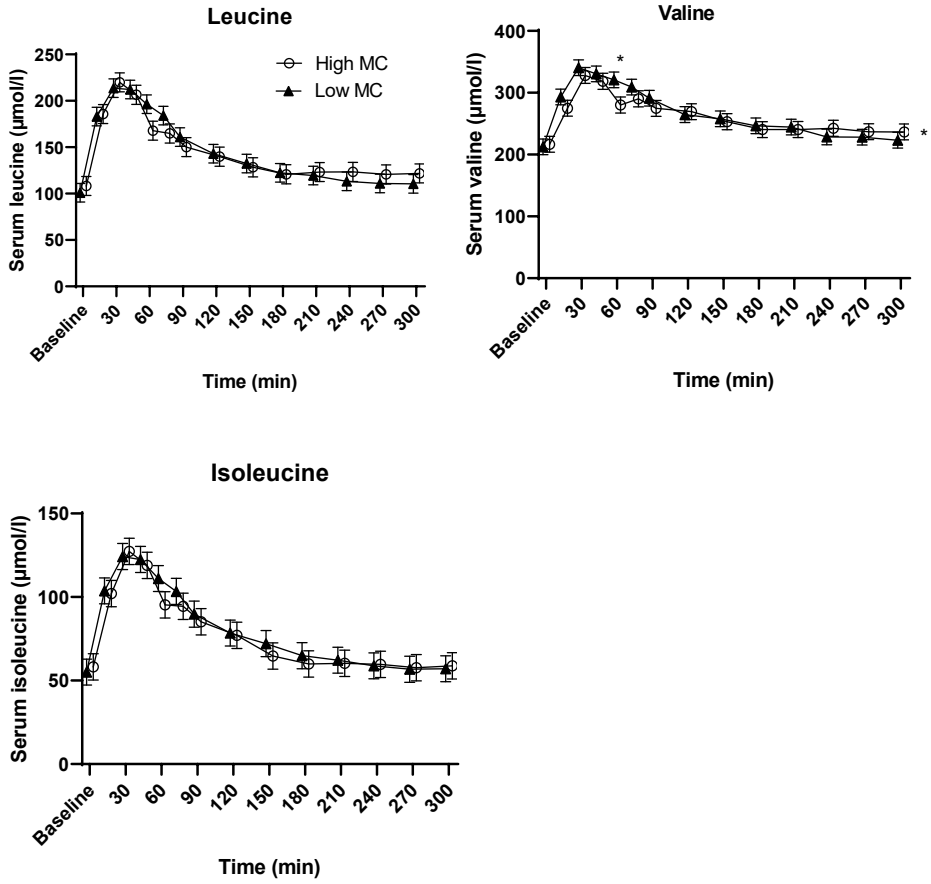
Supplementary figure 3. Examples of T2-weighted magnetic resonance images showing cross-sections of non-coagulating stomach content at T = 5 minutes and coagulating stomach content at T = 80 minutes and the associated image texture metrics.



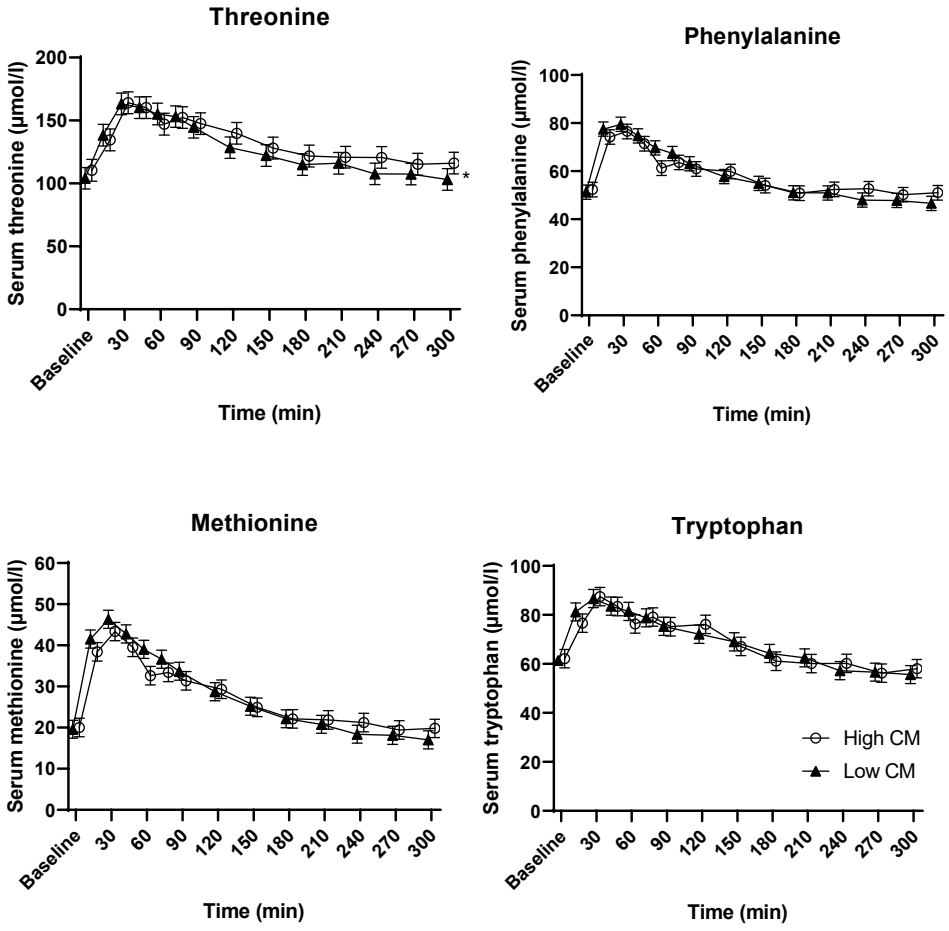
Supplementary figure 4. Examples of T2-weighted magnetic resonance images showing cross-sections through an empty stomach after an overnight fast (baseline) and after 600 ml skimmed milk consumption. The red line delineates stomach content. At T = 40 and 90 minutes milk protein coagulation can be observed by darker and lighter voxels.



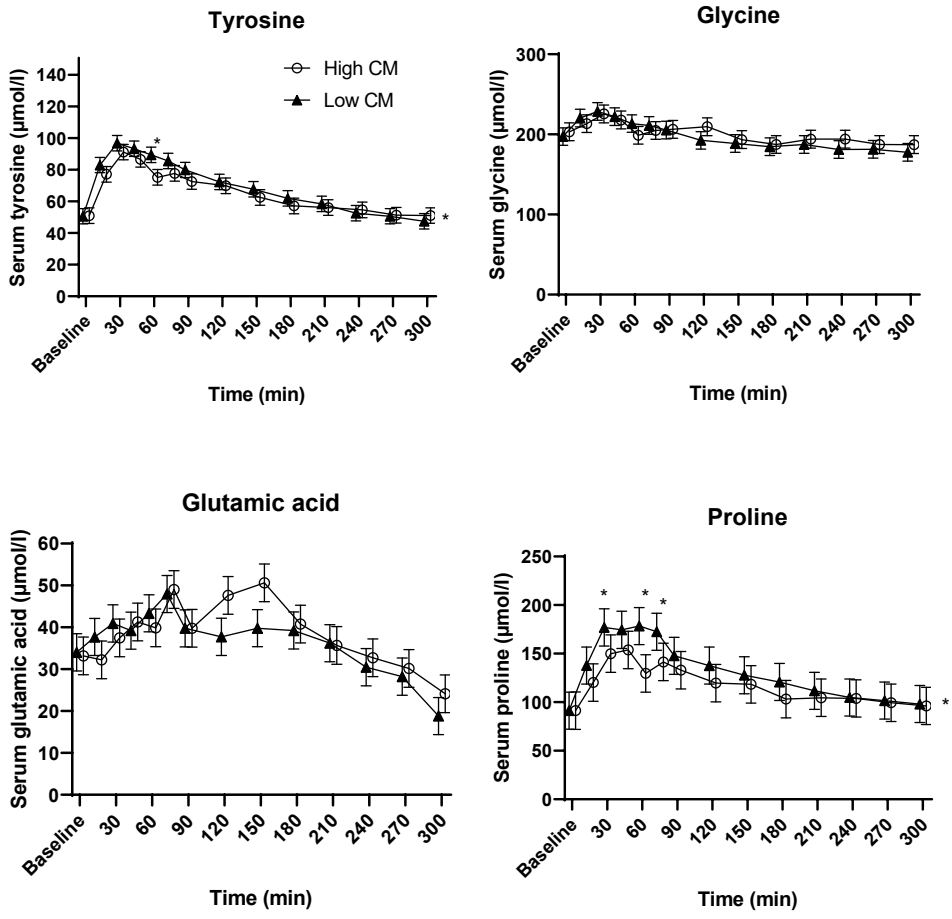
Supplementary figure 5. Examples of T2-weighted magnetic resonance images showing cross-sections through a stomach at T = 60 min with A showing the original image and B the voxels of stomach content colored: liquid as blue and semi-solid as red after applying the thresholding method.



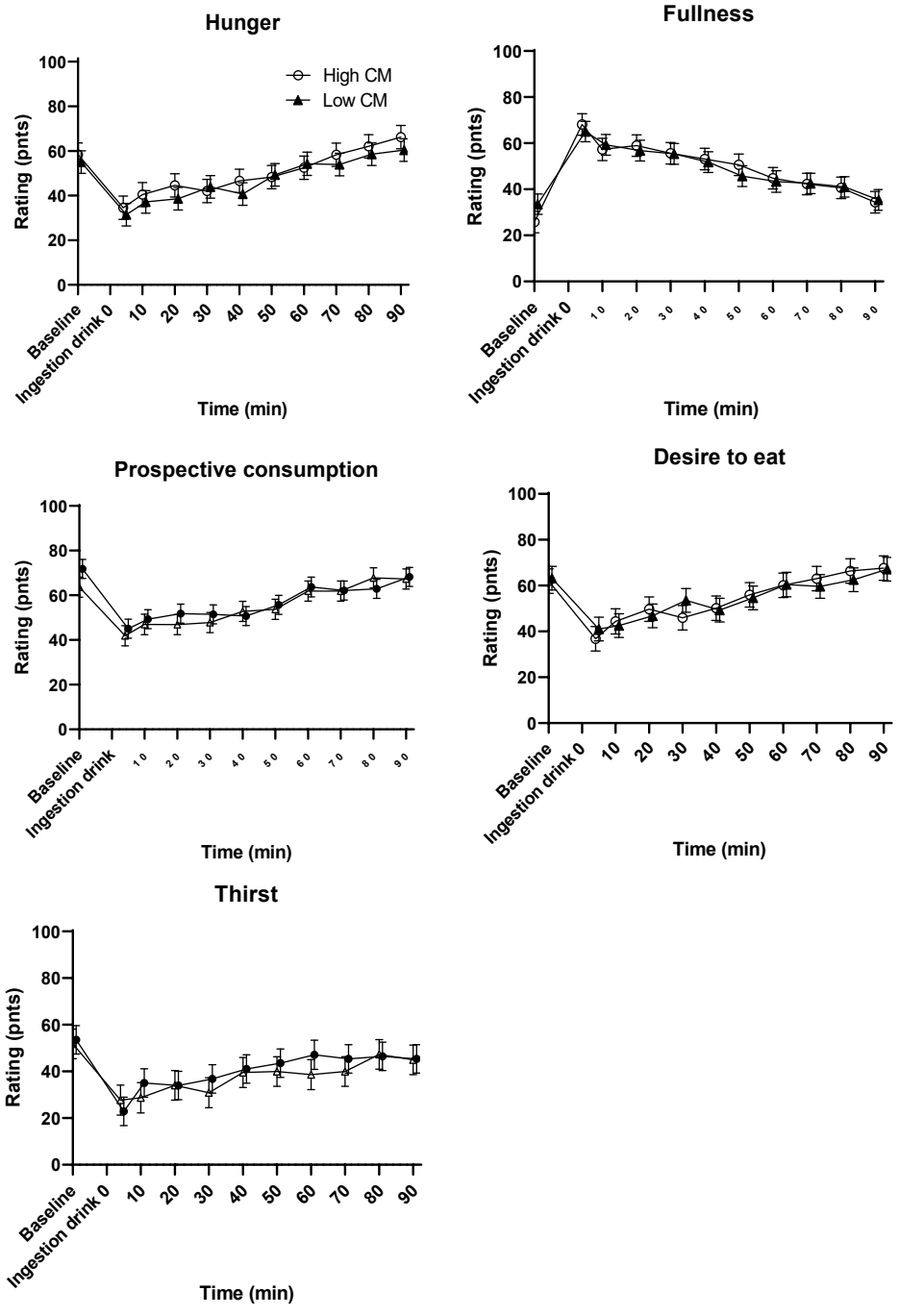
*Supplementary figure 6. Mean ± SEM of serum branch chained amino acids over time (n=15). *p < 0.05 placed above the value denotes a significant time point, at the right of the graph it denotes a significant treatment effect.*



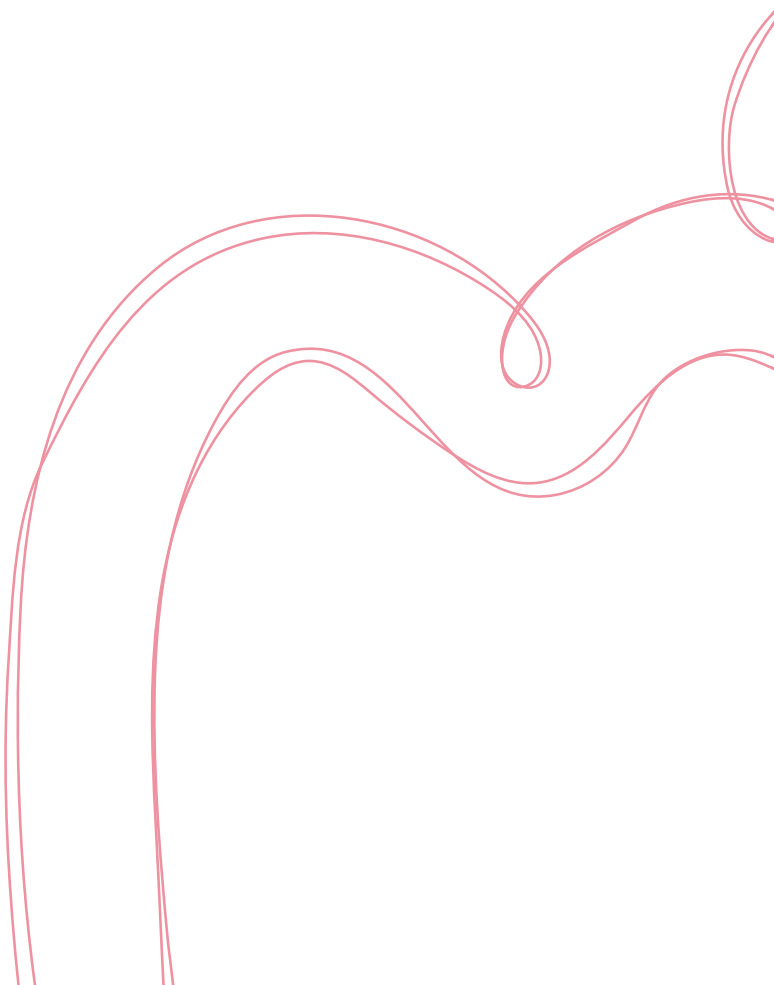
*Supplementary figure 7. Mean ± SEM of serum essential amino acids over time (n=15). *p < 0.05 placed above the value denotes a significant time point, at the right of the graph it denotes a significant treatment effect.*



*Supplementary figure 8. Mean ± SEM of serum non-essential or conditionally essential amino acids over time (n = 15). *p < 0.05 placed above the value denotes a significant time point, at the right of the graph it denotes a significant treatment effect.*

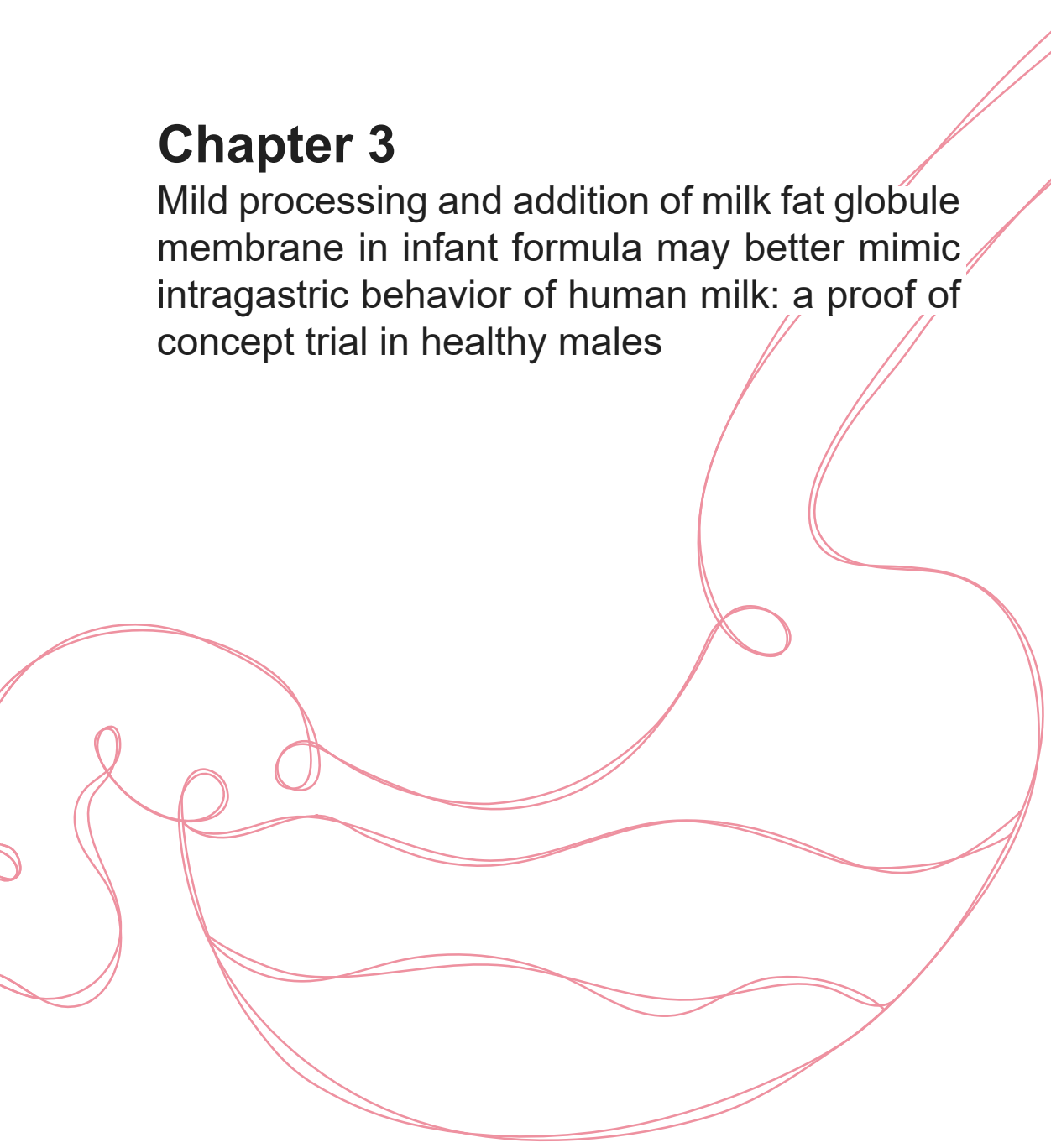


Supplementary figure 9. Mean ± SEM of appetite ratings hunger, fullness, prospective consumption, desire to eat and thirst (n=15).



Chapter 3

Mild processing and addition of milk fat globule membrane in infant formula may better mimic intragastric behavior of human milk: a proof of concept trial in healthy males



This chapter is published as:
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ABSTRACT

Background: During breastfeeding the macronutrient composition of breastmilk changes gradually from relatively low-fat (foremilk) to relatively high-fat (hindmilk), initially exposing the gastrointestinal tract to a relatively low fat concentration. In contrast, infant formulae (IF) are homogenous. Mild processing and addition of milk fat globule membrane (MFGM) may impact gastric emulsion instability, potentially impacting the phased release of nutrients as observed during breastfeeding.

Objective: To assess gastric emulsion stability, gastric emptying, and the postprandial plasma metabolome of an experimental minimally processed IF (EF) with an altered fat-globule interface and a control IF (CF).

Methods: Twenty healthy males participated in this double-blind randomized crossover trial. Gastric MRI scans and blood samples were obtained before and after consumption of 600 ml CF or EF over a 2-h period. Outcomes included gastric top layer formation, total gastric volume, and blood parameters (free fatty acids (FFA), insulin, glucose, and nuclear magnetic resonance (NMR-)metabolomics).

Results: EF showed an earlier onset (13.4 min, p=0.017), smaller maximum volume (49.0 ml, p= 0.033), and a shorter time to maximum top layer volume (13.9 min, p=0.022), but similar AUC (p=0.915) compared to CF. Total gastric volume did not show a treatment*time effect. Insulin concentrations were lower for EF. FFA and glucose did not differ. EF yielded higher serum concentrations of phospholipid- and cholesterol-related metabolites.

Conclusion: An EF displayed faster gastric creaming than a CF, thereby potentially better mimicking the behavior of breastmilk which leads to phased release of nutrients into the intestine. Overall physiological benefits of this difference in gastric behavior remain to be studied further in infants.

Keywords: Infant formula; emulsion stability; gastric behavior; digestion; MRI

1. INTRODUCTION

Breastmilk (BM) provides the best nutrition for the growth, development and health of infants (Victora et al., 2016; WHO, 2003). In case BM is not or not sufficiently available, or in case parents choose to feed their infant otherwise, infant formula (IF) is the only safe alternative to provide adequate nutrition (Dewey, 2003; WHO, 2003). Nevertheless, several reviews and meta-analyses report that differences in infant feeding may have different health effects. For instance, a longer duration of breastfeeding is associated with up to 26% reduced odds of overweight or obesity later in life (Harder et al., 2005; Victora et al., 2016; Weng et al., 2012).

One of the many differences between BM and IF is the change in fat content during a feed. During breastfeeding, the milk fat content and thus the caloric density of BM, slowly increases from low in foremilk, to relatively high in hindmilk (Daly et al., 1993; Forsum & Lonnerdal, 1979; Hytten, 1954; Italianer et al., 2020; Kent et al., 2006). With breastfeeding, the gastrointestinal tract will thus initially be exposed to a lower fat concentration. In addition, it is known that the fat content of BM varies greatly between individuals and shows diurnal variation (Khan et al., 2013). In contrast, the fat content of IF is stable during a feed and throughout the day. Physiologically, these differences may impact feed intake as e.g. the changes in fat content during breastfeeding are thought to influence satiation and overall feed intake (Perez-Escamilla et al., 1995).

Both BM and IF are essentially oil-in-water emulsions (Wang et al., 2023). The fat globules in BM are covered by a biological membrane called the milk fat globule membrane (MFGM). This membrane not only stabilizes the emulsion, but also facilitates digestion (Lopez, 2020; Thum et al., 2022; Wu et al., 2023). In contrast to BM, the fat globules in IF are primarily stabilized by proteins such as whey proteins and caseins (McCarthy et al., 2012). Under gastric conditions, as a result of pepsin-mediated protein digestion, these emulsions will destabilize, causing coalescence and creaming of the milk fat globules which results in a high-fat top layer (Kunz et al., 2005). Due to their different fat globule interface and coherent emulsion

stabilities, BM and IF destabilize at different rates in the stomach (Bourlieu et al., 2015; Camps et al., 2021; Chai et al., 2022).

In addition to protein composition, formula processing also influences emulsion stability, mainly by heating-induced protein denaturation (Ye et al., 2020). Particularly whey proteins are sensitive to this and form complexes with caseins upon heating (Anema et al., 2004; Fox et al., 2015; Guyomarc'h et al., 2009; Mulet-Cabero et al., 2019). The formation of these complexes results in a higher emulsion stability because more whey proteins are absorbed at the fat-globule interface stabilized by caseins (Raikos, 2010). More severe heat treatment of IF may thus impact gastric emulsion stability resulting in slower coalescence and creaming in the stomach. This study therefore studied if mild processing and addition of MFGM will induce earlier coalescence and creaming, and thus the formation of a high-fat top layer. Thereby potentially mimicking the aforementioned initial exposure to a relatively low fat concentration along the gastrointestinal tract as observed with breastfeeding. Other studies have already shown that a decreased emulsion stability can result in quicker gastric emptying (Marciani et al., 2009; Marciani et al., 2007) and an altered post-prandial response in lipids (Acevedo-Fani & Singh, 2022).

Overall, BM is known to have a faster gastric emptying rate compared to IF (Meyer et al., 2015; van Den Driessche et al., 1999; Wang et al., 2023). This was also confirmed by Camps et al. (2021) with the use of Magnetic Resonance Imaging (MRI) in adults. The advantage of using MRI in digestive research is that it allows for visualization and quantification of intragastric processes such as gastric emptying and emulsion stability (Smeets et al., 2021). However, due to ethical constraints, MRI cannot be used to assess gastric processes in healthy infants for research purposes.

Therefore, in this proof-of-concept study, gastric emulsion stability, gastric emptying, and post-prandial plasma metabolomics of an experimental IF, produced with a reduced heat-load and enriched in MFGM, was compared with a control IF in healthy adults. To complement this *in vivo* study, *in vitro* digestions were performed under

both simulated infant and adult conditions to investigate emulsion stability of the formulae under gastric conditions. Since we were not able to perform *in vivo* measurements in infants, the *in vitro* digestion allowed us to compare adult and infant conditions. Moreover, this allowed for validation with *in vivo* findings. It was hypothesized that the different fat-globule interface resulting from differences in formula composition and processing would result in faster gastric emulsion instability thereby potentially better mimicking the phased-release of nutrients in the intestine as observed with breastfeeding.

2. PARTICIPANTS AND METHODS

2.1. In vitro digestion

In vitro gastric digestion was performed using a semi-dynamic digestion model based on the international INFOGEST standard simulating infant and adult gastric conditions as described in Lambers et al. (2023). Digestion units contained 2.5 ml (infant), or 6 ml (adult) simulated gastric fluid (SGF), containing 30 mM HCl (Sigma-Aldrich) and 300 U/ml pepsin (Sigma-Aldrich, P6887) for infant or 100 mM HCl and 1000 U/ml pepsin for adult conditions, at the start of the experiment to simulate the fasting state. 60 ml formula was added immediately (adult) or with a feed flow of 3 ml/min (simulating a typical infant feeding time of 20 min) and SGF with a flow of 0.39 ml/min (infant) or 0.72 ml/min (adult) was added until sampling pH (i.e. pH = 5.75, 5.5, 5.0, and 4.5) using preprogrammed DAS-box scripts. Subsequently, protease inhibitors (Pepstatin A, 5 µM, Sigma-Aldrich) were added to stop the enzymatic reactions and visually analyze layer formation over time at t = 0, 5, 15, and 30 min. During the simulated digestion, samples were taken at pH values representing both the early and later phases of gastric digestion (pH = 5.75, 5.5, 5.0, and 4.5 (Bourlieu et al., 2014)) and layer formation was visually assessed with the use of photographs.

2.2. *In vivo* trial

2.2.1. Design

This study was a double-blind randomized crossover trial in which healthy men underwent gastric MRI scans and blood sampling at baseline and after consumption of two formulae. The primary outcome was gastric top layer formation resulting from emulsion instability. Secondary outcomes were total gastric volume, and blood parameters related to metabolic responses (Roelofs et al., 2024; van Eijnatten et al., 2023), including free fatty acids (FFA), glucose, insulin, and a range of (Nuclear Magnetic Resonance (NMR)-based) metabolites (Soininen et al., 2015; Würtz et al., 2017). In addition, subjective ratings of appetite (hunger, fullness, thirst, desire to eat, and prospective consumption) and nausea were collected (Noble et al., 2005). The study procedures were approved by the Medical Ethical Committee of Wageningen University in accordance with the Helsinki Declaration of 1975 as revised in 2013. The study was registered with clinicaltrials.gov under number NCT05224947. All participants signed an informed consent.

2.2.2. Participants

Twenty healthy (self-reported) males aged 18-45 y and with a BMI between 18.5 and 25 kg/m^2 were included. Participants were excluded if they reported an allergy or intolerance for cow's milk, lactose, soy and/or fish, gastric disorders, or regular gastric complaints, used medication that affects gastric behavior, smoked more than 2 cigarettes per week, had an alcohol intake >14 glasses per week, or had a contra-indication to MRI scanning (including but not limited to pacemakers and defibrillators, ferromagnetic implants, and claustrophobia). Since female sex hormone levels are known to influence gastrointestinal function, only males were included in the study (Gonenne et al., 2006; Lajterer et al., 2022; Soldin & Mattison, 2009). Participants were recruited via digital advertisements (e-mail and social media). In total, 20 men participated in the study (age: 25.5 ± 5.8 y, BMI: 21.9 ± 1.5 kg/m^2) (**Supplementary Figure 1**).

2.2.3. Treatments

A routine cow's milk-based formula (control IF, CF) and an experimental mildly processed formula (i.e. low-temperature pasteurization) comprised of skimmed milk, a native whey protein concentrate (Hiprotal® Milkserum 60 Liquid, FrieslandCampina), and a MFGM-enriched whey protein concentrate (Vivinal® MFGM, FrieslandCampina, containing 69-76% (m/m) protein, 6-10% (m/m) phospholipids) (experimental IF, EF) were used. Both products met the nutritional requirements of infants (0 to 6 months) (**Table 1**), were produced specifically for this study, and were produced under food-grade conditions. Participants consumed 600 ml of the formulae at approximately 37 °C as prescribed in the preparation instruction of both formulae.

Table 1. Nutrient composition of the experimental mildly processed formulae containing MFGM and control formulae.

Composition per 100 ml	Control	Experimental
Energy (kcal (kJ))	66 (276)	67 (280)
Protein[N*6.25] (g)	1.4	1.3
Casein[N*6.25] (g)	0.5	0.5
Whey protein[N*6.25] (g)	0.9	0.8
Fat (g)	3.5	3.4
Carbohydrate (g)	7.0	7.3
Galacto-oligosaccharides (g)	0.4	0.4
Phospholipids (mg)	29	49

2.2.4. Study procedures

Participants were instructed to consume the same meal the evening prior to both test days. After this the overnight fasting period of minimally 12 hours started. Drinking water was allowed up to 2 hours prior to their visit. Upon arrival at Hospital Gelderse Vallei (Ede, The Netherlands), a cannula was placed in an antecubital vein, a blood sample was taken, a baseline MRI scan was performed, and subjective ratings on appetite and nausea were obtained. Subsequently, participants consumed one of the two formulae from a cup while in an upright position. Mean (± SD) ingestion time was 2.1 (± 0.9) min (CF: 2.3 ± 0.9 min, EF: 2.0 ± 0.9 min).

Subsequently, gastric MRI scans were performed at 5-minute intervals during the first 30 minutes after the start of consumption. After that, scans were made every 10 minutes, up until 2 hours. Blood samples were taken at t = 15, 30, 45, 60, 75, 90 and 120 min. In addition, participants were asked to verbally rate their appetite and nausea on a scale from 0 (not at all) to 100 (very much) every 10 minutes, up to 90 minutes (Noble et al., 2005) (**Figure 1**).

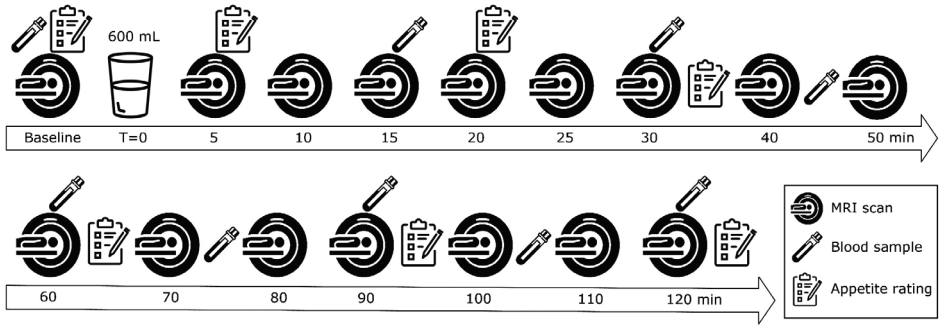

Figure 1. Overview of a test session.

2.2.5. MRI scanning

Participants were scanned in a supine position with the use of a 3 Tesla Philips Ingenia Elition X MRI scanner (Philips, Eindhoven, The Netherlands). A 2-D Turbo Spin Echo sequence (37 4-mm slices, 1.4 mm gap, 1 x 1 mm in-plane resolution, TR: 550 ms, TE 80 ms, flip angle: 90 degrees) was used with breath hold command on expiration to fixate the position of the diaphragm and the stomach. The scan lasted approximately 20 seconds.

2.2.6. MRI image analysis

Gastric top layer volume and total gastric content volume were manually delineated with the use of the program MIPAV (Medical Image Processing, Analysis and Visualization Version 7.4.0, 2016) to obtain the number of voxels located within the stomach (**Figure 2**) (McAuliffe et al., 2001). Top layer volume and total gastric volume were calculated for each time point by multiplying the number of voxels with the voxel size, taking into account gap distance (MATLAB (2021b)). A representative

example of a complete time series for both formulae is shown in the **Supplementary Figure 2**.

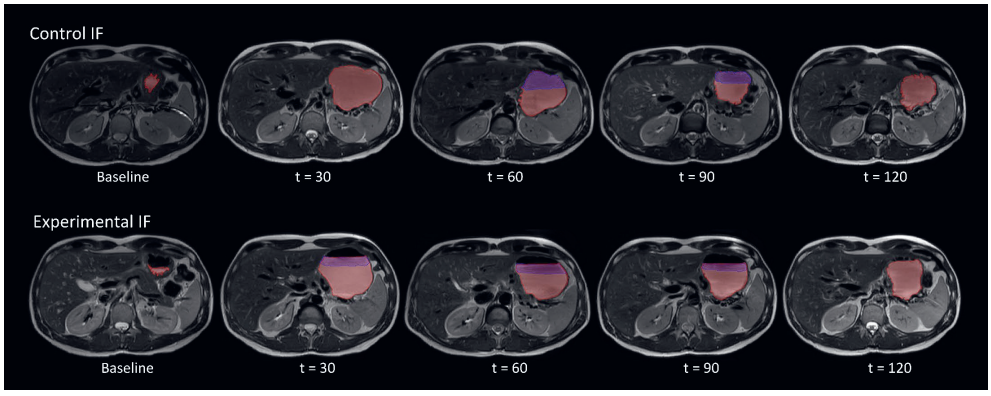

Figure 2. Illustration of a stomach MRI time series from one participant with top layer volume (blue) and total gastric volume (light red) delineated for the control IF and experimental IF.

The stomach contents were independently delineated by two researchers. To ensure that both researchers delineated total gastric volume and top layer in the same manner, the scans of both sessions of three participants were assessed by both researchers. The delineations were then discussed, and consensus agreements were made to ensure similar assessment by both observers. After this, scans were delineated by one of the two researchers. 15% of the sessions were analyzed in duplicate to check for potential differences. For top layer volume this resulted in an interclass correlation coefficient of 0.903 (95% CI: 0.849 – 0.938) which indicates good reliability. For total gastric content volume this was 0.997 (95% CI: 0.993-0.998), indicating excellent reliability (Koo & Li, 2016).

2.2.7. Blood sample collection and analysis

Blood samples were drawn from the IV cannula into sodium-fluoride, serum-, and EDTA tubes. After collection, the serum tubes were allowed to clot for 60 min. All tubes were centrifuged at 1000 g for 10 min. The sodium-fluoride and serum tubes were centrifuged at 22°C and the EDTA tubes at 4°C. The aliquots were stored at -80°C until they were analyzed in bulk. To determine glucose concentrations, the sodium-fluoride plasma samples were processed using the Atellica CH Glucose

Hexokinase_3 (GluH_3) assay kit and quantified using the Atellica CH analyzer (Siemens Healthineers, Netherlands) by a clinical chemistry laboratory (Ziekenhuis Gelderse Vallei, Ede, The Netherlands). The lower detection limit was 0.2 mmol/l and a maximum intra-assay CV of 4.5%. The EDTA plasma samples were processed and insulin was quantified using an enzymatic immunoassay kit (ELISA, Mercodia AB, Sweden). The lower detection limit was 1 mU/L and inter-assay CVs ranged from 3.3-19.1%. For the quantification of FFA, EDTA plasma samples were processed and quantified with an enzymatic kit (InstruChemie, Delfzijl, The Netherlands). The lower detection limit was 0.008 mmol/l and inter-assay CVs ranged from 0.2-20.9%. Quantification of 250 (NMR-based) metabolites was performed in the serum samples using ^{1}H-NMR metabolomics (Nightingale Health Ltd, Helsinki, Finland, https://nightingalehealth.com/) (Soininen et al., 2015; Würtz et al., 2017).

2.2.8. Sample size

Sample size was calculated for the primary outcome top layer formation. The calculation was based on Camps et al. (2021) who found that an intake volume of 200 ml IF resulted in a top layer of 17 ± 2.3 ml in adults. Therefore, it was expected that intake of 600 ml IF would result in a top layer of approximately 50 ml. With a larger intake volume, the magnitude of individual differences was expected to increase. Thus, it was estimated that the deviation from control would increase to approximately 13 ml. The minimal MRI-detectable difference in top layer deemed clinically relevant was estimated at 10 ml. Based on a two-sided test, an α of 0.05 and a power of 0.9, it was estimated that 20 complete datasets were needed. The calculation was done using: http://hedwig.mgh.harvard.edu/sample_size/js/js_crossover_quant.html.

2.2.9. Statistical analysis

For gastric top layer formation, the time at which the top layer appeared (onset) was compared between treatments. In addition, the maximum top layer volume and time to maximum top layer volume was identified for each individual. All three parameters were compared between treatments with a paired t-test and with a linear mixed

model that included baseline gastric volume as covariate. AUC of the top layer and total gastric volume over time were also calculated for each individual using the trapezoidal rule and compared with paired t-tests. Pearson correlation coefficients were calculated for baseline gastric volume and top layer characteristics.

Differences in gastric top layer volume over time were tested with the use of a generalized linear mixed model using a zero-inflated Poisson distribution, testing for main effects of time, treatment, and treatment*time interactions. Baseline gastric volume was included as a covariate, due to its effect on digestion (Camps et al., 2021). Differences in total gastric volume over time were tested using linear mixed models, with time, treatment, and treatment*time interactions as fixed factors and baseline gastric volume as a covariate. Tukey HSD corrected post-hoc t-tests were used to compare individual time points.

Differences in plasma concentrations of FFA, glucose, insulin, serum NMR-based metabolites, and subjective ratings over time were tested by using linear mixed models, testing for main effects of time, treatment, and treatment*time interactions. Baseline values were added as covariate. False discovery Rate (FDR) correction was used for the NMR-based metabolites (Storey, 2002).

Normality of the data was confirmed with quantile-quantile (QQ) plots of the residuals. For insulin, FFA and prospective consumption a logarithmic transformation was applied to create a normal distribution. All statistical analyses were performed using the R statical software (version 4.0.2). The significance threshold was set at $p = 0.05$. Data are expressed as mean ± SE unless stated otherwise.

3. RESULTS

3.1. In vitro digestion

To investigate emulsion stability of the EF and CF under gastric conditions, *in vitro* digestions were performed under both simulated infant and adult conditions

(**Supplementary Figure 3**). Under both simulated infant and adult conditions, the EF displayed an earlier, i.e., at a higher pH, onset of layer formation compared to the CF. In addition, the formed lipid layer in the EF appeared to be more dense compared to the CF.

3.2. *In vivo* trial

3.2.1. Gastric top layer formation

Onset time of the high-fat top layer, maximum top layer volume, and time to maximum top layer volume are described in **Table 2**. The onset of the top layer was 13.4 ± 5.1 min earlier for the EF compared to the CF ($p = 0.017$, 26.1 ± 4.4 min, and 39.5 ± 3.3 min for the EF and CF respectively) (**Supplementary Figure 4**). When baseline gastric volume was added as covariate the effect became more significant with a mean difference of 13.6 ± 3.6 min ($p = 0.001$). Time to maximum top layer volume was shorter and maximum top layer volume was lower for the EF ($p = 0.022$ and 0.033, respectively). Top layer AUC did not differ between treatments ($p = 0.915$).

Although the AUC was similar for both formulae, top layer volume over time showed a significant treatment by time interaction ($p < 0.001$) with lower volumes for the EF at all time points except t = 100. The individual curves for top layer volume over time can be found in **supplementary figure 5**.

Table 2. Differences in top layer formation between the two formulae (mean ± SE).

Characteristic	Control	Experimental	Mean difference (95% CI)	P-value
Onset time (min)	39.5 ± 4.4	26.1 ± 3.3	13.4 (2.7 – 24.1)	0.017
Maximum top layer volume (ml)	203.4 ± 23.5	154.6 ± 10.9	49.0 (4.4 – 93.6)	0.033
Time to maximum top layer volume (min)	41.3 ± 4.7	27.4 ± 3.5	13.9 (2.3 – 25.7)	0.022
AUC (ml*min)	5163 ± 642	5090 ± 444	72.7 (-1343 – 1488)	0.915

3.2.2. *Total gastric volume*

Total gastric volume showed a linear decline over time for both formulae (**Figure 3**), with higher volumes for the EF (treatment effect, mean difference = 15.0 ml, $p < 0.001$). However, treatment by time interaction was not significant ($p = 0.323$). Total gastric volume AUC tended to be lower for the CF ($p = 0.063$).

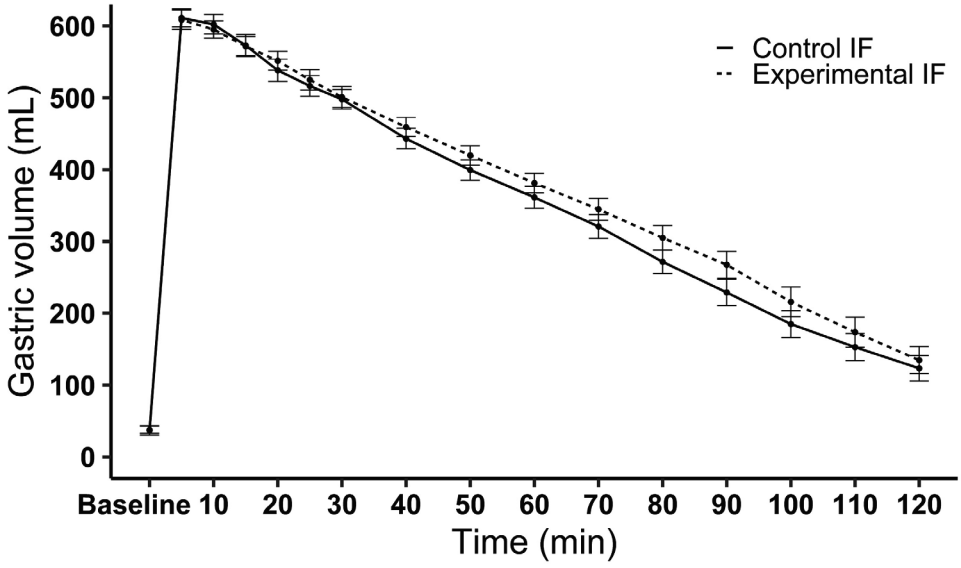

Figure 3. Mean ± SE total gastric volume over time of the two formulae.

3.2.3. *Correlations*

Baseline gastric volume correlated with the top layer onset time for both the EF ($r = -0.82$, $p < 0.001$) and the CF ($r = -0.72$, $p < 0.001$) (**Supplementary Figure 6**). Baseline gastric volume were also correlated with maximum top layer volume ($r = 0.65$ for EF, $p < 0.001$ and 0.46 for CF, $p < 0.001$) and time to maximum top layer ($r = -0.80$ for EF, $p = 0.002$ and $r = -0.71$ for CF, $p = 0.046$).

3.2.4. Postprandial blood response

No treatment effects were found for postprandial plasma FFA and glucose (p = 0.763 and 0.325, respectively) (**Supplementary Figure 7)**. However, plasma insulin concentrations were lower for the EF (p = 0.040) (**Figure 4**). No treatment by time interaction was observed for these three parameters (p = 0.835, 0.881, and 0.547, respectively).

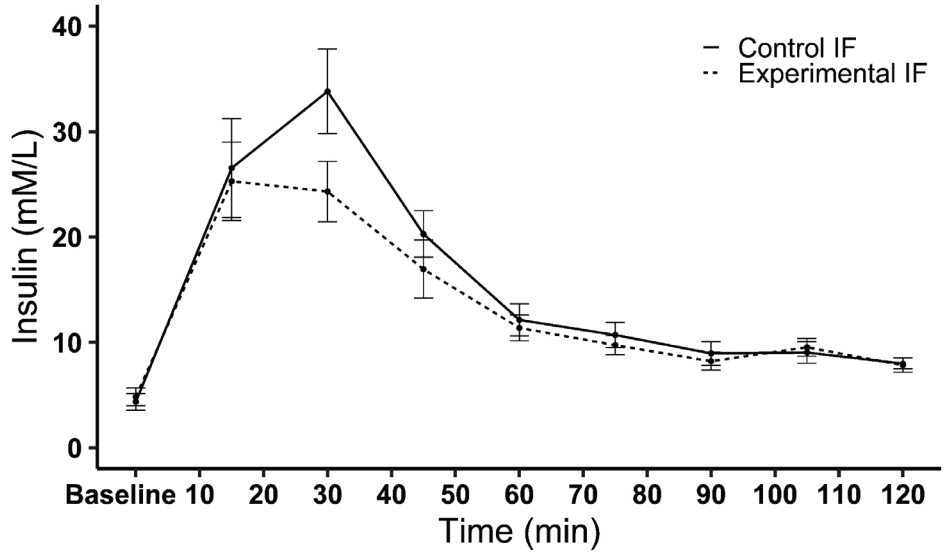

Figure 4. Mean ± SE plasma insulin concentrations over time after ingestion of the formulae.

Treatment effects were found for 63 of the NMR-based metabolites (**Supplementary Figure 8)**. Differences were mainly found in cholesterol- and phospholipid-related metabolites, which all had lower levels for the CF. None of the metabolites showed a significant treatment by time interaction**.**

3.2.5. Appetite & nausea ratings

There were no significant treatment effects for hunger, fullness, prospective consumption, and thirst (p = 0.751, 0.340, 0.706, and 0.757 respectively). Desire to

eat tended to be lower for the CF (p = 0.078). Nausea was overall rated lower for the EF (p = 0.023), although all scores remained around zero. None of the ratings showed a significant interaction between treatment and time (p = 0.820, 0.575, 0.257, 0.662, 0.718, and 0.890 respectively). The graphs are shown in **Supplementary Figure 9.**

4. DISCUSSION

This study compared gastric top layer formation, gastric emptying, and post-prandial plasma metabolomics of an experimental minimally processed IF containing MFGM with that of a control IF. Onset of the high-fat gastric top layer was earlier for the EF and top layer volume was lower compared to the CF. For both formulae, total gastric volume emptied almost linear over time, with a higher volume for the EF. No treatment differences were found in post-prandial plasma concentrations of FFA, insulin, and glucose. NMR-based metabolomics mainly showed differences in postprandial serum phospholipid and cholesterol related metabolomics, with higher concentrations for the EF.

Gastric top layer volume over time was lower for the EF and the layer was formed ~13 minutes sooner for the EF. This is in line with the observations in the *in vitro* digestion, which demonstrated layer formation at higher pH (i.e., in an earlier phase of gastric digestion) for the EF under both simulated infant and adult conditions. The lower gastric volumes for the EF are likely the result of the earlier top layer formation, resulting in a more even distribution over time. This earlier onset of emulsion destabilization is in line with our hypothesis and can most likely be explained by the mild processing. As a result of the lower heating compared to the CF, fewer whey proteins are denatured and complexed with caseins at the fat globule interface. This in turn causes the emulsion to be less stable under gastric conditions as caseins, resulting from pepsin-mediated hydrolysis, will coagulate quickly under gastric conditions (Anema et al., 2004; Fox et al., 2015; Guyomarc'h et al., 2009; Raikos, 2010). In the CF top layer formation occurred later, most likely as a result of a slower hydrolysis of the casein-whey complex that stabilizes the emulsion. A faster gastric lipid layer formation may potentially better mimic the phased-release of nutrients in

the intestine overall as observed with breastfeeding where the gastrointestinal tract will initially be exposed to a relative low-fat fraction (foremilk) followed by exposure to a relatively high-fat fraction (hindmilk) (Daly et al., 1993; Forsum & Lonnerdal, 1979; Hytten, 1954; Kent et al., 2006; Saarela et al., 2005).

For both formulae, gastric emptying over time followed a linear pattern, with lower gastric volumes for the CF after 30 min. However, individual timepoints did not show a significant difference and the AUC did not significantly differ between treatments, indicating that the effect is small. Moreover, this small effect also did not result in significant differences for FFA and glucose. Therefore, it should be questioned whether this small treatment difference in gastric volume is clinically relevant.

Plasma insulin showed a higher initial postprandial peak for the CF. As total levels of carbohydrates were only slightly higher in the EF, these observations are likely explained by the insulinotropic effect of proteins (Rietman et al., 2014). Most likely, the slower destabilization of the emulsion for the CF led to a more protein-rich fraction initially reaching the intestine compared to the EF, thereby initially promoting insulin secretion more prominent. Surprisingly, few data is available with respect to post- prandial insulin responses after consumption of human milk, likely resulting from ethical challenges of (serial) blood sampling in infants. Interestingly, an adult study comparing, amongst others, human milk with bovine milk, human milk displayed the lowest insulin response (Gunnerud et al., 2012).

As identified previously by Camps et al. (2021), baseline (fasted) gastric juice volume was correlated with top layer formation. Higher baseline gastric juice volume was strongly associated with an earlier onset of the top layer. This is likely explained by its low pH and the presence of pepsin. When there is more gastric juice, the gastric pH will initially be lower and more pepsin will be available, resulting in an overall quicker coalescence and creaming. Relative to Camps et al. (2021) (200 ml), a larger ingestion volume (600 ml) was chosen to minimize the influence of baseline gastric juice. Nevertheless, a strong correlation between fasting gastric juice volume and subsequent top layer formation was also observed in the current study. This

underscores the relevance of taking baseline gastric content volumes into consideration, also because these show large day-to-day and intra-individual variability (Grimm et al., 2018). Interestingly, one of the participants did not show any top layer formation for both formulae. Although this participant did not show abnormal baseline gastric juice volumes, it could have been that this participant had low pepsin activity. It is known that there are large interindividual variabilities in pepsin activity (Walther et al., 2019).

Post-prandial concentrations of phospholipid and cholesterol related metabolites were higher for the EF. Most likely, this can be explained by the addition of MFGM-enriched whey to this formula, which, as compared to normal whey, is enriched with phospholipids and cholesterol (Venkat et al., 2022). No treatment differences were found in post-prandial plasma FFA and glucose concentrations. This is likely due to the overall similar nutritional composition of the two formulae concerning fat and carbohydrate source. In addition, especially for the FFA, the measurement time was relatively short in the current study. Plasma FFA concentrations rise during fasting and drop as soon as food is ingested. This drop happens due to the meal-induced secretion of insulin, which suppresses intracellular lipase and thereby lowers the release of FFA into the circulation (Albrink & Neuwirth, 1960; Fielding, 2011; Lairon et al., 2007). This is in line with the plasma FFA concentration changes found in this study, which showed an initial decrease after ingestion of the IF. Therefore, it is often recommended to measure FFA concentrations for a longer period. For example, Lairon et al. (2007) recommend a period of 6-8 h.

One of the limitations of the study is the use of adults instead of infants because of ethical considerations associated with MRI. The gastrointestinal tract of infants is not yet completely developed and therefore differs from that of adults. Amongst others, the minimum gastric pH of infants is higher compared to that of adults (3 - 4 compared to 0.5 - 2.3) and less pepsin is secreted (Poquet & Wooster, 2016). Since the pH influences the activity of pepsin and gastric lipase, this impacts digestive processes in the stomach, among which the coalescence and creaming into a high-fat top layer. The higher pH in infant gastric conditions, would most likely result in a slower destabilization of the emulsion. However, as both formulae were measured

in adults, it is expected that the differences between treatments will remain similar. Moreover, in the semi-dynamic *in vitro* digestion model the difference between both formulae were observed under both infant and adult digestion conditions. Combined, these results thus suggest that the differences in coalescence and creaming that were observed will also occur in infants.

MRI usually requires participants to be scanned in a supine position. Studies have shown that sitting in an upright position accelerates gastric emptying compared to a supine position (Jones et al., 2006; Spiegel et al., 2000). However, these effects are small and since participants were scanned in the same position for both treatments, we expect the differences between treatments to remain similar. Moreover, from the perspective of infant nutrition a supine position after feeding is realistic.

In conclusion, this study shows that an experimental minimally processed infant formula containing MFGM-enriched whey had an accelerated gastric creaming as compared to a control formula. No effects on overall nutrient absorption over time were found, except for the cholesterol- and phospholipid-related metabolites which can most likely be attributed to the presence of MFGM-enriched whey in the experimental formula. Although the overall physiological consequences remain to be identified, a faster high-fat gastric top layer formation of infant formulae may potentially better mimic the phased-release of nutrients in the intestine as observed with breastfeeding where the gastrointestinal tract will initially be exposed to a relative low-fat fraction (foremilk) followed by exposure to a relatively high-fat fraction (hindmilk).

5. AUTHORS' CONTRIBUTIONS AND ACKNOWLEDGEMENTS

Julia J.M. Roelofs: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Reina S. Tjoelker**: Conceptualization, Writing – review & editing. **Tim T. Lambers**: Conceptualization, Writing – review & editing. **Paul A.M. Smeets**: Conceptualization, Writing – review & editing, primary responsibility for the final content. All authors read and approved the final manuscript.

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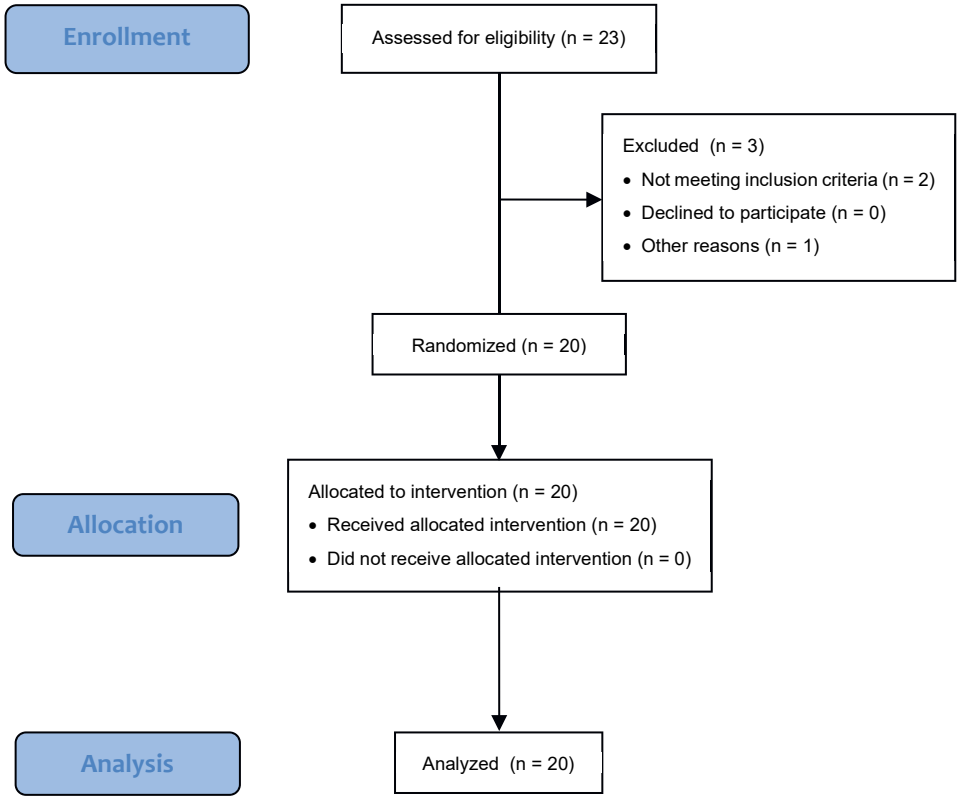
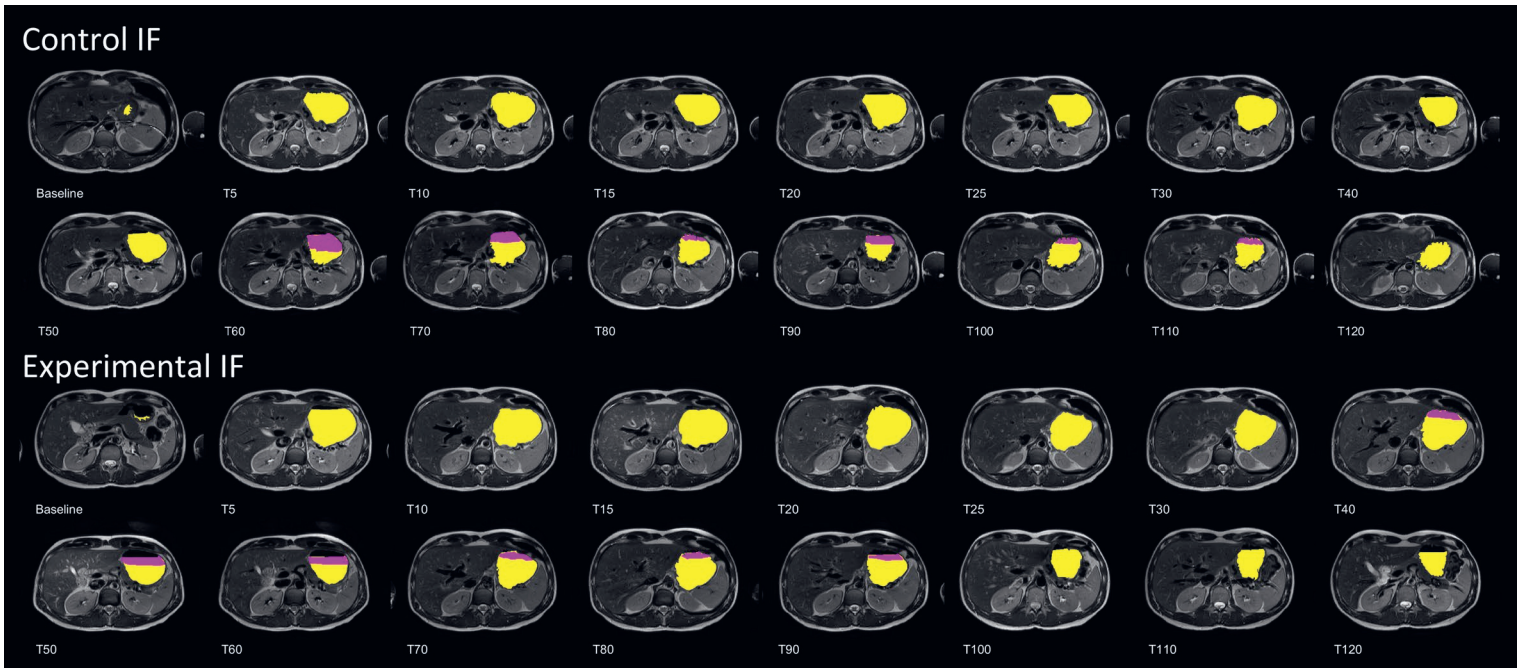
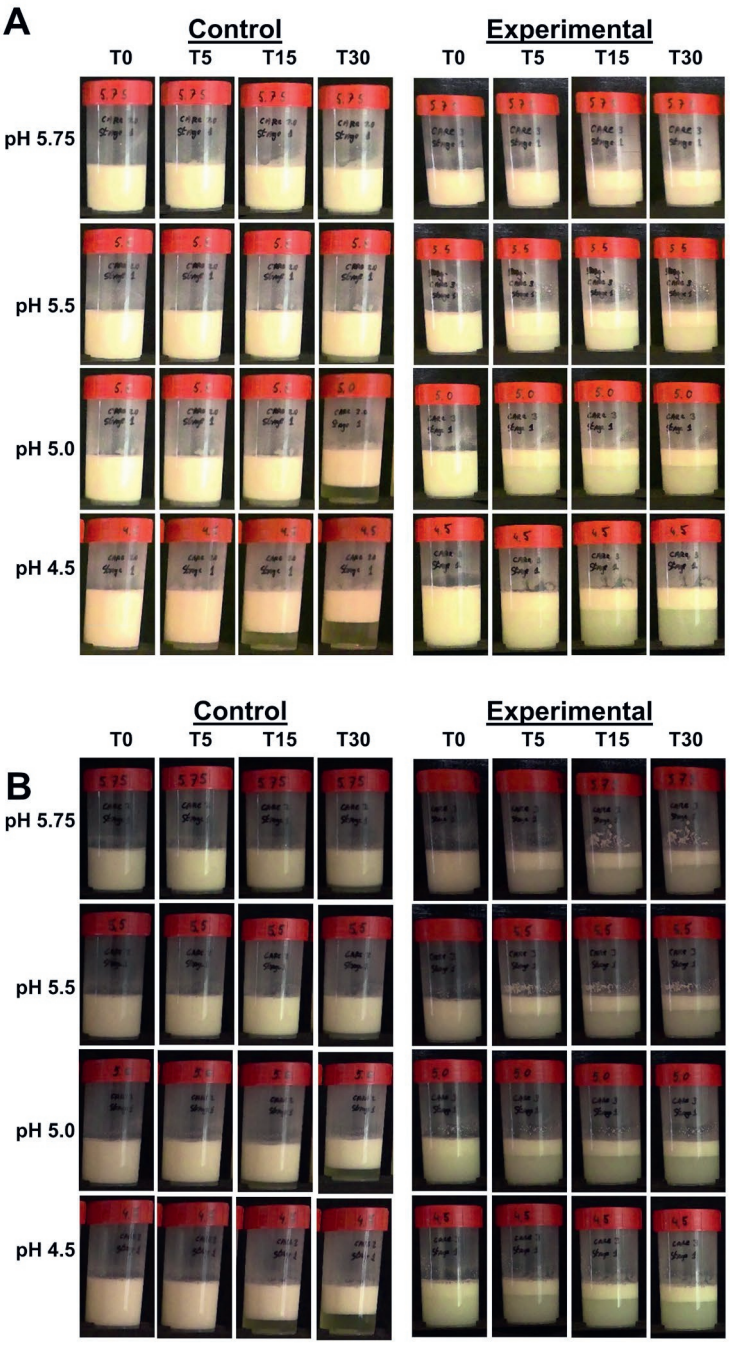
7. SUPPLEMENTARY MATERIALS



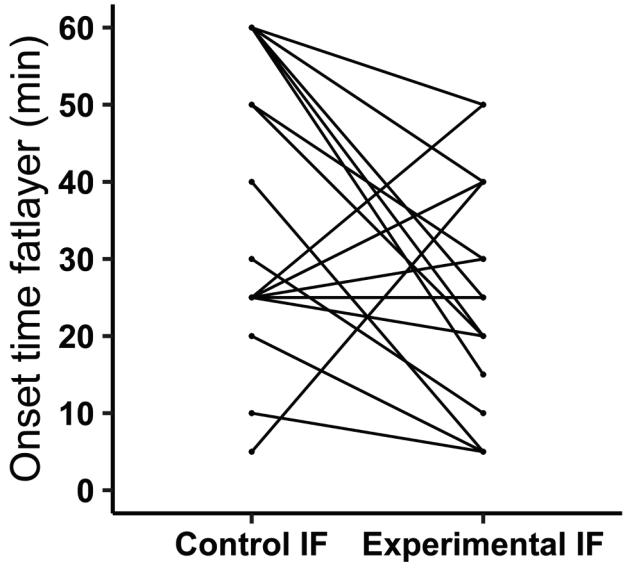
Supplementary Figure 1. Flow diagram for inclusion, treatment allocation and analysis.



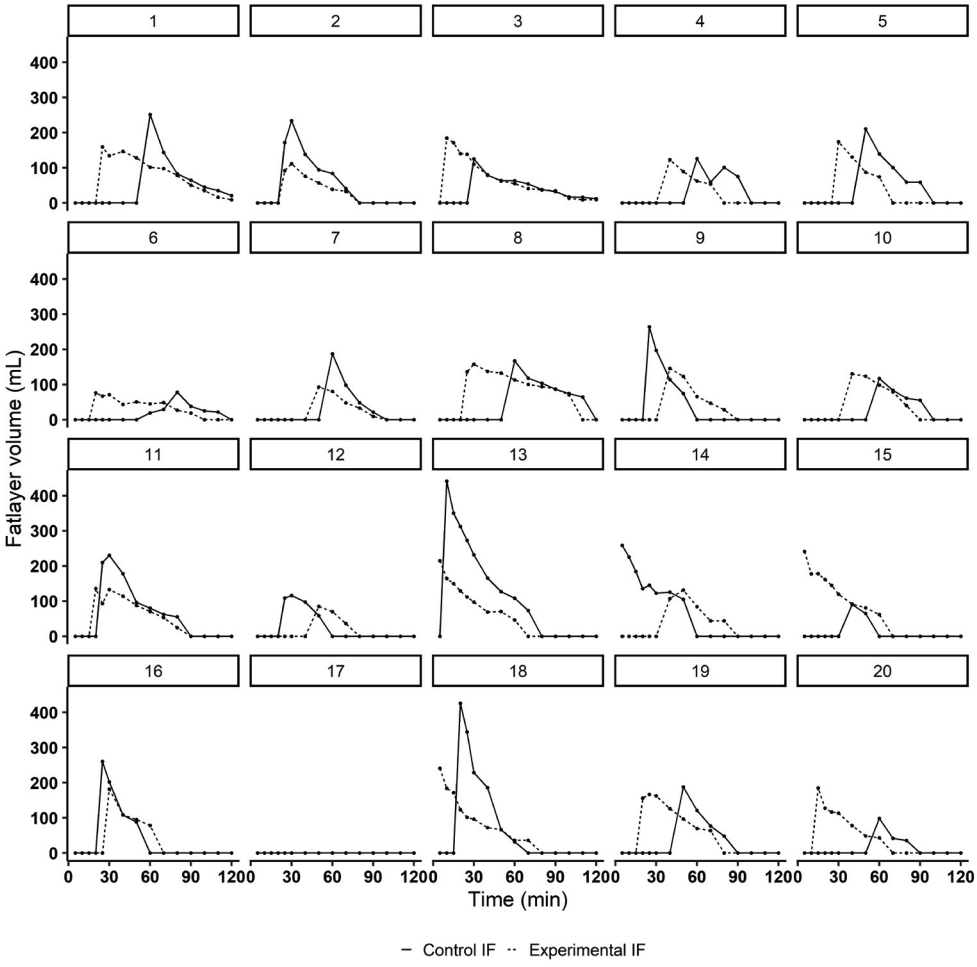
Supplementary Figure 2. Illustration of a stomach MRI time series from one participant with high-fat top layer volume (pink) and total gastric volume (yellow + pink) delineated for the CF and EF.



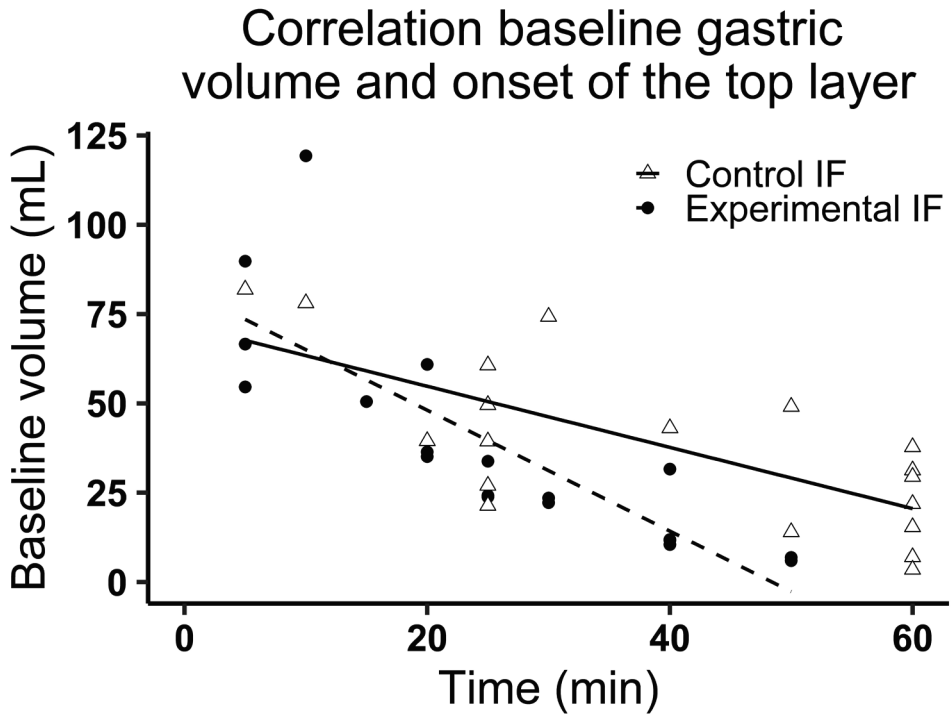
Supplementary Figure 3. Emulsion stability of EF and CF over time after in vitro digestion under simulated infant (A) and adult (B) conditions at different pH.



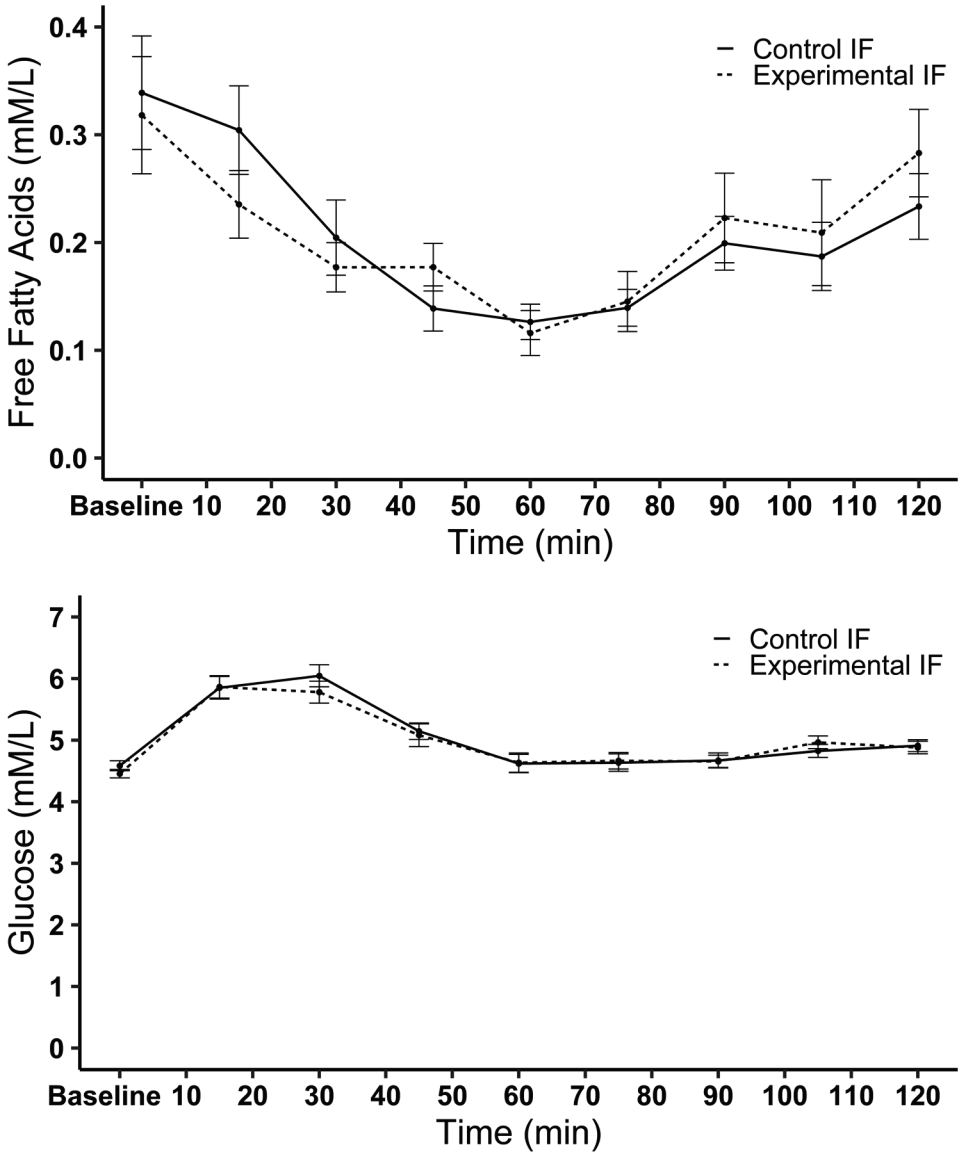
Supplementary Figure 4. Onset time of the gastric top layer (min) per participant for both formulae, CF and EF.



Supplementary Figure 5. Individual curves of the high-fat gastric top layer volume over time for CF and EF.

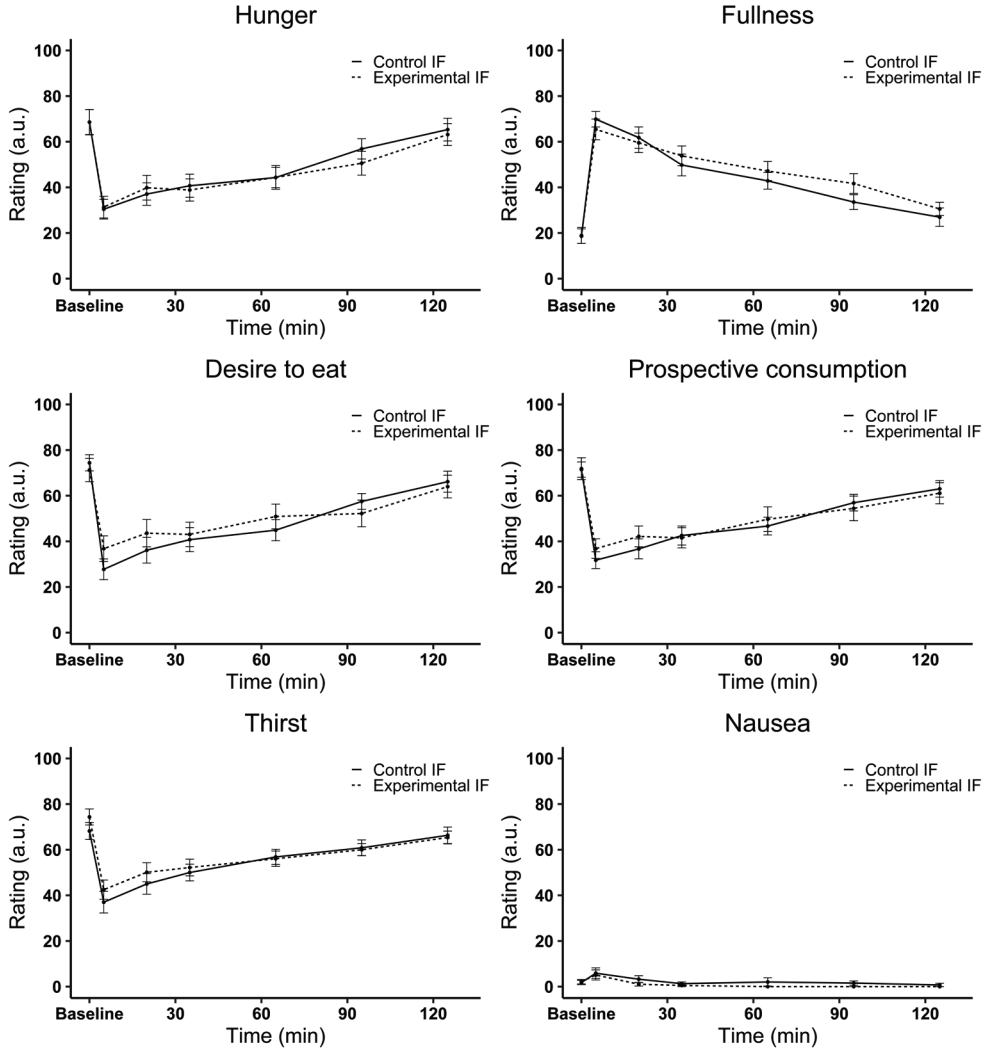


Supplementary Figure 6. Scatterplot of onset time of the gastric top layer (min) and baseline gastric volume of control IF (r = -0.72) and EF (r = -0.82).

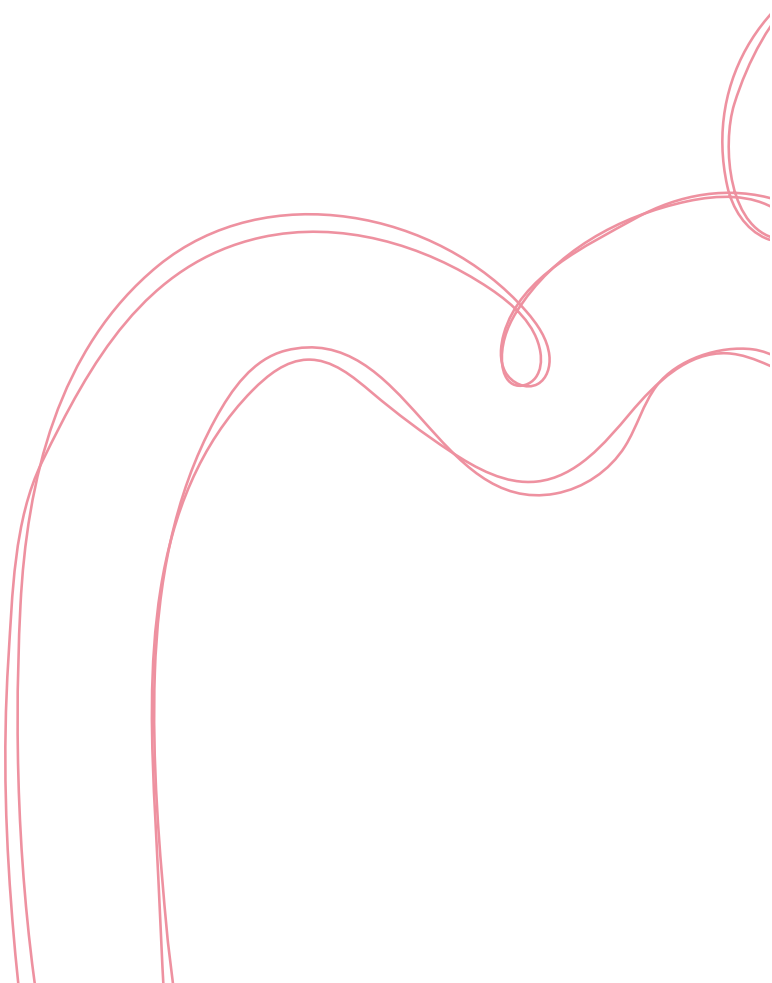


Supplementary Figure 7. Mean ± SE plasma FFA and glucose concentrations over time during the test session after ingestion of the formulae. No treatment effects were found for both postprandial FFA and glucose (p = 0.763 and 0.325, respectively). Moreover, there were no treatment by time interactions (p = 0.835 and 0.881, respectively).

Supplementary Figure 8. NMR-based metabolomics over time that differ significantly ($p < 0.05$) between the control IF and EF. No significant effect of treatment by time was found.

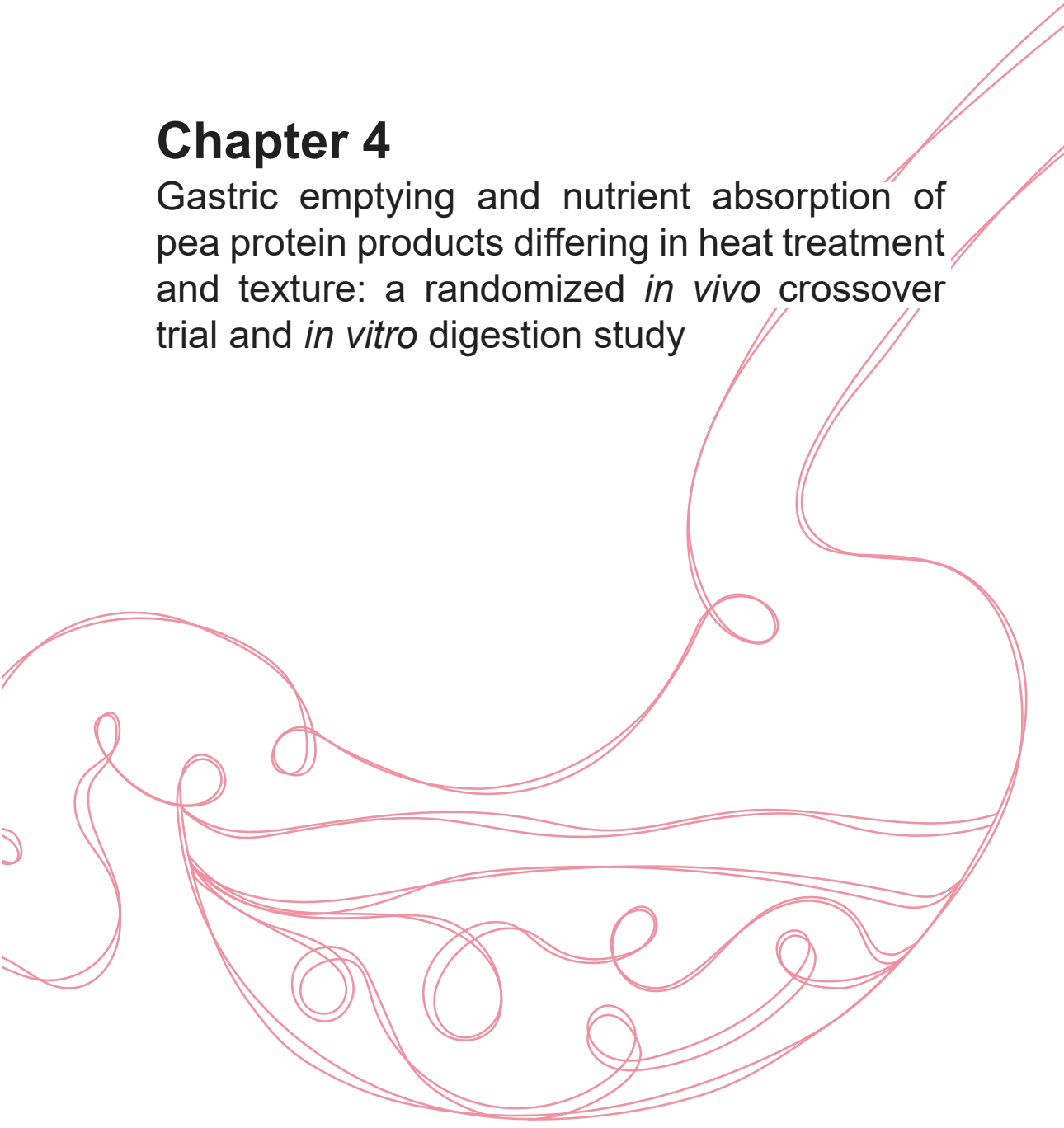


Supplementary Figure 9. Mean ± SE for appetite and nausea ratings over time per treatment. No significant differences were found for hunger, fullness, prospective consumption, and thirst (p = 0.751, 0.340, 0.706 and 0.757 respectively). Desire to eat tended to be lower for the control IF (p = 0.078). Nausea was overall rated lower for the EF (p = 0.023). None of the ratings showed a significant interaction between treatment and time (p = 0.820, 0.575, 0.257, 0.662, 0.718 and 0.890 respectively).



Chapter 4

Gastric emptying and nutrient absorption of pea protein products differing in heat treatment and texture: a randomized *in vivo* crossover trial and *in vitro* digestion study



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ABSTRACT

Pea protein is an interesting alternative for animal-based proteins due to its good availability, low cost, and relatively balanced amino acid (AA) profile. Its digestibility may be affected by heat treatment and food texture. Our aim was to study *in vivo* AA absorption kinetics and gastric behavior of pea protein products differing in heat treatment and texture and compare this with *in vitro* digestion. We included fourteen males in this randomized crossover trial with three iso-caloric and iso-volumetric treatments: a 420 mL heated drink, 420 mL unheated drink and 105 g heated gel (semi-solid) consumed with 315 mL water, all containing 20 g pea protein. Gastric MRI scans were made until 90 minutes post-prandial. Blood samples were collected at baseline and up to 5h. All treatments were tested with an *in-vitro* digestion model (INFOGEST). Heat treatment did not alter AA absorption kinetics and gastric emptying. Time to maximum peak was delayed for the gel treatment (total AAs: 66.9 versus 48.0 min for both drinks, essential AAs: 75.4 versus 50.0 and 46.6 min for the drinks). For the gel treatment initial emptying was faster due to the rapid passage of water. *In vitro*, the degree of hydrolysis was highest for the unheated drink in the gastric phase and for the gel treatment in the intestinal phase. In conclusion, heat treating pea protein products does not affect digestion. In contrast, texture of pea protein products can be altered to influence the rate of gastric emptying and AA absorption without affecting total AA absorption.

Keywords: pea protein, digestion, gastric behavior, amino acid absorption, MRI

1. INTRODUCTION

Protein is an essential building block for the growth and renewal of tissue (Atherton & Smith, 2012). For certain populations, such as older adults, athletes and critically ill, it can be difficult to obtain the necessary amount of protein from the diet (Coelho-Júnior et al., 2018; Liao et al., 2017; Sieber, 2019). It is therefore important that the protein we ingest is properly digested and absorbed, so that it can be used for protein synthesis (Fardet et al., 2019; Mahe et al., 1996; van Vliet et al., 2015). Digestion consists of a series of mechanical, physiological, and biochemical processing steps leading to the breakdown of food structures that eventually allows for absorption and utilization of nutrients (Mackie, 2019). Despite all these processing steps, some proteins are still poorly digested. This is especially the case for plant-based proteins, which often have a lower digestibility (Berrazaga et al., 2019; Pasiakos et al., 2015). However, due to the growing population worldwide, animal-based protein puts a strain on the environment (Katz, 2019). Because of this, the demand for more sustainable plant-based proteins is rapidly growing. Therefore, it is important to explore how the digestibility of plant-based proteins may be improved.

The lower digestibility of proteins from plants is explained by the intact cell wall that hinders direct contact between intracellular macronutrients and the digestive enzymes. This slows down or even completely prevents the access of proteases to the cell contents and limits intracellular protein hydrolysis. Thus, the digestibility of plant-based proteins depends on the fraction of broken cells generated during their processing (Zahir et al., 2018). Food processing such as the isolation of proteins, alters the chemical and physical characteristics and can increase the nutritional value of food products (Joye, 2019). Moreover, plant-based proteins are known for their lower solubility compared to animal-based proteins, which influences digestibility (Rivera del Rio et al., 2020). Plants also contain anti-nutritional factors. These are compounds that reduce nutrient utilization and/or food intake of plants or plant products used as human foods which can be removed or inactivated by processing (Thakur et al., 2019). In addition, the quality of plant-based protein is often lower compared to animal-based protein. Animal-based protein has the highest

protein quality as determined by the Digestible Indispensable Amino Acid Score (DIAAS). The DIAAS of animal-based proteins is typically greater than 100, indicating excellent quality, while for plant-based proteins it is generally below 75, indicating lower quality (Herreman et al., 2020).

Several plant-based proteins, from crops such as wheat, soy, and pea, are increasingly used in foods. With its good availability, low cost and relatively good quality for a plant-base protein (DIAAS = 70), pea protein is one of the better alternatives for animal-based proteins in functional food applications (Bailey et al., 2023; Lu et al., 2020). Although there is ample information about the digestion of traditional protein sources, the digestion of pea protein and the influence of intensive processing on its digestion is not known in detail (Rivera del Rio et al., 2020). This is essential to evaluate its potential as a nutritious sustainable protein source.

Digestion of food products is predominantly studied with *in vitro* digestion models (Muttakin et al., 2019). Although these models are based on *in vivo* data, they obviously do not account for all factors, such as the mixing of the food in the stomach. Therefore, *in vivo* research is needed to understand to what extent *in vitro* models represent *in vivo* digestion. Magnetic Resonance Imaging (MRI) allows for visualization and quantification of gastric processes such as gastric emptying, emulsion stability and coagulation (Smeets et al., 2021). In addition, measuring AA concentrations provides information on differences in absorption kinetics. Although it is not possible to directly relate gastric emptying with subsequent AA absorption because of all intermediate processes involved, combining these measurements does provide more insight in the overall differences between products.

Gastric emptying is largely determined by the chemical characteristics of food, such as the energy density and macronutrient content, but also by physical characteristics, such as texture (Camps et al., 2016; Marciani et al., 2001; Roy et al., 2022). The food matrix plays an important role in digestibility because of its influence on the kinetics of transit and hydrolysis of macronutrients. For example, liquids empty

faster from the stomach compared to semi-solid foods (Camps et al., 2016; Clegg & Shafat, 2014; Mackie et al., 2013; Zhu et al., 2013).
The isolation of plant-based proteins often includes a thermal denaturation step. Thermal denaturation of proteins may either improve or decrease their digestibility, depending on the type of protein and severity of the heat treatment. Proteins either lose their tightly folded structure, resulting in a higher accessibility of the peptide chain for enzymes, or they will aggregate, thereby impairing digestion (Joye, 2019). *In vitro* work on pea protein showed that heating disrupts the structure, thereby increasing the number of smaller better digestible particles. Conversely, these heat-induced aggregates are up to 50% less digestible compared to before the heat treatment (Mulet-Cabero et al., 2019; Rivera del Rio et al., 2020).

The aim of this study was to measure *in vivo* AA absorption kinetics and gastric behavior of pea protein products differing in heat treatment and texture. In addition, we aimed to compare *in vitro* digestion data with the *in vivo* data.

2. METHODS

2.1. *In vivo* trial

2.1.1. Design

The study was a randomized crossover trial in which healthy men underwent gastric MRI scans and blood sampling before and after consumption of three pea protein products. Primary outcomes were plasma AA absorption kinetics and gastric volume over time. Secondary outcomes included plasma glucose and insulin concentrations and appetite and nausea ratings (hunger, fullness, thirst, desire to eat, prospective consumption and nausea). In addition, potential MRI markers of digestion (T_2 relaxation time (Deng et al., 2020; Deng et al., 2023; Deng et al., 2022) and the Magnetic Transfer Ratio (MTR)) were explored (Mayar et al., 2022; Mayar et al., 2023). However, these data are not reported in the current paper. The procedures followed were approved by the Medical Ethical Committee Arnhem-Nijmegen in accordance with the Helsinki Declaration of 1975 as revised in 2013. This study was registered with the Dutch Trial Registry under number NL9413. The record can be

retrieved from the International Clinical Trials Registry Platform at https://trialsearch.who.int/Trial2.aspx?TrialID=NL9413. All participants signed informed consent.

2.1.2. Participants

Healthy (self-reported) males aged 18-55 y and with a BMI between 18.5-25.0 kg/m^2 were included (**Supplementary Figure 1**). Participants were excluded if they reported a pea allergy, gastric disorders or regular gastric complaints, used medication that affects gastric behavior, used recreational drugs within 1 month prior to the study screening day, smoked more than 2 cigarettes per week, had an alcohol intake >14 standard units per week, or had a contra-indication to MRI scanning (including but not limited to pacemakers and defibrillators, ferromagnetic implants and claustrophobia). Since female sex hormones are known to influence gastrointestinal function, only males were included in the study (Gonenne et al., 2006; Lajterer et al., 2022; Soldin & Mattison, 2009). Participants were recruited via digital advertisements (e-mail and social media).

2.1.3. Sample size

A priori sample size was estimated for both primary outcomes, i.e., AA absorption kinetics and gastric volume over time. The estimation for postprandial AA was based on the peak value and the total free AA assessed in the serum after consumption of protein products. For the peak value, a difference of 100 µg/mL was regarded as relevant with an individual difference in peak values of 100 µg/mL (Farnfield et al., 2009; He et al., 2013). Given an α of 0.05 and a power of 0.9, we estimated a requirement of 11 participants.

For gastric emptying the sample size estimation was based on gastric emptying half times of liquids from Camps et al. (2016), and gels from Hoad et al. (2009) taking into account intake volume and caloric content. We estimated 10 min as the minimum detectable difference which is physiologically relevant, and an average SD of 11 minutes. With a two-sided test, an α of 0.05 and a power of 0.9, this resulted in a minimum of 12 participants. To accommodate drop-out, we aimed to include 14

participants. The calculations were done using software from: http://hedwig.mgh.harvard.edu/sample_size/js/js_crossover_quant.html.

2.1.4. Treatments

The three treatments were a 420-mL unheated pea protein drink, 420-mL heated pea protein drink and 105 g heated semi-solid pea protein food (gel) consumed with 315 mL water (Table 1). All treatments contained 20 g of pea protein isolate (Nutralys® F85M, Roquette, France) and were iso-caloric (153 kcal) and iso-volumetric (420 mL). In addition to pea protein isolate and water, the test foods contained vanilla aroma, chocolate aroma, cocoa powder, and sweetener (See **Supplement** for exact product preparation). The heated treatments were heated in a steam oven at 90 °C for 30 minutes. After preparation, the products were stored overnight at 4 °C.

Table 1. Treatment overview.

Treatment	Texture	Volume/ weight	Heat treatment	Water consumed separately (mL)
Unheated drink	Liquid	420 mL	None	0
Heated drink	Liquid	420 mL	90 ˚C – 30 min	0
Heated gel	Semi-solid	105 g	90 ˚C – 30 min	315

2.1.5. Study procedures

The evening before the test day participants consumed a standard pasta meal (Iglo Green Cuisine Linguine Bolognese) after which their overnight fast started. During the fasting period of at least 12 hours, participants were allowed to drink water and herbal tea up to 1.5 hours prior to their visit. Participants were instructed to keep their level of exercise in the 24h before the test session identical for each of the three sessions. In addition, they were instructed to use the same mode of transportation for each session. Upon arrival at Hospital Gelderse Vallei (Ede, The Netherlands), a cannula was placed, a baseline MRI scan was performed, appetite and nausea

ratings were obtained, and a blood sample was drawn. Subsequently, participants consumed one of the three treatments. For the drink, the participants were instructed to consume it over a period of 5 minutes through a straw to ensure an eating time comparable to that of the gel (mean ingestion time was 4.7 ± 0.6 and 4.7 ± 0.9 min for the heated and unheated drink, respectively). For the gel treatment, participants were instructed to consume it within 10 minutes and alternate eating and drinking (mean ingestion time 6.7 ± 1.3 min). Subsequently, gastric MRI scans were performed at t = 10, 15, 20, 30, 40, 50, 60, 70, 80 and 90 minutes after the start of ingestion. Blood samples were taken at t = 30, 60, 75, 90, 120, 150, 180, 240 and 300 min. In addition, participants verbally rated their appetite and nausea on a scale from 0 to 100 every 10 minutes, up to 90 minutes (Noble et al., 2005). These ratings were written down by the researcher (**Figure 1**).

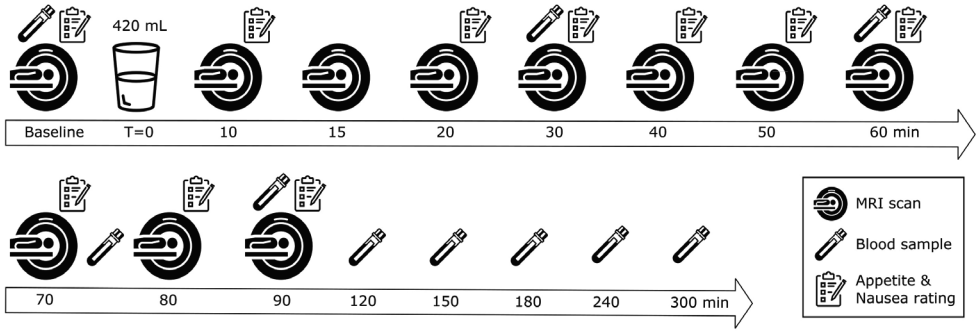

Figure 1. Overview of a test session.

2.1.6. MRI

Participants were scanned in a supine position with the use of a 3-Tesla Philips Ingenia Elition X MRI scanner (Philips, Eindhoven, The Netherlands). A 2-D Turbo Spin Echo sequence (37 4-mm slices, 1.4 mm gap, 1 x 1 mm in-plane resolution, TR: 550 ms, TE 80 ms, flip angle: 90 degrees) was used with breath hold command on expiration to fixate the position of the diaphragm and the stomach. The scan lasted approximately 20 seconds.

Total gastric content was manually delineated on every slice by two researchers with the use of the program MIPAV (Medical Image Processing, Analysis and Visualization Version 7.4.0, 2016) (**Supplementary Figure 2**). When the volumes differed by more than 10% between the two researchers, the segmentation was re-evaluated to reach consensus. Total gastric volume for each time point was calculated by multiplying the number stomach content voxels with voxel volume, taking into account slice thickness and gap distance. The gastric volumes of the two researchers were averaged.

For the gel treatment, volumes of liquid and semi-solid content of the stomach were estimated based on voxel intensity using thresholding (Reddy & Reddi, 2017). The baseline scan was used to calculate the cut-off value for each participant. The cut-off value that was chosen included the 10% voxels with the lowest intensity, since this resulted, on average, in a volume for the semi-solid content at t = 10 minutes close to the volume of ingested (mean solid volume of 102.6 mL, SD = 17.5). This cut-off value was used for all scans in that scan session. An example of a stomach with its semi-solid and solid content marked in red can be found in **Supplementary Figure 3**.

As an approach to quantify gastric coagulation, image texture metrics of the stomach content were calculated with the use of the LIFEx software (version 7.2.10) (Nioche et al., 2018). These image metrics provide information on the spatial patterns of voxel intensity (Thomas et al., 2019). Four metrics were calculated: busyness, coarseness, contrast, and homogeneity. Neighborhood Grey-level Difference Matrix (NGLDM) difference of grey-levels between one voxel and its 26 neighbors in 8 dimensions was used for busyness, coarseness, and contrast. Busyness translates to the spatial frequency of changes in intensity. Coarseness translates to the spatial rate of change in intensity. Contrast is the local variation in grey level. The Gray-Level Co-occurrence Matrix (GLCM) method was used for homogeneity and reflects the differences in voxel intensity between the neighboring voxels. The number of grey levels for texture metric calculation was set at 64, intensity rescaling relative (ROI: min/max) and dimension processing 2D.

These texture metrics were calculated for each slice. Subsequently, a weighted mean was calculated based on gastric volume in each slice, i.e., small stomach volume areas will contribute less to the mean compared to larger areas. In the context of this paper, we interpret changes in image texture metrics as reflecting changes in the degree of coagulation (van Eijnatten, Roelofs, et al., 2023; van Eijnatten, Camps, et al., 2023). An example of two stomachs with their corresponding image texture measures indicating relatively high and low coagulation can be found in **Supplementary figure 4.**

The gel treatment was not considered for this analysis since the presence of dark gel particles in the stomach would yield very different image texture metrics than those of the two drinks. In addition, the analysis was only performed for the postprandial scans. Due to the exploratory nature of these measures, we did not correct for multiple testing.

2.1.7. Clinical chemistry

Blood samples were drawn from the cannula into sodium-fluoride (3 ml) and EDTA (4 ml) tubes. After collection, sodium-fluoride tubes were centrifuged at 1000 g for 10 min at 22°C, to obtain blood plasma. The EDTA tubes were centrifuged at 1000 g for 10 min at 4°C. Following centrifugation, aliquots of 250 µl and 500 µl were pipetted in 2.0 ml cryo-vials and 5 ml tubes and stored at -80°C until they were analyzed in bulk.

Free AA concentrations were analyzed as described previously (Mes et al., 2022) and based on the Waters AccQ Tag method for AA analysis. To determine glucose concentrations, the plasma samples were processed using an Atellica CH Glucose Hexokinase_3 (GluH_3) assay kit and quantified using an Atellica CH analyzer (Siemens Healthineers, Netherlands) by a hospital laboratory (Ziekenhuis Gelderse Vallei, Ede). The lower detection limit was 0.2 mmol/l and inter-assay CVs were at most 4.5%. The plasma samples were processed and quantified using an enzymatic immunoassay kit (ELISA, Mercodia AB, Sweden) to determine insulin

concentrations. The lower detection limit was 6 pmol/l and inter-assay CVs ranged between 0.3 and 20.0%.

2.2. *In vitro* digestion

A static *in vitro* digestion was performed using the INFOGEST digestion protocol for all three treatments (Brodkorb et al., 2019). Gastric digestion was performed for 2 hours followed by 2 hours of intestinal digestion. The degree of hydrolysis and size distribution of the soluble peptides were measured at 30-minute intervals in the gastric phase and at 60-minute intervals in the intestinal phase. Moreover, the unheated and heated drink were tested in a semi-dynamic system. The complete protocol can be found in the **Supplement**.

To measure the particle size of the precipitation in the drinks during digestion, a Mastersizer (Mastersizer 3000, Malvern Panalytical Ltd. United Kingdom) was used. Measurements were taken with an obscuration limit of 4-20%, a reflective index of 1.46 and absorption of 0.1. Non-spherical particle size was selected. The samples were taken after 0, 60 and 120 minutes of static gastric digestion. The samples were not further diluted. Cocoa powder was tested separately to check for any influences on the measurements of the drinks. Results are reported as volume density.

2.3. Statistical analysis

AA concentrations over time were analyzed using the software described in Wehrens et al. (Submitted for publication) (https://github.com/Biometris/aaresponse). In short, peak heights, time to maximum peak and area under the curve of serum AA were calculated for total AAs (TAA) and essential AAs (EAA). For these three parameters of interest, a linear mixed model was used to assess differences between treatments. Analysis was performed in R version 4.1.3.

Further analyses were performed in R statistical software (version 4.0.2). Differences in gastric content volume over time were tested with the use of linear mixed models, testing for main effects of time, treatment, and treatment by time interactions, with baseline gastric volume as a covariate. Tukey HSD-corrected post-hoc tests were

used to compare individual time points. AUC of gastric content volume over time was calculated using the trapezoidal rule. Differences in AUC between treatments were tested by using one-way repeated measures ANOVA.

Differences in the texture metrics (busyness, coarseness, contrast, and homogeneity) of the postprandial gastric volume over time were tested using linear mixed models, with time, treatment, and treatment by time as fixed factors. Tukey HSD corrected post-hoc tests were used to compare individual time points.
Differences in glucose concentrations, insulin concentrations and appetite and nausea ratings over time were tested by using linear mixed models, testing for main effects of time, treatment, and treatment by time interactions. Baseline values were added as covariate. Tukey HSD-corrected post-hoc tests were used to compare individual time points.

For each variable, normality of the data was confirmed with quantile-quantile (Q-Q) plots of the residuals. For insulin, contrast, and nausea a logarithmic transformation was applied to create a normal distribution. The significance threshold was set at $p = 0.05$. Data are expressed as mean ± SD unless stated otherwise.

3. RESULTS AND DISCUSSION

In total, 14 men participated in the study (age: 23.0 ± 3.8 y, BMI: 22.2 ± 1.7 kg/m^2). Two participants dropped out after one test session. Hence, two additional participants were recruited. Two participants completed only two test sessions due to Covid-19 infection related quarantine.

3.1. Blood amino acid kinetics

Figure 2 shows the curves of TAA and EAA over time. AUC did not differ between the three treatments, indicating that total absorption is comparable (**Figure 3 and Supplementary Figure 5**). Individual curves of TAA and EAA can be found in **Supplementary Figure 6**. For the individual AA, only tyrosine showed a significant lower AUC for the gel treatment compared to unheated and heated drink (234 µM*min (CI: 201 – 268) compared to 304 µM*min (CI: 249 – 359) and 311 µM*min

(CI: 262 – 360) respectively). AUCs of the other AAs did not differ significantly. Curves of the individual AAs are shown in **Supplementary Figure 7**.

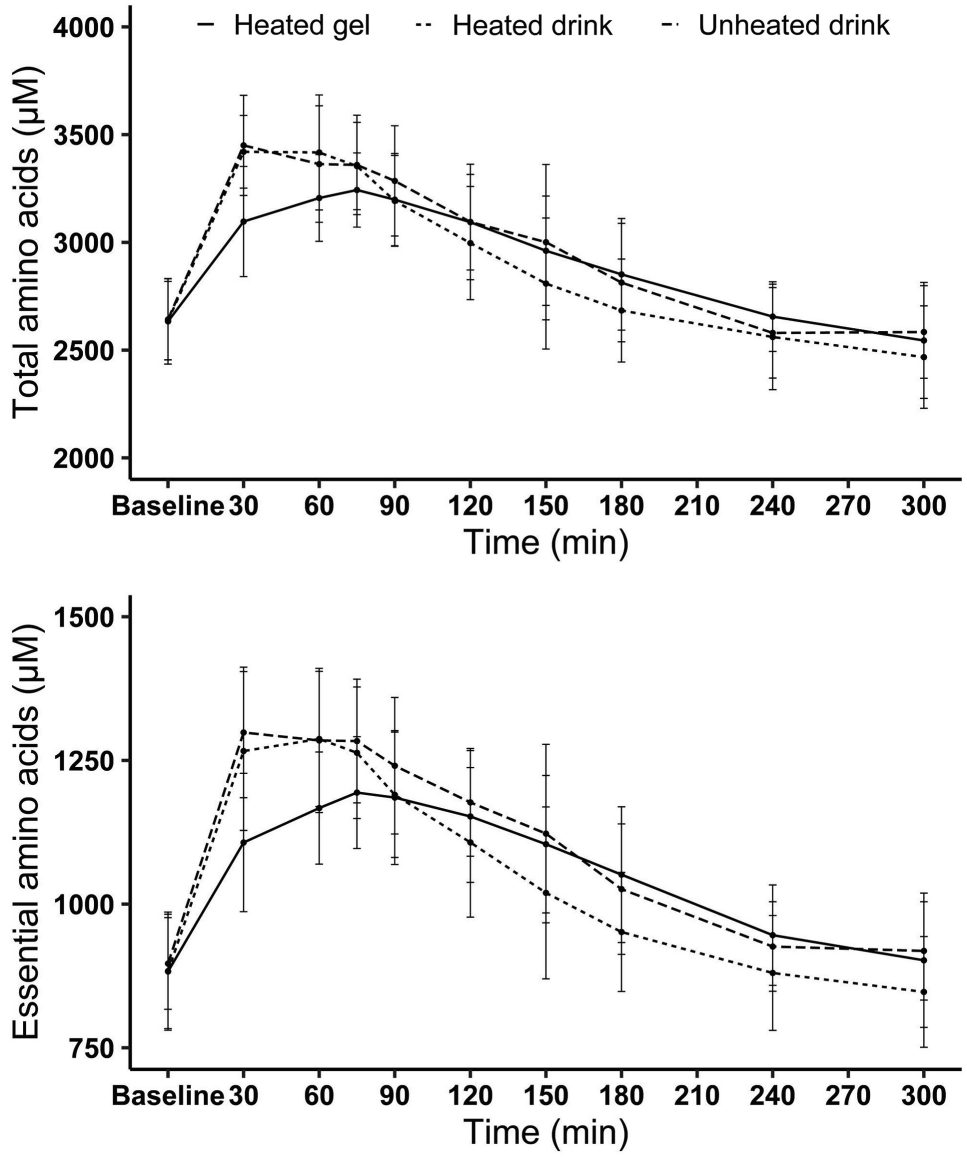

Figure 2. Total amino acids (top) and essential amino acid (bottom) levels over time after consumption of the three pea protein products (mean ± SD).

Maximum peak height for EAA was 131 and 145 μM lower for the gel treatment compared to the unheated and heated drinks (330 μM (CI: 290 – 369) compared to 460 μM (CI: 389 – 531) and 475 μM (CI: 407 – 543) respectively). No difference in maximum peak height was found for TAA. Eleven individual AAs showed a significantly 21.8 – 33.6% lower maximum peak height for the gel treatment compared to the unheated drink.

In addition, the time to maximum peak for TAA absorption was 18.8 and 18.9 minutes later for the gel treatment compared to the unheated and heated drink respectively (66.9 min (CI: 59.2 – 74.6) compared to 48.0 min (CI: 37.9 – 58.2) and 48.0 ± 8.4 min (CI: 41.0 – 55.0) respectively). For EAA, time to maximum peak was significantly delayed by 25.4 and 28.8 minutes for the gel treatment compared to the unheated and heated drink respectively (75.4 min (CI: 66.8 – 83.9) compared to 50.0 min (CI: 39.9 – 60.0) and 46.6 min (CI: 38.4 – 54.9)) (**Supplementary Figure 5**). Of the 19 individual AAs measured, 14 AAs showed a 14.4 – 30.3-minute later time to maximum peak height for the gel treatment compared to both drinks.

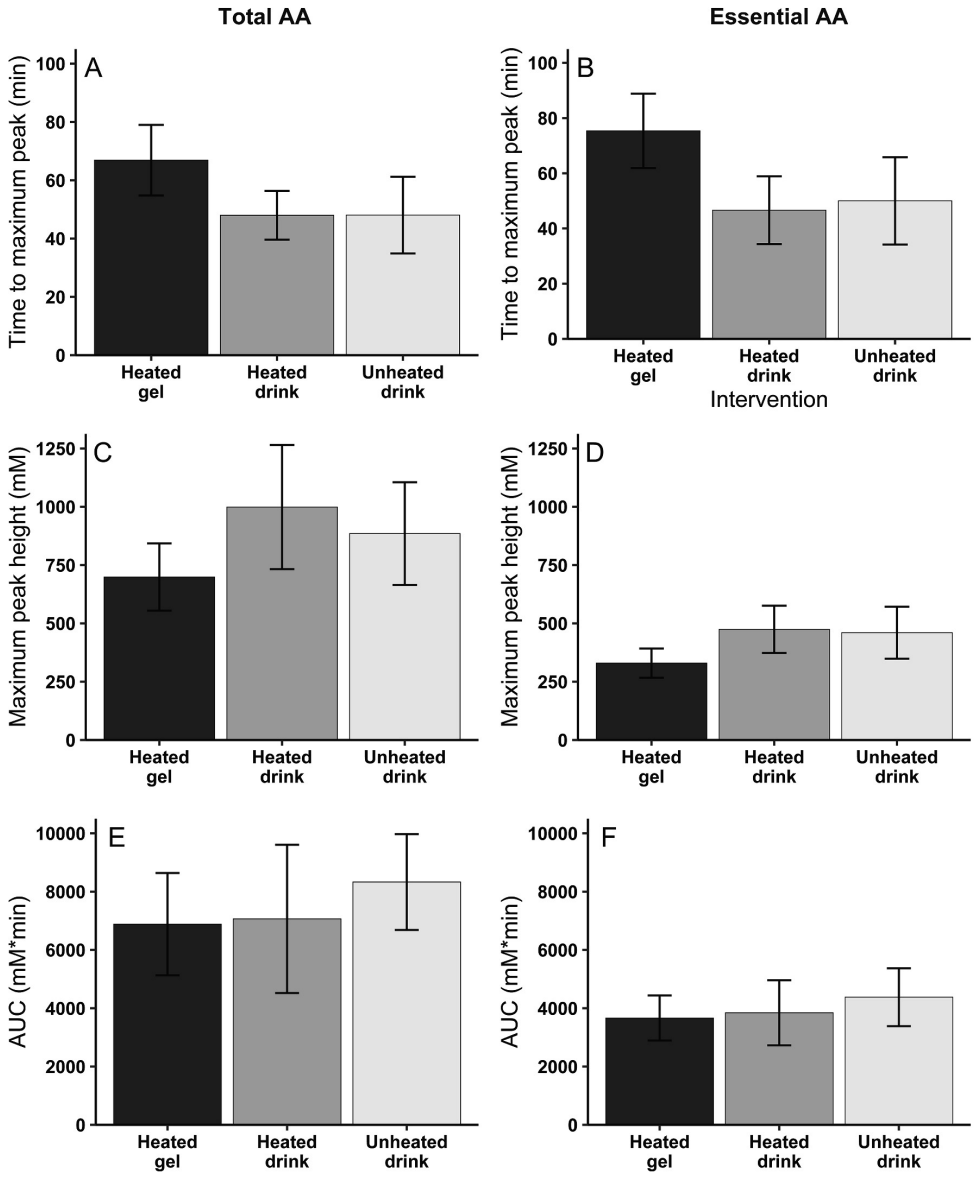

*Figure 3. Mean ± SD time to maximum peak (min) (A and B), maximum peak height (μM) (C and D) and AUC (mM*min) (E and F) of the three pea protein products for total AA (left panel) and essential AA (right panel).*

These results show that heat treatment did not affect AA absorption kinetics, but that the gel treatment resulted in a lower, delayed maximum peak. The industrial processing required to manufacture pea protein isolate includes heat treatment. Since the heat-treated drink did not show altered AA absorption kinetics, additional heat treatment did not further affect digestibility.

The attenuated rise in postprandial AA concentrations after consumption of a solid versus liquid food was reported in multiple studies (Conley et al., 2011; de Hart et al., 2021; Hermans et al., 2022; Horstman et al., 2021), which is in contrast to our findings. However, these studies compared products that did not only differ in texture, but also macro- and micronutrient composition, protein composition and/or volume. In contrast, van Lieshout et al. (2023) compared liquid vs solid iso-caloric and iso-volumetric products based on whey isolate and calcium caseinate and found no difference in postprandial AA concentrations in healthy females. This difference might be explained by the type of the proteins. Animal-based proteins, especially caseins, are known to coagulate, thereby delaying gastric emptying (Huppertz & Chia, 2021) and AA absorption kinetics (Horstman & Huppertz, 2022), while this is not the case for plant-based proteins.

3.2. Gastric emptying

Baseline gastric volume was 36.2 ± 19.5 mL for the gel treatment, 26.3 ± 26.7 mL for the unheated and 40.0 ± 26.4 mL for the heated drink ($p = 0.373$). An example time series for the unheated drink and the gel treatment is shown in **Figure 4**.

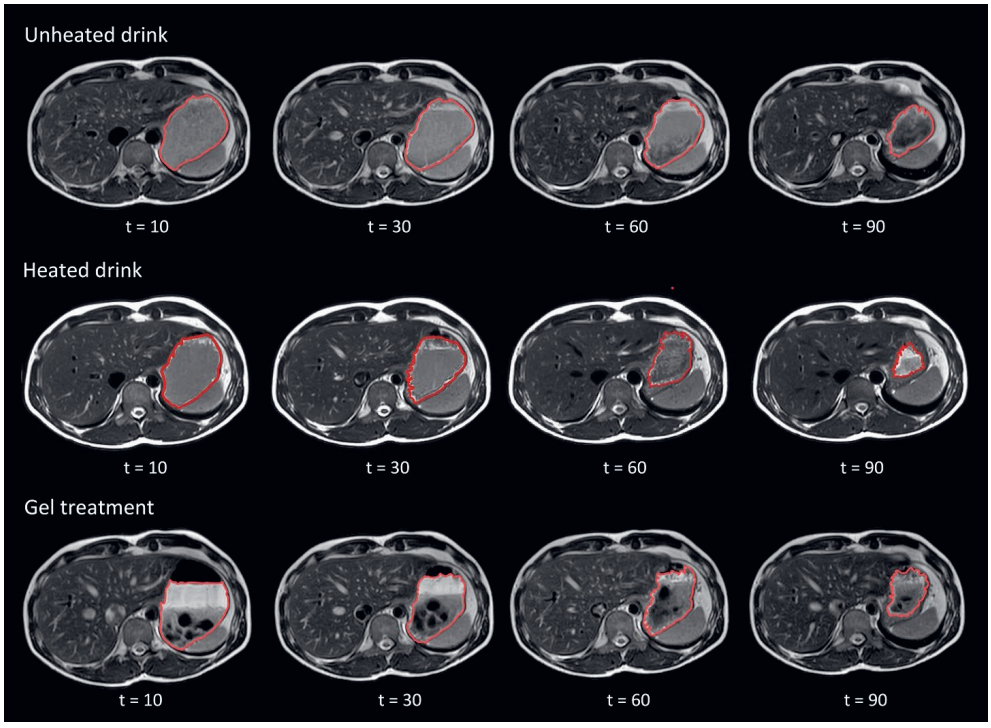

Figure 4. Illustration of gastric emptying over time showing axial MRI images including the stomach for the three treatments over time. The stomach content is delineated in red. S indicates the spine. The figure shows that gastric content volume decreases over time for all treatments. For the two drinks the gastric content is relatively homogenous. For the gel treatment the first scan (t = 10 min) shows a bright layer on top, which is the water that was consumed. The black particles at the bottom, are gel pieces.

Figure 5 shows an almost linear emptying for the drinks, while the gel treatment shows a quick initial emptying. No differences were found between the heated and unheated drink. *In vitro* work by Rivera del Rio et al. (2020) showed that heat treatment of pea protein isolate not only results in small and suspended particles that can be better hydrolyzed by pepsin in the stomach but also induces aggregates, which are less digestible. Thus, although they found that heat treatment of pea protein isolate affects the structure of the proteins, it did not significantly affect the overall *in vitro* gastric digestibility, which is in line with our findings *in vivo*.

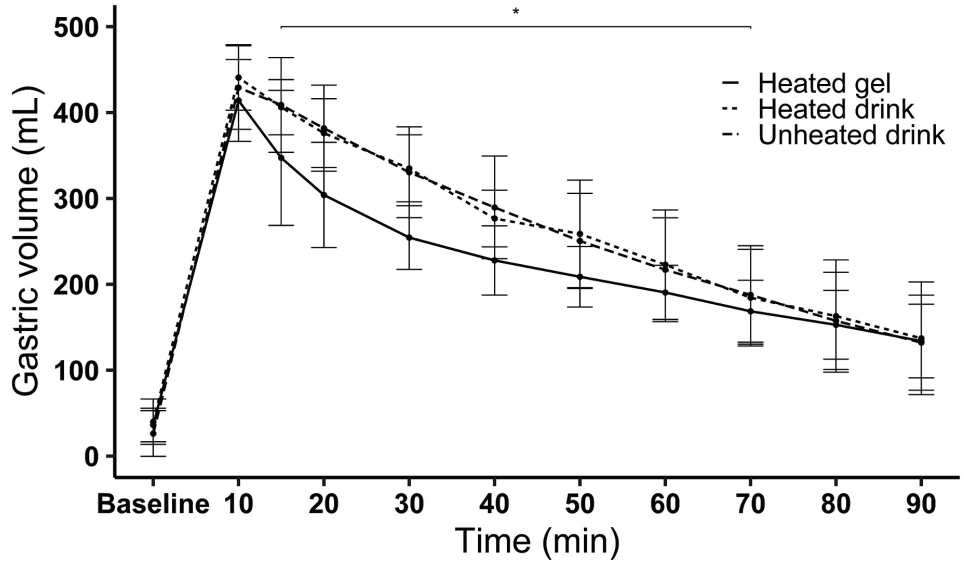

*Figure 5. Mean ± SD gastric volume over time of pea protein products. *p < 0.05, as analyzed with a linear mixed model and Tukey HSD correction for multiple comparison. There was a significant treatment effect for the gel treatment compared to both drinks at t = 10 until t = 70 min.*

There was a significant treatment by time effect for gastric volume, with a lower volume for the gel treatment (p = 0.002). This effect was driven by timepoints t = 15 up to 70 minutes. The AUC of gastric volume over time showed a trend toward a treatment effect (p = 0.071). On average, AUC of the gel treatment was 16% and 15% lower compared to the heated and unheated drink respectively (17608 ± 3059 mL*min compared to 21037 ± 3999 mL*min and 20605 ± 3892 mL*min, p = 0.086 and 0.149, respectively) (**Figure 6**). There was no difference between the two drinks (p = 0.959). Since we do not expect large differences in gastric juice production, and because, if anything, the gel consumption might induce greater gastric juice release due to the greater sensory exposure, we think that the AUC trend towards faster emptying is caused by the relatively rapid gastric passage of the watery fraction.

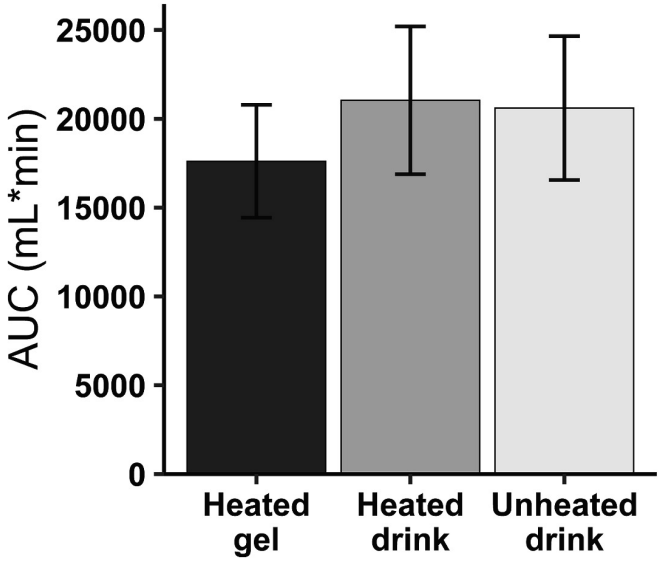

Figure 6. Average AUC ± SD of gastric volume over time for the three treatments. One-way ANOVA showed no significant difference between treatments.

Figure 7 shows that for the gel treatment the liquid content of the stomach emptied quickly during the first 30 minutes, while the solid content emptied slower. Over 90 minutes, the liquid volume decreased from 300 ± 15 mL to 66 ± 8 mL (78.1% decrease). The solid volume, that is, the protein gel fraction, decreased from 103 ± 5 mL to 69 ± 9 mL (32.4% decrease). For the unheated and heated drink, the decrease of liquid volume over 90 minutes was 69.2% and 68.9% respectively. This is in line with previous research of Mackie et al. (2013) who found slower gastric emptying after consumption of a semi-solid compared to an iso-caloric liquid meal containing animal-based proteins (grated gouda cheese and low-fat yogurt consumed with water compared to a homogenous liquid mixture of sunflower oil, sodium caseinate, whey protein isolate and sugar). In addition, Marciani et al. (2012) showed that when the solid and water fraction are not homogenized, the water sieves past the gastric content and empties quickly. When the same meal was blended into a soup, gastric content volume decreased more slowly in a linear fashion (Marciani et al., 2012). This is in line with our results for the pea protein drinks, which had an

approximately linear emptying curve. In addition, the lower accessibility of pepsin to penetrate a food bolus explains why hydrolysis of a semi-solid protein food was slower compared to that of the protein drinks, leading to slower gastric emptying (Bornhorst et al., 2016; Luo et al., 2015). No clear correlation was found between the slower emptying of the pea protein gel and AA absorption kinetics. However, the delay that was found in gastric emptying was also reflected in the absorption of AA in the blood, which showed an 18.8 – 28.8 min delay and lower maximum peak compared to the drinks. This is in line with our expectation that delayed gastric emptying results in delayed AA absorption.

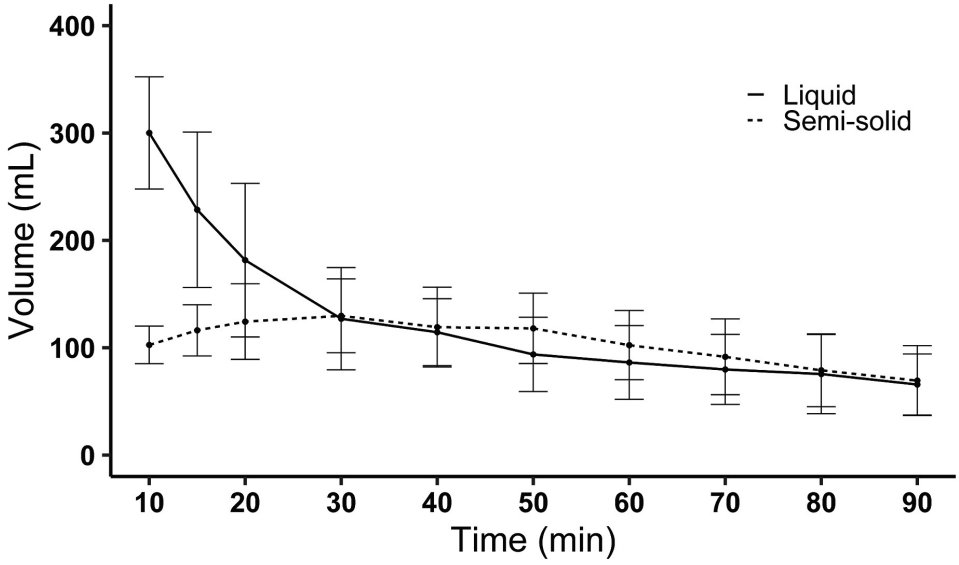

Figure 7. Mean ± SD liquid and semi-solid gastric volume over time of the gel treatment after ingestion of 105 g of pea protein gel with 315 mL water.

3.3. Gastric behavior

Although no coagulates were visible on the MRI images, **Figure 8** shows an overall change in the texture metrics over time (all $p < 0.001$). Busyness and homogeneity decreased, and coarseness and contrast increased over time for both drinks. This suggests a higher degree of coagulation for both drinks in this time frame.

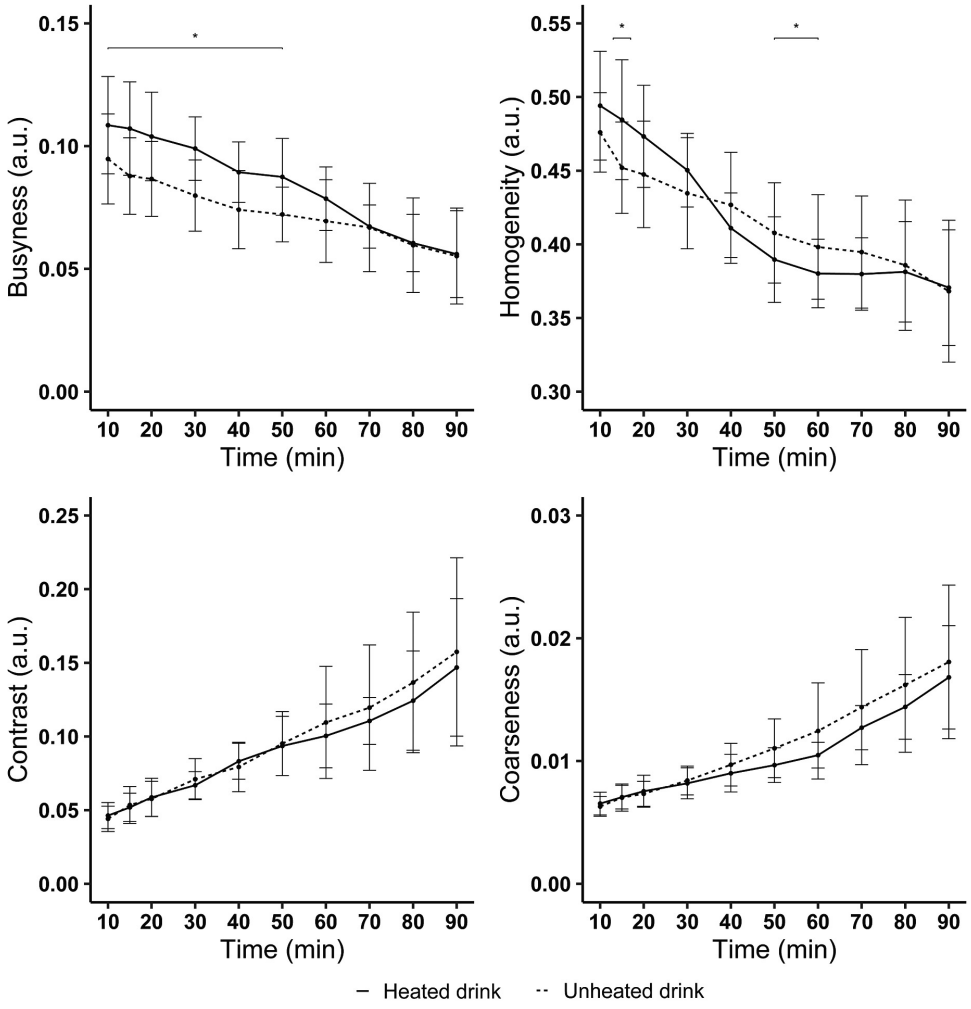

*Figure 8. Mean ± SD texture metrics (arbitrary units) of the stomach contents over time for the unheated and heated drinks. *P < 0.05, as analyzed with a linear mixed model and Tukey HSD correction for multiple testing.*

No treatment or treatment by time interaction effect was found for contrast (p = 0.204 and p = 0.973) and coarseness (p = 0.295 and p = 0.564). However, for homogeneity a treatment by time interaction was found (p = 0.002). It was lower for the unheated drink at t = 15 min, while it was higher at t = 50 and 60 min compared to the heated drink. Treatment by time interaction was also significant for busyness, with higher

values for the heated compared to the unheated drink (p = 0.019). This was driven by timepoints t = 10 until t = 50 min. Based on the latter two, one might conclude that the unheated drink showed a greater degree of coagulation in the first ~60 minutes compared to the heated drink.

In literature, the results of *in vitro* digestion research on pea protein coagulation are inconsistent. An *in vitro* study by Overduin et al. (2015) showed that a 3% solution of the same pea protein isolate as used in this study forms coagulates of 50-500 µm within 2 hours. This is in agreement with our *in vitro* measurements that showed a maximum particle size of 500 µm. Coagulates of this size will not be visible on the MRI images, with a resolution of 1 by 1 by 4 mm. However, formation of such small coagulates could still affect the intensity of these T_2-weighted scans. This might explain the observed changes in the image texture metrics. In addition, since these texture metrics look at intensity contrast in the stomach, gastric juice might influence these metrics, since it appears as a high image intensity. This requires further validation. However, even when these small coagula would be present, this is not likely to affect gastric emptying since particles <1-2 mm can be emptied through the pylorus (Kong & Singh, 2008). This is in line with our results, where no differences in gastric emptying and AA absorption were observed between both drinks. Based on these findings we conclude that even if pea protein isolate coagulates in the stomach, this does not affect further digestion.

3.4. Glucose and Insulin

For glucose there was a trend towards lower concentrations for the unheated drink (p = 0.069). However, the interaction with time was not significant (p = 0.602) (**Supplementary Figure 8**). For insulin, there was a trend towards lower concentrations over time for the gel treatment (p = 0.058), driven by t = 30 min (**Supplementary Figure 9**). Since carbohydrate levels were similar for the products, this is in line with our expectations. The trend for insulin might be explained by the delay in AA absorption kinetics, as proteins are known to have an insulinotropic effect (Rietman et al., 2014).

3.5. Appetite and nausea

Appetite and nausea ratings are shown in **Supplementary Figure 10**. There was a treatment effect for hunger, fullness, desire to eat and, prospective consumption ($p < 0.001$, $p = 0.018$, $p = 0.003$ and $p < 0.001$, respectively). Hunger (MD -8.3 and -7.1), desire to eat (MD -6.7 and -5.2) and prospective consumption (MD -7.3 and -8.3) were all lower for the gel treatment compared to the unheated and heated drink, respectively ($p < 0.001$ for all differences). Fullness was higher for the gel treatment compared to the unheated drink (MD 4.8, $p = 0.014$), but not the heated drink (MD 3.5, $p = 0.108$). However, the interaction with time was not significant for hunger, fullness, desire to eat and prospective consumption ($p = 0.714$, $p = 0.960$, $p = 0.999$ and $p = 0.998$, respectively). Thirst did not differ between treatments (treatment effect: $p = 0.359$, treatment by time interaction $p = 0.998$). Nausea showed a treatment effect ($p = 0.003$) with lower levels for the gel treatment compared to the unheated drink (MD -2.9, $p = 0.002$), but not the heated drink (MD -1.5, $p = 0.230$). However, there was no interaction with time ($p = 0.283$).

These results indicate that consumption of a semi-solid food results in increased feelings of satiety compared to the consumption of iso-caloric and iso-volumetric liquid foods. This is in contrast to a study of Marciani et al. (2012) that showed that a mixed solid/liquid food is less satiating compared to the same meal in homogenized form. They suggested that this might be due to the quick initial emptying, which reduces gastric volume and thus lowers sensation of fullness. This lower sensation of fullness is in line with our findings for the gel treatment. However, a study of Zijlstra et al. (2009) found that consuming semi-solids was more satiating compared to liquids. In addition, Camps et al. (2016) also showed that increasing viscosity increased satiation. One explanation for this is the greater degree of oral exposure when consuming the gel. Longer mastication for an isocaloric load leads to higher feelings of satiety (Forde & Stieger, 2022; Lasschuijt et al., 2021; Wanders et al., 2013). However, it should be noted that overall differences between the semi-solid treatment and drinks were small, with a mean difference <10, which is often considered as a cut-off point for clinical relevance (Flint et al., 2000).

3.6. *In vitro* digestion

Figure 9 shows the *in vitro* degree of hydrolysis of the three treatments for 2 hours of gastric digestion (0 – 120 min) and 2 hours of intestinal digestion (120 – 240 min). The digestibility, expressed as degree of protein hydrolysis of the unheated drink was slightly higher compared to that of the heated drink and gel treatment during the gastric phase (9.6% compared to 5.2% and 3.0% at 120 min, respectively). In the intestinal phase, the gel treatment had a higher degree of hydrolysis compared to the unheated and heated drinks (57.6 compared to 38.3 and 38.9% at 240 min respectively). This suggests, that in the stomach, the gel structure reduces the access of pepsin. However, after two hours in the gastric phase the gel might have swollen, leading to a looser structure that is more accessible for trypsin in the intestine. In addition, the peristaltic movements in the intestine might result in increased fractionation, creating a larger surface area. This is in agreement with the *in vivo* results, where the gel treatment showed lower AA concentrations during the first ~60 minutes, but comparable concentrations after that.

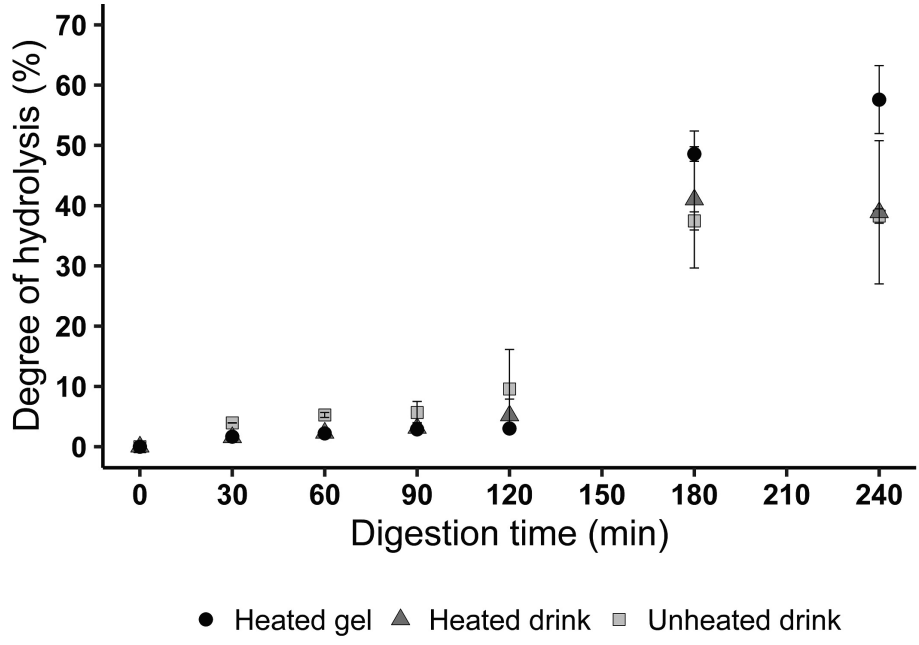

Figure 9. Degree of hydrolysis (%) of the three pea protein products measured via a static in vitro protocol.

Precipitation of the drinks was similar at t = 0. This initial precipitation can be explained by the low solubility that plant-based proteins are known for. For the heated drink precipitation increased over time indicating higher levels of aggregation. However, precipitation was stable for the unheated drink (**Supplementary Figure 11**). This was confirmed with the semi-dynamic digestion model (**Supplementary Figure 12**). This is in contrast with our *in vivo* measures of gastric behavior that indicate a greater degree of coagulation over time for both treatments.

Moreover, the heated drink had larger particles compared to the unheated drink (**Supplementary Figure 13**). Supplementary Figure 13-B and 13-C show the size distribution of the particles in both drinks at 0, 60 and 120 minutes after the start of digestion. In both drinks, most particles were around 10 µm. Both drinks showed a decrease over time for larger particles, while the volume density for smaller particles increased. The maximum particle size for the unheated drink was 270 µm, while the heated drink showed particles sizes up to 500 µm. This is in line with previous research that showed coagulates of 50-500 µm for the same product (Overduin et al., 2015).

In the gastric phase (0-120 min), the heated drink showed a higher number of soluble peptides (**Supplementary Figure 14**). The higher solubility of the heated drink is likely a result of the heating process (Rivera del Rio et al., 2022). In addition, for the gel treatment it took about an hour in the gastric phase until the same amount of dissolved peptides was present. During the intestinal phase (180 and 240 min), the AUC was higher for the gel treatment compared to the unheated and heated drink (8415 compared to 5660 and 5791 mAu*min at 240 min, respectively), which is in agreement with the higher degree of hydrolysis (**Figure 9**). For the drinks, the number of large molecules decreased over digestion time and more small size peptides became soluble (**Supplementary Figure 14**).

3.7. Limitations

This study used MRI to examine gastric behavior. This requires participants to be scanned in a supine position. Although the effect is small, studies have shown that

protein ingestion in an upright sitting position accelerates gastric emptying and increases the postprandial rise in plasma AA availability by increasing protein digestion and AA absorption rates compared to a supine position (Holwerda et al., 2016; Holwerda et al., 2017; Jones et al., 2006; Spiegel et al., 2000). The study of Holwerda et al. (2017) showed a higher peak plasma leucine concentration for upright sitting compared to a supine position (213 ± 15 compared to 193 ± 12 μmol/L, $P < 0.05$). However, the participants were scanned in the same position for all treatments. Therefore, the relative differences between treatments are expected to remain the same.

4. CONCLUSION

To conclude, this study demonstrates that heat treatment of pea protein isolate does not affect gastric emptying or AA absorption. However, consuming pea protein isolate in a product with a semi-solid texture slowed down both gastric emptying and subsequent AA absorption compared to liquids but did not affect total absorption kinetics. These results suggest that texture influences the rate at which pea protein is absorbed, but not total absorption. In addition, comparison with *in vitro* data showed that *in vitro* digestion models gave additional support and insight to *in vivo* digestion results.

5. AUTHORS' CONTRIBUTIONS AND ACKNOWLEDGEMENTS

Julia J.M. Roelofs: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Elise J.M. van Eijnatten**: Conceptualization, Writing – review & editing. **Patteela Prathumars**: Investigation, Writing – review & editing. **Joris de Jong**: Data curation, Investigation, Writing – review & editing. **Ron Wehrens**: Data curation, Formal analysis, Writing – review & editing. **Diederik Esser**: Conceptualization, Writing – review & editing. **Anja E.M. Janssen**: Conceptualization, Writing – review & editing. **Paul A.M. Smeets**: Conceptualization, Writing – review & editing, primary responsibility for the final content. All authors read and approved the final manuscript. None of the authors declare a conflict of interest. We thank Caya Lindner for assisting with data collection. The use of the 3T MRI was made possible by WUR Shared Research Facilities.

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7. SUPPLEMENT

7.1. Supplementary methods – Product preparation

Pea protein gel

Ingredients (104.5g):

25 g pea protein isolate (Nutralys® F85M)
60 mL water
8 pcs cyclamate and saccharin sweeteners (Kruger)
7 mL vanilla aroma (Dr. Oetker)
5 mL chocolate aroma (Nielsen Massey Chocolate-extract)
7.5 g cacao

Preparation:

Combine and thoroughly mix all ingredients
Cover with plastic foil and poke a few holes in the plastic
Steam it in the steam oven for 30 min on 90 ^{o}C
Store the product immediately (4 ^{o}C)

Pea protein drinks

Ingredients (419.5g):

25 g pea protein isolate (Nutralys® F85M)
60 mL water
8 pcs cyclamate and saccharin sweeteners (Kruger)
7 mL vanilla aroma (Dr. Oetker)
5 mL chocolate aroma (Nielsen Massey Chocolate-extract)
7.5 g cacao

Preparation:

Combine and thoroughly mix all ingredients

Unheated drink

Store the product immediately (4 ^{o}C)

Heated drink:

Cover with plastic foil and poke a few holes in the plastic

Steam it in a steam oven for 30 min at 90 °C

Store the product immediately at 4 °C.

7.2. Supplementary methods – In-vitro digestion model

The three treatments were analyzed using the static *in vitro* digestion model. For the drinks, a load of 5 mL was used and for the gel treatment 1.25 g of gel was used together with 3.75 mL water.

Gastric phase

The volume ratio of drink to SGF (simulated gastric fluid) included pepsin (2000 U/mL final activity) was 1:1 for the gastric phase of the digestion. The gastric digestion was performed for 2 hours at 37°C and separate tubes were used for each sampling time point. After sampling, digestion was stopped by adding a predefined amount of NaOH to reach pH 7 to inactivate pepsin and the tube was placed in an ice bucket.

Intestinal phase

This step is a follow-up of the gastric digestion. The gastric digestion was stopped by adding a predefined amount of NaOH to get pH 7.0. Subsequently, SIF (simulated intestinal fluid) was added in a volume ratio of 1:1. Next to that, pancreatin (final trypsin activity 100 U/mL) was added to start the intestinal digestion. The intestinal digestion was performed at 37°C for 2 hours with individual tubes for 60 and 120 minutes. To stop the digestion, the tubes were heated at 95°C for 5 minutes and afterwards placed in an ice bucket.

Peptide characterization

High Performance Size-Exclusion Chromatography (HPSEC) was used to analyze the peptide size distribution during digestion. An UltiMate 3000 chromatographic system (ThermoFischer Scientific Inc., USA) is used with a two TSK gel columns G3000SWXL and G2000SWXL. The eluent consisted of 30% v/v acetonitrile and

0.1% v/v trifluoro acetic acid and the flow rate was 1.5 mL/min. The UV-detector was set at 214 nm. Details on the analysis were described by Rivera del Rio et al. (2020).

Degree of hydrolysis

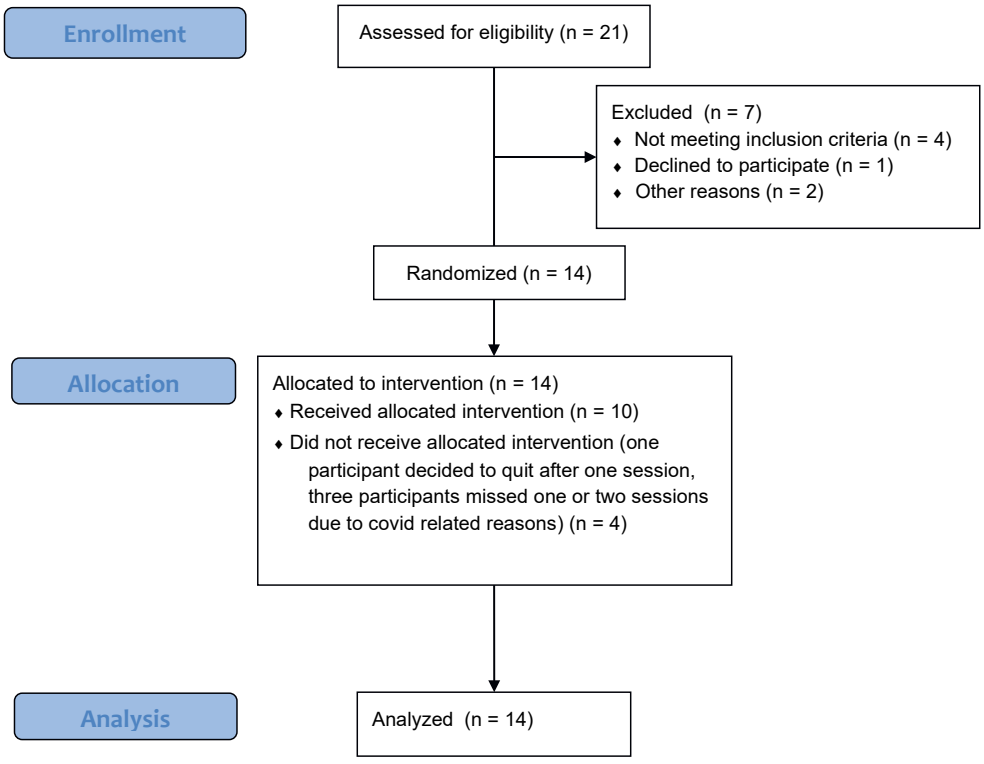
The degree of hydrolysis is the number of cleaved peptide bonds divided by the total number of peptide bonds, expressed as percentage. It can be calculated using this equation: $DH = \frac{h}{h_{tot}} \cdot 100\%$. Where h_{tot} is the total number of peptide bonds in 1 kg protein in meq/g. The number of peptide bonds cleaved in 1 kg protein, h, can be calculated as follows: $h = \frac{\frac{[NH_{2,free}]}{[Protein]}}{\alpha}$. Where [$NH_2$, free] is the calculated concentration of free amino groups in the samples by using a standard curve, [Protein] is the initial protein concentration and α stands for the relationship between [NH_2, free] and the color intensity measured and is 1 for pea protein.

The concentration of free amino groups is measured via a colorimetric reaction with o-phthaldialdehyde (OPA). The method has been described by Rivera del Rio et al. (2022). The calibration curve was prepared with a leucine standard solution. The experiments were done in duplicate.

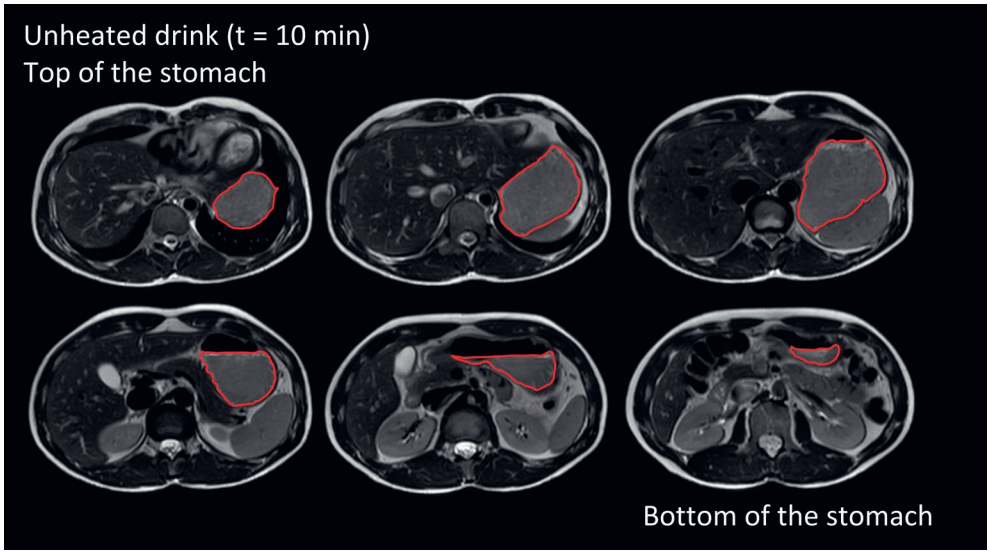
Semi-dynamic in vitro digestion model

The unheated and heated drink were tested in a semi-dynamic system, where 1.21 mL of SGF (simulated gastric fluid) containing 4000 U/mL pepsin was added at 4 time points: t = 0, 30, 60, 90 min. After adding SGF, the tube was shaken. No gastric emptying was included.

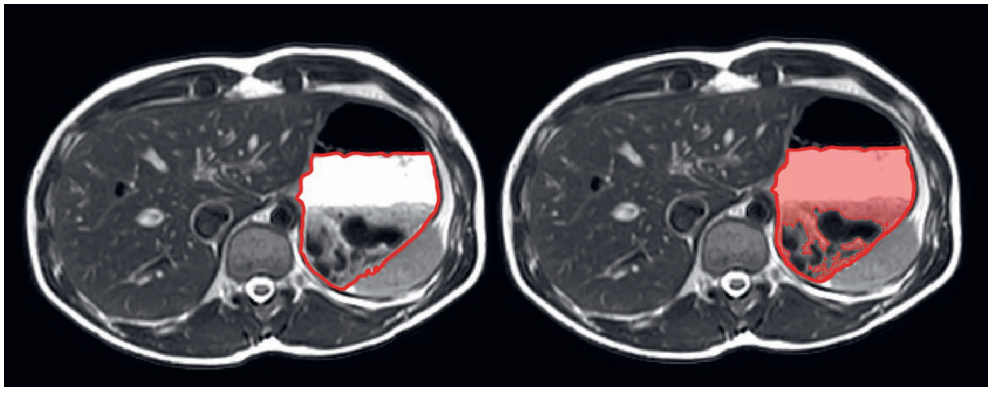
7.3. Supplementary Figures



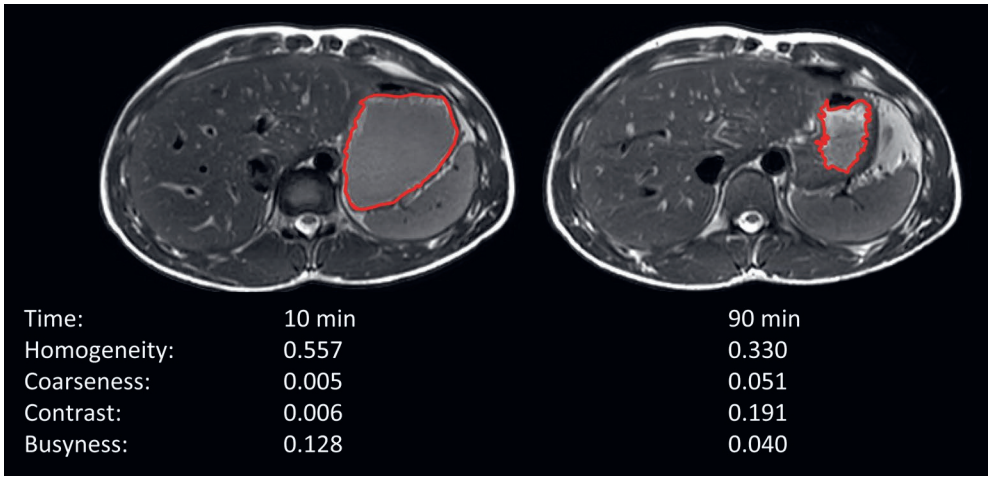
Supplementary Figure 1. Flow diagram for inclusion, treatment allocation and analysis.



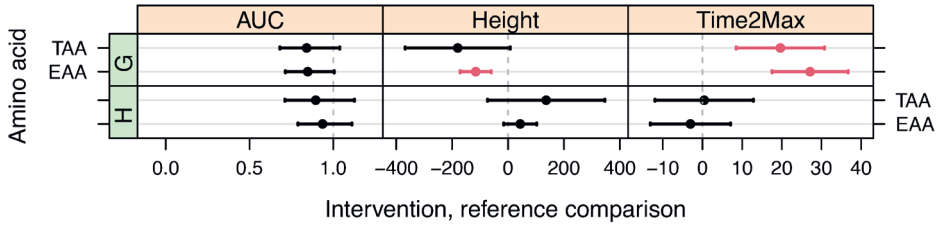
Supplementary Figure 2. Axial MRI scan cross-sections with delineated stomach content after ingestion of the unheated drink (420 mL) at 10 min after the start of ingestion from top to bottom.



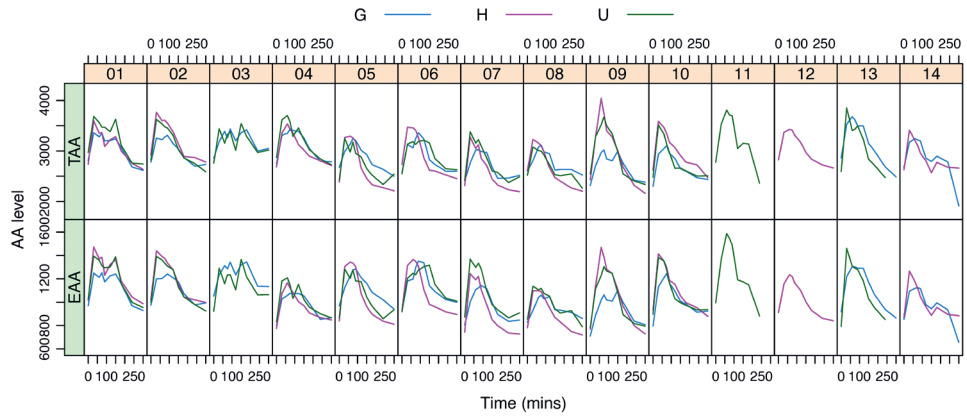
Supplementary Figure 3. Example of two identical T_2-weighted MRI images with total gastric volume delineated (left) and total gastric volume with liquid content marked in red and semi-solid content unmarked based on voxel intensity using thresholding (right).



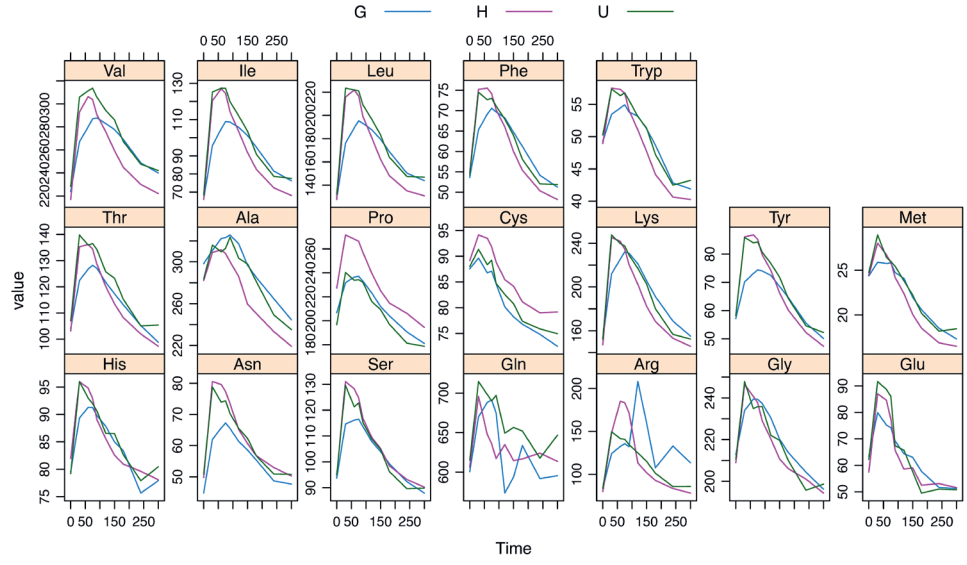
Supplementary Figure 4. Example of two T_2-weighted MRI images with their associated image texture metrics indicating relatively low (t = 10 min) and high (t = 10 min) coagulation.



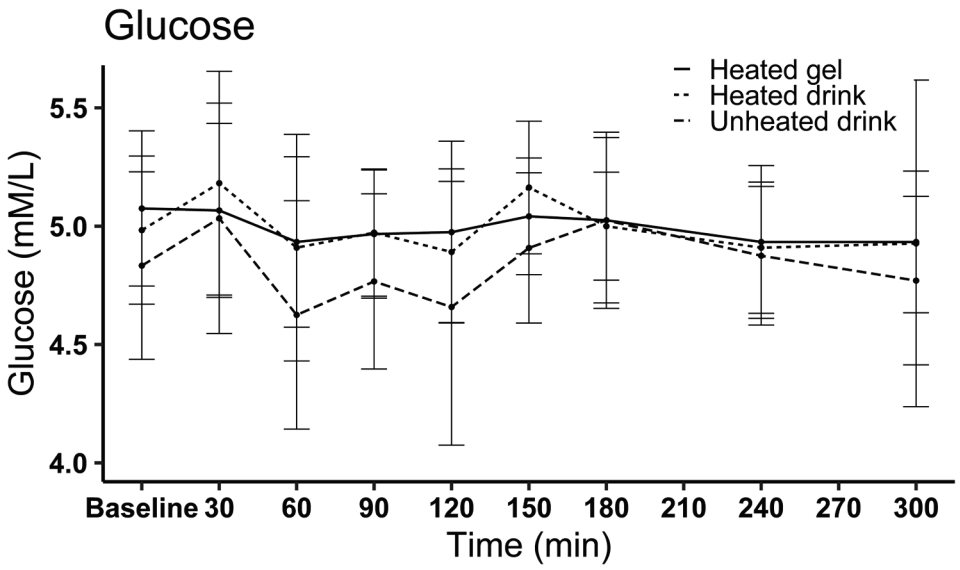
Supplementary Figure 5. Mean differences ± SD in AUC (fold change), maximum peak height (μM) and time to maximum peak (min) of AA of the gel treatment and heated drink compared to the unheated drink. Significant differences are indicated in red. G = gel treatment, H = heated drink, TAA = total amino acids, EAA = essential amino acids.



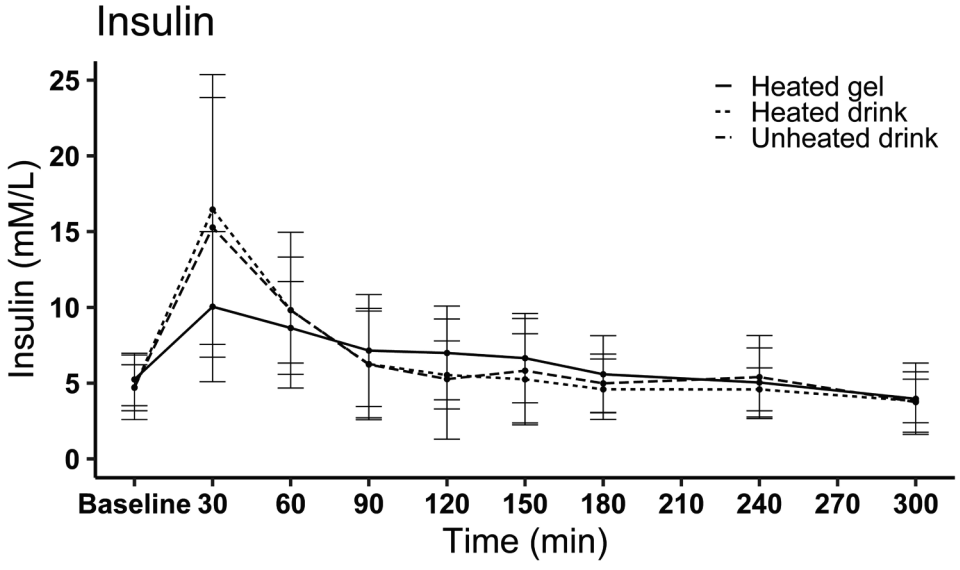
Supplementary Figure 6. Total amino acid (top) and essential amino acid (bottom) levels over time of the participants (01-14) after consumption of the three pea protein products. G = gel treatment, H = heated drink, TAA = total amino acids, EAA = essential amino acids.



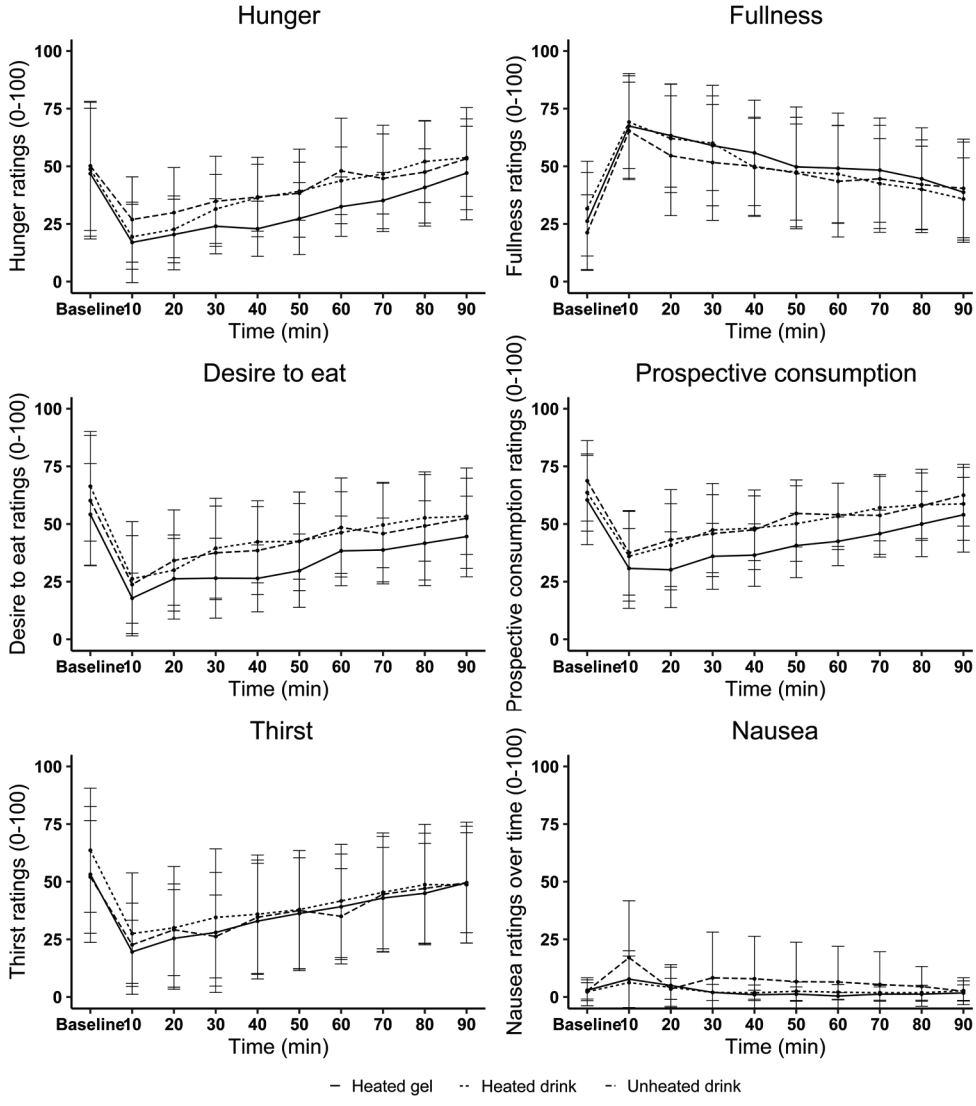
Supplementary Figure 7. Mean amino acid levels over time after consumption of the three pea protein products. G = gel treatment, H = heated drink.



Supplementary Figure 8. Mean ± SD glucose concentration over time for three treatments.

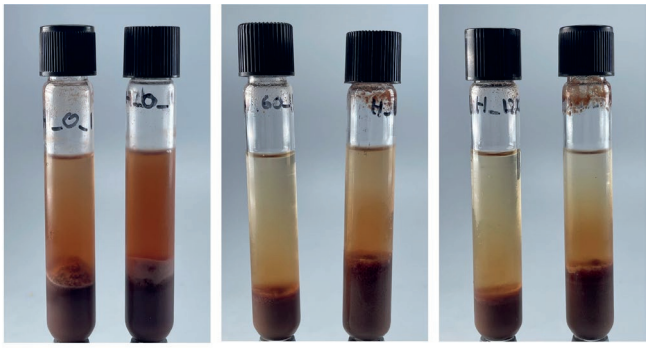


Supplementary Figure 9. Mean ± SD insulin concentration over time for three treatments.



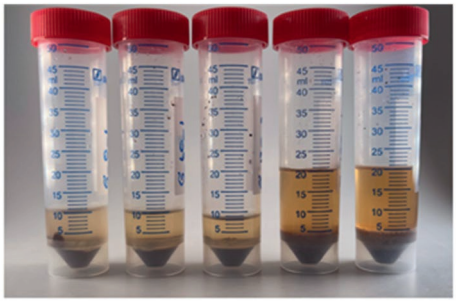
Supplementary Figure 10. Appetite and nausea ratings over time during the test session per treatment. Graphs depicting mean ± SD for hunger, fullness, appetite, expected prospective consumption, thirst and nausea over time after ingestion of pea protein products.

A



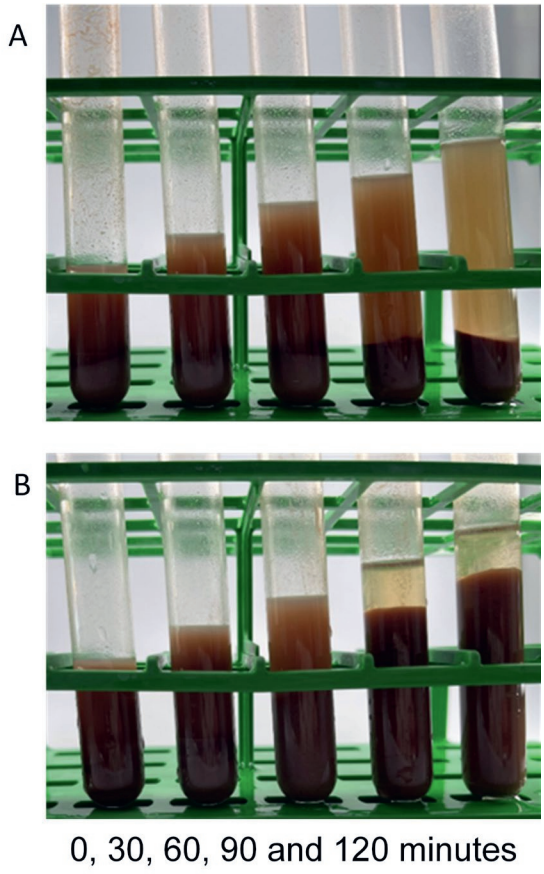
0 minutes 60 minutes 120 minutes

B

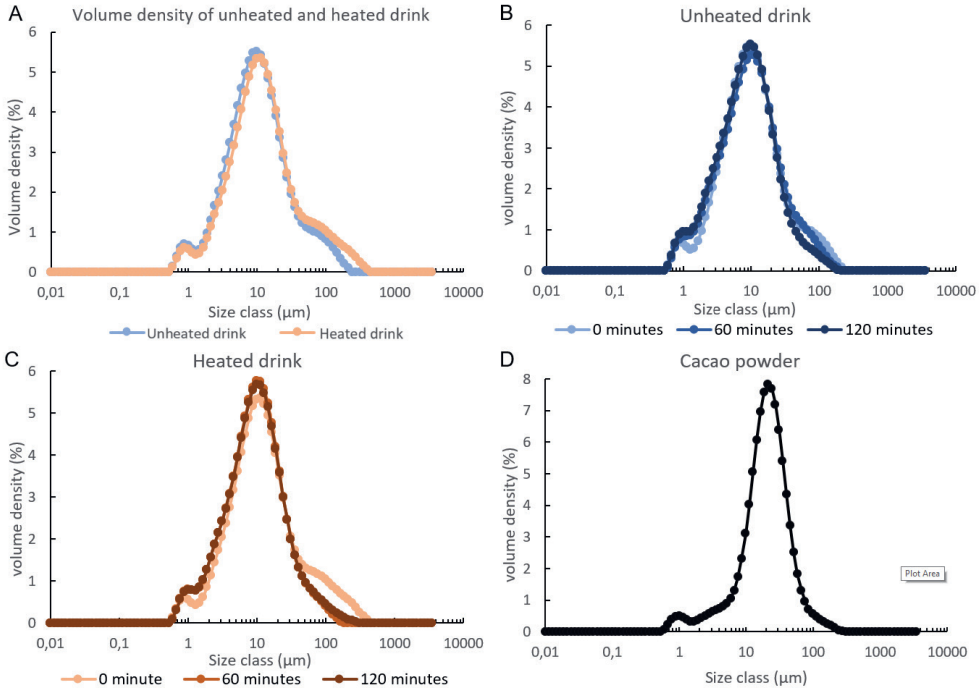


0, 60, 120, 180 and 240 minutes

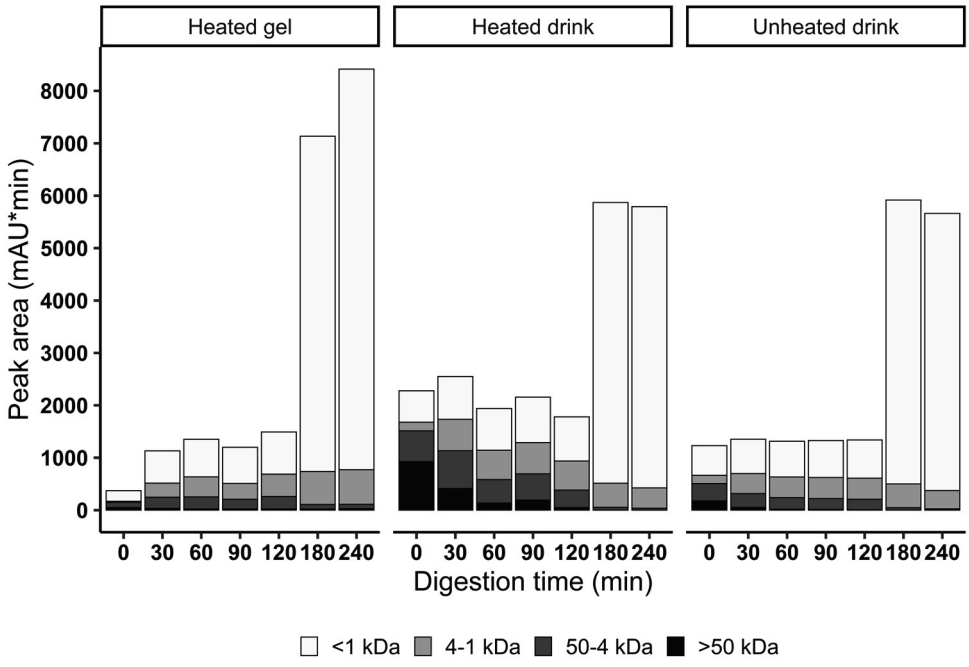
Supplementary Figure 11. Precipitation during the digestion process: (A) precipitation of the unheated (left tube) and heated (right tube) drinks and (B) precipitation of gel and water 0, 60, 120, 180, 240 minutes from left to right.



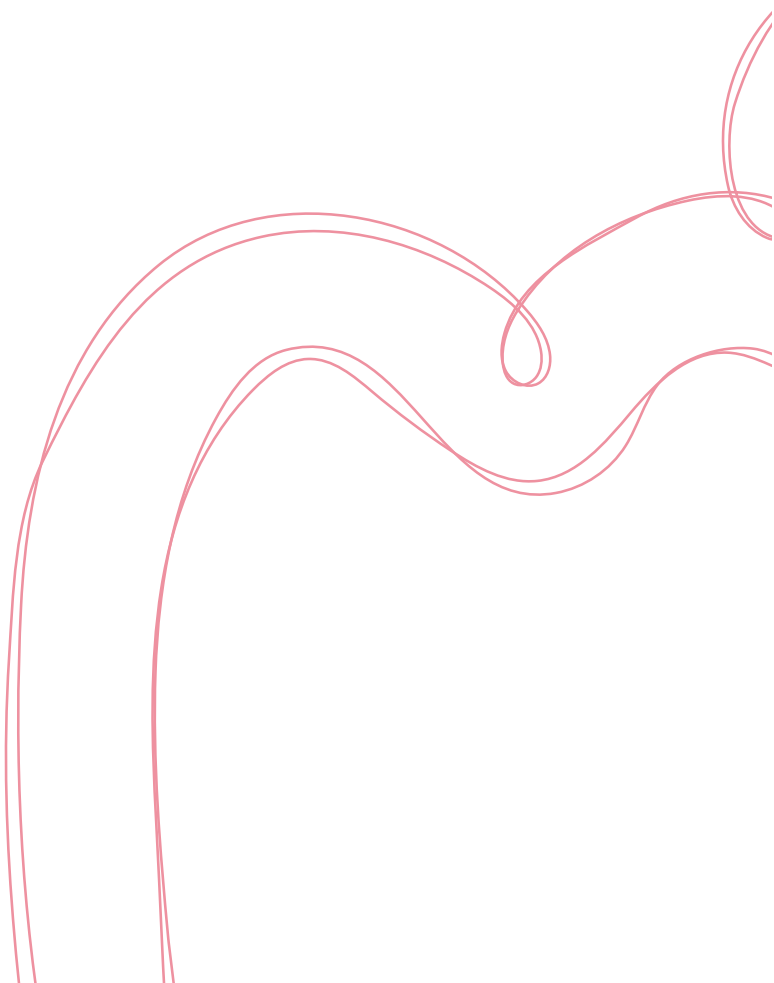
Supplementary Figure 12. Precipitation of unheated (A) and heated (B) drinks over digestion time (0, 30, 60, 90 and 120 minutes from left to right) during semi-dynamic in vitro digestion.



Supplementary Figure 13. Volume density (%) of particle size distribution for the A) unheated and heated drink at the start of digestion (0 min), B) of the unheated drink at 0 min, 60 min and 120 minutes after the start of digestion, C) of the heated drink at 0 min, 60 min and 120 minutes after the start of digestion, C) of cacao powder.

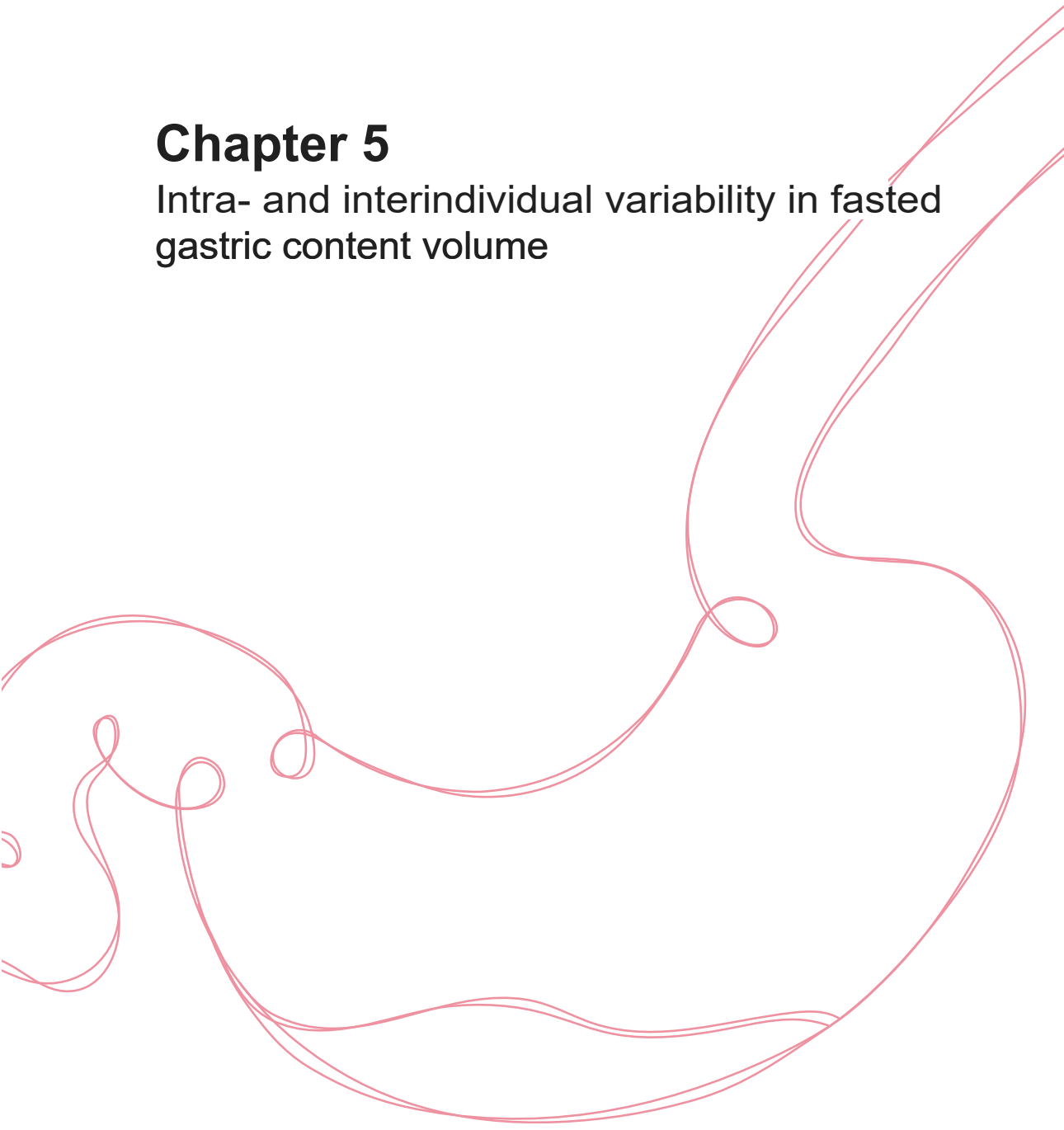


Supplementary Figure 14. Size distribution of the peptides over time (gastric phase: 0 – 120 minutes, intestinal phase: 180 and 240 minutes).



Chapter 5

Intra- and interindividual variability in fasted gastric content volume



This chapter is under review for publication and is available as a pre-print:
Roelofs, J. J. M., Camps, G., Leenders, L. M., Marciani, L., Spiller, R. C., van Eijnatten, E. J. M. , Alyami, J., Deng, R., Freitas, D., Grimm, M., Karhunen, L. J., Krishnasamy, S., Feunteun, S. L., Lobo, D. N., Mackie, A. R., Mayar, M., Weitschies, w., & Smeets, P. A. M. Intra- and interindividual variability in fasted gastric content volume. MedRxiv, https://doi.org/10.1101/2024.03.12.24304085

ABSTRACT

Background: Gastric fluid aids in food digestion, therefore, the amount of gastric fluid present in a fasted state may influence subsequent digestion. We aimed to describe intra- and interindividual variation in fasted gastric content volume and to determine the association with age, sex, and body size characteristics.

Methods: Data from 24 MRI studies measuring fasted gastric content volume in healthy, mostly young individuals after an overnight fast were pooled. Analysis included 366 participants with a total of 870 measurements. Linear mixed model analysis was performed to calculate intra- and interindividual variability and to assess the effects of age, sex, weight, height, weight*height as a proxy for body size, and body mass index (BMI).

Results: Fasted gastric content volume ranged from 0 to 156 mL, with a mean (± SD) value of 33 ± 25 mL. The overall coefficient of variation within the study population was 75.6%, interindividual SD was 15 mL, and the intraindividual SD was 19 mL. Age, weight, height, weight*height, and BMI had no effect on fasted gastric content volume. Women had lower volumes compared to men (MD: -6 mL), when corrected for the aforementioned factors.

Conclusion: Fasted gastric content volume is highly variable, with higher intraindividual compared to interindividual variability, indicating that fasted gastric content volume is subject to day-to-day and within-day variation and is not a stable personal characteristic. This highlights the importance of considering fasted gastric content volume when studying digestion and drug dissolution. Exact implications remain to be studied.

Keywords: gastric juice, gastric secretion, gastric volume, biological variation, digestion, MRI

1. INTRODUCTION

Digestion is the breakdown of food into particles that can be absorbed by the body. This process starts with the oral phase, where mastication and secretion of saliva lead to the formation of a food bolus that can be swallowed safely, and continues in the gastrointestinal tract (Koç et al., 2013; Witt & Stokes, 2015). Digestion is a series of mechanical, physiological, and biochemical processing steps that eventually allows for absorption and utilization of nutrients (Mackie, 2019). These biochemical processing steps include the breakdown by acid and enzymes present in gastric secretions. Gastric fluid serves two main functions: it acts as a first line of defense against infection by killing swallowed microorganisms, and it aids in digestion by initiating the breakdown of food (Martinsen et al., 2019). Gastric fluid is a combination of water, hydrochloric acid (HCl), gastric lipase, pepsin, intrinsic factor, ions (Na^+, K^+, and Cl^-), and mucus (Chew, 2004; Martinsen et al., 2019). These components are continuously secreted by endocrine cells in the stomach wall to maintain an acidic environment with a pH between 1.4 and 2.0 in the fasted state (Chew, 2004; Mennah-Govela et al., 2021). The fasted gastric secretion rate is approximately 1 mL min^{-1} but after food ingestion it can increase to up to 9 mL min^{-1} (Mennah-Govela et al., 2021; Wilson & Stevenson, 2019).

The main zymogen in gastric fluid is pepsinogen (Heda et al., 2019; Wilson & Stevenson, 2019). Under acidic conditions, pepsinogen converts into its active form, pepsin (Chew, 2004; Wilson & Stevenson, 2019). A pH ≤ 2.0 allows pepsin to function optimally, while a pH > 7.2 irreversibly denatures it. The secretion of HCl is therefore essential for the activity of pepsin (Heda et al., 2019). Pepsin is important for the digestion of proteins as it breaks them down into smaller peptides (Wilson & Stevenson, 2019). Due to its role in protein digestion, the amount of gastric fluid present in the fasted stomach will influence gastric protein digestion by affecting the pH and amount of pepsin available. Camps et al. (2021) found that fasted gastric content volume affected gastric layering and gastric emptying of infant formula. In line with this, Roelofs, Tjoelker, et al. (2024) found that fasted gastric content volume was highly correlated with the destabilization of infant formula in the stomach: a higher fasted gastric content volume correlated with earlier gastric phase separation

of the emulsion. Since the emulsion was stabilized with casein-whey complexes, this effect is likely explained by increased protein hydrolysis and flocculation due to the low pH and the presence of pepsin in the stomach. When food is ingested, gastric pH increases due to the buffering effect of the meal (Gardner et al., 2002). However, with a higher fasting gastric content volume, the gastric pH will initially be lower for a given buffering effect. In addition, more pepsin will also be available. Since pepsin activity is higher at low pH(Pearson et al., 1986), a higher fasted gastric fluid volume is thus associated to both a higher amount of pepsin and a higher pepsin activity, leading to increased protein hydrolysis in the early stages of digestion (Mennah-Govela et al., 2021). In addition to the digestion of food, fasted gastric content volume is also a crucial parameter in oral drug delivery. Changes in fasting volumes and composition contribute to intra- and interindividual variability in drug plasma profiles (Koziolek et al., 2016; van den Abeele et al., 2017). Data on the variability are therefore much needed in order to optimize *in vitro* and *in silico* models for the development of novel drugs and dosage forms (Grimm, Koziolek, Kühn, et al., 2018).

Many studies that investigated variability in fasted gastric fluid have used gastric aspiration to measure the stomach content. Gastric aspirates are taken through a nasogastric- or endoscopic tube and is usually done over a 15 to 60-minute period. Nasogastric intubation is an invasive procedure which could perturb the baseline physiological state and the progression of the tube from the throat to the stomach is often aided with water swallows. Gastric emptying also depends on the actual positioning of the tube ports within the stomach. Although total gastric content can be measured with this method, results are often reported as secretion rates (Miraglia et al., 2018). More recently, magnetic resonance imaging (MRI) has been used to measure gastric content volume as this method is less invasive and the technique is inherently suited to visualize body fluids with excellent spatial resolution. In contrast to gastric aspirates taken over a longer period, the use of MRI yields measurement of gastric volume at specific time points.

Other studies found that various individual characteristics may affect gastric acid secretion. Goldschmiedt et al. (1991) found a trend towards an association of aging

with gastric acid secretion and a higher basal acid output in older (44-71 y) compared to younger (23-42 y) adults (5.8 vs. 3.2 mmol h^{-1}, p = 0.05). When the sexes were tested separately, older men (45-71 y) had significantly higher acid outputs than younger men (27-37 y, output: 8.4 vs. 3.8 mmol h^{-1}). Older women (44-65 y) tended to have slightly higher acid outputs than younger women (23-42 y, p = 0.09) and older men had a higher output compared to older women. In contrast, Feldman et al. (1996) found no difference in fasted gastric acid secretion between young (18-34 y), middle-aged (35-64 y) and elderly (65-98 y). Moreover, studies showed that, on average, women have lower fasted gastric acid secretion rates compared to men. Feldman and Barnett (1991) found that basal acid output was almost twice as high in men compared to women. It has been suggested that this is due to hormones, lower body weight or smaller stature of women, thus having smaller stomachs, and associated decreased parietal cell mass (Baron, 1969; Feldman & Barnett, 1991; Freire et al., 2011; Hassan & Hobsley, 1971; Kekki et al., 1982; Vakiland & Mulekar, 1965; Whitfield & Hobsley, 1987). Moreover, body weight was found to be weakly correlated with fasted gastric acid secretion rate in young adults (n = 176, r = 0.184) (Novis et al., 1973). Altogether, these findings suggest a possible role of age, sex, and body size characteristics on fasted gastric content volume. However, these studies all reported gastric acid secretion rate as measured by gastric aspiration as opposed to gastric fluid secretion rate or gastric volume. Moreover, taking gastric aspirates might result in underestimation as some of the gastric acid might be lost due to gastric emptying. In addition, removing the gastric juice from the stomach itself can reinforce the secretion (Ghosh et al., 2011). Next to these personal characteristics, the migrating motor complex (MCC) cycle is known to cause temporal changes in gastric motility and secretion (van den Abeele et al., 2017) and might therefore contribute to the variation in fasted gastric content volume.

Grimm, Koziolek, Kühn, et al. (2018) compared fasted gastric content volumes from 5 MRI studies with 1-6 visits. They found a mean fasted volume of 25 ± 18 mL (n = 120), with a range from 1 to 96 mL. The interindividual and intraindividual variability were comparable, namely 49 ± 19 mL and 44 ± 18 mL, respectively.

To date, several MRI studies have reported fasting gastric content volumes. However, the sample sizes in these studies are generally small. The aim of this study was therefore to gather all available data from the community and combine it to provide unique insights on the intra- and interindividual variation in fasted gastric content volume and to explore possible associations with age, sex, and body size characteristics.

2. METHODS

2.1. Study selection

Studies were selected by contacting members of the *INFOGEST and UNGAP Imaging Working Group* for available data and by emailing authors of eligible studies. A PubMed search was performed in May 2023 to identify eligible studies using the following search string: (all fields): "(gastric AND (emptying OR retention) AND ("Magnetic Resonance Imaging" OR MRI) [full text, clinical trial, human, English]". Inclusion criteria for studies were: 1) data on fasted gastric content volume was determined from MRI images, 2) the study was published in a peer reviewed journal and/or registered in a public trial registry, 3) the study was published in the past 15 years (in or after 2008) and 4) the study was conducted in healthy participants. Studies that did not include a fast of at least 10 h prior to the assessment of fasted gastric content volume were excluded.

All volunteers provided written informed consent for the specific study procedures and subsequent use of anonymized volume data for comparative investigations. Authors who agreed to participate provided data on fasted gastric content volume and individual participant characteristics (sex, age, weight, and height). Information on in- and exclusion criteria was extracted from the paper or the trial registration. This analysis was preregistered at OSF under code BDQS4 (https://doi.org/10.17605/OSF.IO/BDQS4).

2.2. Statistical analysis

Fasted gastric content volume data was characterized by means, medians, standard deviation, range, and overall coefficient of variation. A linear mixed model was used to assess intra- and interindividual variability and the effects of age, sex, and body size characteristics. Participant ID and study site were added as random factors. Age, sex, weight, height, weight*height, and BMI were added as fixed factors in two different models. That is, a model with age, sex, weight and height, and a model with age, sex, weight*height, and BMI. Non-collinearity of the variables was confirmed in both models with the Variance Inflation Factor (VIF) (all < 3) (Sheather, 2009). In addition, the intraindividual range (maximum value – minimum value) was calculated for each person to be able to compare our results to the study of Grimm, Koziolek, Kühn, et al. (2018).

To determine what a normal range of variation in fasted gastric content volume might be, we calculated mean ± 3 SDs, which should cover 99.7% of the data. Since our data on gastric content volumes followed a right-skewed distribution, we applied this method to our square root transformed data and back transformed the outcome.

Normality of the data was assessed with quantile-quantile (QQ) plots of the residuals, which showed a roughly normal distribution. Using a square root transformation led to a slight improvement in the distribution but had no effect on our outcomes. Transforming the data leads to difficulty in interpreting the data, and because linear mixed models are capable of dealing with violation of the distributional assumptions (Schielzeth et al., 2020), we chose to report the results of the non-transformed data. Analysis was performed in R version 4.1.3, using the nlme (Pinheiro et al., 2017) and car package (Fox et al., 2012). The significance threshold was set at $p = 0.05$. Data are expressed as mean ± SD unless stated otherwise.

3. RESULTS

3.1. Included studies

In total, data of 31 studies was received. Four studies were excluded due to a fasting period of less than 10h and two due to missing data. Data of four unpublished studies were received (*ClinicalTrials.gov NCT05575687*, Unpublished; *ClinicalTrials.gov NCT05854407*, Unpublished; *Trialsearch.who.int NL8137*, Unpublished) via the *INFOGEST and UNGAP Imaging Working Group* , of which one was excluded due to unavailability of a trial registration. This led to inclusion of 24 studies in total, performed at 5 research sites in the UK, Germany, France, and the Netherlands (**Figure 1**).

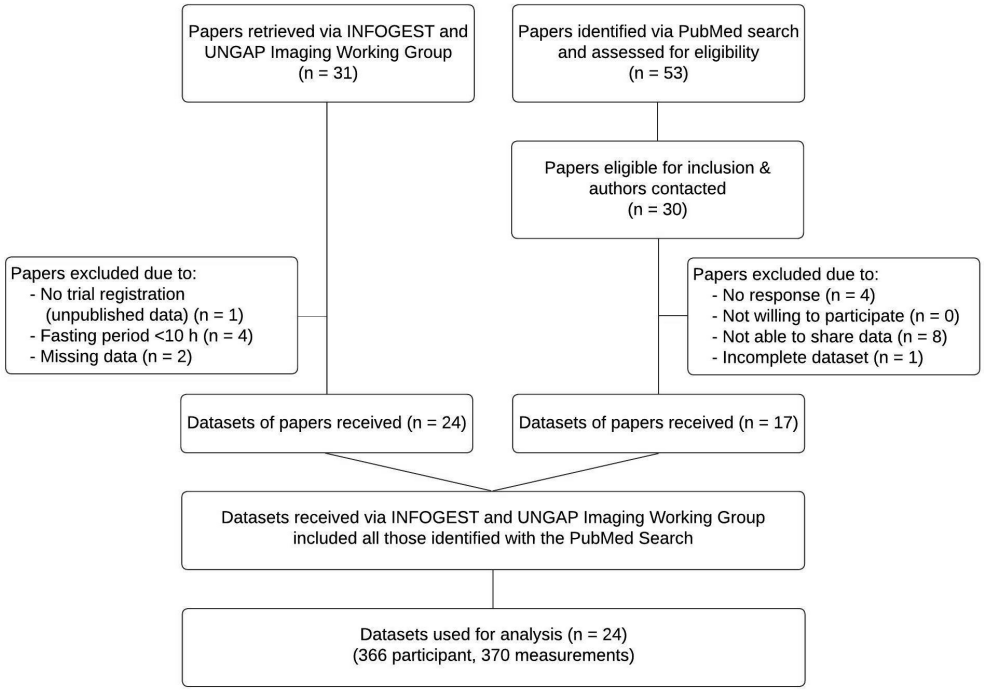


Figure 1. Flowchart.

3.2. Study designs

The fasting regimen differed somewhat between the studies; details can be found in **Supplementary Table 1**. In short, all studies included an overnight fast of at least 10 hours (the selection criterion). In some studies, participants were not allowed to drink during the fast, while others could drink water (or non-caloric, non-caffeinated drinks) up until 1-2 hours before their visit. Five studies standardized the evening meal before the fasting. Fourteen studies included instructions on physical activity, either not allowing heavy exercise or keeping it constant before and on all test days. Studies that did not exclude participants based on medication use required the participants to quit the medication for at least the duration of the overnight fast.

In- and exclusion criteria differed slightly between studies. Details can be found in **Supplementary Table 2**.

3.3. MRI measurements

All studies scanned the participants during a breath hold to fixate the position of the diaphragm and the stomach and prevent respiratory motion artefacts. Most studies scanned participants in a supine position, but two scanned participants in a supine tilted position with their left side slightly raised (Alyami et al., 2019; Murray et al., 2015) and one scanned participants in a right decubitus position (Freitas et al., 2022). This was not expected to influence gastric secretion, however, no literature could be identified on this. A study comparing gastric emptying of a soup in the right versus left decubitus position found no difference (Boulby et al., 1997).

Since the studies were performed at different research facilities, scanning protocols slightly deviated from each other. However, this is not expected to influence the results. Details of the scan sequences used can be found in **Supplemental Table 3**. All studies acquired transverse slices, except for Grimm, Koziolek, Saleh, et al. (2018) who acquired coronal slices.

Three studies analyzed gastric content volumes semiautomatically using an intensity-based thresholding technique (Grimm, Koziolek, Saleh, et al., 2018;

Krishnasamy et al., 2020; Mudie et al., 2014). All other studies manually delineated the stomach contents on each slice of the MRI scan to calculate the total gastric content volume.

3.4. Participants

In total, 366 participants were included in the analysis, 146 women and 220 men. Participants were 25.5 ± 6.8 years old and had a BMI of 22.7 ± 2.3 kg m^{-2} (**Table 1 & 2**). In total, 870 individual measurements were collected, of which one measurement was considered an outlier and excluded for analysis. This participant had a volume of 373 mL, which is 12.3 SDs above the mean, which seems highly unlikely caused by natural variation. In addition, the same participant showed a fasting gastric content volume of 21 and 26 mL on other days. Therefore, this high volume of 373 mL was deemed unrealistic. It might have been caused by non-compliance to the fasting period or a rare case of gastroparesis. The analysis was performed both on the complete dataset, as well as the set without outlier. Results of the analysis with the outlier are reported in **Supplementary Tables 4 & 5**.

The studies included up to six measurements of fasted gastric content volume per participant, with the majority of the participants having two or three measurements. There were 47 participants with one measurement, 184 with two, 115 with three, 6 with four, and 14 with six measurements.

Table 1. Overview of studies and their participant characteristics.

Study	Sex (# F/M)	Age (years)	BMI (kg m^{-2})	# measurements per participant
Alyami et al. (2019)	13/6	28.2 ± 10.6	23.4 ± 3.1	2
Camps et al. (2018)	0/19	-	21.7 ± 1.4	2
Camps et al. (2021)	16/0	30.9 ± 3.7	22.6 ± 3.9	2
ClinicalTrials.gov NCT05575687 **(Unpublished)**	6/6	24.1 ± 4.3	22.2 ± 2.2	2
ClinicalTrials.gov NCT05854407 **(Unpublished)**	10/6	22.8 ± 1.8	22.1 ± 2.0	6
Coletta et al. (2016)	7/5	27.3 ± 8.4	22.7 ± 1.8	3
Deng et al. (2023)	0/18	27.1 ± 4.9	22.8 ± 1.6	3
Freitas et al. (2022)	0/10	32.8 ± 10.4	23.0 ± 1.8	3
Grimm, Koziolek, Saleh, et al. (2018)	3/3	25.2 ± 2.1	23.4 ± 1.2	4
Hussein et al. (2015)	0/11	23.8 ± 4.1	24.4 ± 3.2	3
Juvonen et al. (2015)	0/4	39.0 ± 8.0	25.2 ± 1.0	2
Krishnasamy et al. (2020)	14/4	24.7 ± 4.2	22.7 ± 3.5	3
Lobo et al. (2009)	10/10	29.4 ± 7.8	23.4 ± 1.9	2
Marciani et al. (2010)	8/8	22.8 ± 3.7	22.0 ± 2.3	2
Marciani et al. (2012)	9/9	20.3 ± 0.8	22.2 ± 2.2	2
Marciani et al. (2013)	4/8	22.3 ± 4.4	23.0 ± 2.9	2
Marciani et al. (2015)	5/8	20.8 ± 0.7	22.6 ± 1.7	2
Mudie et al. (2014)	8/4	21.3 ± 2.2	22.1 ± 2.0	1
Murray et al. (2015)	0/17	25.3 ± 5.5	23.8 ± 2.7	3
Roelofs, Tjoelker, et al. (2024)	0/20	25.5 ± 5.8	21.9 ± 1.5	2
Roelofs, van Eijnatten, et al. (2024)	0/14	23.0 ± 3.8	22.2 ± 1.7	3
Trialsearch.who.int NL8137 **(Unpublished)**	0/18	25.9 ± 8.3	22.7 ± 1.6	2
van Eijnatten, Roelofs, et al. (2023)	0/15	30.9 ± 13.8	22.3 ± 2.0	2
van Eijnatten, Camps, et al. (2023)	30/0	25.1 ± 5.2	22.4 ± 2.3	1
Total	146/220	25.5 ± 6.8	22.7 ± 2.3	

Table 2. Mean ± SD age and body mass index (BMI) of study participants, stratified by sex.

	Women (n = 146)			**Men (n = 220)**			**Total (n = 366)**		
	Mean ± SD	*Median*	*Range*	*Mean ± SD*	*Median*	*Range*	*Mean ± SD*	*Median*	*Range*
Age (y)	24.9 ± 6.0	23	18-57	25.9 ± 7.2	23	18-55	25.5 ± 6.8	23	18-57
BMI (kg m^{-2})	22.2 ± 2.2	22.1	18.0-30.4	23.0 ± 2.3	22.7	18.3-33.0	22.7 ± 2.3	22.5	18.0-33.0
Weight (kg)	61.9 ± 7.8	62.5	44.0-90.0	75.4 ± 9.4	74.0	58.0-108.0	70.4 ± 11.0	70.0	44.0-108.0
Height (m)	1.67 ± 0.07	1.67	1.49-1.90	1.81 ± 0.08	1.80	1.54-2.02	1.76 ± 0.1	1.77	1.49-2.02
Height*Weight (m*kg)	103.6 ± 16.2	103.2	66.0-156.6	136.9 ± 21.2	134.0	89.3-199.0	124.6 ± 25.3	123.7	66.0-199.0
Fasted gastric content volume (mL)	31 ± 21	27	0.0-122	33 ± 27	27	0-156	33 ± 25	27	0-156

3.5. Fasted gastric content volume

Fasted gastric content volume ranged from 0 mL to 156 mL, with a mean value of 33 ± 25 mL (**Figure 2, Table 2**). An example of a relatively low, medium and high fasted gastric content volume is shown in **Figure 3**. The median volume was 27 mL (IQR = 15 – 45 mL). Women had a slightly lower fasted gastric content volume compared to men (31 ± 21 mL vs. 33 ± 27 mL). The overall coefficient of variation within the study population was 75.6%. The interindividual standard deviation, corrected for study site, was 15 mL, while the intraindividual standard deviation was 19 mL. The intraindividual range varied between 0 and 108 mL, with a mean of 24 mL and a SD of 21 mL.

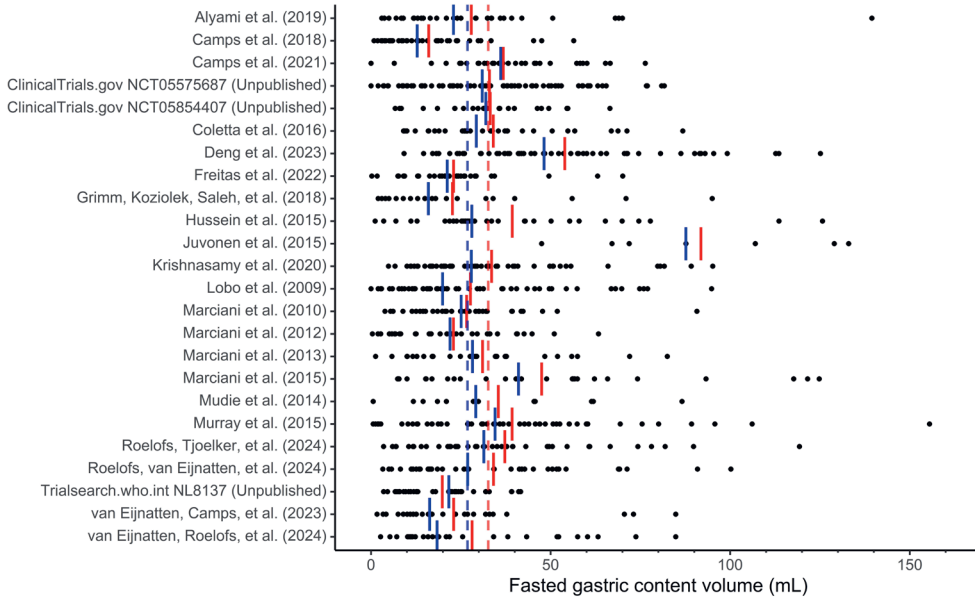


Figure 2. Fasted gastric content volumes for each study and for all individual visits (black dots). Red lines denote the mean volume. Blue lines denote the median. The dashed lines denote the overall mean (red) and median (blue).

Calculating the mean ± 3 SD, results in a fasted gastric content volume that would range between 0-138 mL. This is in fair agreement with the volumes in our data, only 2 measurements are outside of this range (0.2% of the values).

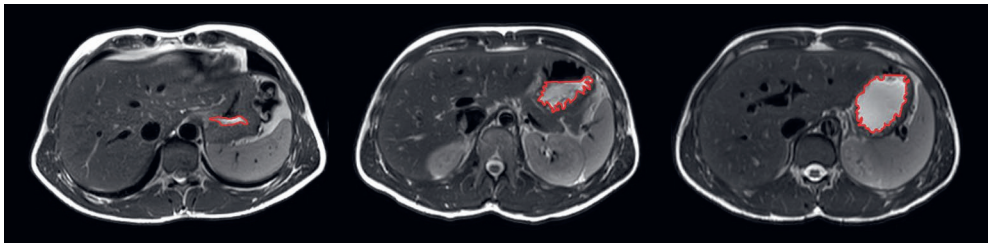


Figure 3. Illustration of variation in fasted gastric content volume as apparent on MRI images (left to right: 4 mL, 61 mL, and 119 mL).

3.6. Age, sex, and body size characteristics

There was no effect of age, weight, height, weight*height, and BMI on fasted gastric content volume (**Table 3**). For both models, men showed a significantly higher volume compared to women (estimate: -6 mL, p = 0.045 and p = 0.043, for the model with weight & height and the model with weight*height and BMI, respectively). Scatterplots of age, sex, and BMI with fasted gastric content volume are shown in **Figure 4**, those of weight, height, and weight*height can be found in **Supplementary Figure 2**.

Table 3. Results of the linear mixed model analysis of the effect of age, sex, and body size characteristics on fasted gastric content volume (mL unit^{-1}).

	Age, Sex, Weight & Height		**Age, Sex, Weight*Height & BMI**	
	Estimate	*p-value*	*Estimate*	*p-value*
Age	0 mL y^{-1}	0.178	0 y^{-1}	0.164
Sex	-6 mL	0.045	-6 mL	0.043
Weight	0 mL kg^{-1}	0.652		
Height	0 mL m^{-1}	0.229		
Weight*Height			-1 mL (kg*m)$^{-1}$	0.215
BMI			1 mL (kg m^{-2})$^{-1}$	0.220

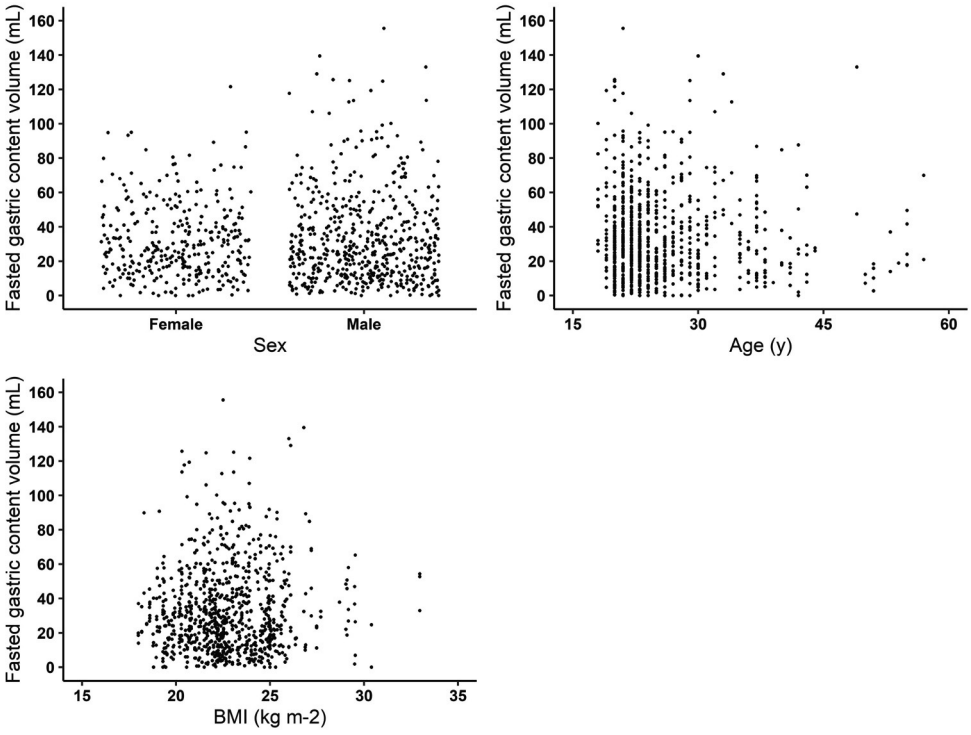


Figure 4. Scatterplots of fasted gastric content volume by age, sex, and body mass index (BMI). Linear mixed model analysis showed that women had lower fasted gastric content volume compared to men (~6 mL, $p < 0.05$). There was no effect for age and BMI.

4. DISCUSSION

With this analysis we aimed to establish the intra- and interindividual variation in fasted gastric content volume, and to assess to what extent this variation is associated with age, sex, and body size. Our analysis, based on 366 participants, established that there is large variation in fasted gastric content volume within healthy adults, with a mean of 33 mL and overall coefficient of variation of 75.6%. When corrected for age, sex, and body size, interindividual variability was 15 mL and intraindividual variability 19 mL. Age, weight, height, weight*height, and BMI were not associated with fasted gastric content volume. Men were found to have a ~6 mL

higher fasted gastric content volume compared to women, although clinical relevance remains to be studied.

4.1. Intra- and interindividual variability

The mean fasted gastric content volume of 33 ± 25 mL is comparable to the mean volume of 25 ± 18 mL (n = 120) found by Grimm, Koziolek, Kühn, et al. (2018). Their volumes ranged from 1 to 96 mL, while ours ranged up until 156 mL. Although our upper limit is more than 1.5 times as high, only 1.8% of our volumes were above 100 mL (i.e., 16 out of 869) indicating that the majority of our data was within the same range as theirs. Based on our results, a normal fasted gastric content volume can range from 0-138 mL. The coefficient of variation was 75.6%. Data in literature is limited, Grimm, Koziolek, Kühn, et al. (2018) reported values ranging from 39 to 159%, although sample size was very small (n = 6 each). Our mean intraindividual range was 24 ± 21 mL, which is much lower compared to that of Grimm, Koziolek, Kühn, et al. (2018) of 44 ± 18 mL but might be explained by their low sample size (n = 8 subjects), which is more susceptible to individuals with high variability.

Interindividual variation was slightly lower with 15 mL compared to the intraindividual variation of 19 mL. This indicates that fasted gastric content volume is subject to day-to-day and within-day variation and cannot be seen as a stable personal characteristic. Part of the variation might be explained by the MMC cycle. Studies have shown that the MMC causes temporal variations in gastric secretion. The different phases of the MMC cycle are associated with increases and decreases in secretion rate(van den Abeele et al., 2017) with differences up to 78% (Vantrappen et al., 1979). Therefore, the variability in both intra- and interindividual fasted gastric content volume might partly be explained by the phase of the MMC cycle at the time of measurement. However, a review of van den Abeele et al. (2017) found that MMC cycle durations varied between 96 and 172 minutes and Parkman et al. (1998) found that only 1 in 3 individuals had antral contractions during a 60-minute period. Moreover, Kellow et al. (1986) reported that while MMC cycles occurred every 1-2 hours, only 1 in 3 originated in the stomach. Goetze et al. (2009) used MRI to repeatedly measure fasted gastric content volume over 90 minutes but found no

changes over this period (n = 12, average slope: 0.0018 mL min^{-1}, $p > 0.05$)[59]. In addition to gastric secretion rates, the peristaltic, phasic contractions also change during the MMC cycle, resulting in an increased liquid emptying rate during phase III (van den Abeele et al., 2017). This increase in gastric secretion rate happens prior to phase III contractions, which roughly corresponds to late phase II/phase III contractions in the stomach (van den Abeele et al., 2017). This might therefore (in part) counteract the effect of the increased secretions during this phase. As gastric volume is the result of gastric secretion and emptying, this might explain why Goetze et al. (2009) found no difference in gastric volume over time. Altogether this indicates that more prolonged studies are needed to better capture the change in fasted gastric content volume over time during the MMC cycle.

Comparison of our results with literature is difficult. Previous studies often report fasted gastric acid secretion rate as measured by gastric aspiration as opposed to gastric fluid secretion rate or gastric volume. The gastric aspirates are often taken over a period of 30-60 minutes and might therefore capture temporal fluctuations. A disadvantage is that this method might result in underestimation as some of the gastric acid might be lost due to gastric emptying. Moreover, removing the gastric juice from the stomach itself can reinforce the secretion (Ghosh et al., 2011). This difference in assessment might account for some of the discrepancies between our findings and those in literature. One study has been identified that reported both volume and secretion rate. Goyal et al. (1966) collected gastric fluids over a period of 60-minute during continuous suction of gastric fluid and found a mean volume of 63.5 mL and a gastric acid secretion rate of 2.99 mmol h^{-1}. It is important to note that they urged the participants to spit out their saliva during collection. This is in contrast to measurements with MRI where the stomach might also contain small amounts of saliva. Saliva flow rate during fasting (6 hours) is 0.1 mL min^{-1}, so effects are expected to be minimal (Rahim & Yaacob, 1991).

4.2. Biological variations and other factors influencing fasted gastric content volume.

Our results show no effect of age on fasted gastric content volume (-0.2 mL, $p > 0.05$), although it should be noted that our study population was relatively young, ranging 18-57 y with only 10 participants (2.7%) aged 44 years or older. Literature is inconclusive on the effect of age. Feldman et al. (1996) found no difference between young (18-34 y), middle-aged (35-64 y) and elderly (65-98 y) while Goldschmiedt et al. (1991) found a trend for higher fasted gastric content volume in older (44-71 y) compared to younger (23-42 y) adults (5.8 vs. 3.2 mmol h^{-1}, $p = 0.05$). Based on our findings, it can be concluded that within healthy, mainly young adults, age has no effect on fasted gastric content volume when corrected for age, sex, and body size characteristics.

Fasted gastric content volumes were lower for women compared to men (~6 mL, $p < 0.05$), which is in agreement with previous studies that found higher fasted gastric secretion rates in men compared to women. It has been suggested that this might be due to their lower body weight or smaller stature, thus having smaller stomachs, and associated decreased parietal cell mass or due to hormonal differences (Baron, 1969; Feldman & Barnett, 1991; Freire et al., 2011; Hassan & Hobsley, 1971; Kekki et al., 1982; Vakiland & Mulekar, 1965; Whitfield & Hobsley, 1987). Since we corrected for weight and BMI in our models, this could not have been the explanation for the difference between men and women. Novis et al. (1973) found that body weight was weakly correlated with fasted gastric acid secretion rate (n = 176, range: 45-105 kg, r = 0.184), however, we did not find an effect of either weight or BMI. Since it is known that the volume present in the stomach is the result of both secretion and gastric emptying and that those with a higher body weight have faster gastric emptying (Acosta et al., 2015), these effects might counterbalance the increase in secretion. Thus, based on our findings it can be concluded that weight, within a healthy range, does not influence fasted gastric content volume.

In an attempt to correct for differences in stomach size, height and weight*height were included as a proxy for body size. No effect was found for these measures. It

is, however, noteworthy that literature is inconclusive on whether stomach size is associated with weight and height. A study looking at mucosal surface area of the stomach *post-mortem* found that, on average, men have a 10% larger stomach size compared to women. However, variations in stomach size were not related to age, body height or weight(Cox, 1945). In contrast, Lee et al. (2016) found that sex, age, height and body weight were associated with the length of the lesser curvature, and sex and weight with the length of the greater curvature. They found that men have longer stomachs compared to women, although it is noteworthy that this study was performed on stomachs removed during total gastrectomy in gastric cancer patients. Moreover, these studies included many overweight participants (>50%), raising the question whether these findings would be similar for healthy participants. Thus, although we corrected for body size by using height and weight*height, this might not have been very accurate for controlling for differences in stomach size. Whether stomach size, and associated parietal cell mass, is (partly) responsible for lower fasted gastric content volume in women remains to be studied.

In addition to body size, hormones have been suggested as an explanation for sex differences in gastric acid secretion. Studies on the effect of sex hormones on gastric acid secretion are inconclusive. Goldschmiedt et al. (1991) did not find an effect of menstrual phase on fasted gastric acid secretion rate (n = 10). However, Sakaguchi et al. (1991) showed that gastric acid secretion is decreased in the premenstrual phase, showing an inverse correlation with plasma estradiol concentrations (n = 24, r = 0.629). An animal study showed that estrogen might inhibit gastric acid secretion by binding to estrogen receptors on the parietal cells in the stomach(Campbell-Thompson et al., 2001). These findings might also explain the lower gastric acid secretion in women compared to men (Campbell-Thompson et al., 2001; Sakaguchi et al., 1991). This effect of menstrual phase is something that has not been taken into account by all of the studies included in this analysis and might thus have contributed to the variation in fasting gastric volumes.

Gastric acid secretion is influenced by many more factors. Examples include, sex hormones, the circadian rhythm, smoking, exercise, stress levels, the use of

medication, and the presence of *Helicobacter pylori*. Gastric acid secretion has been shown to have a distinct circadian cycle in the absence of food stimulation, with the highest secretion rate in the evening and the lowest in the morning(Moore & Englert, 1970). Smokers have higher fasted gastric acid secretion compared to non-smokers (4.1 vs. 2.7 mmol h^{-1}, respectively) (Feldman et al., 1996). Interestingly, not all studies mentioned the exclusion of smokers. Since the prevalence of smoking is higher among men (Eurostat, 2022), this might have contributed to the difference we found between women and men. However, no data on the prevalence of smoking was available for the studies.

Studies on exercise and gastric acid secretion report a decrease in fasted gastric acid secretion either during exercise or during restitution (Canelles et al., 1990; Markiewicz et al., 1977; Zach et al., 1982). Furthermore, physical stress is known to induce a 3-fold increase in fasted gastric acid secretion (Oektedalen et al., 1984). Moreover, certain medications are known to influence gastric acid secretion, such as protein pump inhibitors, antacids, and histamine, which might impact further digestion. For example, protein pump inhibitors are known to slow down the gastric emptying of solids, which is suggested to be due to impairment of intragastric peptic digestion (Sanaka et al., 2010). Moreover, a review of Maideen (2023) found that long-term use of protein pump inhibitors is associated with micronutrient deficiencies (e.g. iron, B12, calcium).

Most of these factors are not expected to have influenced our results. Studies were all performed in the morning after an overnight fast, thereby minimizing the influence of the circadian rhythm and food and beverages that were consumed previously. In addition, the use of medication was either an exclusion criteria or stopped for at least the fasting period.

Moreover, it is known that a *Helicobacter pylori* infection can initially cause hypergastrinemia and gastric hypersecretion, while later in life it can cause gastric atrophy with impaired gastric secretion (Calam, 1999). Since an infection is commonly asymptomatic, prevalence in Europe is around 34% (Hooi et al., 2017),

and participants were not tested for this, this might explain some of the variance that we found.

4.3. Fasted gastric content volume and digestion

Gastric secretion is of key importance for (peptic) digestion. Naturally, the volume present in the stomach will affect the digestion due to the presence of enzymes and HCl and their effect on the breakdown of nutrients, specifically proteins. Greater amounts of enzymes present and a higher concentration of HCl can both facilitate digestion in the early stages of gastric digestion. The association between fasted gastric content volume and protein digestion was already shown (Camps et al., 2021; Roelofs, Tjoelker, et al., 2024), where higher volumes were associated with earlier destabilization of emulsions, indicating increased protein hydrolysis. However, the sole effect of fasted gastric content volume on digestion is difficult to establish given the many variables that are involved. Whether the difference in fasted gastric content volume can be considered as clinically relevant also depends on the size of a meal: the larger the meal, the smaller the effects will likely be. Moreover, it not only depends on the initial amount of gastric juice present, but also on the increase in secretion that happens when food is ingested. This increase depends on multiple factors and can already start by the sight and smell of food, and increases further when tasting and chewing the food (Feldman & Richardson, 1986). After that, the presence of the food and distention of the stomach will further stimulate gastric acid secretion. It was found that gastric secretion tends to increase with meal size. Furthermore, the constituents of food can affect the gastric acid secretion, e.g. it is known that peptides and amino acids stimulate secretion, but also alcohol, calcium, and lemon juice (Chew, 2004; Freitas et al., 2022; Lennernäs, 2009; Varum et al., 2013)

Limited literature is available on the correlation between fasting and meal-induced gastric secretion. Goyal et al. (1966) found no correlation between basal and histamine-stimulated peak total secretion ($r = 0.345$, $n = 22$). Moreover, they reported ratios of 4.7-45.3% between basal and histamine-stimulated maximal acid output ($n = 22$), with all, except one, below 35%. Other studies found ratios between 9.9 and

30.7% (Goyal et al., 1966). Thus, based on fasted gastric content volume alone, it is difficult to predict the exact effect on digestion. This highlights the need for methods to estimate gastric acid secretion after ingestion of food. Marciani et al. (2001) used T_2 relaxation time measurements to monitor the process of dilution by gastric secretions and mixing of viscous meals and Goetze et al. (2009) used fast T_1 mapping techniques for the quantification of intra-gastric dilution and distribution of orally applied gadolinium-based paramagnetic contrast agents showing that there is potential for estimating gastric secretion volumes with MRI.

Moreover, it is important to question what volume can be considered as a clinically relevant difference in fasted gastric content volume and will impact digestion kinetics. Recently, an *in vitro* digestion model was developed that considers sex differences in the gastrointestinal tract, accounting for the lower fasting and meal-stimulated gastric acid secretion rates and higher pH in women. Subsequently, they studied the breakdown of whey proteins with both the female and male digestion model and found differences in proteolysis of these proteins (Lajterer et al., 2022). Although the model takes into account more factors than the fasted gastric content volume, it does show that the differences in gastric acid secretion between women and men are of clinical relevance to our digestion. It also highlights the importance of taking into account sex differences, both in *in vitro* digestion studies as well as *in vivo*.

In addition to the digestion of food, fasted gastric content volume is also a crucial parameter in drug release and absorption of oral drugs (Koziolek et al., 2016; van den Abeele et al., 2017). The fasting gastric content volume at the time of oral drug administration influences the dissolution of the drug. Especially since drugs are usually taken with a glass of water, the fasting volume will affect the pH. For example, a low initial volume will cause a less acidic environment, resulting in better solubility for acidic drugs (Grimm, Koziolek, Kühn, et al., 2018). These findings on individual variability can be used to optimize *in vitro* and *in silico* models for the development of novel drugs and dosage forms (Grimm, Koziolek, Kühn, et al., 2018).

4.4. Conclusion

To conclude, fasted gastric content volume is highly variable and should range between 0-138 mL in healthy individuals. After correction for age, sex, and body size characteristics, intraindividual variability was 19 mL compared to 15 mL for interindividual variability. This indicates that fasted gastric content volume is subject to day-to-day and within-day variation and is not a stable personal characteristic. No associations were found with age, body weight and size, within healthy, relatively young individuals who mostly had a healthy weight. Men had a ~6 mL higher fasted gastric content volume compared to women, after correction for the aforementioned factors. Differences in fasting gastric content are expected to affect both digestion and drug dissolution. Exact implications of the observed variations, including the difference between women and men, remain to be studied further. Our results highlight the importance of considering (variations in) fasted gastric content volume when studying digestion.

5. AUTHORS' CONTRIBUTIONS AND ACKNOWLEDGEMENTS

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Julia J.M. Roelofs: Conceptualization, Formal analysis, Writing – original draft. **Guido Camps**: Conceptualization, Writing – review & editing, primary responsibility for the final content. **Louise M. Leenders**: Data collection. **Paul A.M. Smeets**: Conceptualization, Writing – review & editing, primary responsibility for the final content. All authors critically reviewed and approved the final manuscript.

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Supplementary Table 1. Fasting instructions for the participants of each study.

Study	Fasting	Drinking	Exercise	Medication	Meal
Alyami et al. (2019)	>10h	No drinking,		Excluded	
Camps et al. (2018)	Overnight			Excluded	
Camps et al. (2021)	>10h	Water		Excluded	
ClinicalTrials.gov NCT05575687	After 20PM	Water		Excluded	
ClinicalTrials.gov NCT05854407	After 22PM	Water		Excluded	Same meal
Coletta et al. (2016)	>12h	No drinking,	18h	18h	
Deng et al. (2023)	After 22PM	Water		Excluded	
Freitas et al. (2022)	>10h		No	Excluded	
Grimm, Koziolek, Saleh, et al. (2018)	>10h			Stopped	
Hussein et al. (2015)	After 20PM		18h	24h	
Juvonen et al. (2015)	10-12h		24h no	Excluded	
Krishnasamy et al. (2020)	After 22PM	No drinking,	18h	Stopped	
Lobo et al. (2009)	Overnight		18h	24h	
Marciani et al. (2010)	Overnight	Not allowed	overnight	Overnight	
Marciani et al. (2012)	>12h		18h	18h	
Marciani et al. (2013)	>13h	No drinking,	18h	18h	
Marciani et al. (2015)	After		18h	24h	light, non-
Mudie et al. (2014)	>10h	Water		Excluded	Same size
Murray et al. (2015)	After 22PM	Water	24h	Excluded	
Roelofs, Tjoelker, et al. (2024)	>12h	Water	Keep	Excluded	Same meal
Roelofs, van Eijnatten, et al. (2024)	>12h	Water	Keep	Excluded	Standardize
Trialsearch.who.int NL8137	>12h	Water	Keep	Excluded	
van Eijnatten, Roelofs, et al. (2023)	>12h	Water and		Excluded	Standardize
van Eijnatten, Camps, et al. (2023)	After 20PM	Non-caloric,		Excluded	

Supplemental Table 2. In- and exclusion criteria.

	Inclusion criteria			Controlled for:						
Study	**Sex**	**Age**	**BMI**	**Medication**	**GI surgery**	**GI disorders**	**Alcohol**	**Smoking**	**Recreational drugs**	**Weight gain/loss**
Alyami et al. (2019)	F/M	18-65	18-25 & <120kg	Yes	Yes	Yes	>21 units/w			>10% in last 6 months
Camps et al. (2018)	M	18-35	18-25		Yes	Yes				>5 kg in last 2 months
Camps et al. (2021)	F	None	None	Yes		Yes		Yes		
ClinicalTrials.gov NCT05575687 (Unpublished)	F/M	18-45	18,5-25	Yes	Yes	Yes	>7 units/w	>2/w	<1 w	>5kg in last month
ClinicalTrials.gov NCT05854407 (Unpublished)	F/M	18-30	18,5-25	Yes	Yes	Yes	>14 units/w	>1/d	<1 w	
Coletta et al. (2016)	F/M	18-55	<120 kg	Discontinued	Yes	Yes	Dependence	Discontinued		
Deng et al. (2023)	M	18-45	18.5-25	Yes	Yes	Yes				
Freitas et al. (2022)	M	18-60	18-25		Yes	Yes	Abusive	Start/stopped		>3 kg in last 3 months
Grimm, Koziolek, Saleh, et al. (2018)	F/M			Discontinued			Discontinued			
Hussein et al. (2015)	F/M									
Juvonen et al. (2015)	F/M		Normal	Yes			>21 units/w	Yes		
Krishnasamy et al. (2020)	F/M	18+	<120 kg	Discontinued	Yes	Yes	>35 units/w	No		
Lobo et al. (2009)	F/M	18-45	20-26							
Marciani et al. (2010)	F/M	21-28								
Marciani et al. (2012)	F/M		Normal							
Marciani et al. (2013)	F/M									
Marciani et al. (2015)	F/M									
Mudie et al. (2014)	F/M	18-55	18,5-25	Yes	Yes	Yes	>21 units/w	Yes	If history of	
Murray et al. (2015)	M	18-60	20-35	Yes	Yes	Yes	>21 units/w	Yes		>10% in last 6 months
Roelofs, Tjoelker, et al. (2024)	M	18-45	18,5-25	Yes	Yes	Yes	>14 units/w	>2/w		>5 kg in last 2 months
Roelofs, van Eijnatten, et al. (2024)	M	18-55	18,5-25	Yes	Yes	Yes	>14 units/w	Yes	<4 w	>5 kg in last month
Trialsearch.who.int NL8137 (Unpublished)	M	18-55	18-25	Yes		Yes		Yes		
van Eijnatten, Roelofs, et al. (2023)	M	18-55	18,5-25	Yes	Yes	Yes	>14 units/w	Yes	<4 w	>5 kg in last 2 months
van Eijnatten, Camps, et al. (2023)	F	18-60	18,5-30	Yes	Yes	Yes	>14 units/w	>4/d		>5kg in last month

Supplementary Table 3. MRI details.

Study	Magnetic Field (T)	Type of scan sequence	# Slices	Resolution† (mm)	Interslice gap (mm)
Alyami et al. (2019)	1.5	Balanced turbo field echo	25		
Camps et al. (2018)	3.0	Turbo spin-echo	24	1.19*1.19*6	2.4
Camps et al. (2021)	3.0	Turbo spin-echo	24	1.19*1.19*6	2.4
ClinicalTrials.gov NCT05575687 (Unpublished)	3.0	2-D Turbo Spin Echo	33	1.00*1.00*5	1.4
ClinicalTrials.gov NCT05854407 (Unpublished)	3.0	Turbo Spin Echo	28	0.63*0.63*4	1.4
Coletta et al. (2016)	1.5	Balanced turbo field echo	25	2.01*1.76*10	0
Deng et al. (2023)	1.5	Spin-echo	33	0.78*0.78*6	
Freitas et al. (2022)	1.5	Turbo spin-echo	72	1.67*1.71*3	0
Grimm, Koziolek, Saleh, et al. (2018)	1.5	Turbo spin-echo	40‡	1.76*1.7*5.1	0.77
Hussein et al. (2015)	1.5	Balanced turbo field echo	40	1.56*1.56*7	0
Juvonen et al. (2015)	1.5	Balanced fast field echo		1.56*1.56*5	0
Krishnasamy et al. (2020)	1.5	Balanced turbo field echo	50	1.56*1.56*5	0
Lobo et al. (2009)	1.5	Balanced turbo field echo	40	2.5*1.56*10	
Marciani et al. (2010)	1.5	Balanced turbo field echo	20	1.56*1.56*10	0
Marciani et al. (2012)	1.5	Balanced turbo field echo	24	1.56*1.56*10	0
Marciani et al. (2013)	1.5	Balanced turbo field echo	20	1.56*1.56*10	0
Marciani et al. (2015)	1.5	Balanced turbo field echo	24	1.56*1.56*10	0
Mudie et al. (2014)	1.5	Balanced turbo field echo	50	2.00*1.77*5	0
Murray et al. (2015)	1.5	Single-shot balanced-gradient echo	30	1.56*1.57*10	0
Roelofs, Tjoelker, et al. (2024)	3.0	2-D Turbo Spin Echo	37	1.00*1.00*4	1.4
Roelofs, van Eijnatten, et al. (2024)	3.0	2-D Turbo Spin Echo	37	1.00*1.00*4	1.4
Trialsearch.who.int NL8137 (Unpublished)	3.0	Turbo spin-echo	24	1.19*1.19*6	2.4
van Eijnatten, Roelofs, et al. (2023)	3.0	2-D Turbo Spin Echo	37	1.00*1.00*4	2
van Eijnatten, Camps, et al. (2023)	3.0	Turbo spin-echo	24	1.19*1.19*6	2.4

†Without gap; ‡coronal slices

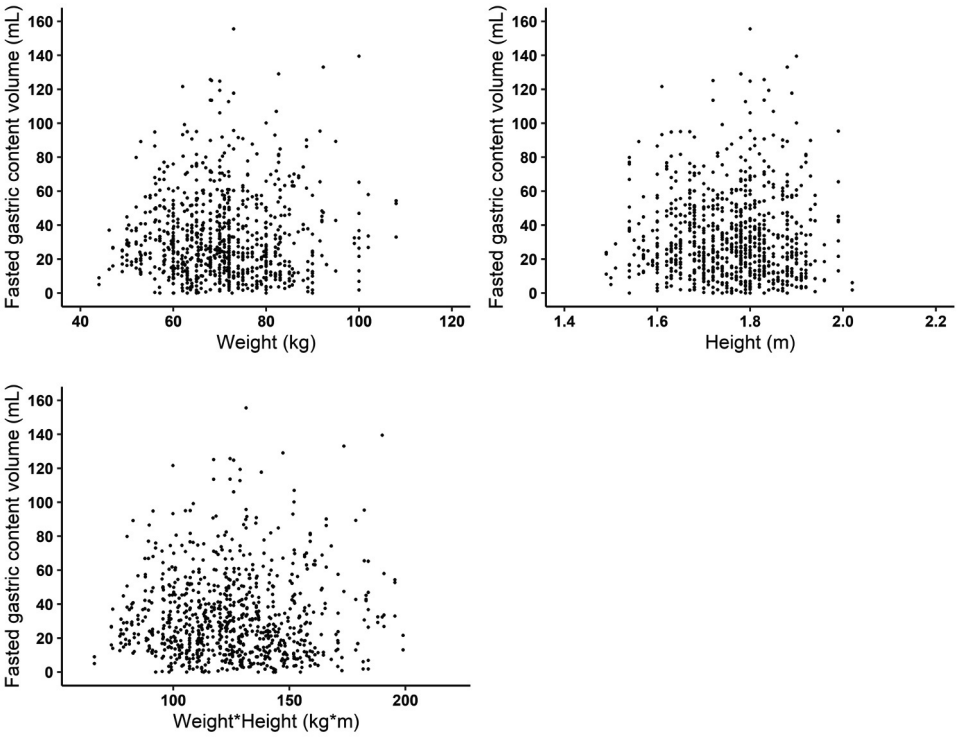
Supplementary Table 4. Mean fasted gastric content volume and variability with outlier.

Mean	33 mL
Median	27 mL
Range	0.0 – 373 mL
Coefficient of variation	82.5%
Interindividual variability	14 mL
Intraindividual variability	23 mL

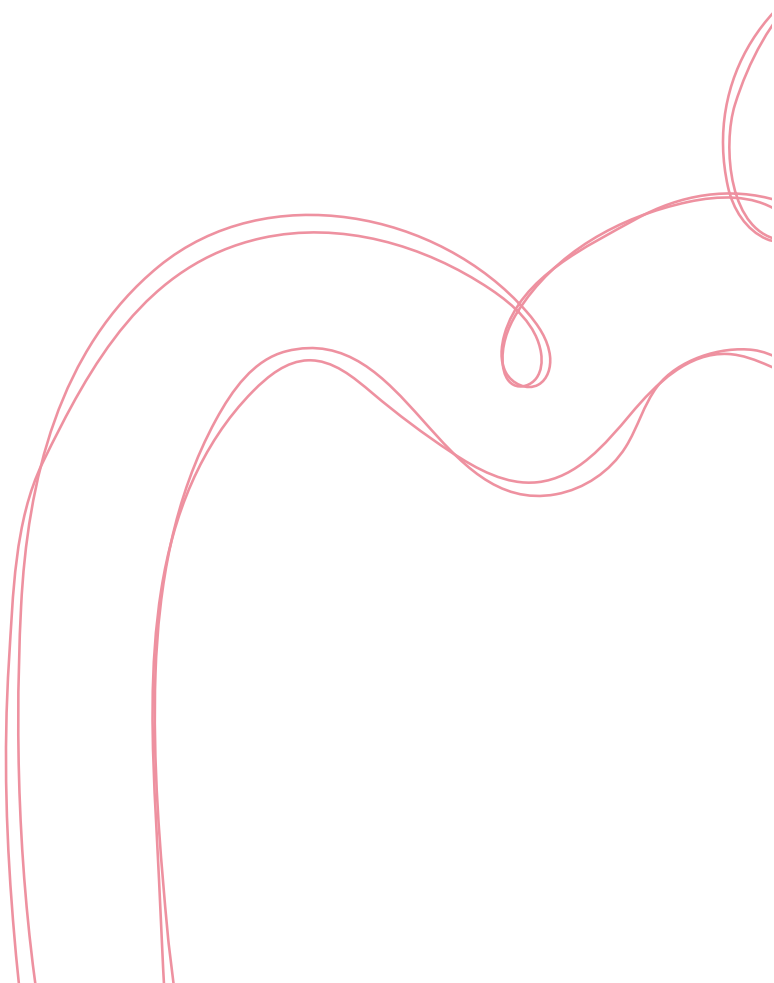
Supplementary Table 5. Results of the linear mixed model analysis of the effect of age, sex, and body size characteristics on fasted gastric content volume (mL unit^{-1}) with outlier.

	Age, Sex, Weight & Height		**Age, Sex, Weight*Height & BMI**	
	Estimate	*p-value*	*Estimate*	*p-value*
Age	0 mL y^{-1}	0.205	0 y^{-1}	0.189
Sex	-7 mL	0.027	-7 mL	0.024
Weight	0 mL kg^{-1}	0.796		
Height	0 mL m^{-1}	0.261		
Weight*Height			0 mL (kg*m)$^{-1}$	0.186
BMI			1 mL (kg m^{-2})$^{-1}$	0.259

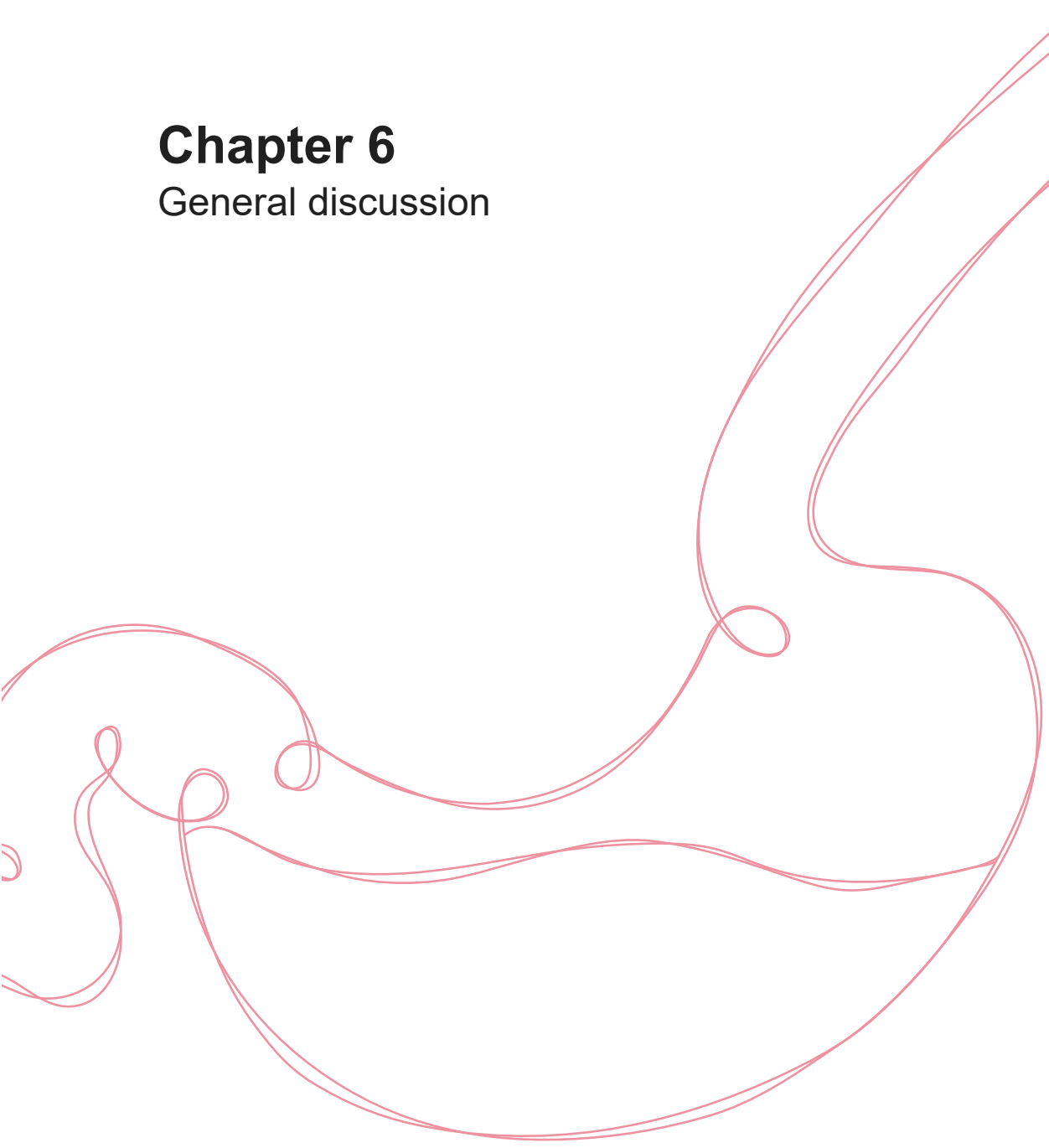
7.2. Supplementary Figure



*Supplementary Figure 1. Scatterplots of fasted gastric content volume by weight, height, and weight*height (all p > 0.05).*



Chapter 6
General discussion



This thesis describes a series of studies conducted to improve our fundamental understanding of gastric digestion, and how this is affected by the properties of the food consumed. The overall aim was to obtain a better understanding of how food composition, processing, and texture affect intragastric behavior (gastric coagulation and layering), gastric emptying, and postprandial nutrient absorption, as measured using *in vivo* MRI and blood sampling, in combination with *in vitro* digestion models. Moreover, we studied intra- and interindividual variability in fasted gastric content volume, as it is increasingly recognized that there are large variations that may influence digestion.

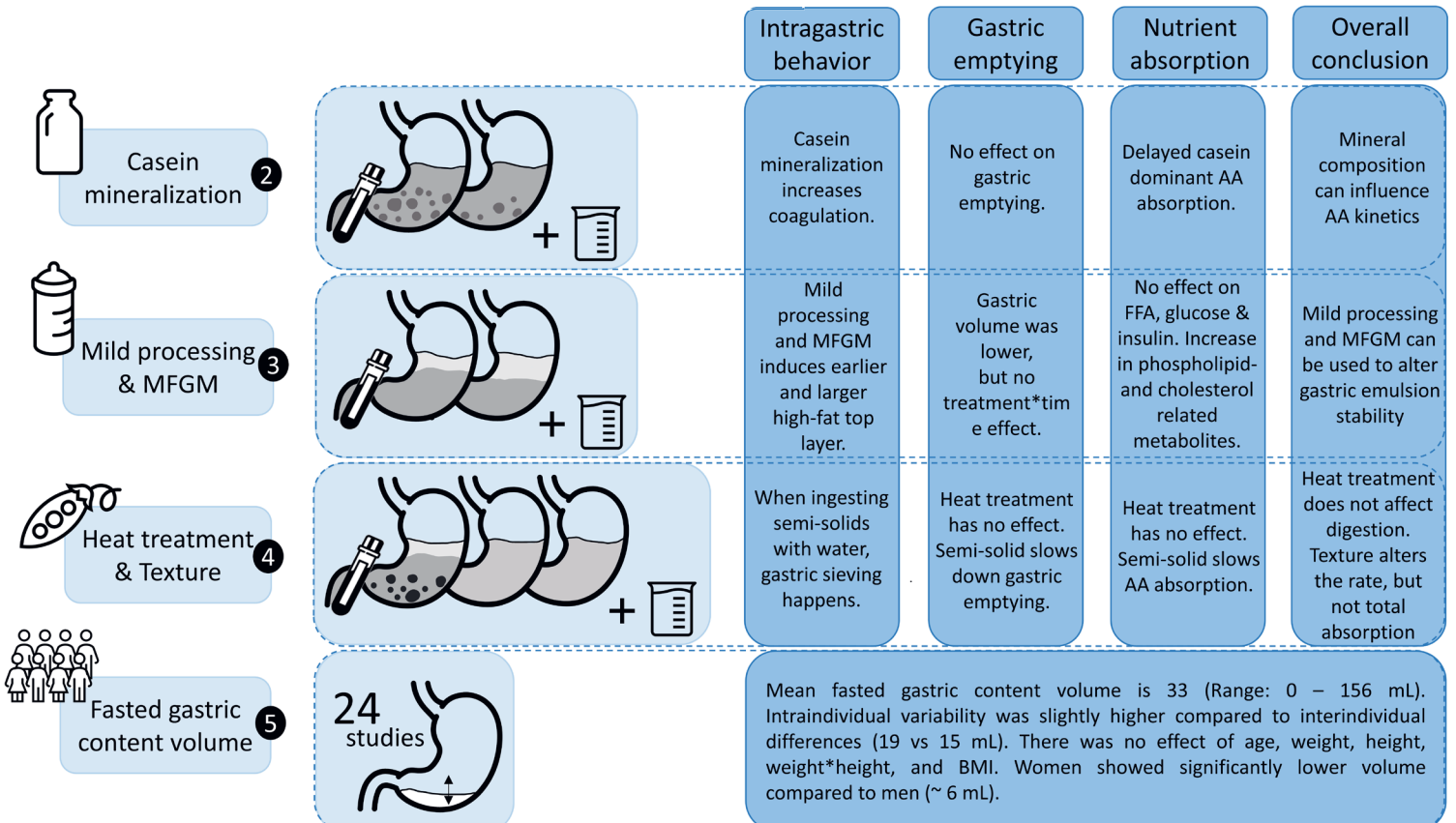
In the following sections the main results will be summarized and placed in the context of other literature. Moreover, strengths, limitations and implications for future research will be discussed. The chapter will end with an overall conclusion.

1.1. Main findings

A schematic overview of the main findings of this thesis is shown in **Figure 1**. When studying the effect of mineral composition on coagulating properties of milk, we found that consumption of milk with high casein mineralization resulted in increased coagulation (**Chapter 2**). Although overall gastric emptying was not affected, the increased coagulation resulted in quicker emptying of the liquid fraction, while the coagulum persisted. This effect was also reflected in amino acid absorption kinetics, where appearance of amino acids dominant in caseins were delayed in the milk with high casein mineralization. This shows that the food matrix, in this case the mineral composition of milk, can influence coagulating properties and therefore digestion kinetics. For infant formula, we found that milder processing and the addition of milk fat globule membrane (MFGM) is an effective way of altering the fat-globule interface (**Chapter 3**). This altered interface resulted in a reduced emulsion stability under gastric conditions and induced early formation of a high-fat top layer in the stomach. This confirmed our findings of the *in vitro* digestion model, under both infant and adult conditions. Moreover, we found that fasted gastric content volume was negatively correlated with the onset time of this fat layer. This demonstrates that this initial gastric volume is able to influence digestion. For pea protein, we showed that

heat treating pea protein isolate does not affect gastric digestion or postprandial amino acid absorption (**Chapter 4**). A semi-solid texture, on the other hand, slowed down gastric emptying and subsequent amino acid absorption kinetics compared to a liquid. However, total absorption was not affected, suggesting that only the rate of digestion is influenced. In addition, comparison with *in vitro* data showed that *in vitro* digestion models gave additional support to the *in vivo* digestion results regarding texture, but not heat treatment effects. That fasted gastric content volume is highly variable was confirmed with an analysis based on data from 24 studies (**Chapter 5**), which found that that intraindividual variability is slightly higher compared to interindividual differences (19 vs. 15 mL). This indicates that fasted gastric content volume is subject to day-to-day and within-day variation and cannot be attributed as a stable personal characteristic. Although no associations were found with age, sex, body weight and size, women were found to have a lower gastric content volume compared to men (~6 mL on average).

Figure 1. Summary of the main findings discussed in this thesis.



1.2. Food properties and gastric behavior

Food is not simply a sum of its components. The way in which these components are organized and interact with each other within the food matrix determines its properties and affects the digestion (Dupont et al., 2018). In this thesis we further studied how food properties impact digestion, by studying the effects of mineral composition, heat treatment, and texture. The findings will be discussed and put into context of other literature below.

1.2.1. Composition

The composition of food has a major influence on digestion. Each food component has its own functionality and behavior. However, when it is embedded in a food matrix it may result in interaction with other components and change its digestion (Aguilera, 2019). The role of the food matrix in digestion was confirmed in **Chapter 2**, where a different mineral composition in skimmed milk resulted in increased degree of coagulation. In this case, the caseins in the milk form casein micelles with calcium, phosphate, and magnesium. Changing the mineral composition alters the coagulating properties, and therefore the degree of gastric coagulation. As expected, the milk with higher casein mineralization resulted in higher degree of coagulation and denser coagula structure, which is in line with *in vitro* digestion studies (Huppertz & Lambers, 2020). In contrast to our expectations, this difference in coagulation did not affect overall gastric emptying. This is likely explained by the differential emptying of the fractions. In this case, the semi-solid coagulates persist in the stomach, while the liquid fraction empties (Ye et al., 2016). This was confirmed in **Chapter 2**.

1.2.2. Processing

Food processing, such as enzymatic hydrolysis and heating, alters the chemical and physical characteristics and can significantly alter digestion under gastric conditions (Joye, 2019; Loveday, 2022). In many cases, processing is much needed as it can eliminate pathogens, remove anti-nutritional factors, and prolong shelf-life. In addition, it can be used to improve palatability, or induce certain intragastric behavior as shown in **Chapter 3**.

In this thesis we studied the effect of heat treatment. That the effect of heat treatment depends on the type of food is illustrated by our findings in **Chapter 3 and 4**. When looking at foods containing pea protein isolate, heat treating did not affect gastric emptying or post-prandial amino acid absorption. This could be explained by the prior heat treatment during the manufacturing of pea protein isolate, after which additional heat treatment does not further affect digestibility. In contrast, in **Chapter 3** we show that in infant formula mild processing changes gastric emulsion stability. This is likely explained by a change in the denaturation of proteins. Milder heat treatment results in less denaturation, leading to a decreased formation of complexes which decreases emulsion stability (Raikos, 2010). This contrast in findings highlights the fact that the effect of processing depends on many factors, such as the type of food, the food matrix, prior processing, and the intensity of the treatment. In addition, the findings in **Chapter 3** highlight that processing is not only useful in preparing food for consumption but can actively be used to induce certain gastric behaviors.

1.2.3. Texture

Food texture influences food intake (Bolhuis & Forde, 2020). Increased viscosity results in increased oral processing time, but also delayed gastric emptying (Camps et al., 2016). In **Chapter 4** we studied the effect of texture on pea protein digestion, comparing a liquid with a mixed meal consisting of a semi-solid and water with equal overall nutrient density. In line with literature, we found that consumption of a semi-solid with water resulted in rapid initial emptying of water, a phenomenon called gastric sieving. Emptying of the semi-solid protein fraction was slower, which can be explained by the mechanical breakdown that is needed before a semi-solid can pass the pylorus since it can only pass particles smaller than 0.5 – 2 mm (Kelly, 1980; Marciani et al., 2001; Meyer, 1980). This demonstrates potential of modulating the rate of digestion by changing the texture.

The above clearly demonstrates how food properties modify subsequent gastric digestion and postprandial absorption kinetics. This knowledge can be used in order to adapt or design foods to specific nutritional needs.

1.3. Linking gastric behavior with postprandial absorption

Gastric emptying determines the rate at which nutrients are delivered to the intestine and thus the rate at which nutrients can be absorbed (Hellström et al., 2006). For homogenous foods, the gastric emptying rate might be a good predictor for nutrient absorption. However, for non-homogenous foods, or meals, this is not always the case. Especially under gastric conditions where certain intragastric behaviors are induced, such as coagulation and gastric layering, gastric volume may not always be a good predictor for nutrient absorption. This is apparent from **Chapter 2 and 4**. In **Chapter 2**, no differences were found in overall gastric emptying. However, the postprandial amino acid response was different between both drinks, as explained by the differences in coagulation. In **Chapter 4**, consumption of a semi-solid food together with water led to an initial quick emptying of the water. However, postprandial amino acid response was delayed, as explained by gastric sieving. This shows that intragastric behaviors might be more relevant in predicting postprandial response than overall gastric content volume. Moreover, this highlights the added value of using MRI and thereby being able to visualize the processes happening in the stomach, to quantification the different fractions and see how those empty into the intestine.

1.4. Looking at the individual: Taking into account interindividual differences

Chapter 5 shows that there is both large intraindividual as well as interindividual variability in fasted gastric content volume, indicating that it is subject to day-to-day and within-day variation and cannot be seen as a stable personal characteristic. It has been suggested that part of this variation might be explained by the migrating motor complex (MMC) cycle. The MMC cycle is associated with increases and decreases in the secretion of gastric fluid and with changes in peristaltic contractions, resulting in increased and decreased gastric emptying (van den Abeele et al., 2017), which will ultimately affect fasted gastric content volume. Moreover, it is known that a *Helicobacter pylori* infection can initially cause hypergastrinemia and gastric hypersecretion, while later in life it can cause gastric atrophy with impaired gastric secretion (Calam, 1999). Since an infection is commonly asymptomatic, prevalence

is around 34% in Europe (Hooi et al., 2017), and participants are usually not tested for this, this might explain some of the variance.

Fasted gastric content volume is known to influence gastric behavior (**Chapter 3**) (Camps et al., 2021). Thus, when food is consumed during a phase of the MMC cycle where there is an increase in gastric secretions, this may affect further digestion. This emphasizes the need for assessment of fasted gastric content volume prior to other measurements. Moreover, this assessment can act as a compliance check for the fasting conditions. In addition to fasted gastric content volume, meal-induced gastric secretion is also highly variable, highlighting the need for methods to estimate gastric acid secretion after ingestion of food. Marciani et al. (2001) used T_2 relaxation time to monitor the process of dilution by gastric secretions and mixing of viscous meals and Goetze et al. (2009) used fast T_1 mapping techniques for the quantification of intra-gastric dilution and distribution of orally applied gadolinium-based paramagnetic contrast agents showing that there is potential for estimating gastric secretion volumes with MRI.

Intra- and interindividual variability is not only present in gastric fluid secretion, but also in gastric emptying, transit times, and nutrient absorption and should be taken into consideration. For example, the study of van Dam et al. (2023) shows that there is large inter-individual variation in plasma amino acid concentrations after consumption of different protein drinks. Some individuals only have a slight increase in amino acid concentrations, while others show a substantial increase in response to the same drink. Moreover, some individuals show a similar amino acid response for each protein drink (pea, milk, casein, and casein-pea), while others respond very differently to the different protein sources. This shows that there is large variation within and between individuals in the digestion of food, emphasizing the need to be careful with generalization. Studying individual differences and an individual's digestive function can contribute to further development of personalized nutrition (Dallas et al., 2017).

1.5. Using MRI to study digestion

MRI has proven to be a useful, non-invasive tool to measure intragastric behavior of food and gastric emptying (Smeets et al., 2021; Spiller & Marciani, 2019). With anatomical scans, we are able to accurately measure gastric volume. Additionally, because different matrices emit different radiofrequency pulses, these will appear on the MRI scan with different intensities. Therefore, in addition to quantifying the content volume, these scans allow for visual differentiation between matrices. For example, on the T_2-weighted anatomical scan, water appears with a higher intensity compared to fat, whereas air appears black (Smeets et al., 2021). Although time consuming, it is relatively easy to separately segment air in the stomach, or gastric layering (**Chapter 3**). In addition to manual segmentation, thresholding techniques have been applied to the anatomical scans. With these techniques, volumes of different fractions can be quantified based on grey levels (Otsu, 1979). This is a useful technique, especially for matrices such as coagulates or gel particles, which are difficult to segment manually.

One application for this approach is the quantification of coagulum volume. Previously, coagulation was often visually graded, and therefore prone to subjectivity (Coletta et al., 2016). When proteins coagulate, there is a shift from a liquid to a semi-solid state. These coagulates will thus appear with a different intensity on an MRI scan compared to the liquid content of the stomach. This principle can be used in order to quantify coagulation more objectively. This thresholding technique was already successfully used in *in vitro* studies (Mayar et al., 2022; Mayar et al., 2023) and now also applied *in vivo* (**Chapter 2)**. Moreover, **Chapter 4** shows that this thresholding technique works well for segmenting liquid versus solid content when a mixed meal containing liquids and semi-solids is ingested. In real life situations, a meal usually consists of different textures, often including both solids and liquids. This technique would allow us to estimate which fraction is emptied.

Moreover, an increasing number of studies explore the use of MRI techniques to study digestion at a molecular level. These may provide markers for digestive processes, such as texture changes and nutrient breakdown (Smeets et al., 2021).

Two promising digestion markers are T_2 relaxation time (T_2) and Magnetization Transfer Ratio (MTR). T_2 is determined by the relaxation behavior of water protons in different environments. This marker has recently been used to monitor digestion of whey protein *in vitro* (Deng et al., 2020; Deng et al., 2022). MTR is a Magnetic Resonance technique used to create a contrast between tissues. The protons in tissues are present in three different states: in free water, bound to semi-solid macromolecules and as water in the hydration layer between macromolecules (Mayar et al., 2022; Mayar et al., 2023; Smeets et al., 2021). When proteins coagulate during gastric digestion, it changes from a liquid to a (semi-)solid, which might be tracked by MT measurements. Although promising, these techniques were only recently applied in an *in vivo* digestion study for the first time (Deng et al., 2023) and should be further explored. These techniques therefore require further development and exploration in their potential to track dilution, phase changes, and digestion in the stomach to be able to apply these successfully *in vivo* and to study more complex meals.

1.6. Linking *in vivo* and *in vitro* digestion research

Digestion is studied both *in vitro* and *in vivo*. Although only *in vivo* digestion studies can show real-life digestion, they come with limitations: they should conform ethical regulations, they are expensive, and take a lot of time. Therefore, *in vitro* digestion models that mimic human digestion are frequently used to study digestion. *In vitro* studies are relatively quick, cheap, and reproducible. Various *in vitro* digestion models have been developed, ranging from simple to multiple compartment static, semi-dynamic, and fully dynamic models (Zhou et al., 2023) with most studies using the standardized INFOGEST model for healthy young adults (Brodkorb et al., 2019). Although some of these models are highly advanced, they have various limitations regarding important aspects of human gastric digestion, such as the fluctuations in pH, gastric motility, and gradual gastric emptying, and can therefore not fully replicate the complex human digestion.

In vitro studies are very useful during early phases of product development, to study the effects of food composition and processing on digestion. Especially when

studying relatively new protein sources or processing techniques. During these developmental stages, many formulations can easily be studied under controlled conditions. This provides details on the effects of enzymatic processes on the physical and chemical characteristics of foods during digestion (Smeets et al., 2021), as shown in **Chapter 3 and 4**. Thus, despite their limitations, *in vitro* studies can provide useful information that might be difficult or even impossible to obtain *in vivo*. For example, in the case of coagulation. MRI allows us to visualize coagulation and therefore quantify the volume of the coagula. However, with *in vitro* methods, it is much easier to study the structure of the coagula. Thus, *in vitro* studies are useful in helping us understand the *in vivo* findings. In addition, with *in vitro* models it is possible to study digestion under different conditions. That is, you can use models specifically tailored to study digestion in, for example, infants, elderly, or clinical populations without ethical constraints (Bourlieu et al., 2014; Menard et al., 2023).

Combining *in vitro* digestion models with *in vivo* studies is getting more common (van Eijnatten et al., 2023). In this thesis, static and semi-dynamic models were used to study the food products to study coagulation (**Chapter 2 & 4**), to validate findings under infant conditions (**Chapter 3**), and overall agreement. Further exploration and bridging *in vitro* and *in vivo* will allow us to further validate the results and understand to what extent and in which situations *in vitro* models are best able to represent *in vivo* digestion. Moreover, MRI might be used to bridge this gap between *in vitro* digestion and *in vivo* studies, as discussed in section 1.5.

1.7. Methodological considerations

1.7.1. Supine position

MRI usually requires the participants to be in a supine position. This is, in most cases, different from the real-life situation. Effects of posture on differences in gastric digestion should be considered, as studies have shown that a supine position may affect gastric digestion. As a consequence of gravity, the supine position can affect the intragastric distribution of the fat layer. Boulby et al. (1997) studied the effect of body position on the intragastric distribution and gastric emptying of a soup with added oil. They found that the right decubitus position resulted in immediate

emptying of oil, while there was a profound lag phase in the emptying of the oil layer in the left decubitus position, with virtually no emptying of oil in 60 minutes. Similar findings were reported by Horowitz et al. (1993) who found that oil emptied more slowly from the stomach in a sitting position compared to the left decubitus position. They also reported that in the decubitus position, oil emptied faster than the aqueous phase. This is likely explained by the oil being retained in the distal stomach compared to the proximal stomach in the decubitus versus the sitting position, resulting in emptying of this oil fraction. The same principle might apply to the supine position, where the fat layer might empty quicker compared to an upright sitting position. In **Chapter 3**, the ingestion of infant formula resulted in the formation of such a high fat top layer. Although the supine position might thus have influenced the intragastric distribution, from the perspective of infant nutrition, the supine position is actually more natural.

Several studies on the effect of a supine position on gastric emptying have been conducted. Spiegel et al. (2000) found that a supine position slows down gastric emptying rate after consumption of soup with a sandwich compared to a seated position. Another study found that gastric emptying of milk in a supine or −20° head-down tilted position resulted in a slower gastric emptying compared to an upright sitting position (Holwerda et al., 2016; Holwerda et al., 2017). In contrast, Jones et al. (2006) found no difference in gastric emptying after consumption of a glucose solution compared to sitting position. There are also studies comparing other postures. Horowitz et al. (1993) found that total volume emptied after 180 min of a soup with oil was not different in a right decubitus position compared to sitting. A study comparing left and right decubitus positions found no difference in gastric emptying of a soup. However, when oil was added to the soup, the gastric emptying half time was slower in the left compared to the right decubitus position (Boulby et al., 1997), likely caused by the change in intragastric distribution as explained above. In this position, the more nutrient-dense fat layer on top flows more easily into the duodenum resulting in increased nutrient sensing compared to the right decubitus position. Consequently, the nutrient-based feedback mechanism slows down gastric emptying (Brener et al., 1983; Calbet & MacLean, 1997; McHugh & Moran, 1979;

Shahidullah et al., 1975). The effect of posture might therefore also depend on the type of food consumed. That is, for a homogenous liquid the effects might be small, but in case of gastric layering it might have a more pronounced effect.

Consequently, a decrease in gastric emptying can change postprandial absorption kinetics. Studies have indeed shown that protein ingestion in an upright sitting position increases the postprandial rise in plasma amino acid availability by increasing protein digestion and amino acid absorption rates compared to a supine position, mediated by a decrease in gastric emptying rate (Holwerda et al., 2016; Holwerda et al., 2017). However, effects are small. The study of Holwerda et al. (2017) showed a higher peak plasma leucine concentration for upright sitting compared to a supine position (213 compared to 193 μmol/l, $p < 0.05$).

Thus, although effects are small, a supine position might slow down gastric emptying and postprandial amino acid absorption. Since all participants were scanned in the same position for all treatments, the relative differences between treatments are expected to remain the same.

1.7.2. Study population

The trials discussed in this thesis solely include young, healthy, male participants. It is known that the gastrointestinal physiology differs between women and men. In **Chapter 5** we showed that men have lower fasted gastric content volume. In **Chapter 3**, we showed how this fasted gastric content volume can affect further digestion. In addition to fasted gastric content volume, more differences exist. Studies have shown that women have higher gastric pH, lower gastric acid secretion in response to a meal, and higher gastrointestinal transit times compared to men (Freire et al., 2011; Lajterer et al., 2022). The latter also includes slower gastric emptying for women. Moreover, ageing is also known to affect the gastrointestinal tract, and is associated with a slight decrease in gastric emptying (Menard et al., 2023; Stillhart et al., 2023) and in post-prandial amino acid response in elderly (van der Heijden et al., 2024).

Furthermore, our study population included only healthy individuals. However, in the general population there can be many circumstances in which gastrointestinal digestion is affected. This includes, being over- or underweight, having had surgery on the gastrointestinal tract, or the use of certain medication, which can result in altered transit time, different pH conditions or enzyme concentrations (Verkempinck et al., 2023). Thus, although these studies in healthy males have a valuable contribution to our fundamental understanding of digestion, the external validity of the findings is limited. This highlights the need for more research in women, elderly, and clinical populations. Especially the latter two could benefit from more research because they frequently struggle to consume sufficient nutrients and could therefore benefit from improved digestion and optimal utilization of nutrients. When the effect of these specific conditions is studied, this might lead to more tailored and appropriate food choices.

1.8. Future digestion research

1.8.1. The bigger picture

Ideally, when studying digestion, the whole digestive tract should be considered, from the oral phase until the gut. It has already been acknowledged that oral behavior affects nutrient release and utilization during digestion (Chen et al., 2021). Ideally, current research should therefore be expanded by including the oral phase. Furthermore, others already showed that it is possible to image the small intestine and the colon with MRI (Spiller & Marciani, 2019). It is, for example, possible to measure small bowel water content to measure water fluxes (Hoad et al., 2007) or use dynamic MRI to measure motility and transit in the gastrointestinal tract (De Jonge et al., 2018). Another valuable addition would be assessing appetite regulation by measuring postprandial hormonal responses. Connecting and taking into account these different phases would result in a more complete overview of the digestion of food.

The studies discussed in this thesis are among the first to provide visualization and quantification of gastric digestion and to combine this with postprandial nutrient

responses. However, it is important to be aware that these postprandial amino acid concentrations do not reflect the absolute absorption of the foods consumed. These concentrations are rather a result of absorption and clearance rates in tissues. More reliable methods to analyze absolute protein absorption use labelling techniques, however, these techniques are costly and more difficult. Although measuring the appearance of postprandial nutrients does not provide information on absolute absorption, they do provide information on de relative differences between treatments, and they generally reflect the amino acid profile of the consumed food (Mes et al., 2022). Moreover, the use of MRI digestion markers, T_2 relaxation time (T_2) and Magnetization Transfer Ratio (MTR), should be further developed to measure digestion on a molecular level as discussed in section 1.5.

Moreover, many studies are limited to studying isolated ingredients, or very simple foods. However, this is not how we consume food in real life. The food matrix plays an important role in digestibility because of its influence on the kinetics of transit and hydrolysis of macronutrients. Research should therefore not solely focus on isolated nutrients, but also study more complex foods and meals.

This fundamental knowledge on how composition, processing, and texture of can modulate the digestion in each different phase can then be applied to optimize digestion, and thereby utilization of nutrients. It can be used to tailor foods to specific needs, whether that is a specific clinical population, elderly, athletes, or in regard to sustainability. For example, developing infant formulae that mimics the digestion of breast milk even better, or developing better digestible plant-based protein foods. However, tailoring to specific needs also calls for more research in specific target populations, as discussed above.

1.8.2. Further development and validation of in vitro digestion models

In vitro digestion models have significantly advanced our understanding of digestion of foods. Despite their limitations, there can be fair agreement between *in vivo* and *in vitro* findings. However, this is especially true for simple (protein) solutions, stable emulsions, or starch. For more complex, or realistic foods, agreement is limited

(Bohn et al., 2018; Egger et al., 2017). Moreover, it is increasingly recognized that there are large interindividual differences in human food digestion (**Chapter 5**). The growing evidence supporting these differences has resulted in the development of more tailored *in vitro* digestion models (Lesmes, 2023). Specific models have already been developed for infants and elderly (Bourlieu et al., 2014; Ménard et al., 2018; Menard et al., 2023). Recently, an *in vitro* model was developed that accounts for the sex differences in the gastrointestinal tract (Lajterer et al., 2022).

In addition to these tailored *in vitro* models, more advanced dynamic systems are being developed. One of the more recently developed system is the NEar Real Digestive Tract (NERDT). This dynamic system was designed to mimic the peristaltic contractions, gastric morphology, gastric secretions and emptying as close to the real-life situation as possible (Li et al., 2020; Peng et al., 2021; Zhang et al., 2023). While *in vivo* trials are often used to validate *in vitro* findings, systems like these benefit from *in vivo* data as input to tailor their settings. For example, when *in vivo* data is available on gastric emptying, the operating parameters, such as stomach tilting angle, frequency of the peristaltic contractions, and squeezing frequency of the pylorus can be altered to accurately represent the real life situation. Wang et al. (2019) already demonstrated that this system was able to generate consistent gastric emptying profiles for both the solid and liquid fractions of a mixed meal consisting of beef stew and orange juice. Systems like these are useful in predicting how foods might behave and might even reduce the need for *in vivo* research. Since these systems can be tailored to specific conditions, they might be specifically beneficial for studying certain populations, who are difficult to study *in vivo.* Although systems like this are very useful, they are also very expensive, and accessibility might therefore be limited.

Moreover, recently MRI digestion markers have been validated for the first time to study *in vitro* protein digestion (Deng et al., 2020; Deng et al., 2022; Mayar et al., 2022; Mayar et al., 2023). Applying these same markers to study *in vivo* digestion might help with validating *in vitro* digestion models (Deng et al., 2023). So far, these studies are often performed with relatively simple foods to explore if, and how these

techniques can be used. Ideally, more complex foods and whole meals should be studied too.

Another approach is the development of MRI-compatible *in vitro* models. Deng et al. (2022) developed a semi-dynamic gastric simulator that can control gastric secretion, emptying and mixing at body temperature. With this, it is easier to directly bridge *in vitro* and *in vivo* and to compare findings. Moreover, this is useful in validating our thresholding technique and image texture metrics, as these are not yet validated for this application. With this *in vitro* system we are both able to scan the coagulates, use the thresholding technique and determine the image texture metrics, and compare those by analyzing the coagulates. Especially for the image texture metrics, the different metrics and their interpretation require additional research for validation and understanding what information these metrics exactly provide regarding coagulation.

1.8.3. Protein transition

Animal-based proteins are quite well studied, however, studies on plant-based proteins are still relatively limited, especially *in vivo*. The need to transition from animal-based proteins towards more sustainable proteins requires additional investigation of the digestion of these plant-based proteins, especially due to their suboptimal amino-acid composition and lower digestibility (Herreman et al., 2020). This lower quality and suboptimal digestion are usually no problem for healthy adults. However, when looking at those that already struggle to ingest sufficient protein or those that have increased needs for protein, such as elderly, athletes and critically ill, improving digestibility and utilization will be very beneficial.

Many different plant-based proteins are available, all with different amino acid compositions and digestibility. Wheat, soy, and pea are among the most studied plant-based proteins; however, many more types are available which all have a different amino acid composition, digestibility, and might have beneficial properties. Moreover, many plant-based proteins are often studied in isolation. Combining multiple plant-based proteins can improve the overall amino acid profile.

Nevertheless, the limited digestibility still remains a challenge. Applying appropriate processing techniques might improve this digestibility. Many plant-based proteins are consumed via concentrates or isolates, which require heavy processing in order to improve functionality and bioavailability. This often results in denaturation of the proteins and thus affects digestibility (Rivera del Rio et al., 2022). This shows that processing techniques should be carefully evaluated whether they not only result in functional improvements, but also do not decrease digestibility. Especially since many of these plant-based proteins are still relatively new, this has not yet been extensively studied (Day et al., 2022). This highlights the need to further research processing methods of plant-based proteins in order to improve bioaccessibility and bioavailability.

Furthermore, the use of plant-based analogues for meat, seafood, and dairy, formulated by a variety of plant-based proteins, such as legumes, nuts, and oils, has increased over the last years. These products are often developed to have certain sensory properties in order to mimic the animal-based products. However, often they do not mimic their nutritional profile (Sridhar et al., 2023). These inherent structural differences hamper direct substitution. Ideally, plant-based products should therefore contain similar protein and micronutrient content of animal-based products. By carefully studying the characteristics of different types of plant-based proteins, and using processing to modify their properties and digestibility, we will have the ability to maximize their utilization and design products that are acceptable to the consumer.

Thus, deepening our knowledge on the digestion and the impact of processing of the various plant-based proteins available, *in vitro* as well as *in vivo* will allow us to improve the digestibility and increase the utilization of these nutrients.

Furthermore, insects are also of increasing interest in regard to the protein transition. Insects are protein rich and have good essential amino acid profiles. The production of insects as food is considerable more sustainable compared to the production of other animal proteins (Pyett et al., 2023). Direct use of insects as food is often

disliked by consumers, especially in the western world. However, when insects are consumed in unrecognizable form, acceptability increases. Moreover, insects often contain high levels of non-protein components such as chitin, which have a negative impact on digestibility. To increase digestibility, processing is needed (Sweers et al., 2022). However, studies on the exact effects of processing are limited (Rodríguez-Rodríguez et al., 2022), thus, showing great potential for further research.

1.9. Conclusion

To conclude, this thesis contributed to our understanding of how nutrient composition, processing, and texture impact gastric digestion and nutrient absorption, and how interindividual differences might play a role. In this thesis, it was confirmed that food properties have significant impact on gastric digestion and subsequent absorption rates. We confirmed that the food matrix and heat treatment can influence intragastric behavior, by inducing coagulation and gastric layering. Gastric emptying was affected by texture, but, in contrast to our expectation, not by coagulation and gastric layering. However, coagulation resulted in emptying of a different fraction. Postprandial amino acid absorption was affected by coagulation and texture, but not by heat treatment. This shows that changing the food properties can significantly affect subsequent intragastric behavior, gastric emptying, or postprandial absorption kinetics. This knowledge can be used in order to adapt or design foods to specific nutritional needs. For example, by designing foods in such a way that they will undergo structural modifications under gastric conditions, such as coagulation or gastric layering, thereby changing their susceptibility to be broken down and alter gastric emptying and absorption kinetics. Moreover, large intra- and interindividual variability in fasted gastric content volume were shown, highlighting the need to consider individual differences when studying digestion. Fair agreement was shown between *in vitro* digestion models and our *in vivo* findings, although these digestion models could still benefit from further development.

These studies are among the first that combine the assessment of intragastric behavior, gastric emptying, and postprandial nutrient absorption and show the added value combining these measurements. MRI provides important information

regarding intragastric behavior on top of gastric emptying, which can be used to further underpin differences in postprandial absorption kinetics. Moreover, MRI also has great potential to study digestion on a more molecular level, for validating *in vitro* digestion models, and to bridge the gap between *in vitro* and *in vivo* research. However, there is still much potential in digestion research, for example, by including the oral phase and the complete gastrointestinal tract. The ongoing efforts to further elucidate the fundamental processes underlying digestion, with both *in vitro* and *in vivo* digestion studies, will contribute to improving our ability to improve digestion, optimal utilization of nutrients, tailoring foods for specific needs, or even personalized nutrition. Moreover, it can further contribute to the current transition towards consumption of more sustainable proteins.

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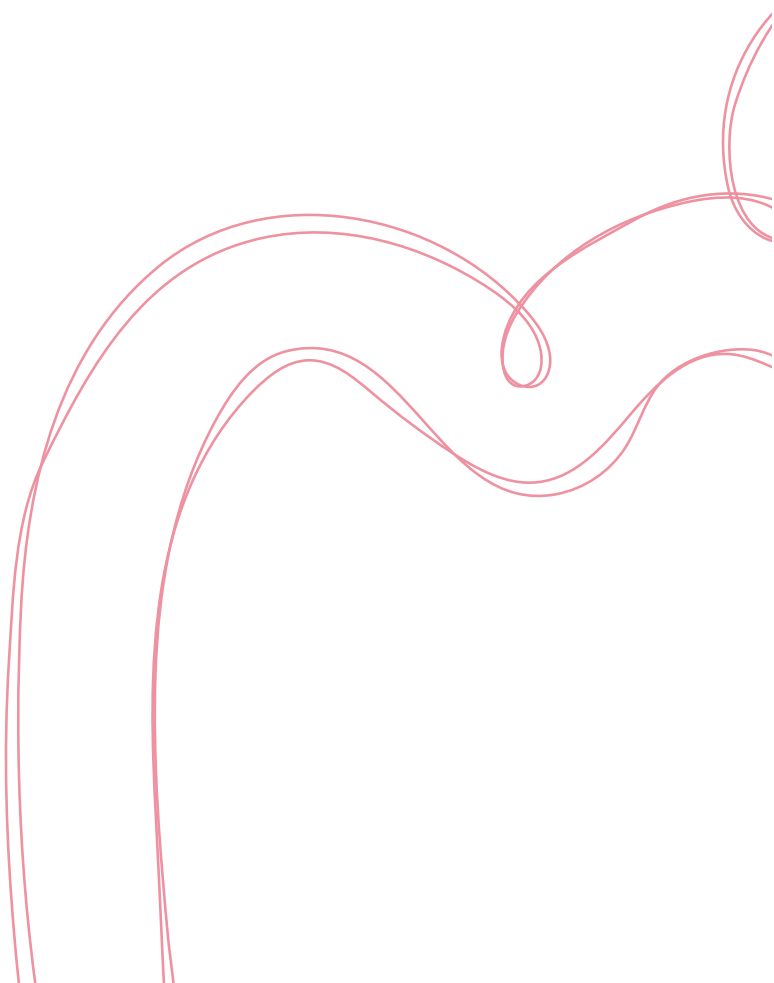
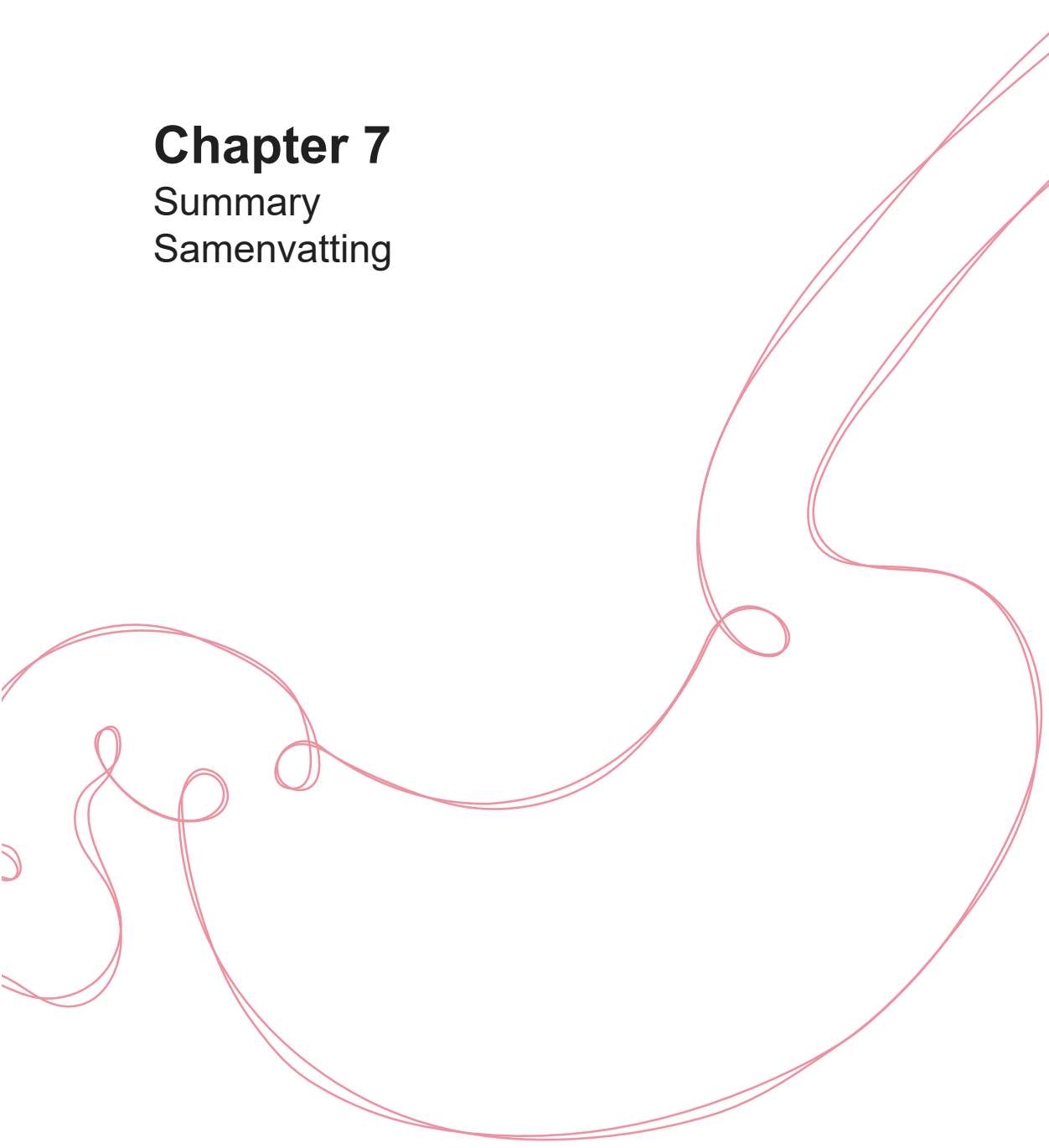


Chapter 7

Summary

Samenvatting



This thesis describes a series of studies conducted to improve our fundamental understanding of gastric digestion, and how this digestion is affected by the properties of the food consumed. The overall aim was to obtain a better understanding of how food composition, processing, and texture affect intragastric behavior (gastric coagulation and layering), gastric emptying, and postprandial nutrient absorption.

Gastric coagulation

In **Chapter 2** we studied the effect of mineral composition on coagulating properties. Milk with high casein mineralization resulted in increased coagulation. Overall gastric emptying was not affected, however, this resulted in quicker emptying of the liquid fraction, while the coagulum persisted. This effect was also reflected in amino acid absorption kinetics, where appearance of amino acids dominant in caseins were delayed in the milk with high casein mineralization. This shows that mineral composition of milk can influence coagulating properties and therefore digestion.

Gastric layering

In order to induce earlier onset of a high-fat top layer in infant formula, we explored if mild processing and the addition of milk fat globule membrane (MFGM) is an effective way of altering the fat-globule interface and therefore the emulsion stability to induce this behavior (**Chapter 3**). We showed that this resulted in earlier onset of a high-fat top layer and higher maximum fat layer volume. Gastric volume over time was lower but did not show significant treatment by time interaction. In addition, fasted gastric content volume was found to be negatively correlated with onset time of the high-fat top layer. Moreover, post-prandial absorption kinetics of free fatty acids and glucose were not affected, but insulin levels were lower. Moreover, cholesterol- and phospholipid-related metabolites were increased, which could be explained by the addition of MFGM. The *in vitro* model showed similar results under both infant and adult conditions, i.e., earlier onset of the fat layer.

Texture and heat treatment

In **Chapter 4** we studied the effect of heat treatment and texture of pea protein products on amino acid absorption kinetics and gastric behavior. We showed that heat treating products containing pea protein isolate does not affect gastric emptying or amino acid, glucose, and insulin absorption kinetics. On the other hand, pea protein isolate product with a semi-solid texture consumed together with water slowed down gastric emptying and subsequent amino acid absorption kinetics compared to liquids. However, total absorption was not affected, suggesting that only the rate of digestion is influenced. In addition, comparison with *in vitro* data showed that *in vitro* digestion models gave additional support to the *in vivo* digestion results.

Inter- and intraindividual variability

As previous studies have shown the large variability in fasted gastric content volume and the impact on gastric behavior, we assessed this inter- and intraindividual variability based on the data of 24 studies (**Chapter 5**). This multi-study analysis showed that intraindividual variability is slightly higher compared to interindividual differences (19 vs. 15 mL). This indicates that fasted gastric content volume is subjected to day-to-day variation and cannot be attributed as innate to a person. No associations were found with age, weight, height, weight*height, and BMI. However, women had lower gastric content volume compared to men (6 mL).

To conclude, this thesis contributed to our understanding of how nutrient composition, processing, and texture impact gastric digestion and nutrient absorption, and how interindividual differences might play a role. In this thesis, it was confirmed that food properties have significant impact on gastric digestion and subsequent absorption rates. We confirmed that the food matrix and heat treatment can influence intragastric behavior, by inducing coagulation and gastric layering. Gastric emptying was affected by texture, but, in contrast to our expectation, not by coagulation and gastric layering. However, coagulation resulted in emptying of a different fraction. Postprandial amino acid absorption was affected by coagulation and texture, but not by heat treatment. This shows that changing the food properties can significantly affect subsequent intragastric behavior, gastric emptying, or

postprandial absorption kinetics. This knowledge can be used in order to adapt or design foods to specific nutritional needs. For example, by designing foods in such a way that they will undergo structural modifications under gastric conditions, such as coagulation or gastric layering, thereby changing their susceptibility to be broken down and alter gastric emptying and absorption kinetics. Moreover, large intra- and interindividual variability in fasted gastric content volume were shown, highlighting the need to consider individual differences when studying digestion. Fair agreement was shown between *in vitro* digestion models and our *in vivo* findings, although these digestion models could still benefit from further development.

These studies are among the first that combine the assessment of intragastric behavior, gastric emptying, and postprandial nutrient absorption and show the added value combining these measurements. MRI provides important information regarding intragastric behavior on top of gastric emptying, which can be used to further underpin differences in postprandial absorption kinetics. Moreover, MRI also has great potential to study digestion on a more molecular level, for validating *in vitro* digestion models, and to bridge the gap between *in vitro* and *in vivo* research. However, there is still much potential in digestion research, for example, by including the oral phase and the complete gastrointestinal tract. The ongoing efforts to further elucidate the fundamental processes underlying digestion, with both *in vitro* and *in vivo* digestion studies, will contribute to improving our ability to improve digestion, optimal utilization of nutrients, tailoring foods for specific needs, or even personalized nutrition. Moreover, it can further contribute to the current transition towards consumption of more sustainable proteins.

Samenvatting

Goede voeding van voldoende kwaliteit is essentieel voor een gezond lichaam en het functioneren hiervan. Voordat ons lichaam de voedingstoffen uit deze voeding kan gebruiken moet deze eerst verteerd worden. De vertering van voedsel wordt beïnvloed door verschillende factoren, waaronder de samenstelling, de manier waarop het bewerkt is, de textuur en fysiologische verschillen tussen personen. Deze factoren kunnen de vertering zowel positief als negatief beïnvloeden. De onderzoeken in dit proefschrift zijn erop gericht om onze fundamentele kennis van de vertering in de maag en de factoren die daarbij een rol spelen, te vergroten. Hierbij was het doel om meer inzicht te krijgen in de manier waarop de samenstelling, de verwerking en de textuur van ons voedsel van invloed kunnen zijn op de vertering in de maag (coagulatie en laagvorming), de maaglediging en de opname van voedingstoffen.

Coagulatie

In **Hoofdstuk 2** is het effect van mineraalsamenstelling op de coagulerende eigenschappen van melk onderzocht. Melk met hoge caseïne mineralisatie resulteerde in meer coagulatie in de maag. Omdat er meer caseïne coaguleerde, zorgde dit er ook voor dat de opname van de aminozuren dominant in caseïne vertraagd was. Dit laat zien dat de mineraal samenstelling van melk invloed heeft op de coagulerende eigenschappen in de maag en de opname in het bloed.

Laagvorming in de maag

In Hoofdstuk 3 is onderzocht of een mildere verhitting en het toevoegen van milk fat globule membrane (MFGM) aan zuigelingenvoeding een effectieve manier is om de emulsiestabiliteit te verlagen en om op deze manier een snellere laagvorming te induceren in de maag na consumptie. Dit onderzoek liet zien dat deze laagvorming inderdaad sneller ontstaat en dat deze toplaag ook een groter maximaal volume heeft. Het totale maagvolume was lager voor deze experimentele zuigelingenvoeding, maar toonde geen significante interactie met tijd. Daarnaast was er een negatieve correlatie tussen het nuchtere maagvolume en de tijd waarop de laagvorming ontstond. Er was geen verschil in de opname van vrije vetzuren en glucose. Insuline levels waren lager voor de experimentele voeding, maar toonde

geen significante interactie met tijd. Daarnaast waren ook de concentraties van cholesterol- en fosfolipide gerelateerde metabolieten in het bloed verhoogd, wat verklaard kan worden door de toevoeging van MFGM aan de voeding. Het *in vitro* model vertoonde vergelijkbare resultaten onder zowel zuigelingen- als volwassen omstandigheden, namelijk, een vroegere vorming van de vetlaag.

Textuur en verhitting

Hoofdstuk 4 was gericht op het effect van verhitting en textuur op de vertering van producten met erwteneiwit. Hier laten we zien dat verhitting van deze producten geen effect heeft op de vertering. Echter, een product met een vastere textuur resulteerde in een vertraagde maaglediging en de daaropvolgende aminozuur opname in vergelijking met een vloeibaar product. De totale aminozuuropname werd niet beïnvloed, wat suggereert dat de textuur enkel de snelheid van vertering beïnvloed.

Inter- en intra-individuele variabiliteit

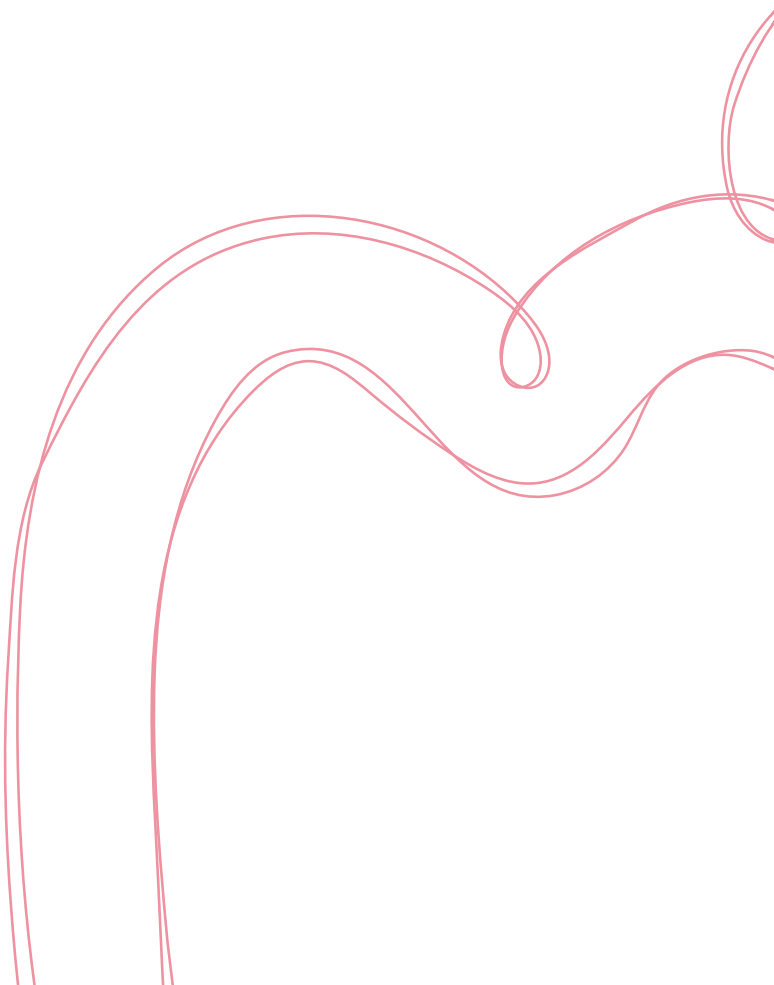
Voorgaande studies hebben laten zien dat er grote variatie is in de maaginhoud in nuchtere staat en dat dit volume invloed heeft op de vertering in de maag. In **Hoofdstuk 5** is daarom gekeken naar de inter- en intra-individuele variabiliteit op basis van de data van 24 studies. Deze multi-studie analyse toonde aan dat de intra-individuele variabiliteit hoger is dan de interindividuele variabiliteit (19 versus 15 mL). Dit laat zien dat de nuchter maaginhoud onderhevig is aan dagelijkse variatie en geen stabiele persoonlijke eigenschap is. Er zijn geen associaties gevonden met leeftijd, lichaamsgewicht en grootte. Bij mannen was deze maaginhoud, na correctie voor leeftijd, lichaamsgewicht en grootte, gemiddeld ~6 mL meer. Verschillen in de nuchtere maaginhoud worden verwacht invloed te hebben op zowel vertering van voeding als de opname van medicijnen.

De studies in dit proefschrift dragen bij aan een beter inzicht in hoe de samenstelling, de verwerking en de textuur van onze voeding de vertering in de maag en de opname van voedingsstoffen beïnvloedt en hoe individuele verschillen een rol kunnen spelen. Dit proefschrift bevestigd dat deze eigenschappen van onze voeding

een significante invloed hebben op de vertering in de maag, de maaglediging en de daaropvolgende opname van voedingsstoffen. Bovendien bevestigt dit proefschrift dat de voedselsamenstelling en verhitting de vertering in de maag beïnvloeden, door het induceren van coagulatie en laagvorming. Maaglediging werd beïnvloed door textuur, maar in tegenstelling tot onze verwachting niet door coagulatie en laagvorming. Wel zorgde coagulatie voor een andere opname van aminozuren. Een vastere textuur zorgde voor een vertraagd opname van aminozuren, maar verhitting had geen effect. Dit laat zien dat het veranderen van de voedseleigenschappen gevolgen kan hebben voor de vertering. Deze kennis kan worden gebruikt om voedingsmiddelen te veranderen of te ontwerpen voor specifieke voedingsbehoeften. Bijvoorbeeld door voedingsmiddelen zo te ontwerpen dat ze in de maag structurele veranderingen ondergaan, zoals coagulatie of laagvorming, waardoor de vatbaarheid voor verteringsenzymen, de maaglediging of de opname ook veranderen. Bovendien toonden de grote intra- en interindividuele variabiliteit in de nuchtere maaginhoud aan dat het cruciaal is om individuele verschillen in overweging te nemen bij verteringsonderzoek. Er was redelijke overeenstemming tussen *in vitro* verteringsmodellen en de *in vivo* resultaten, hoewel deze verteringsmodellen nog steeds baat zouden hebben bij verdere doorontwikkeling.

Deze studies behoren tot de eerste studies die de vertering in de maag, de maaglediging en de opname van voedingsstoffen combineren en laten zien wat de toegevoegde waarde is van het combineren van deze metingen. MRI resulteert, naast het accuraat kunnen bepalen van de maaglediging, in belangrijke informatie over de vertering in de maag, wat kan worden gebruikt om verschillen in de opname van voedingsstoffen verder te onderbouwen. Bovendien heeft MRI ook potentie om de vertering op een moleculair niveau te bestuderen, om *in vitro* verteringsmodellen te valideren en om de kloof tussen *in vitro* en *in vivo* onderzoek te overbruggen. Er is nog veel potentieel in verteringsonderzoek, bijvoorbeeld door ook te kijken naar wat er in de mond gebeurt, en om het volledige spijsverteringskanaal mee te nemen in deze onderzoeken. Het verder verduidelijken en beter begrijpen van de fundamentele processen van de vertering, draagt bij aan ons vermogen om de vertering te beïnvloeden, te zorgen voor optimale benutting van voedingsstoffen, het

aanpassen van voedingsmiddelen aan specifieke behoeften of zelfs het ontwikkelen van gepersonaliseerde voeding. Bovendien kan dit verder bijdragen aan de huidige transitie naar de consumptie van duurzamere eiwitten.



Acknowledgements
Dankwoord

En toen zat het er al weer op, de afgelopen drie jaar zijn voorbij gevlogen. In tegenstelling tot mijn verwachtingen (veel stress en lange dagen) heb ik er vooral van genoten en het onwijs naar mijn zin gehad. En dat heb ik mede te danken aan alle mensen die hier de afgelopen jaren onderdeel van waren, die de studies mogelijk hebben gemaakt, input en expertise hebben geleverd of deze tijd gewoon een stuk leuker hebben gemaakt.

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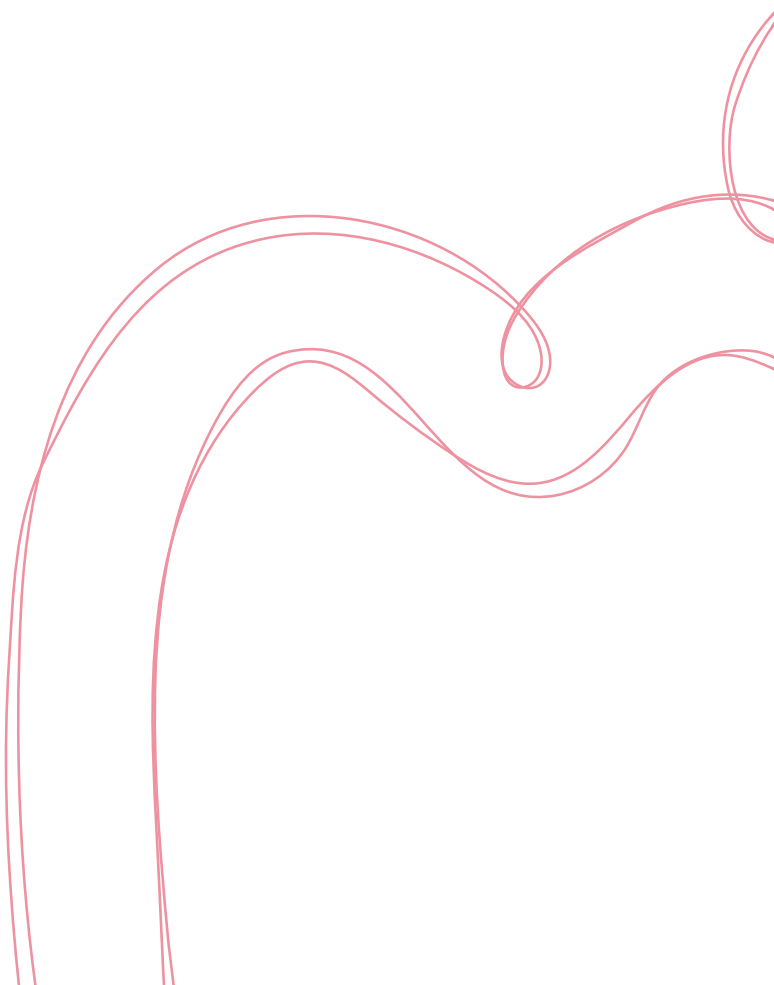
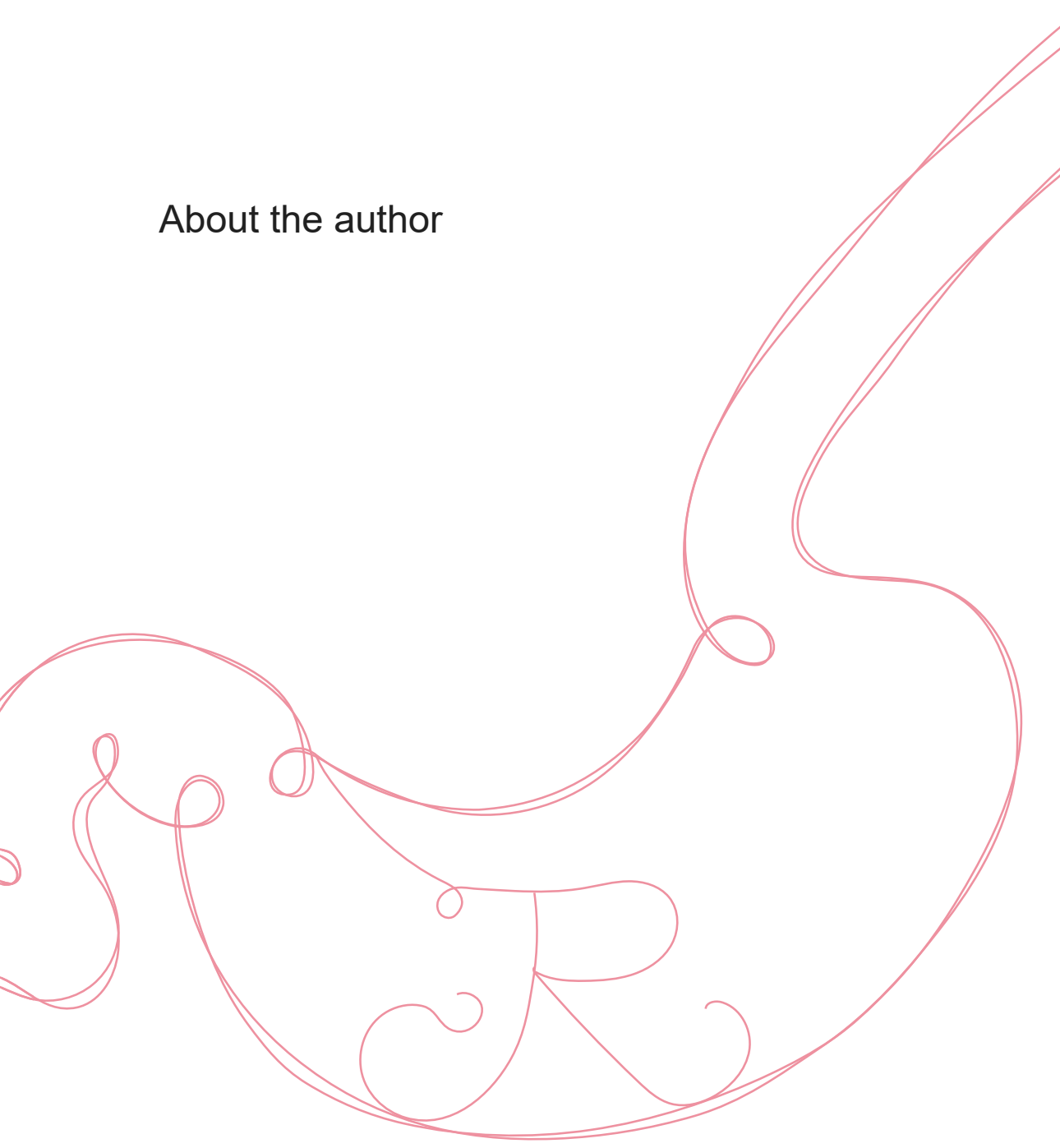


About the author



About the author

Julia Roelofs was born on the 4th of June 1996 in Zutphen, the Netherlands. After completing secondary school in 2014, she started studying the Bachelor of Science program in Nutrition and Health at Wageningen University & Research (WUR). During her BSc she did a minor Neuroscience at the University of Groningen, the Netherlands. After her BSc she continued with the Master of Science program Nutrition and Health, specializing in Physiology and Health Status. She did an internship at Radboud University Medical Center in Nijmegen where she studied the effect of nutritional intake on gut related problems in children with anorectal malformations. This was followed with a MSc thesis on pouch emptying in patients with a Roux-en-Y gastric bypass.

After finishing her MSc in 2019, Julia started working as a research and teaching assistant in the chair group of Sensory Science and Eating Behaviour, part of the Division of Human Nutrition and Health at Wageningen University. In 2021, Julia was appointed as a PhD candidate at this chair. Her research focused on the effect of food composition, processing, and texture on intragastric behavior, gastric emptying, and postprandial nutrient absorption. During her PhD, Julia attended several (international) conferences and courses and was involved in teaching and supervising master's students.

Publications

PUBLICATIONS IN PEER REVIEWED JOURNALS

Roelofs, J. J. M., van Eijnatten, E. J. M., Prathumars, P., de Jong, J., Wehrens, R., Esser, D., Janssen, A. E. M., & Smeets, P. A. M. (2024). Gastric emptying and nutrient absorption of pea protein products differing in heat treatment and texture: A randomized in vivo crossover trial and in vitro digestion study. *Food Hydrocolloids*, *149*. https://doi.org/10.1016/j.foodhyd.2023.109596

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PAPER UNDER REVIEW

Roelofs, J. J. M., Camps, G., Leenders, L. M., Marciani, L., Spiller, R. C., van Eijnatten, E. J. M. , Alyami, J., Deng, R., Freitas, D., Grimm, M., Karhunen, L. J., Krishnasamy, S., Feunteun, S. L., Lobo, D. N., Mackie, A. R., Mayar, M., Weitschies, w., & Smeets, P. A. M. Intra- and interindividual variability in fasted gastric content volume. *MedRxiv*, https://doi.org/10.1101/2024.03.12.24304085

Overview of completed training activities

Discipline specific activities	Organizing Institute(s)	Year
Virtual International Conference of Food Digestion (ICFD)	INFOGEST	2021
Research Conference Plant-Based Foods & Proteins Europe	Bridge2Food	2021
Nutritional Science Days 2021	Nederlandse Academie van Voedingswetenschappen	2021
Gerrit Grijns Initiative (GGI) webinars	Gerrit Grijns Initiative	2021-2023
International INFOGEST Webinars on Food Digestion	INFOGEST	2021-2023
International Conference on Food Digestion (ICFD) 2022	INFOGEST	2022
Symposium 'Gastric food digestion: a focus on protein breakdown'	Sensory Science and Eating Behaviour Group, WUR	2022
Symposium 'Consumer Dimensions of Future Foods'	Massey University	2022
In-vivo NMR course	UMC Utrecht	2022
Nutritional Science Days 2022	Nederlandse Academie van Voedingswetenschappen	2022
Symposium: "First Bites; Eating Behaviour and Healthy Growth in Children"	Sensory Science and Eating Behaviour Group, WUR	2022
The protein transition: Diverse perspectives	PE&RC	2023
International symposium: Dietary protein for human health	FAO, Riddet Institute, WUR, and IAEA	2023
2nd Wageningen Child Eating Behaviour Symposium	Sensory Science and Eating Behaviour Group, WUR	2023

General courses	Organizing Institute(s)	Year
PhD Carousel	WGS	2021
Rmarkdown	WGS	2021
VLAG PhD week	VLAG	2021
Introduction to R	VLAG	2022
Applied statistics	VLAG	2022
Scientific Writing	WGS	2022

Teaching and supervision activities	Organizing Institute(s)	Year
Basic Sensory Science (HNH-29803)	Sensory Science and Eating Behaviour Group, WUR	2021-2023
Psychobiology of Food Choice and Eating Behaviour (HNH-30306)	Sensory Science and Eating Behaviour Group, WUR	2021
Nutrition Behaviour (HNH-20306)	Sensory Science and Eating Behaviour Group, WUR	2022
Principles of Sensory Science (HNH-30506)	Sensory Science and Eating Behaviour Group, WUR	2022-2023
Supervision of MSc students	Sensory Science and Eating Behaviour Group, WUR	2021-2023

Other activities	Organizing Institute(s)	Year
Preparation of research proposal	Sensory Science and Eating Behaviour Group, WUR	2021
Biweekly Tasty talks	Sensory Science and Eating Behaviour Group, WUR	2021-2024
Divisional Seminar	HNH, WUR	2021-2023
Voeding en ons brein (tv-program)	Omroep Max	2022
Reviewing 2 chapters of book “Our Future Proteins - A Diversity of Perspectives”	FSE, WUR	2022
Reviewing 4 papers	HNH, WUR	2023
PhD study tour Australia	HNH, WUR	2024

Colophon

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