# Protein digestion kinetics

## a proxy for postprandial amino acid responses

Evan ABRAHAMSE

**Propositions**

1. Caseins are not slow proteins.
   (this thesis)

2. Postprandial plasma amino acid peak concentrations are determined by gastrointestinal protein hydrolysis kinetics.
   (this thesis)

3. Mineral bioavailability is not determined by the solubility of the mineral source under intestinal conditions.

4. Fasting period maximization for glycogen storage disease (type I) patients is not achieved through maximally slowing down starch digestion.

5. To be efficacious anti-regurgitation infant formula needs to be thicker than standard infant formula not only in the bottle and but also in the stomach.

6. Freedom of speech and self-censorship are not mutually exclusive.

7. The enjoyment of music is determined by the quality of the sonic (re)production.

Propositions belonging to the thesis, entitled

**Protein digestion kinetics, a proxy for postprandial amino acid responses**

Evan Abrahamse
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# Protein digestion kinetics, a proxy for postprandial amino acid responses

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# Protein digestion kinetics, a proxy for postprandial amino acid responses


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**Thesis**

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*Voor mijn Moeder*

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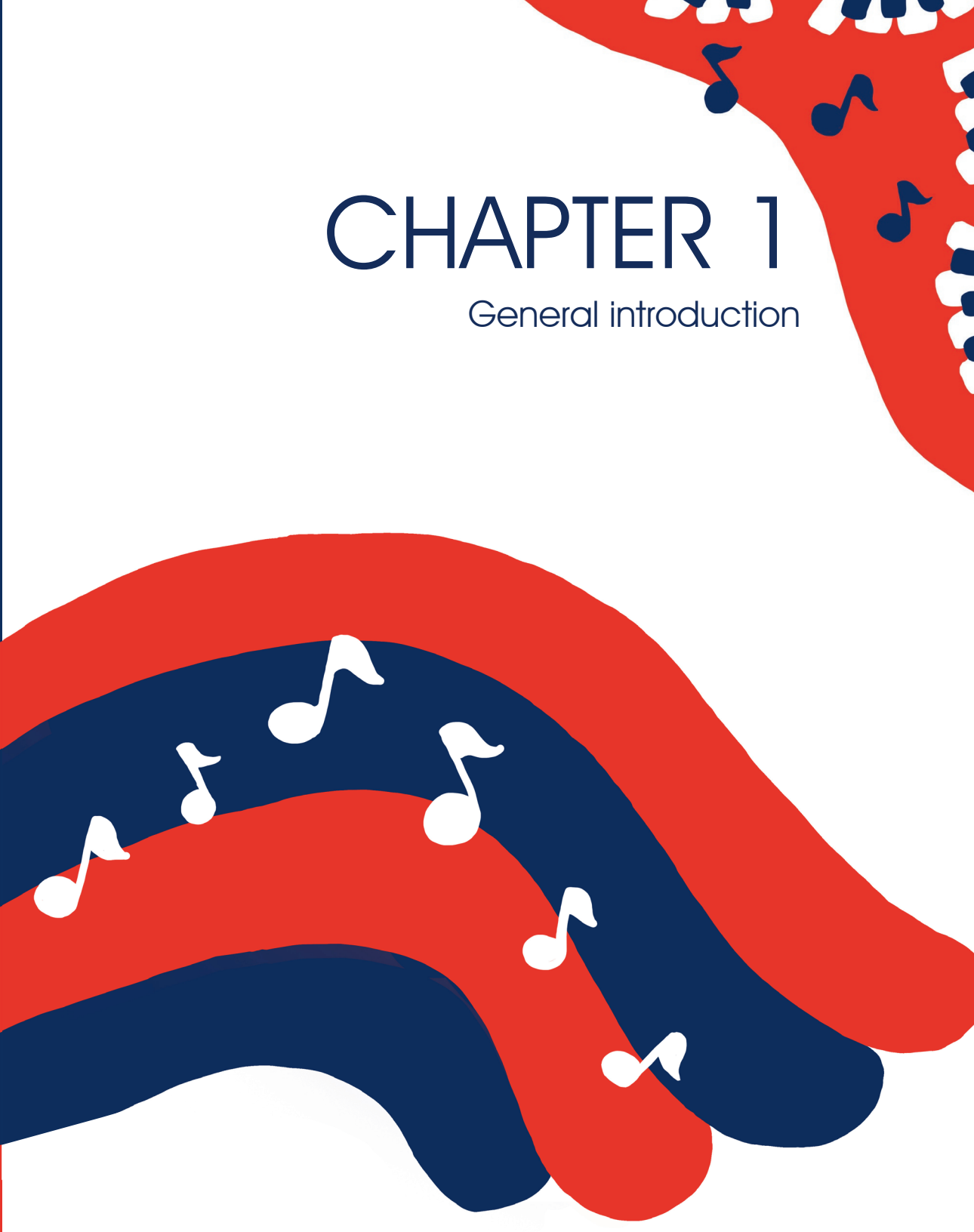
# CHAPTER 1

## General introduction



## Milk proteins in human nutrition and physiology

Dietary intake of proteins is required to meet amino acid (AA) and nitrogen (N) demands for metabolism and maintenance, growth in infants and children, and pregnancy and lactation in women [1]. The age-dependent daily protein requirements are reflected in intake quantity recommendations and reference AA compositions. Protein quality scores have been developed that describe the digestibility and ratios of AA compared to the reference AA composition. To contribute to metabolism and growth, AA need to be absorbed in the small intestine (and not fermented in the colon or excreted in the feces). The two most commonly used protein quality scores are the protein digestibility corrected AA score (PDCAAS) and the more recently introduced digestible indispensable AA score (DIAAS) [1, 2]. Three key improvements in the DIAAS vs the PDCAAS are: 1) the use of ileal vs fecal digestibility values, 2) the use of individual AA vs N digestibility values, and 3) reporting untruncated values vs truncation of values above 1. When a protein has a lower quality score, the quantity that needs to be consumed to meet the daily requirements is higher. Bovine milk proteins typically have high quality scores. For example; bovine whey protein concentrate, bovine casein and bovine milk protein concentrate all have DIAAS values >1 [3, 4]. Yet, clinical outcomes differ greatly between bovine milk protein-based foods [5, 6].

In addition to determining the amount of AA that are available systemically, total protein digestibility also determines the amount of protein reaching the colon. Protein not digested to tripeptides or smaller reaches the colon and can be fermented by the colonic microbiota, which is unwanted and negatively affects gut health, because potentially harmful compounds, gases and microbial proteases are formed [7]. Problems with protein digestion in infancy can cause short-term and long-term adverse consequences, such as pain, diarrhea, intolerance, allergy, malabsorption, and constipation [8]. Particularly in infancy the incidence of gut health related functional gastrointestinal disorders is high [9]. Limitation of colonic protein flow is thus of importance in infancy and consequently protein in infant formula (IF) is desired to have high ileal digestibility.

After ingestion of a protein-containing meal, the AA concentration in blood increases to a peak value and decreases again to baseline, typically in about 4 hours. This pp AA peak concentration has been shown to play an important signaling role in AA metabolism and tissue protein synthesis. As such, it has been shown in healthy adults that muscle accretion of AA depends on the pp AA peak concentration [10]. Especially the pp essential AA (EAA) and leucine peaks correlated with muscle protein synthesis rates, possibly due to the activation of mammalian target of rapamycin (mTOR) pathway [10-12]. The stimulation of muscle protein synthesis is especially important in elderly people: a considerable part of the elderly population is frail and suffering from sarcopenia, and thus experiences an increased loss of muscle mass and strength. A complicating factor here is that muscle protein synthesis

stimulation in elderly requires higher pp leucine concentrations than in young adults [13, 14].

In contrast to adults and elderly, less is known about the optimal pp AA concentrations in infants. However, also in this population plasma leucine concentrations appeared to be a major regulator of muscle protein synthesis via mTOR activation [15]. Human milk (HM) is regarded as the optimal nutrition for infants; compared to IF, HM brings benefits in growth and development, and protection against diseases in early and later life [16]. HM protein quality is considered as optimal, and as such its AA composition is used as reference in protein quality scores for foods for infants [1, 17]. Typically, IF protein originates from bovine milk, which has a lower protein quality than HM, therefore the quantity of protein in IF is higher than in HM [18]. Higher protein in IF has been suggested to program the metabolism towards overweight and obesity in later life, known as the early protein hypothesis [19, 20]. The proposed mechanism of this early protein hypothesis is that increased protein intake leads to increased concentrations of insulin- and IGF-1-releasing AA. Interestingly, in a study where preterm infants were fed either HM or cow's milk based IF iso-proteinaceously, it was observed that the pp EAA peak concentration of IF was 18% higher than that of HM (**Figure 1.1**) [21], suggesting that other factors besides quantity of protein ingestion may trigger insulin and IGF-1 release.

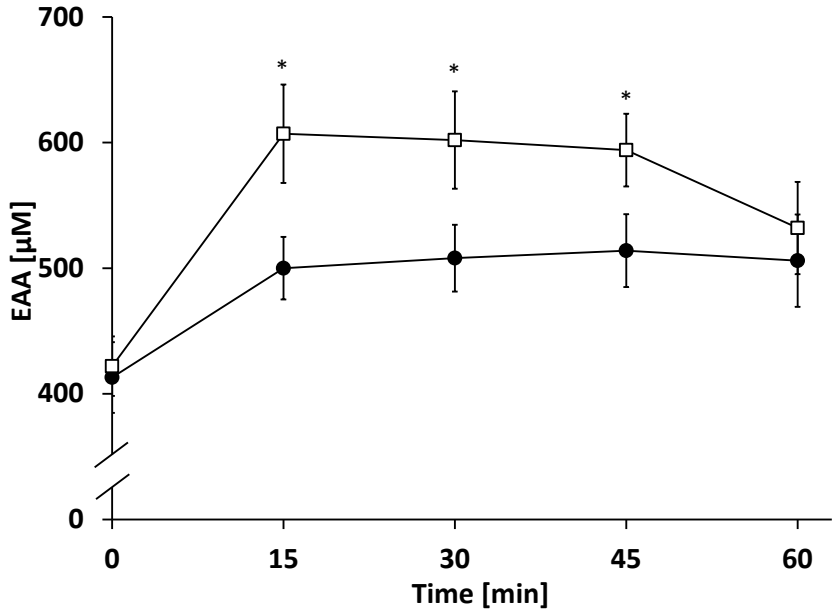



**Figure 1.1: Postprandial plasma EAA concentrations of HM and humanized cows' milk based IF in preterm infants. Infants were fed equal protein quantity from • HM and □ IF. Displayed are means±SEM (n=6). *significant difference between HM and IF; p<0.05. Recalculated and drawn using data from [21].**

Hence, besides AA composition and digestibility (as captured in protein quality scores) also the pp AA concentrations are to be considered when developing nutritional solutions to best meet the protein requirements of elderly and infants.

## Determinants of postprandial plasma amino acid peak concentrations and colonic protein flow

Key determinants of pp AA peak concentrations and colonic protein flow lie in physiological, physical, and chemical processes that occur in the stomach and small intestine, such as: gastric emptying rate, protein hydrolysis kinetics and total protein digestibility. Proteins are digested in the gastrointestinal tract by proteases and peptidases, ultimately leading to free AA and di- and tripeptides, which can be absorbed by the small intestinal epithelium [22]. After absorption the epithelium releases AA into the portal blood flowing towards the liver (**Figure 1.2**).

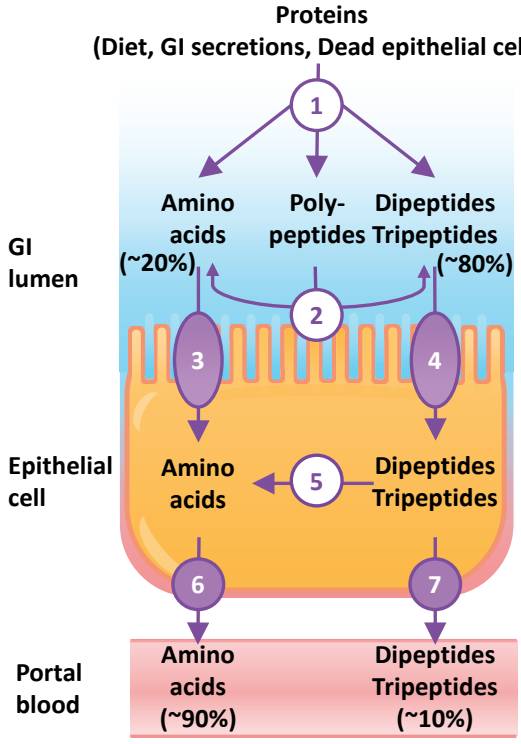



**Figure 1.2: Simplified overview of protein digestion, absorption, and trafficking in the gastrointestinal tract. (1) Gastric and pancreatic proteases and peptidases, (2) brush border peptidases, (3) brush border amino acid transporters, (4) brush border peptide transporter (5) cytoplasmic peptidases, (6) basolateral amino acid transporters, and (7) basolateral peptide transporters. GI = gastrointestinal. Drawn inspired by and using data from [23].**

Systemic plasma AA concentrations are a net-result of influx of AA from the intestine, systemic protein turnover and metabolic demands. The rate of influx of AA from the intestine is determined by the overall protein digestion kinetics. In turn, differences in protein digestion kinetics are assumed to be determined by (1) the gastric emptying and intestinal transit rates, (2) the hydrolysis rate (in gastric and intestinal phase), and (3) the efficiency of the absorption of the formed AA/di-/tripeptides.

Gastric emptying rate is the speed with which the stomach empties its contents into the small intestine where successive hydrolysis and absorption of digestion products take place. Because there is no absorption of protein digestion products in the stomach, the gastric emptying rate paces further hydrolysis and absorption kinetics [24, 25]. The stomach delivers chyme into the intestine at a rate that matches its processing capability, hence it fulfils a "reservoir" function [26]. One aspect is "liquification" where stomach antral grinding decreases solid particle size to smaller than 1-2 mm, which allows escape from pyloric sieving and subsequent entrance into the duodenum [27]. Protein hydrolysis kinetics are the rate at which enzymes hydrolyze the peptide bonds in proteins, which ultimately results in the release of absorbable digestion products. Total protein digestibility is the fraction of the protein that is digested and absorbed as peptide or AA during the journey from the mouth until the ileum (and hence does not reach the colon) and is the factor used in the aforementioned DIAAS.

Protein hydrolysis starts in the stomach where the aspartic endopeptidase pepsin cleaves the peptide bonds of non-terminal AAs, its preferential cleavage sites (CS) being listed in **Table 1.1.**

**Table 1.1: Cleavage sites of gastric and pancreatic proteases and peptidases.**[1]

| Enzyme | Cleavage sites | EC number |
|---|---|---|
| Pepsin | Phe, Tyr, Leu | 3.4.23.1 |
| Trypsin | Arg, Lys | 3.4.21.4 |
| Chymotrypsin | Aromatic AA, Neutral AA | 3.4.21.2 |
| Elastase | Leu, Met, Phe | 3.4.21.71 |
| Carboxypeptidase A | Aromatic AA | 3.4.17.1 |
| Carboxypeptidase B | Arg, Lys | 3.4.17.2 |

[1]Cleavage sites and enzyme commission (EC) numbers from the BRENDA online enzyme database [84].

Pepsin activity is dependent on high gastric acidity as its pH optimum is 1.8-3.5 [22]. Protein hydrolysis continues in the duodenum through the action of pancreatic proteases and peptidases that are released from the pancreas. These include the serine endopeptidases trypsin, chymotrypsin, and elastase, and exopeptidases carboxypeptidase A and B, with their preferential CS also listed in **Table 1.1**. These pancreatic enzymes are secreted as

zymogens and are dependent on intestinal enterokinase for conversion into their active form in a chain reaction involving trypsin [28]. Chyme entering the duodenum from the stomach is neutralized by bicarbonate-rich pancreatic and biliary secretions [23]. Fittingly, the pH optima of pancreatic proteases and peptidases are above neutral: trypsin: 7.5-8.5 [29], chymotrypsin: 8 [28], elastase: 7.5-10.5 [28], carboxypeptidase A: 7.5-8 [28], and carboxypeptidase B: 7.65 [28]. The final phase of protein hydrolysis to free AA and di- and tripeptides is performed by a broad range of (amino)peptidases present at the small intestinal brush border membrane (BBM). At least 12 different BBM peptidases have been identified so far [30]. Their substrates were previously believed to be oligopeptides up to 8 AA residues in length (as this was the largest substrate tested) [22]. However, more recent studies have shown that porcine BBM extracts hydrolyze milk protein derived peptides up to 3500 Da (~30 AA residues) [31, 32]. The main products of intestinal protein digestion by all proteases and peptidases in concert are di- and tripeptides (~80%), while ~20% is free AA [22, 23, 33] (**Figure 1.2**).

Epithelial absorption of AA occurs via a multitude of specific transporters, while all di- and tripeptides are absorbed via one transporter: peptide transporter-1 [22]. Absorption of AA residues occurs at a higher rate in di- and tripeptide form than in free AA form [34]. Once within the enterocyte, cytoplasmic peptidases hydrolyze most of the absorbed peptides (~90%) to free AA, which is then released in the portal bloodstream [22, 23]. Considerable amounts of absorbed AA are used by first pass splanchnic extraction and do not appear systemically. Splanchnic extraction of AA by the portal drained viscera can reach, depending on the specific AA, 20-90% as has been shown in pigs [35]. Human adult studies using stable isotope labelled dietary protein and leucine, phenylalanine and tyrosine infusion, demonstrated that in total ~55% of the dietary AA reaches the systemic circulation [36]. In neonates, large differences in the proportion that reaches systemic circulation have been found between AA; ranging from 0-20% for threonine and several nonessential AA to 70-85% for lysine [37].

## Population specific gastrointestinal function

The specific gastrointestinal functions important for protein digestion are not stable throughout the entire lifespan. In elderly, the gastric function is decreased compared to young adults. Aging per se has only been demonstrated to be associated with decreased pepsin output [38]. Furthermore, a considerable portion of the elderly is suffering from gastritis, a condition affecting gastric functioning. Overall this results in a ~53% decrease of peak pepsin (U/min) and ~35% decrease of peak acid output (mmol/min) compared to healthy young adults [38]. The decreased acid output in elderly results in considerably slower gastric acidification compared to young adults; the time to return to pH=4 after a

neutral mixed meal ingestion was ~52 vs ~14 min, respectively [39]. Obviously, this will impact the physicochemical and hydrolysis processes occurring in the stomach and thereby the pacing of overall protein digestion.

Although the neonates' gastrointestinal tract (GIT) is able to digest macronutrients, its functioning is not fully mature yet and many differences relevant for protein digestion exist compared to the GIT of children and adults [40-42]. Gastric pepsin concentration (mg/mL gastric content) at 0-6 months is only 14-41% of that of adults [41, 43]. Gastric acidification is lower as well, resulting in a postprandial pH above 5 for up to 60 min [44-53]. Pancreatic secretion in early infancy showed lower protease activity in duodenal fluids compared to children (≥2 y); trypsin 90-100%, chymotrypsin 50-60% and carboxypeptidase B 15-25% of activity in children [54, 55]. In addition, intestinal enterokinase activity in young infants is only 20% of that in children [56]. All in all, this infant specific gastrointestinal functioning will impact the physicochemical and hydrolysis processes occurring in the gastrointestinal tract and with that the pacing of overall protein digestion and the total protein digestibility.

## Food characteristics: impact on postprandial plasma amino acid concentrations and gastrointestinal processes

Postprandial plasma AA peak concentrations and gastrointestinal processes were shown to be influenced by food characteristics, such as protein composition, heat processing and product matrix. Protein composition determines the variety of primary structures (AA sequences and CS) and secondary and tertiary structures (3D structure including folding) present. Product heat processing includes the industrial heating for microbiological safety, drying purposes and physicochemical and techno functional properties of the end product. Product matrix includes the presence of other nutrients and components, as well as their structural organization within the food product.

### Milk protein composition

Proteins in milk from different mammalian species vary in concentration and AA composition. The protein content of bovine milk is approximately 21–35 g/L, whereas HM contains only 8–15 g/L [57]. Milk protein composition, irrespective of species, consists of two distinct protein types; globular serum proteins (whey proteins) and unstructured proteins organized in colloidal particles (caseins) [58]. The bovine milk that is used as protein source for human nutrition is typically a mixture of the milk of many cows, which attenuates the dynamics in composition due to stage of lactation and results in a mixture of ~20% whey protein and ~80% caseins (**Figure 1.3**). HM protein composition changes over time, with a high whey protein proportion (>80%) directly after birth of the infant, which decreases to ~50% at late lactation [59]. HM is referred to as mature from 30 days postpartum [59]. Typically, protein

in IF consists of bovine milk protein with humanized composition, i.e. the whey protein and caseins proportions are adapted to 60% and 40%, respectively, as an approximation of the ratio during the normal course of human lactation [59].

The individual proteins that constitute the casein and particularly the whey protein fractions in human or bovine milk are very different. For instance, HM is devoid of β-lactoglobulin (BLG), while in bovine milk it is the most abundant whey protein. Moreover, HM is rich in lactoferrin and secretory immunoglobulin A (sIgA), especially in early lactation. In mature HM, still these two proteins constitute about 54% of the whey fraction, while in bovine milk this is only about 14% (**Figure 1.3**) [57].

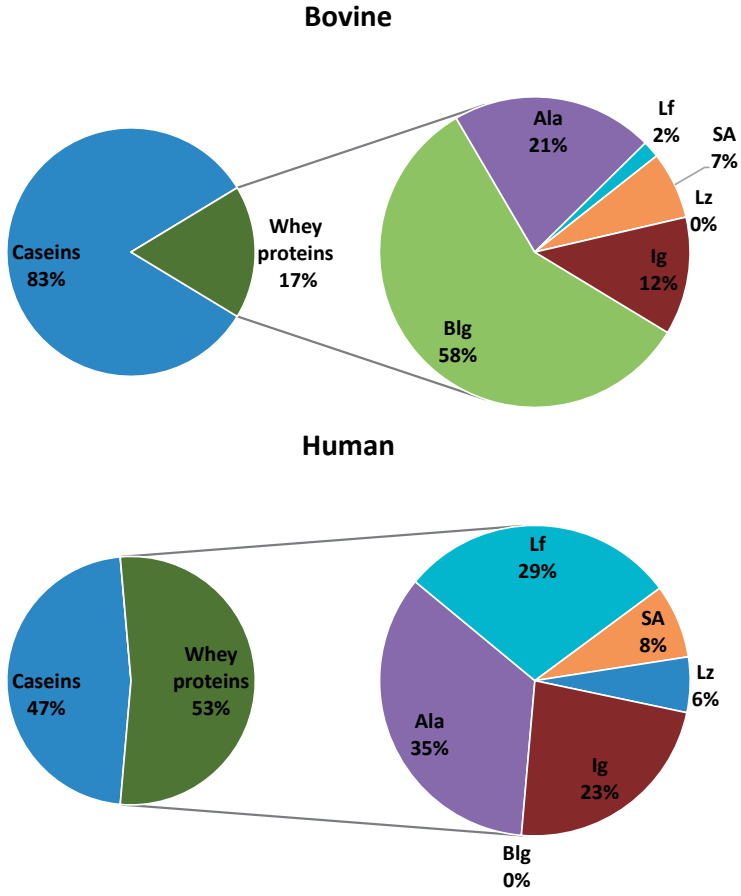



**Figure 1.3: Protein composition of bovine and human milk. Calculated using data from [57]. Human and bovine milk composition represents mature composition. Ala = α-lactalbumin, Blg = β-lactoglobulin, Lf = lactoferrin, SA = serum albumin, Lz = lysozyme, Ig = immunoglobulins.**

Postprandial plasma AA peak concentrations appear to be protein composition dependent; whey protein ingestion resulted in a considerably higher pp AA peak than casein ingestion

in healthy adults [10]. Interestingly, it has also been shown that whey protein ingestion, resulting in a higher pp AA peak concentration, stimulates muscle protein synthesis better than caseins in older subjects [6]. The difference in pp AA peak concentrations is attributed to the difference in gastric emptying rate as influenced by the distinct physicochemical behaviour of the proteins under gastric conditions [5]. Caseins clot under acidic gastric conditions to particles that are larger than what can pass the gastric pyloric sieve, while native whey proteins remain soluble under these conditions. Therefore, in the case of clotting of caseins, stomach antral grinding is needed to decrease particle size to smaller than 1-2 mm, similar to when solid food is ingested [27]. The gastric emptying kinetics of solid foods are typically slower than that of liquid foods [60]. When caseins are pre-hydrolyzed, the gastric physicochemical behaviour is changed, i.e. large clots are not formed anymore, resulting in a considerably increased plasma EAA peak concentration as compared to intact caseins [6]. The distinct physicochemical behaviour of whey proteins and caseins is the hypothetical basis of the fast/slow protein concept that was introduced by Boirie [5].

As mentioned above, in one study the pp EAA peak concentration of IF was found to be 18% higher than that of iso-proteinaceous HM in preterm infants (**Figure 1.1**) [21]. Currently, it is unknown what the relative contribution of gastric emptying rate and protein hydrolysis rate are in overall protein digestion kinetics in infants. Studies investigating the gastric emptying rate of HM compared to IF generally found similar or faster rates for HM [40]. Together this suggests that protein hydrolysis kinetics and absorbable product release and/or overall digestibility of protein in IF is higher than that of HM. There is thus a knowledge gap with respect to the effect of infant milk protein composition on protein hydrolysis kinetics. Furthermore, it is unknown if in infancy differences in protein hydrolysis kinetics can result in differences in pp AA concentrations, without affecting gastric emptying rate.

The AA sequence (i.e., protein primary structure) and tertiary structure of each individual milk protein is unique, in addition, as discussed earlier, the GIT proteases and peptidases have preferential CS, therefore the progression of hydrolysis is unique for each individual milk protein as well. This may translate to differences in protein hydrolysis kinetics between protein mixtures differing in composition. It is known that the hydrolysis of intact milk proteins is protein type dependent, i.e. intact bovine caseins were earlier undetectable with SDS-PAGE compared to bovine whey proteins [61], which is currently mainly attributed to the differences in secondary and tertiary (i.e. 3D) structure.

HM protein composition includes several proteins that have functionality beyond delivering AA and N, arising from their bioactivity, which partly depends on the intactness of their structure. Indeed, intact lactoferrin and sIgA from HM were still detectable (<10% of intake) in fecal samples from infants [62]. It has been hypothesized that human lactoferrin and sIgA may therefore be relatively resistant to enzymatic hydrolysis as a result of their tertiary

structure and specific post translational modification [63, 64]. Altogether this shows a gap in knowledge on how kinetics of protein hydrolysis between IF and HM compare.

## Heat processing

In most milk protein food applications, industrial heating is employed to ensure microbiological safety, to dry in the production of powdered foods and ingredients, and to obtain desired physicochemical and techno functional properties of the product. However, heat treatment can impact protein structurally, which may influence physiological outcomes after ingestion. Key examples are protein denaturation and aggregation and AA chemical modification [65]. Protein denaturation is a modification of protein tertiary structures to a differently folded state than the native form. Of the milk proteins, only globular whey proteins denature upon heating, whereas caseins do not because of their unstructured nature [66]. Upon heating above denaturation temperature (i.e., >70°C for whey proteins), folded proteins unfold and upon cooling subsequently either fold back to native like, or non-native like structures, and/or form aggregates [67]. The degree to which these consequences occur depend on the heating conditions, such as temperature, duration, protein concentration and presence of other components. Denaturation-related aggregation is induced by intermolecular hydrophobic interactions and/or formed covalent links such as disulphide bonds [65]. The most common chemical modification of amino acids is glycation of the free amino groups via the Maillard reaction. Specifically, the free amino groups of lysine and arginine residues, or N-terminal amino acids react with the carbonyl group of a reducing sugar molecule (e.g., lactose) to initially form Amadori products, thereby changing the protein's primary structure. Further reactions generating advanced glycation end-products (AGEs) can take place depending on the heating conditions; additionally glycation can also induce protein aggregation [68].

Ultra-high temperature (UHT) processing of milk protein has been shown to result in a significantly higher pp AA, and urea concentrations compared to unheated or pasteurized milk in healthy adults [69]. These pp AA deamination losses (as reflected in increased urea concentrations) were ~40% higher in the UHT group than in the other two groups. The higher anabolic use of UHT milk protein strongly suggests that these differences were due to modifications of protein digestion kinetics and further AA metabolism [69]. It is unclear to what degree increased denaturation of whey protein, increased protein glycation, or lower gastric coagulation individually contributed to the found effect.

Heat processing-induced glycation of humanized cows` milk protein has been shown to dose dependently lower pp lysine availability, but not other AA, in healthy adults [70]. Indeed, glycated lysine molecules were shown to not become available as lysine in the bloodstream [71]. Currently, there is a knowledge gap on the effect of heat-induced protein denaturation on pp AA concentrations of milk protein. Heat treatment has been shown to lower the consistency of gastric casein clots. The higher the temperature (pasteurization vs UHT),

the lower the consistency, thereby influencing gastric emptying of protein by increasing protein release from the clot in the beginning of digestion [72]. Heat-induced whey protein aggregation can impact whey protein solubility, which in turn may impact gastric emptying kinetics as well, because it may lead to inhomogeneity of the stomach content if the mixing action of the stomach is insufficient.

*In vitro* digestion studies have shown differential impact of heat treatment on milk protein hydrolysis kinetics. Enzymatic protein hydrolysis is dependent on the accessibility of the CS in the protein for the enzyme. Heat processing-induced changes to protein structure or its AA can influence CS accessibility. Heat induced protein unfolding (denaturation) increases the accessibility of CS that are hidden in the native whey protein molecule. Whey protein denaturation is described to result in a faster loss of intact proteins during *in vitro* digestion [73]. In contrast, heat treatment minimally affects the release of small protein digestion products *in vitro* [74, 75]. Dupont et al. observed that after heat treatment BLG became less hydrolysis resistant, while some caseins became more resistant to enzymatic hydrolysis under infantile digestion conditions [76]. Others have shown that specific oligomers of BLG could be formed during heat treatment that are particularly resistant to enzymatic hydrolysis [77].

Glycated CS were shown to be resistant to hydrolysis by trypsin, while glycated binding sites prevented α-chymotrypsin from hydrolyzing adjacent CS [78]. Furthermore, glycated lysine molecules may inhibit BBM aminopeptidases leading to less rapid appearance of amino acids in the plasma, as demonstrated in animals [79]. Moreover, it has been found in human adolescents that the apparent fecal protein digestibility was decreased when dietary glycation product concentrations were increased [80]. Currently, little is known about the individual contributions of heat processing induced protein denaturation, aggregation, and glycation on protein hydrolysis kinetics of milk protein.

## Product matrix

Milk protein is typically consumed as part of a mixed meal that includes other components. These mixed meals are typically emulsions or suspensions, and milk proteins are often part of this structural organization of the food product.

Combining milk protein solutions with carbohydrates and lipids to form mixed meals increases their energy density. Meal energy density is one of the most important factors influencing gastric emptying, as the stomach appears to pace emptying to ~2.25 kCal/min [26]. Therefore, the differences in pp AA peak concentrations between whey protein and caseins may be altered when they are consumed as part of a mixed meal. The addition of carbohydrates and fats may also influence the rate of protein hydrolysis, as it has been shown, for example, that the physical association between protein and lipids could impact

protein digestion by partial unfolding of the protein or by spheric inaccessibility [81]. Currently, little is known about the effect of a mixed meal including whey proteins and caseins on pp AA concentrations and potential mechanisms related to gastric emptying and protein hydrolysis kinetics.

It is known that the milk protein matrix can influence ileal protein digestibility. For example, pig studies have shown that addition of soluble dietary fiber decreases protein digestibility more than that the addition of insoluble fiber [82]. It is currently unclear if, and to what extent, IF product matrix can influence colonic protein flow. For one specific IF (a fermented IF), it was shown that colonic gas production after consumption was decreased in infants compared to a standard IF. This suggests that the used fermentation rendered the protein easier to digest resulting in higher ileal digestibility and lower colonic protein flow [83]. However, the lactic acid bacteria used are known to have little to no proteolytic activity; the protein is therefore not expected to be hydrolyzed during the fermentation process and thus to remain structurally unchanged. Currently it is unknown if indeed the total digestibility of the specific fermented IF is higher than that of a standard IF, and which mechanism may lie underneath.

## Aim and outline

Altogether, the above-mentioned aspects of protein digestion comprise relationships between food characteristics, gastrointestinal processes, and their systemic and colonic consequences; these are summarized in **Figure 1.4**.

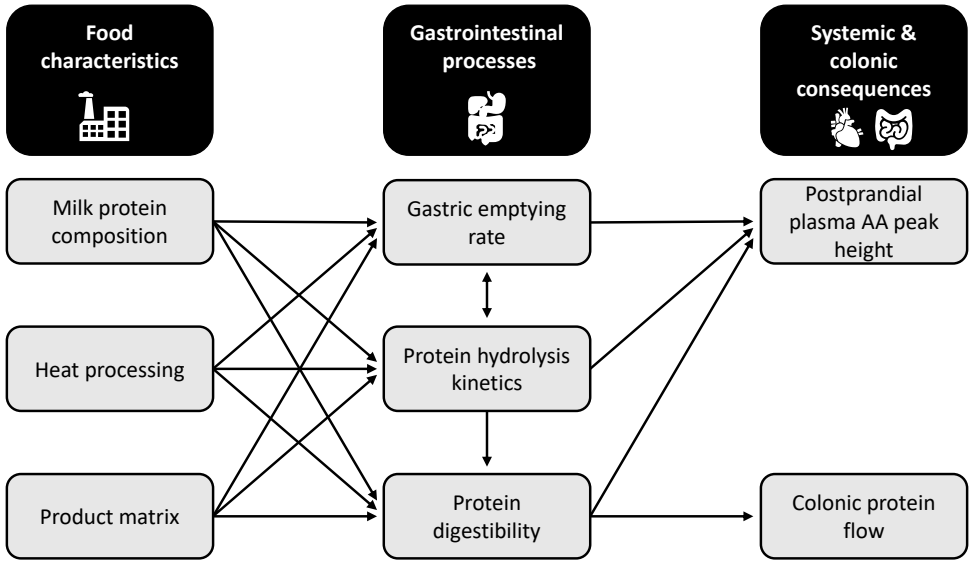



**Figure 1.4: Relationships between food characteristics, gastrointestinal processes and systemic and colonic consequences.**

A better understanding of the relationships between food characteristics, gastrointestinal processes and their systemic and colonic consequences will increase the ability to formulate milk products with optimal protein quality specifically tailored to the needs of vulnerable consumers such as infants or elderly people.

The overall research question addressed in this thesis is: How do food characteristics, i.e. protein composition, processing and the product matrix, impact overall protein digestion, and how is this reflected in systemic and colonic consequences, such as pp AA peak concentrations and colonic protein flow? Postprandial plasma AA peaks and colonic protein flow are best investigated *in vivo*; however, the gastrointestinal processes can be difficult to measure *in vivo* and may be investigated using *in vitro* models of the gastrointestinal tract. Therefore, the research in this thesis combines *in vitro* and *in vivo* studies in humans and piglets to gain insights in the relationships between food characteristics, gastrointestinal processes, their systemic and colonic consequences, and their interplay. Specifically, the aims were to:

**1)** Gain insights in the relative importance of gastric emptying and protein hydrolysis kinetics in determining the postprandial AA peak.
**2)** Differentiate the individual effects of the mentioned food characteristics on gastrointestinal processes.
**3)** Assess and improve correlation between *in vitro* and *in vivo* studies.

In **chapter 2** the matrix effect of increased energy density on the pp AA concentrations of whey protein and casein was investigated in healthy elderly. Furthermore, to better understand the relative importance of gastric emptying and protein hydrolysis, *in vitro* investigations in a newly developed semi-dynamic model of the gastrointestinal tract (SIM) simulating elderly conditions were undertaken. In **chapter 3** the effect of whey protein denaturation, as well as the effect of changing native milk protein composition by incorporation of non-clotting casein, on pp AA concentrations in neonatal piglets is studied. Furthermore, gastric emptying measurement and pharmacokinetic modelling of pp AA data was performed to gain insights in the role of protein hydrolysis. To further elucidate mechanisms of the observations in chapter 3, in **chapter 4** protein hydrolysis and absorbable product release kinetics and mechanisms as affected by milk protein composition or denaturation were investigated in SIM and a dynamic *in vitro* model of the gastrointestinal tract (TIM-1), both simulating infant conditions. In addition, directions for improved correlation between *in vitro* digestion measures and *in vivo* pp AA concentrations was sought. **Chapter 5** provides detailed insights in the differences in kinetics of protein hydrolysis and absorbable product release of human milk and cow's milk based IF in SIM simulating infant conditions, which offers directions to bring IF closer to HM. In **chapter 6** IF protein digestibility and colonic protein flow as affected by formula protein composition and matrix was investigated in ileal cannulated piglets. Lastly, in **chapter 7** all observations are discussed, and future directions are proposed.

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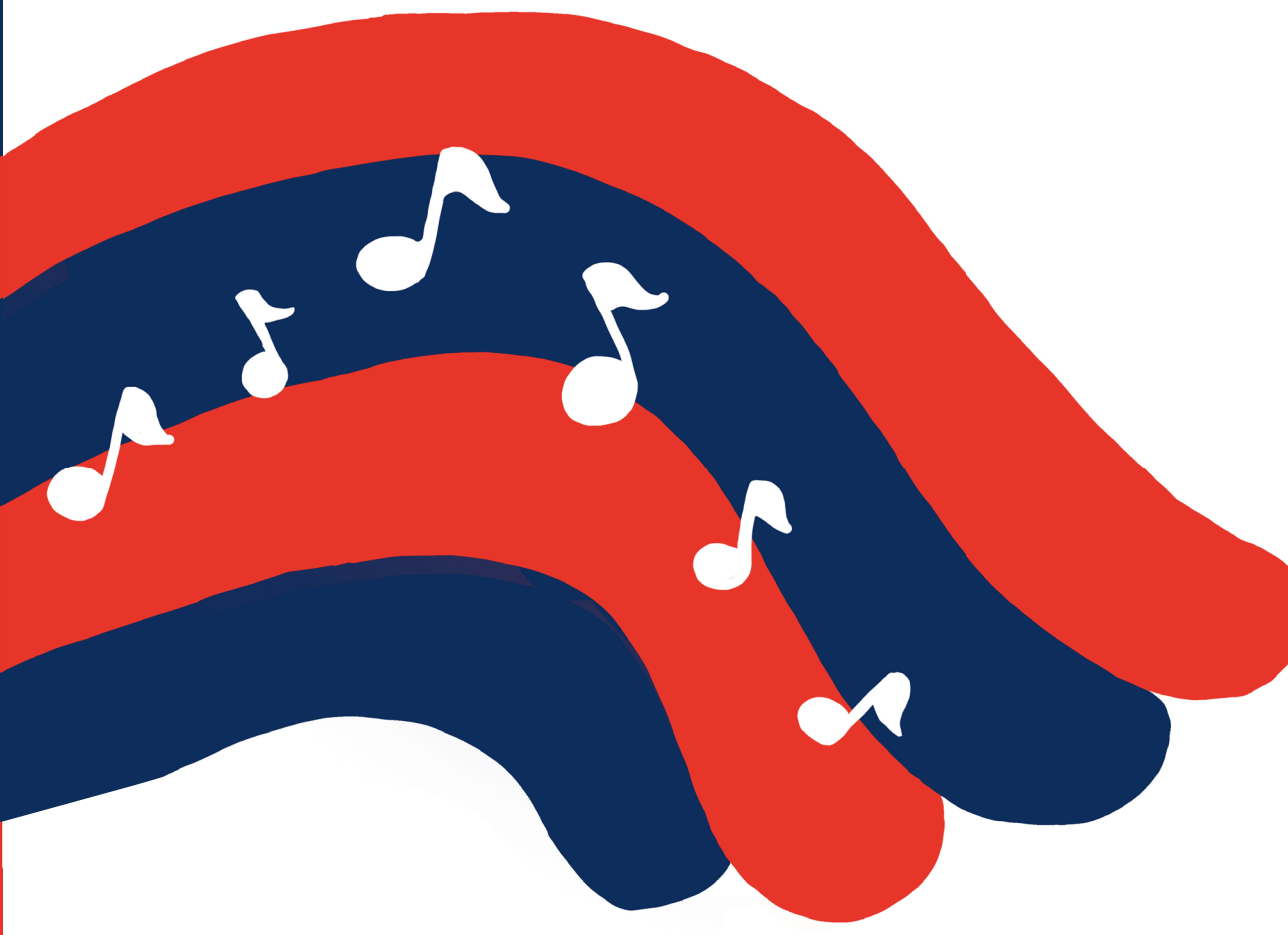
2

# CHAPTER 2

## Protein type and caloric density of protein supplements modulate postprandial amino acid profile through changes in gastrointestinal behaviour: a randomized trial



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## Abstract

The requirement of leucine and essential amino acids (EAA) to stimulate muscle protein synthesis increases with age. To target muscle anabolism, it is suggested that higher postprandial blood levels of leucine and EAA are needed in older people. The aim was to evaluate the impact of oral nutritional supplements with distinct protein source and energy density, resembling mixed meals, on serum amino acid profiles and on gastrointestinal behaviour. Four iso-nitrogenous protein (21g) supplements were studied containing leucine-enriched whey protein with 150/320 kcal (W150/W320) or casein protein with 150/320 kcal (C150/C320); all products contained carbohydrates (10 or 32g) and fat (3 or 12g). Postprandial serum AA profiles were evaluated in twelve healthy, older subjects who participated in a randomized, controlled, single blind, cross-over study. Gastrointestinal behaviour was studied *in vitro* by looking at gastric coagulation and cumulative intestinal protein digestion over time. The peak serum leucine concentration was twofold higher for W150 vs. C150 (521±15 vs. 260±15 µmol/L, $p<0.001$), higher for W320 vs. C320 (406±15 vs. 228±15 µmol/L, $p<0.001$), and higher for low-caloric vs. high-caloric products ($p<0.001$ for pooled analyses; $p<0.001$ for interaction protein source*caloric density). Similar effects were observed for the peak concentrations of EAA and total AA (TAA). *In vitro* gastric coagulation was observed only for the casein protein supplements. Intestinal digestion for 90 min resulted in higher levels of free TAA, EAA, and leucine for W150 vs. C150, for W150 vs. W320, and for C150 vs. C320 ($p<0.0125$). A low caloric leucine-enriched whey protein nutritional supplement provides a higher rise in serum levels of TAA, EAA and leucine compared to casein protein or high caloric products in healthy, elderly subjects. These differences appear to be mediated in part by the gastrointestinal behaviour of these products.

## Introduction

Aging often coincides with loss of muscle mass, strength and function, known as sarcopenia [1]. Sarcopenia, a geriatric syndrome closely linked to physical frailty, has a substantial impact on the quality of life of the individual and increases the risk of disability and hospitalization [1]. Sarcopenia has been linked to a decreased muscle anabolic response to insulin and dietary essential amino acids (EAA) [2-5].

Muscle protein synthesis has been shown to respond to extracellular EAA concentration [6], subsequent transport, and intracellular amino acid rate of deposition in muscle [7, 8]. A high amount of EAA and leucine in one single bolus has also been shown to enable stimulation of muscle protein synthesis in older adults [9-12]. Accordingly, higher postprandial blood levels of EAA and leucine correlate to a higher muscle protein synthesis rate [13, 14]. This suggests that the postprandial profile by which amino acids and especially EAA and leucine appear in blood and become available for muscle, is relevant for subsequent stimulation of muscle protein synthesis. This appears to be even more important with advancing age, conditions of inflammation and insulin resistance. The threshold needed to trigger intracellular translation initiation pathways to activate protein synthesis is likely higher under these conditions [15].

The quantity and source of dietary proteins have been shown to impact postprandial blood levels of EAA and leucine [16]. As a result, the concept of “fast/slow” protein was implemented to indicate the postprandial profile of amino acids appearing in the systemic circulation [17, 18]. As an example, the dairy proteins whey and casein contain a similar amount of EAA, but blood EAA levels increased faster and to a higher level after the intake of whey protein [14, 19]. Therefore, whey is considered a “fast” protein, while casein is a “slow” protein. A difference in gastric emptying and digestion and absorption kinetics between casein and whey [14, 20, 21] are suggested as an underlying factor. Distinct factors have been shown to modulate gastric emptying and the gastrointestinal digestion of nutritional compositions. Gastric coagulation of casein with formation of solid particles [17], is an example of a physiological phenomenon that is known to delay gastric emptying of casein [21, 22]. Another example is the difference in sequential release of casein and whey-derived peptides in the jejunum [23], that is indicative of distinct hydrolysis kinetics of casein and whey. Moreover, it is known that the higher the caloric density of nutrition the more it delays gastric emptying [24, 25]. Supplementing sucrose to a casein or milk protein indeed delayed protein digestion and absorption kinetics [26] and increased the oro-ileal transit time [27]. However, the relevance of gastric coagulation and of intestinal digestion rate for postprandial amino acid profiles and systemic amino acid bioavailability of casein and whey protein-containing supplements, i.e., resembling mixed meals of different caloric densities, is less well known.

Our primary study aim was to evaluate postprandial blood amino acids profiles in healthy older people after the intake of a leucine-enriched whey protein nutritional supplement compared to an iso-caloric and iso-nitrogenous control product containing casein protein. While distinct effects of intact whey and casein protein on blood amino acid profiles in older people have been reported, the use of carbohydrates and fat besides protein is less well known. This is relevant as it represents conventional products or a 'mixed meal'. Secondly, we aimed to study the impact of caloric density of the whey and casein products on postprandial amino acid profiles and systemic amino acid bioavailability, which was not studied like this before. Third, we aimed to understand the relevance of distinct gastrointestinal behaviour of the nutritional supplements for postprandial amino acid profiles. While *in vivo* digestion studies with jejunal sampling and/or intrinsically labelled proteins are preferred, these measures are also invasive and require that nutritionally supplements are produced with intrinsically labelled proteins. Therefore, we applied *in vitro* models to compare the coagulation behaviour and protein digestion rate (initial and overall cumulative digestion over time) of the nutritional supplements, under conditions closest to those found in a healthy, elderly population.

# Materials and Methods

## Subjects in clinical study

Fifteen healthy adults that were 65 years or older were screened. A total of 12 subjects (5 male) were enrolled in the study. Subjects who signed the informed consent had a Body Mass Index (BMI) between 21-30 kg/m$^2$ and were willing and able to comply with the protocol. The protocol included adhering to a fasting state from 22:00 h the day prior to the study visits, refraining from alcohol consumption (24 h) and intense physical activities (24 h) before the study visits and not changing dietary habits for the duration of the study. Subjects with a (history of) gastrointestinal disease, or those that had been diagnosed with, or were suspected of having, diabetes mellitus (fasting glucose $\geq$7.0 mmol/L) were excluded from participation. Other exclusion criteria were infection or fever in the past 7 days, medication use (antibiotics within 3 weeks of study entry, current use of corticosteroids or hormones, and the use of antacids or any medication influencing gastric acid production), known allergy to milk or milk products, lactose intolerance and known galactosaemia. Moreover, subjects were excluded when they currently participated in a weight loss or muscle strengthening program or used nutritional supplements that contained proteins or amino acids within one week of study entry, as well as those who had smoked for the past 3 months or abused alcohol or drugs. The Modified Baecke Questionnaire for Older People [28] was completed to measure the normal physical activity level. Body weight and height were measured. **Supplementary Figure S2.1** shows the Consort flow diagram.

## Study products

Four study products, differing in protein source and caloric density (**Table 2.1**), were tested *in vivo* and *in vitro*. Two products composed a leucine-enriched whey protein nutritional supplement (20 g whey protein (from whey protein isolate), 3 g total leucine) with 150 kCal (W150) or 320 kcal (W320), respectively. The other products contained an iso-nitrogenous amount of casein (21 g casein protein (from calcium caseinate)) with 150 kCal (C150) or 320 kCal (C320), respectively. Additional calories were added as fat and carbohydrates, similar to conventional medical nutrition supplements. The osmolality of the 320 kCal products was higher (239-248 mOsm/kg) compared with the 150 kCal products (131-139 mOsm/kg). The pH of the whey products was acidic (4.0), while pH was neutral for the casein products (6.6). Subjects consumed the complete products within 5 minutes. Site staff, not involved in the study, mixed the study products (as powders) with water to a volume of 300 ml before consumption.

**Table 2.1: Composition of the study products.**[1]

| Nutrients | W150 | C150 | W320 | C320 |
|---|---|---|---|---|
| Energy (kcal) | 150 | 150 | 320 | 320 |
| Protein (En%) | 56 | 56 | 26 | 26 |
| Carbohydrates (En%) | 26 | 26 | 40 | 40 |
| Fat (En%) | 18 | 18 | 34 | 34 |
| Total protein (g) | 21 | 21 | 21 | 21 |
| Whey protein (g) | 20 | - | 20 | - |
| Casein protein (g) | - | 21 | - | 21 |
| Free BCAA (g) | 1 | 0 | 1 | 0 |
| Total leucine (g)[2] | 3 | 2 | 3 | 2 |
| Total EAA (g)[2] | 10 | 9 | 10 | 9 |
| Carbohydrates (g)[3] | 11 | 10 | 33 | 32 |
| Fat (g) | 3 | 3 | 12 | 12 |

[1]BCAA = branched chain amino acids; EAA = essential amino acids; [2]Provided by protein and free BCAA; [3]Includes citric acid to obtain an acidic product.

## Clinical study design (*in vivo* study)

This clinical study was a randomized, controlled, single-blinded, cross-over, single center study. Subjects eligible for participation were randomly allocated, by using three 4x4 Latin squares, to obtain a unique order of the study products. Study products were packaged indistinguishably and coded. Aside from a noticeable difference in weight between the low and high-caloric sachets, study staff was blinded to the origin of the study products until completion all data were included in the study database.

Subjects visited the research location in a fasting state on four separate mornings. A flexible 18–22-gauge catheter was placed into a forearm vein for blood sampling. Fasting blood samples were collected at baseline for serum C-reactive protein (CRP), albumin, glucose,

insulin, and amino acid concentrations (t=-15 min), and a second basal sample for amino acid, glucose and insulin concentrations was taken just before product intake. After consumption of the study product (t0), repeated blood samples were drawn over the next 4 h, at 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, and 240 min for analyses of amino acids, glucose and insulin concentrations. Before product consumption and at 240 min after product intake, blood pressure and heart rate were measured, and a tolerance questionnaire was completed to measure the intensity of gastrointestinal symptoms (heartburn, belching, nausea, vomiting, abdominal distension, flatulence, diarrhea, and constipation), light-headedness, headache, thirst and dry mouth.

Visits were planned with intervals of 7 to 10 days between the visits. A follow-up phone call was scheduled approximately one week after the last study visit to identify adverse events and evaluate the tolerance of the intervention. Throughout the study, the use of relevant concomitant medication and nutritional supplement intake was recorded. **Supplementary Figure S2.2** illustrated the clinical study design.

Stopping guidelines with premature discontinuation of participation in the study included cases when further participation was considered a health risk for a subject (at supervising clinicians' discretion), and cases in which a subject had decided to resign from further participation in the study.

## *In vitro* digestion modelling (*in vitro* study)

A semi-dynamic two-step gastrointestinal model was used, as illustrated in **Figure 2.1**. The model has continuous pH control, digestive juice addition and separate pre-defined gastric and intestinal steps. The model consists of eight parallel bioreactors that are computer controlled. Each is equipped with a pH electrode and four dosing lines with separate substrate pumps (Dasgip AG, Jülich, Germany) (**Figure 2.1A and B**).

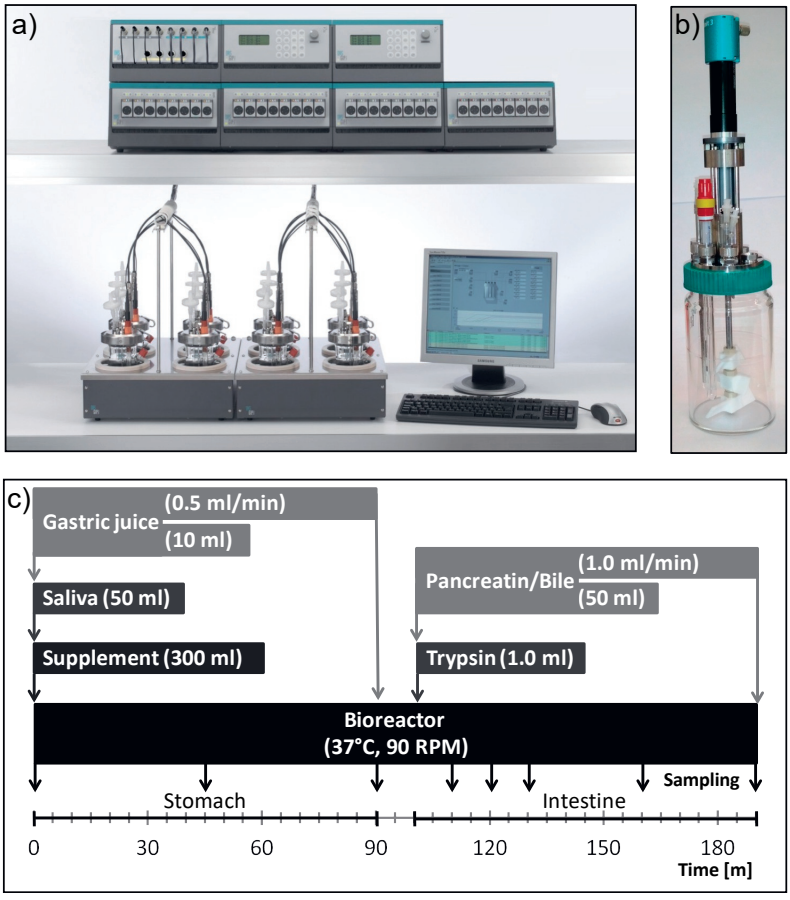


**Figure 2.1: Semi-dynamic model of the gastrointestinal tract. The model consisted of A) Computer controlled parallel bioreactor system, and B) Single bioreactor with overhead agitation. C) Time schedule.**

Gastrointestinal conditions were simulated by pH and substrate pump control at 37°C as described previously [22]. The flow, volumes and compositions of secretions were based on what is used in a dynamic model of the gastro-intestinal tract [29, 30], as described earlier in detail [22]. For the current study, the approach was adapted: a) using reactors with a larger volume (to fit 300mL), therefore no scaling was necessary, b) adding a small intestinal phase, c) adapting gastric secretion to elderly conditions by lowering pepsin and lipase concentration based on the decreased peak pepsin output observed in elderly, i.e. 52%, as compared to healthy young adults [31], and d) using overhead agitation at 90 rpm with three perpendicularly placed 45 degree two-blade impellers, instead of magnetic stirring, for adequate mixing of the study product and digestive juices with lower shear. The four different substrates were a) 1 M HCl and b) 1 M $NaHCO_3$ and 3 M NaOH to adjust the pH, and c) artificial gastric juice and d) pancreatin/bile mixture containing 2% w/v porcine bile

extract (Sigma B8631), 1.75% w/v pancreatin (Pancrex V, Pfizer) supernatant, 0.25% w/v NaCl, 0.03% KCl, and 0.015% w/v $CaCl_2.H_2O$. Artificial saliva was added manually.

Experiments were done in triplicate (n=3) with the investigator blinded for the composition of the nutritional supplements. Gastric digestion was simulated for 90 minutes. During this period the pH was lowered according to a predefined curve that is based on the acid secretion capacity of older people and the pH of the supplements [32]. The pH was lowered from 4.0 to 2.2 within 90 min in whey compositions and from 6.6 to 3.0 in casein supplements within 90 min. In one set of the experiments, the simulation was discontinued, and the gastric contents were analyzed for solid particles, i.e., coagulate fractions. In another set of experiments, the first 90-min phase of gastric digestion was followed by a 90-min simulation phase of small intestinal digestion, allowing the system ten minutes between the 2 phases to bring the pH to the set start pH (6.5) of the intestinal phase. The set intestinal pH curve was the same for all supplements, i.e., an increase to pH 7.2 in 90 min. At the start of the experiment, 50 ml of saliva was added manually (**Figure 2.1C**). The flow rate for gastric juice was 0.5 ml/min. In the first two minutes of gastric digestion, an additional 10 ml was added as a shot to simulate the gastric juice already present in the stomach. The secretion of the pancreatin/bile mixture was at a rate of 1.0 ml/min. At the start of the intestinal phase, 1 ml of trypsin solution (0.2% w/v (sigma T9201)) was added manually to simulate the action of enterokinase on trypsinogen from the pancreatin. In the first 5 min, 50 ml of pancreatin/bile mixture was added as a shot to simulate the intestinal contents at the time of ingestion.

## Sample processing, analyses, and calculations

*Blood samples (*in vivo*)*

Blood was sampled in serum tubes. Serum was obtained by centrifuging whole blood at room temperature for 15 min at 1000*g*. All serum samples were stored at -80°C until further analyses.

*Coagulate fractions (*in vitro*)*

After 90 minutes of simulated gastric digestion, the content of the reactors was poured over three analytical sieves. The mesh widths of the sequential sieves were 2 mm, 1 mm, and 0.25 mm (Retsch, VWR, Amsterdam, Netherlands) in order to separate insoluble particles by particle diameter (D) in three fractions: larger than 2 mm ($D_{>2}$), between 1 and 2 mm ($D_{<1\text{-}2>}$), between 0.25 and 1 mm ($D_{<0.25\text{-}1>}$). The dry matter content of each fraction was determined as described previously [20].

*Protein digestion rate (*in vitro*)*

Sampling over time in the intestinal phase enables us to gain insights into the initial digestion rate (first one-two samples (t10 and t20), early intestinal phase) and the overall digestion rate (later samples, representing cumulative digestion over a maximum 90-min intestinal

period). Measurement of the accumulation of protein digestion products was done with two distinct, complementary methods. The analysis was focused on the end products of pancreatic enzymes. It has been found *in vivo* that the absorbable fraction of protein digestion products consists of free amino acids and peptides built of two to six amino acids, in a ratio of 25:75 mol% of free and peptide-bound amino acids respectively [33]. Therefore, 1kD was chosen as the upper limit as done by others [34], and peptides smaller than 1kD and free amino acids were quantified.

*Peptide analysis (*in vitro*)*
Peptides were quantified using size exclusion chromatography (SEC). The HPLC system (Shimadzu) was equipped with a Superdex Peptide 10/300 column (GE Healthcare 17-5176-01). Detection of peptide bonds in the eluent (25%wt acetonitrile, 0.16%wt trifluoracetic acid) was by done by UV absorption, $\lambda$ = 200 nm. The relationship between elution time and molecular weight was calibrated using ten distinct small proteins and peptides ranging from 12kD to 188D, of which five were smaller than 1 kD. The correlation coefficient of linear fit between elution time and molecular weight was 0.980 between 9.2 and 19.2 min. The AUC was calculated from 15.3 min (1kD) to 23 min to exclude contribution of free aromatic amino acids that absorb at this wavelength.

*Analysis of free amino acids (*in vivo *and* in vitro*)*
Proteins and large peptides were precipitated with perchloric acid. After filtration, the amount of the free amino acids in the supernatant is determined by UFLC using a pre-column derivatization with o-phthalaldehyde and fluorimetry as detection [35].

*Analysis of other blood parameters (*in vivo*)*
Glucose and albumin concentrations were measured using colorimetry. Turbidimetry was used for analysis of CRP. Insulin concentrations were measured by immunoluminometric assay.

*Calculations and primary/secondary outcome parameters (*in vivo*)*
For serum free leucine, EAA (sum of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine), and the sum of all amino acids (TAA: alanine, arginine, asparagine, aspartic acid, citrulline, cysteine, glutamic acid, glutamine, glycine, serine, taurine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine; tyrosine was excluded because of technical problems in the analysis) the following parameters were calculated: the maximum (or peak) concentration, the maximum increment from baseline (delta), the iAUC (from baseline, during 4h after product intake) and the time to reach half the serum leucine iAUC ($t_{½}$). For serum glucose and insulin, the maximum (or peak) and iAUC values were calculated.

The primary outcome parameter of the clinical study was the maximum leucine level; secondary parameters were maximum levels of EAA, TAA, glucose and insulin, the iAUC of leucine, glucose and insulin, and $t_{½}$ of leucine. As a deviation to the protocol but before data analyses, iAUC and $t_{½}$ of EAA and TAA were added as secondary parameters.

## Statistical analysis

Power analysis for the *in vivo* study was based on an estimated 100 μmol/L difference in peak leucine level between W150 and C150 with a standard deviation of difference of 60 μmol/L for whey and 54 μmol/L for casein as estimated from Dangin et al. [19], an α of 0.05, a power of 80% and dropout rate of 33%. All *in vivo* serum variables (amino acids, glucose and insulin concentrations) were analyzed using a mixed model with a random effect for subjects, a fixed effect for the factors protein (2 levels: whey, casein) and caloric density (2 levels: low, high), and a fixed interaction of protein*caloric density. Statistical significance of these pooled analyses was defined as a two-tailed $p<0.05$. In separate mixed model analyses, the difference between W150 and C150, between W150 and W320, between C150 and C320 and between W320 and C320 were analyzed. Statistical significance was defined as a two-tailed $p<0.0125$ in order to correct for multiple comparisons. Baseline value (for leucine, EAA, AA, insulin, or glucose), serum albumin, and serum CRP were included as covariates in the respective mixed model. The concentration at t0 was used for baseline value when concentrations at t-15 and t0 were statistically significantly different using mixed model analysis. In case of no statistical difference, the mean concentration of the 2 time points at baseline was taken. For peak leucine, additional analyses were performed using age, physical activity level, sex or BMI (categories $\leq$25 and >25 kg/m$^2$) as additional covariates in the mixed model. SAS 9.1.3 (SAS Institute; PROC MIXED procedure) software was used. *In vivo* data are presented as estimated marginal mean (EMM) with SE, as derived from the mixed model, unless otherwise stated.

*In vitro* gastric coagulation data were analyzed with Student's t-test, 2-tailed, equal variances assumed, and differences were considered significant when $p<0.05$. *In vitro* protein digestion kinetics data was analyzed using ANOVA univariate analysis with LSD post hoc test. One on the triplicate measures for W150 was excluded from the dataset because of technical malfunction of the bioreactor. Because there were no more supplements available for the study, the statistical interaction model was used to determine estimated means. Differences were considered significant when $p<0.0125$, in order to correct for multiple comparisons. Statistics were performed using SPSS 15.0 for Windows. Data are shown as raw mean with SEM, unless stated otherwise.

# Results

## Subjects

The first subject was included in the study on October 27, 2008. The last subject completed the study on February 19, 2009. Ten out of 12 subjects completed all visits. Two subjects terminated the study early, but had at least 1 study visit completed. None of the randomized subjects violated the eligibility criteria during the study or had other protocol violations. Therefore, all subjects were included in the population analysis, and descriptive and statistical analyses were performed on the 12 subjects (**Supplementary Figure S2.1**). Subjects' characteristics are listed in **Table 2.2**. No related serious adverse events were observed during the study. A few mild to moderate adverse events were reported with no clinically relevant difference in occurrence between the products. There were no clinically relevant differences in incidence of GI symptoms between treatments.

**Table 2.2: Subject characteristics.**[1]

| Characteristic | Baseline | W150 | C150 | W320 | C320 |
|---|---|---|---|---|---|
| Gender: | | | | | |
| Male (n (%)) | 5 (42) | | | | |
| Female (n (%)) | 7 (58) | | | | |
| Age (y) | 67.4±1.8 | | | | |
| Body weight (kg) | 73.1±7.5 | | | | |
| BMI (kg/m$^2$) | 26.0±2.6 | | | | |
| Serum albumin (g/L) | | 41.2±1.89 | 39.8±1.32 | 40.3±2.23 | 40.9±1.87 |
| Serum CRP (mg/L) | | 3.4±2.58 | 5.3±9.27 | 1.7±0.89 | 3.1±2.12 |
| Physical activity level[2] | 13.57±6.65 | | | | |

[1]All data are means±SD, except for sex; n=12 (ITT). [2]Total score, derived from the Modified Baecke Questionnaire for Older People [28].

## Serum concentrations of amino acids, glucose, and insulin

Profiles for serum leucine, EAA, and TAA are illustrated in **Figure 2.2**, and for serum glucose and insulin in **Figure 2.3**. Values and statistical comparison between groups at baseline, peak, delta, iAUC and $t_{½}$ for serum leucine, EAA, and TAA are listed in **Table 2.3**. Baseline values for serum amino acids, glucose and insulin were well balanced between the study visits.

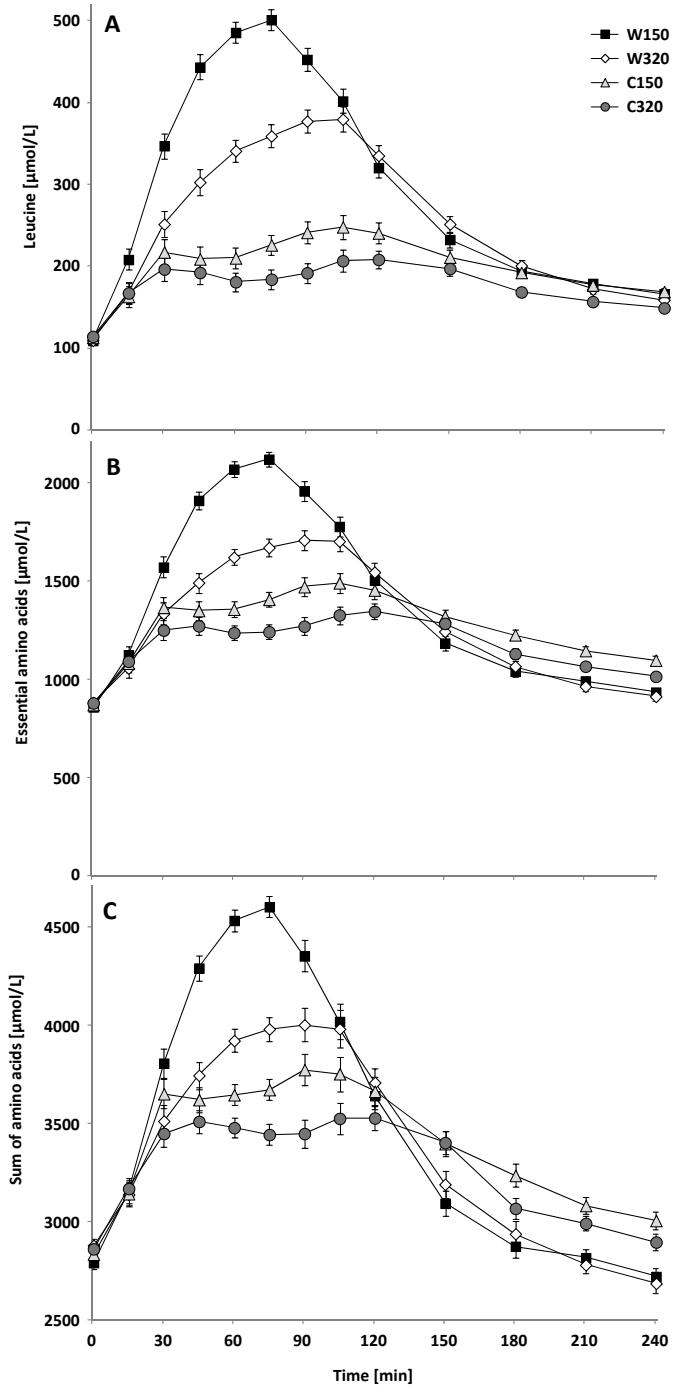


**Figure 2.2: Serum amino acids. Serum levels (EMM±SE) of A) leucine, B) essentials amino acids (EAA), and C) sum of amino acids (TAA) before and after ingestion (at T=0) of the study products: leucine-enriched whey protein, 150kcal (W150); casein protein, 150kcal (C150); leucine-enriched whey protein, 320kcal (W320); and casein protein, 320kcal (C320), in healthy older subjects (n=12).**

Pooled analysis of the low-caloric vs. the high-caloric products (W150 and C150 vs. W320 and C320) showed a significantly higher peak and iAUC of serum leucine, EAA, and TAA for the low-caloric vs the high-caloric products ($p<0.001$). The caloric density effect was stronger for the leucine-enriched whey protein supplements (W150 vs. W320) than for the casein protein supplements (C150 vs. C320) on peak levels of serum leucine (effect size (EMM±SE) of 114±14.3 μmol/L ($p<0.001$) vs 32±14.8 μmol/L ($P=0.042$)), serum EAA (effect size of 395±49.2 μmol/L ($p<0.001$) vs 120±49.6 μmol/L ($p=0.023$), and serum TAA (effect size of 546±79.0 μmol/L ($p<0.001$) vs 247±76.3 μmol/L ($p=0.003$)). This is also reflected by the significant interaction effect between protein source and caloric density ($p<0.001$ for peak levels of leucine and EAA, $p=0.015$ for TAA). Including age, physical activity level, gender or BMI in the mixed model for peak leucine did not change the significance of the effect.

Peak serum glucose concentration was 5.54±0.18 and 6.05±0.19 mmol/L after intake of W150 and C150, respectively ($p=0.013$), and 6.42±0.18 and 6.66±0.18 mmol/L after W320 and C320, respectively ($p=0.195$). Serum glucose iAUC did neither differ between W150 and C150 ($p=0.152$), nor between W320 and C320 ($p=0.577$). Pooled analysis of the low-caloric vs. the high-caloric products showed significantly lower peak and iAUC serum glucose concentration for the low-caloric vs the high-caloric products ($p<0.001$). There was no significant interaction effect between protein source and caloric density for the peak serum glucose concentration ($p=0.314$), nor for serum glucose iAUC ($p=0.494$).

Peak and iAUC serum insulin concentration did not differ between W150 and C150 ($p=0.915$ for peak; $p=0.782$ for iAUC), nor between W320 and C320 ($p=0.989$ for peak; $p=0.261$ for iAUC). Pooled analysis of the low-caloric vs. the high-caloric products showed a significantly lower peak and iAUC serum insulin concentration for the low-caloric vs the high-caloric products ($p<0.001$). The interaction effect between protein source and caloric density for peak serum insulin concentration ($p=0.933$), or for serum insulin iAUC ($p=0.335$) was not significant.

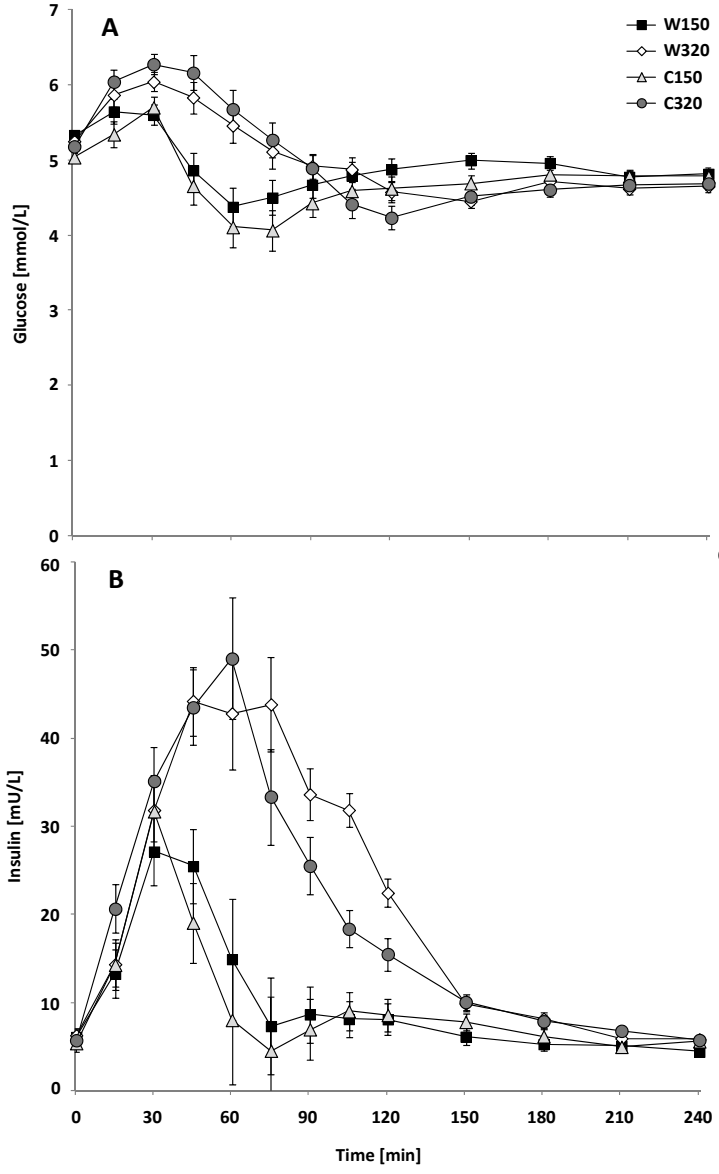


**Figure 2.3: Serum glucose and insulin. Serum levels (EMM±SE) of A) glucose and B) insulin before and after ingestion (at T=0) of the study products: leucine-enriched whey protein, 150kcal (W150); casein protein, 150kcal (C150); leucine-enriched whey protein, 320kcal (W320); and casein protein, 320kcal (C320), in healthy older subjects (n=12).**

**Table 2.3: Serum amino acid concentrations at baseline and after intake of the study products in healthy older subjects (n=12).**[1]

| Parameter | W150 | C150 | W320 | C320 | p-value W150 vs C150 | p-value W150 vs W320 | p-value C150 vs C320 | p-value W320 vs C320 |
|---|---|---|---|---|---|---|---|---|
| Leucine | | | | | | | | |
| Baseline (mmol/L) | 109± 6.3 | 113± 6.5 | 110± 6.3 | 115± 5.7 | | | | |
| Maximum (mmol/L)[2] | 521± 15.1 | 260± 15.4 | 406± 14.5 | 228± 15.2 | <0.001 | <0.001 | 0.042 | <0.001 |
| iAUC (mmol/L*min)[3] | 44588± 1439 | 22207± 1473 | 35952± 1386 | 15793± 1451 | <0.001 | <0.001 | <0.001 | <0.001 |
| $t_{½}$ (min)[4] | 87± 5.4 | 119± 5.6 | 101± 5.2 | 118± 5.5 | <0.001 | 0.015 | 0.867 | 0.003 |
| EAA | | | | | | | | |
| Baseline (mmol/L) | 859± 31.2 | 868± 35.0 | 887± 29.1 | 883± 30.6 | | | | |
| Maximum (mmol/L)[2] | 2187± 57.0 | 1540± 57.5 | 1420± 56.8 | 1792± 55.0 | <0.001 | <0.001 | 0.023 | <0.001 |
| iAUC (mmol/L*min)[3] | 129793± 5690 | 100516± 5765 | 75181± 5685 | 101181± 5471 | <0.001 | <0.001 | <0.001 | <0.001 |
| $t_{½}$ (min)[4] | 83± 5.0 | 115± 5.1 | 117± 5.0 | 94± 4.8 | <0.001 | 0.042 | 0.762 | <0.001 |
| TAA | | | | | | | | |
| Baseline (mmol/L) | 2786± 46.5 | 2824± 37.3 | 2880± 55.1 | 2848± 60.1 | | | | |
| Maximum (mmol/L)[2] | 4687± 111 | 3946± 111 | 4141± 108 | 3699± 110 | <0.001 | <0.001 | 0.003 | <0.001 |
| iAUC (mmol/L*min)[3] | 162702± 12979 | 143018± 12994 | 128047± 12684 | 105525± 12872 | 0.032 | <0.001 | <0.001 | 0.008 |
| $t_{½}$ (min)[4] | 78± 5.2 | 101± 5.3 | 87± 4.9 | 103± 5.1 | <0.001 | 0.143 | 0.769 | 0.007 |

[1]EAA = essential amino acids; AA = sum of amino acids. Study products: leucine-enriched whey protein, 150kcal (W150); casein protein, 150kcal (C150); leucine-enriched whey protein, 320kcal (W320); and casein protein, 320kcal (C320).[2]Maximum (or peak) level. [3]Maximum minus baseline difference. [2]Incremental area under the curve. [4]Time to reach half the serum iAUC. Statistical analysis was done by mixed model including serum albumin, serum CRP, and baseline serum outcome parameter concentration as covariates and subject as random factor. Baseline values were not different between the study products.

## *In vitro* gastric coagulation

The leucine-enriched whey protein supplements did not yield any measurable coagulates after gastric digestion, while casein supplements did. Gastric digestion of C320 yielded a significantly higher total amount of coagulates than C150 (p=0.005). This was due to a higher amount of coagulates of the middle diameter fraction ($D_{<1-2>}$)(p=0.021), and a tendency for a higher amount of coagulates of the smallest diameter fraction ($D_{<0.25-1>}$) (p=0.051). The largest diameter fraction, however, was not significantly different (p=0.691, **Figure 2.4**). The percentage of total coagulate matter in $D_{>2}$ was significantly lower in C320 vs C150 (62.3±9.0 vs 91.0±1.4%, respectively (p=0.035), while the percentage in $D_{<1-2>}$ was significantly higher (15.5±3.6 vs 4.6±0.8%, respectively; p=0.043) and in $D_{<0.25-1>}$ tended to be higher (22.2±6.4 vs 4.4±0.6% respectively; p=0.051).

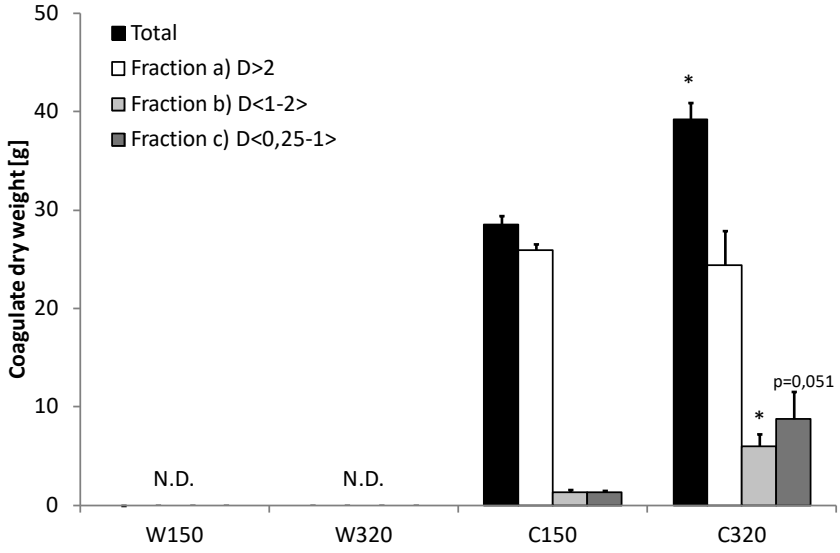


**Figure 2.4: *In vitro* gastric coagulation. Coagulates formed during the gastric phase and differentiated by their particles size (in mm). Means ± pooled standard error (n=3). *significantly different from C150 (Student's t-test; p<0.05). N.D – not detected.**

## *In vitro* protein digestion rate

The vast majority of small peptides were released during the intestinal phase of digestion for all supplements tested. The amount of small peptides present early in the intestinal phase (t10 min) was significantly higher in the casein supplements than the leucine-enriched whey protein supplements of the same caloric density (150 kcal: p=0.003 and 320 kcal p<0.001). For the later samples in the intestinal phase, these amounts were no longer significantly different (p>0.0125). At t90, larger amounts of peptides tended to be present in samples of low caloric supplements; W150 vs W320 (p=0.072) and C150 vs C320 (p=0.049) (**Figure 2.5A**).

There were free AA present in the gastric phase of the leucine enriched whey protein supplements. These are most likely the free branched chain amino acids (leucine, isoleucine, valine; all EAA) that are present in the initial supplement. There was negligible release of amino acids in the gastric phase.

In the early phase of intestinal digestion (t10 and t20), the total amount of free AA was significantly higher for C320 than both C150 ($p<0.0125$) and W320 ($p<0.0125$). From t30 onwards, W150 was significantly higher than W320 ($p<0.0125$) and from t60 onwards also higher than C150 ($p<0.0125$). At the end of the intestinal phase (t90), both C150 and W320 were significantly higher than C320 ($p<0.001$) (**Figure 2.5B**).The amount of free EAA at t10 was significantly higher for C320 than for C150 and W320 ($p<0.001$). From t20 onwards, W150 was significantly higher than C150 ($p<0.0125$) and from t30 onwards also significantly higher than W320 ($p<0.0125$). W320 was significantly higher than C320 at t60 ($p=0.0013$) and t90 ($p<0.001$) (**Figure 2.5C**). After correction for the initial difference in free EAA level between the leucine-enriched whey protein supplements and casein supplement caloric equivalents, the amount of free EAA for W150 was still significantly higher than W320 from t30 onwards ($p<0.0125$), and also higher than C150 at t60 ($p=0.006$) and t90 ($p<0.001$) (**Figure 2.5E**).

The amount of free leucine was significantly higher at all time points for both leucine-enriched whey protein supplements than for their casein supplement caloric equivalents ($p<0.0125$). From t30 onwards, W150 was also significantly higher than W320 ($p<0.0125$). For the casein supplements at t10, C320 was higher than C150 ($p=0.008$), at t20 and t30 there was no significant difference ($p=0.046$ and $p=0.865$, respectively). At t60 and t90, C150 was significantly higher than C320 ($p=0.008$ and $p<0.001$, respectively) (**Figure 2.5D**). After correction for the initial higher leucine level, the amount of leucine in W150 was also significantly higher than W320 from t30 onwards ($p<0.0125$), and also significantly higher than C150 at t90 ($p<0.001$), but not at t60 ($p=0.057$) (**Figure 2.5F**).

SEC chromatograms of t90 samples indicate that peptides smaller than 1kD constitute about 70% (68-72) of all soluble protein and peptides (data not shown). At that time point, there is on average 4.5 gram of free amino acids **(Figure 2.5B)**. Assuming all proteins were dissolved at this point and free AA do indeed not contribute to the signal, the estimated degree of digestion at t90 of the total 21 gr of food protein and 1.3 gr of protein from digestive juices is 76% (calculated as ((22.3-4.5)*0.7 + 4.5)/22.3*100%), of which about 70% are peptides and 30% are amino acids.

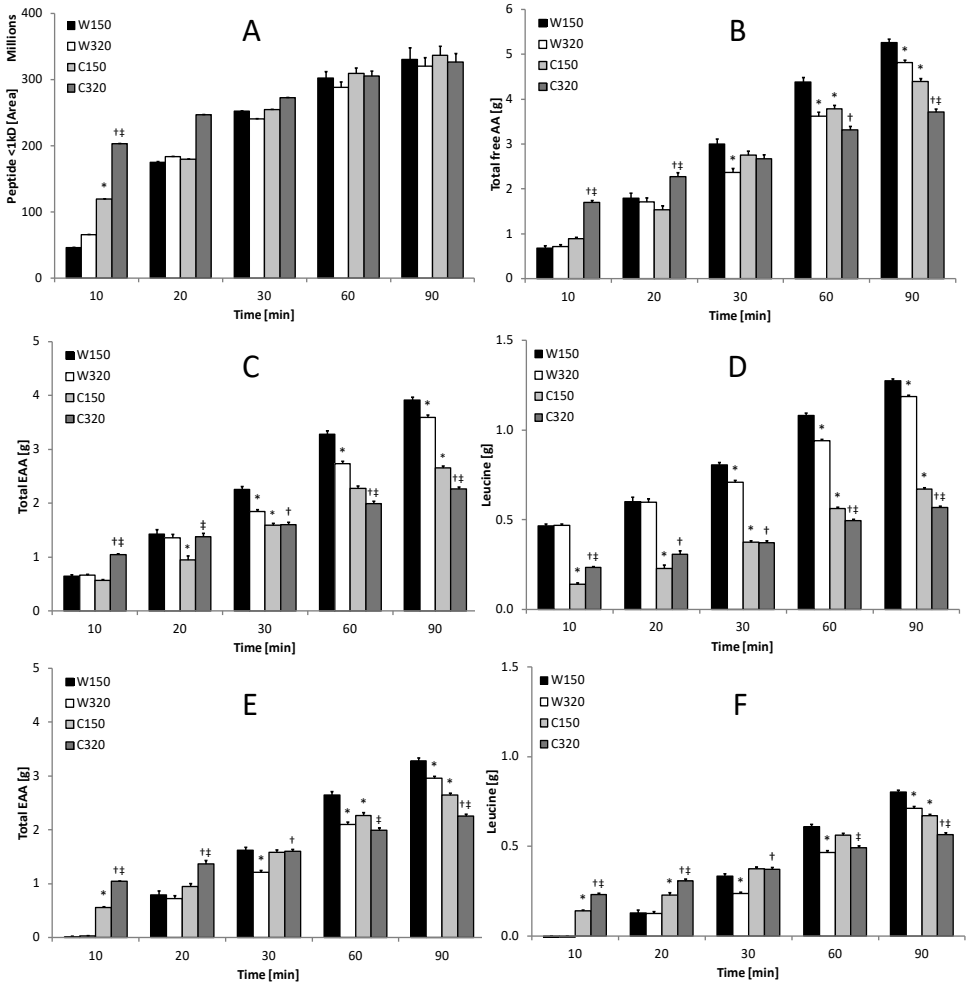


**Figure 2.5:** ***In vitro*** **protein digestion. Accumulation of protein digestion products formed in the intestinal phase over time: A) peptides smaller than 1kD, B) total free AA, C) total free EAA, D) free leucine, E) total free EAA corrected for the initial difference in free EAA in the supplements, and F) total leucine corrected for the initial difference in free leucine in the supplements. Means +/- pooled standard error. *significantly different from W150, †significantly different from W320, ‡significantly different from C150 (ANOVA univariate analysis with LSD post hoc test and correction for multiple comparisons; p<0.0125). The stomach phase is not shown, as negligible amounts of products were formed in this phase.**

## Discussion

In the current study, blood AA profiles after consumption of a leucine-enriched whey protein nutritional supplement or an iso-caloric and iso-nitrogenous casein protein supplement were compared in healthy older men and women. In addition, the impact of the caloric density of the products on the blood AA profiles was analyzed. Higher levels of TAA, EAA, and leucine were observed after the leucine-enriched whey protein supplements compared

to the casein protein supplements. This effect was further augmented by the low-caloric density of the formulation, both for the casein and whey protein-containing supplements. As far as we are aware, this is a novel observation as it resembles realistic protein amounts in mixed meals. In accordance with *in vivo* data, gastric physicochemical behaviour *in vitro* was profoundly protein type dependent; only the casein supplements clotted. The initial intestinal protein digestion rate of casein supplements was higher than that of whey protein supplements. The overall protein digestion rate and cumulative release of AAs over 90-min, however, was higher for whey supplements than their casein counterparts. In addition to the protein type dependency, this was also caloric density dependent, i.e., higher for 150kcal than 320kcal. These overall differences in the *in vitro* data thus nicely fit with the observed differences in *in vivo* blood AA profiles. The results suggest that gastric coagulation, likely impacting gastric emptying, and/or overall protein digestion rate are determinants of amino acid profiles, while initial enzymatic hydrolysis rate in the intestine is less important. The observed differences in postprandial amino acid profiles *in vivo*, therefore, appear to be at least partly mediated by the gastrointestinal behaviour of the products.

## Effect of protein source

The leucine content in the leucine-enriched whey protein supplements was 1.5-fold higher compared to the casein protein supplements. This contributed to the difference in serum leucine levels. However, despite the equal contents of EAA and TAA, these profiles differed between the products and indicate the contribution of other aspects. Delayed gastric emptying of casein due to gastric coagulation of the protein has been demonstrated [22], and subsequent differences in intestinal behaviour between casein and whey have been shown with intra-jejunal sampling [20, 21, 23] and with the use of intrinsically labelled proteins [14]. It has been demonstrated that the particle size has an impact on gastric emptying. Solid matter needs to be broken down to <1–2 mm particles prior to their stomach emptying [36]. Our *in vitro* data confirm gastric coagulation with casein specifically, but also indicate different digestion kinetics for whey and casein protein supplements.

To our knowledge, the intestinal digestive properties of casein and whey protein have not been extensively studied, especially in mixed meals. We observed that, the initial intestinal digestion rate of casein-containing supplements was higher. This is in line with other *in vitro* digestion studies, using human gastric and duodenal aspirates. In these studies, caseins in milk from several species were digested very rapidly after being exposed to either human gastric or duodenal juices, as compared to whey proteins [37, 38].

Moreover, we observed in our study that, the overall protein digestion rate, as reflected by the cumulative levels of TAA, EAA and leucine over the total 90-min intestinal digestion period, was higher with the whey-containing supplements. Thus, digestive properties of

the whey and casein-containing supplements differ, independent from metabolism and feedback mechanisms in the body.

## Effect of caloric density

Interestingly, a significant interaction effect between protein source and caloric density for the peak level of leucine and (essential) amino acids was observed. This means that the effect of calories is even more pronounced for the whey protein supplement than for the casein protein supplement. Most previous studies compared whey and casein products with no added calories from fat and carbohydrates [14, 17, 18], and only one study used a mixed meal [19]. So far, it has only been suggested that, by comparing between studies, the difference in digestion rate for whey versus casein in a mixed meal is less pronounced than when the proteins are given without added calories [16, 19]. In line with our observations, the co-ingestion of carbohydrates with casein protein lowered plasma AA levels in older adults [39], and also delayed digestion and absorption kinetics using intrinsically labelled casein [26]. Only one study in young adults showed lower peak plasma levels of leucine, branched chain amino acids (BCAA) and EAA when whey protein was co-ingested with carbohydrates [40]. Two studies, in young adults with intrinsically labelled milk protein, showed that the addition of carbohydrates and fat delays gastric emptying of milk protein and slows the release of dietary nitrogen to the periphery [27, 41]. A larger caloric load is indeed known to slow gastric emptying [24, 25], but the impact on digestion and absorption is less well known. *In vitro,* we observed that a higher caloric density increased the amount of casein coagulates, and especially the number of smaller sized particles. In addition to this distinctive gastric behaviour, we observed that a higher caloric density decreased the cumulative release of AA from the whey protein supplements significantly over the total 90-min intestinal digestion period. It is possible that the smaller coagulates facilitated the early phase of intestinal digestion with the high-caloric casein product, as reflected in the initial higher level of small peptides and amino acids. This effect was diminished later in the intestinal phase when coagulates had disappeared. Thus, as well as potentially having an effect on gastric emptying rate *in vivo*, caloric density 'slows' overall protein digestion rate *in vitro*.

## Limitations and alternative explanations

While our data demonstrate that the observed differences in serum amino acid profiles are mediated, at least in part, by the gastrointestinal behaviour of the products, we cannot exclude other metabolic effects that impact on serum amino acid levels. This is relevant as the rate of dietary protein digestion and amino acid absorption from the gut also modulate the postprandial metabolic response [17, 42]. This includes a larger inhibition of endogenous protein breakdown [14, 17], and greater splanchnic sequestration [14, 17] of casein compared with whey protein. Both may reduce the systemic appearance of amino acids with casein. Similarly, the addition of carbohydrates and fat lowers whole body protein breakdown,

which is even more pronounced in combination with whey than with casein protein [17, 19]. The observed higher insulin levels after ingestion of the high caloric products could have inhibited protein breakdown to a larger extent [43]. Moreover, added carbohydrates have been shown to reduce protein oxidation [27] and to increase splanchnic sequestration of dietary nitrogen [41, 44]. Thus, we cannot exclude that, besides and maybe partly due to distinct gastrointestinal behaviour, inhibition of protein breakdown and increased splanchnic sequestration with casein and higher caloric content have also contributed to the lower serum amino acid levels. Based on the amino levels only, we cannot quantify how many amino acids came directly from the products over time. Another limitation of the *in vivo* study could be that results have been obtained in a healthy group of people that are 65 years and older. Given that the prevalence and incidence of gastrointestinal dysfunction, i.e. in the form of dysmotility, may increase with age and in disease [45], it could be expected that the differences would be augmented in a population that is not healthy.

*In vitro* models are an approximation of the dynamic situation *in vivo*. When the conditions of gastric and intestinal phases of *in vitro* digestion are fixed, they make it possible to compare between products for gastric coagulation properties and hydrolysis kinetics. Measurement of digestion and absorption *in vivo* is much more invasive or requires the use of intrinsically labelled protein. In our study, the pH was set to be equal for all products in the intestinal phase. However, the 10 min of neutralization between the gastric and intestinal phases were not sufficient to bring the pH to 6.5; the casein products needed 20 min and the whey products 30 min to reach this value. This difference will likely have negatively impacted the initial protein digestion kinetics, especially of the whey products, since the pH was suboptimal for a longer period. Moreover, it is known that the accumulation of products of digestion in a closed system can limit enzymatic hydrolysis to considerably less than 100%. At the end of the intestinal phase (t90), we estimated that 76% was digested to end products, i.e. the absorbable fraction of <1kD [33]. When comparing *in vitro* and *in vivo* observations, the pattern of cumulative *in vitro* digestion of free AA in our study resembled that of the *in vivo* serum AA profile. Besides the free AA, a large abundance of peptides in the absorbable fraction will also and even faster appear as free AA in blood *in vivo* [46] for which we cannot discriminate its origin. However, we did not observe a difference in the cumulative peptide release *in vitro* between the products in our study. A final limitation of the study is that we cannot discriminate between the effect of calories originating from carbohydrates or from fat.

## Relevance of the data

Postprandial blood levels of EAA and leucine are considered relevant to stimulate muscle protein synthesis [13, 14]. With advancing age and disease, the threshold for anabolic stimuli to increase muscle protein synthesis is likely to become higher [8, 15]. The high postprandial levels of EAA and leucine that we observed after ingestion of a leucine-enriched whey protein

supplement in healthy older subjects, therefore, seems preferable to provoke subsequent muscle protein synthesis also in older sarcopenic adults. Moreover, a low caloric product favors higher postprandial blood leucine and EAA levels and faster digestion. The impact of energy density on serum amino acid profiles and gastrointestinal digestion is likely relevant for muscle anabolism and subsequent improvement of muscle mass, strength and function in older sarcopenic adults, in order to counteract the negative impact on quality of life, disability and hospitalization.

In conclusion, it was demonstrated in a healthy population of people of 65 years and older, that consumption of a low caloric leucine-enriched whey protein supplement provides a higher rise in serum levels of TAA, EAA and leucine compared to casein or high caloric products. These differences appear to be mediated at least in part by the gastrointestinal behaviour of these nutritional compositions. This effect on postprandial amino acid profiles is promising for stimulation of muscle protein synthesis in sarcopenic older people with a low caloric leucine-enriched whey protein supplement and for potential long-term effects on muscle mass, strength and function. This requires further study.

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## Statement of authorship

Y.C. Luiking was involved in writing of the protocol, the statistical analysis plan, data interpretation and writing of the manuscript. S. Verlaan initiated the clinical study and was involved in data interpretation. E. Abrahamse and T. Ludwig designed and supervised the *in vitro* experiments, their analysis and interpretation, and wrote parts of the manuscript. Y Boirie was involved in data interpretation. All authors critically reviewed the manuscript.

## Conflict of interest statement

Y.C. Luiking, E. Abrahamse, T. Ludwig and S. Verlaan are employees of Nutricia Research. Y. Boirie is an advisor for Nutricia Research.

## Source of funding

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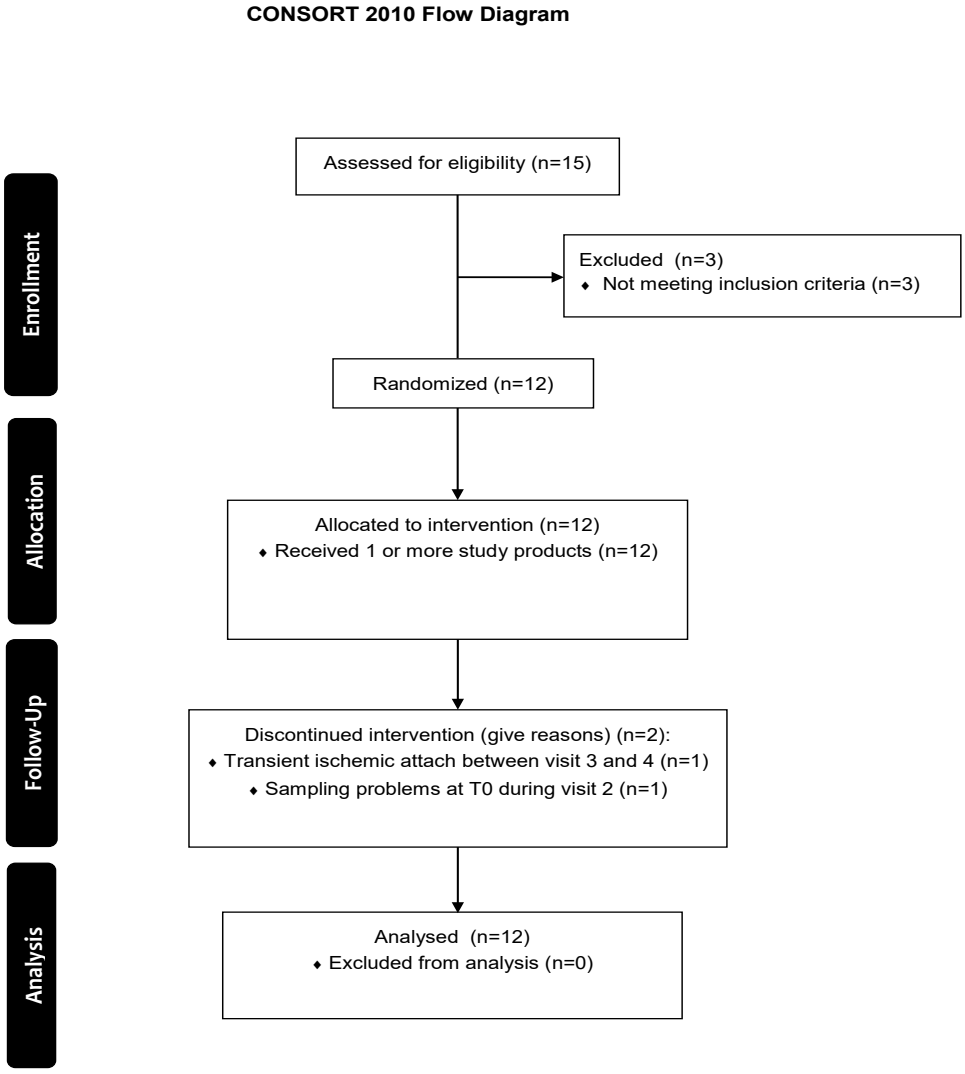
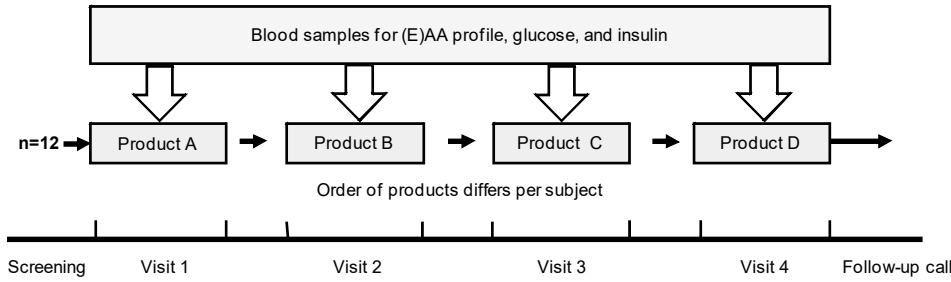
# Supplementary material




**Supplementary Figure S2.1: Consort flow diagram. All subjects were informed of the study procedures and possible risks before signing informed consent. The subject enrolment and study conduction was done by Ampha B.V. (clinical research unit, Nijmegen, the Netherlands) according to ICH-GCP principles, and in complied with the principles of the 'Declaration of Helsinki' (59th WMA General Assembly, Seoul, October 2008) and the local laws and regulations. The Independent Review Board Nijmegen (IRBN), the Netherlands, approved the study. This trial is registered at the ClinicalTrials.gov Trial Register under number NCT02013466.**

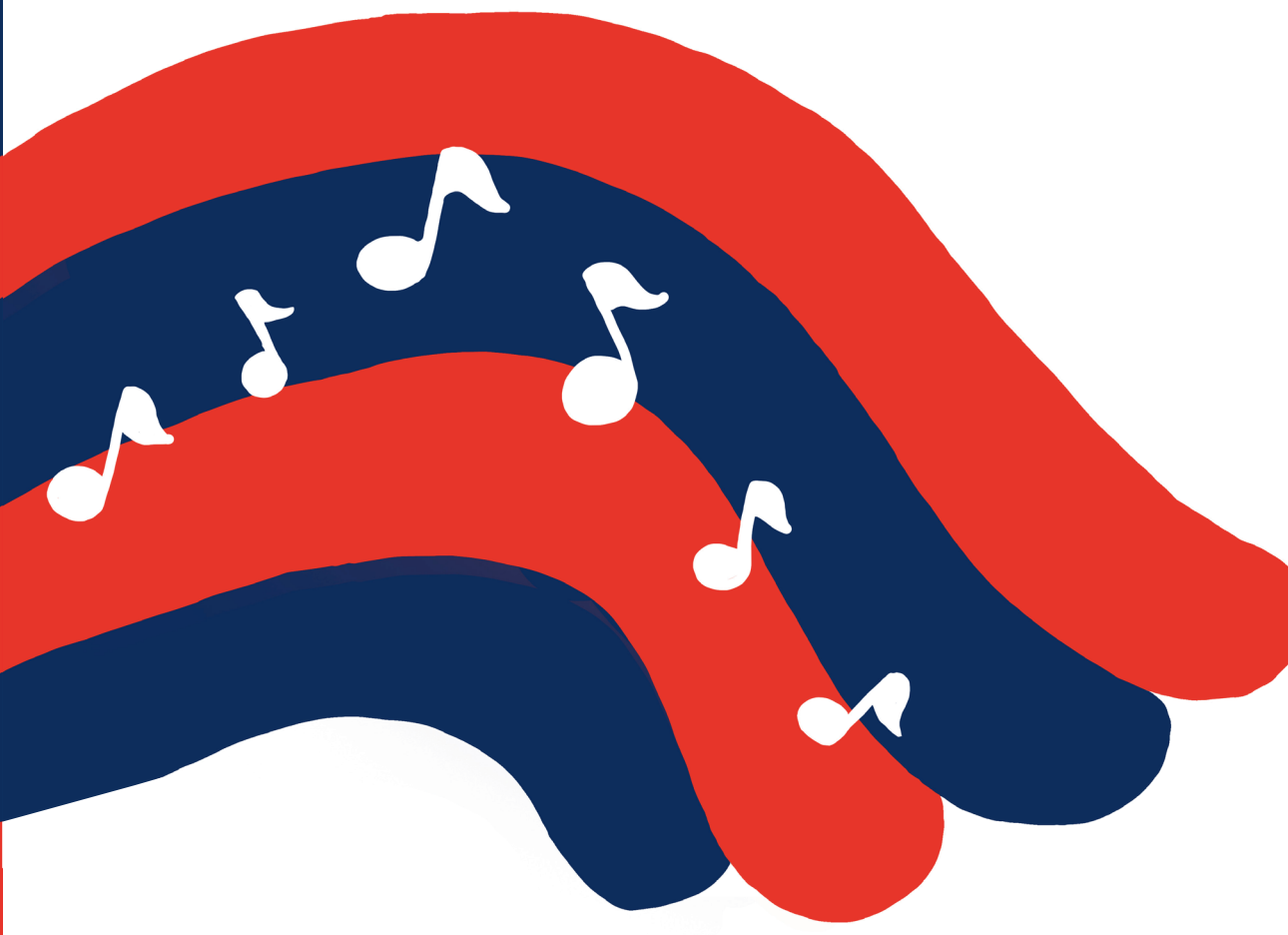



**Supplementary Figure S2.2: Clinical study design. The study included a screening visit, four study visits and a follow-up call. At each study visit, subjects consumed at random one of the study products and blood samples were taken at regular intervals before and during a 4-h period.**


3

# CHAPTER 3

## Postprandial amino acid kinetics of milk protein mixtures are affected by composition, but not denaturation, in neonatal piglets



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## Abstract

Multiple studies have indicated that formula-fed infants show a different growth trajectory compared to breast-fed infants. The observed growth rates are suggested to be linked to higher postprandial levels of branched chain amino acids (BCAA) and insulin related to differences in protein quality. We evaluated the effects of milk protein denaturation and milk protein composition on postprandial plasma and hormone concentrations. Neonatal piglets were bolus fed randomly in an incomplete cross-over design two of three milk protein solutions: native whey protein isolate (NWPI), denatured whey protein isolate (DWPI), or protein base ingredient, comprising whey and casein (PBI). Postprandial plasma amino acids (AA), insulin, GLP-1, glucose, and paracetamol concentrations were assayed. Plasma responses were fitted with a model of first order absorption with linear elimination. DWPI (91% denatured protein) vs NWPI (91% native protein) showed lower essential AA (EAA) (~10%) and BCAA (13-19%) concentrations in the first 30-60 min. However, total AA (TAA) concentration per time-point and AUC, as well as EAA and BCAA AUC were not different. PBI induced a ~30% lower postprandial insulin spike than NWPI, yet plasma TAA concentration at several time-points and AUC was higher in PBI than NWPI. The TAA rate constant for absorption ($k_a$) was twofold higher in PBI than NWPI. Plasma BCAA levels from 60 to 180 min and AUC were higher in PBI vs. NWPI. Plasma EAA concentrations and AUCs in PBI and NWPI were not different. Denaturation of WPI had a minimal effect on postprandial plasma AA concentration. The differences between PBI and NWPI were partly explained by the difference in AA composition, but more likely differences in protein digestion and absorption kinetics. We conclude that modifying protein composition, but not denaturation, of milk protein solutions impacts the postprandial amino acid availability in neonatal piglets.

## Introduction



Breastfeeding is widely recognized as the best nutrition for infants during the first year of life, providing many benefits to the growing infant [1, 2] Infants who are exclusively fed human milk have decreased rates of infectious and allergic diseases, respiratory disorders, and obesity [2-9]. In cases where breastfeeding is not possible, high-quality formulas which fulfil the complex nutrient requirements of the infant must be available as an alternative [4].

The protein content of human milk gradually declines over the course of lactation. Most commercially available infant formulas contain a higher protein content (g/L) than human milk to meet nutritional requirements for infants of all ages [10]. The higher protein concentration in infant formula has been suggested to program the metabolism towards overweight and obesity in later life, known as the early protein hypothesis [5, 7]. The proposed mechanism of the early protein hypothesis is that increased protein intake leads to increased levels of insulin and IGF-1-releasing AA. In particular the BCAA leucine (Leu), valine (Val), and isoleucine (Ile), the aromatic AA (AAA) phenylalanine (Phe) and tyrosine (Tyr), and other essential AA (EAA) such as lysine (Lys), are insulinogenic AA. High levels of insulin and IGF-1 can cause weight gain and adipogenesis potentially leading to obesity [5, 6].

It has been shown in pre-term infants that the postprandial EAA and BCAA peaks after human milk ingestion are lower (~30% and ~25% respectively) and occur later (~30 and ~15 min respectively) compared to bovine protein based infant formula when fed a similar protein quantity [11]. Studies that compared the gastric emptying kinetics of human milk to infant formula generally find a similar or slower half-emptying time for human milk [12], suggesting protein digestion and absorption kinetics are dominant factors in the differences observed in the postprandial time course in preterm infants.

Protein digestion kinetics have been shown to be influenced by several parameters. Firstly, *in vitro* digestion studies have shown differential impact of heat treatment on milk protein hydrolysis rate. Dupont et al. observed that after heat sterilization (120°C, 10 min) of milk, some regions in casein amino acid chains became more resistant to enzymatic hydrolysis, while hydrolysis kinetics of intact β-lactoglobulin, a major whey protein, was increased under *in vitro* infant gastro-duodenal conditions [13]. Possibly, the heat treatment decreased the amount of native whey protein. Denatured (unfolded) proteins are expected to have faster hydrolysis kinetics than native (still folded) proteins [14]. Others have shown that indeed heat treatment (90°C, 120 min) accelerates β-lactoglobulin intact protein hydrolysis kinetics. In particular high molecular weight non-native aggregates were shown to be hydrolyzed faster under *in vitro* gastric conditions. On the other hand, non-native dimers of β-lactoglobulin were formed due to heating, which were particularly resistant to intact

protein hydrolysis [15]. Secondly, it is known that milk protein hydrolysis is protein type dependent, i.e., intact bovine caseins appear to be hydrolyzed faster than bovine whey proteins *in vitro* [16]. However, *in vivo* a lower postprandial AA peak upon casein vs. whey ingestion has been observed, which is attributed to the physicochemical behaviour of casein (i.e., clotting) under the acidic gastric conditions causing slower gastric emptying [17-19].

We aimed to investigate the effect of different protein processing and composition on the postprandial plasma responses *in vivo*. We hypothesized that denaturing whey protein by heating, as well as changing protein composition by including casein, would increase protein hydrolysis kinetics, leading to faster digestion and absorption, and eventually to a higher postprandial plasma AA and insulin peak. We tested this hypothesis in neonatal piglets because they are a well-established animal model of the human infant with respect to body size, gastrointestinal physiology, and amino acid metabolism.

# Material and Methods

## Test proteins

Whey protein isolate (WPI; BiPro™) was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). The protein content of the WPI powder was 92% (w/w) as determined by Dumas method [20] (N x 6.25). The protein composition of WPI was analyzed using reverse phase high pressure liquid chromatography (RP-HPLC) as previously described [21] (**Table 3.1**).

**Table 3.1: Protein composition of the WPI and PBI protein preparations tested.[1]**

| (g/100 g total protein) | WPI | PBI |
|---|---|---|
| α-lactalbumin | 18 | 13 |
| β-lactoglobulin | 68 | 45 |
| $\alpha_{s1}$-casein | n.d. | n.d. |
| β-casein | n.d. | 30 |
| κ-casein | n.d. | n.d. |
| Others | 14 | 12 |

[1]n.d. = not detectable, PBI = protein base ingredient, WPI = whey protein isolate.

Bovine serum albumin (BSA) and immunoglobulins (IGs) cannot be quantified using the RP-HPLC method. However, the supplier of WPI indicates Bovine serum albumin (BSA) and immunoglobulins (IGs) are present, and these proteins could account for the remaining 14% other protein. A protein based ingredient (PBI) for use in infant milk formula was prepared on pilot factory scale using cold membrane filtration as described elsewhere [22]. The composition of PBI powder was 72.7% (w/w) protein as determined by Dumas method (N x

6.25). The PBI powder contained 21.9% (w/w) lactose as determined by high performance liquid chromatography with refractive index detection after pre-clean up with solid-phase extraction C18 cartridge (Eurofins CLF Specialized Nutrition Testing Services, GmbH). PBI protein composition included 30% β-casein as determined by RP-HPLC [21] (**Table 3.1**). The reducing SDS-PAGE analysis reported by McCarthy et al. [22] shows proteins or polypeptide chains which suggest BSA and IGs are present in PBI as well. The 12% by RP-HPLC unidentified protein in PBI could therefore be BSA and IGs as well. The AA compositions of WPI and PBI were determined using ultra pressure liquid chromatography after acid hydrolysis as described previously [23] (**Figure 3.1**).



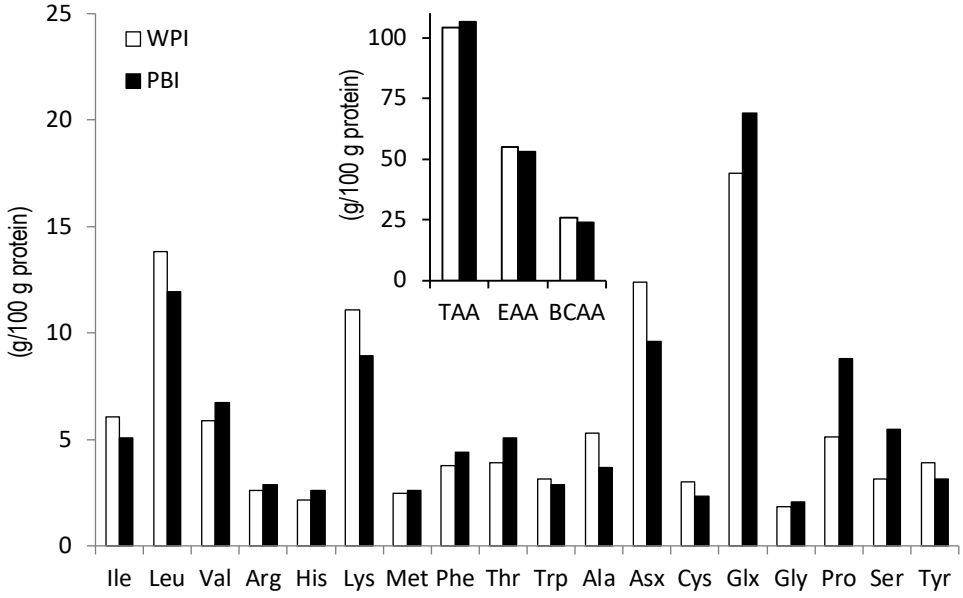


**Figure 3.1: Mean AA composition of the WPI and PBI protein ingredients used to prepare the test solutions as measured in duplicate. PBI = protein-based ingredient, WPI = whey protein isolate. Asx = sum of Asp and Asn, Glx = sum of Glu and Gln, Cys = sum of cysteine and cystine. EAA = sum of Leu, Val, Ile, Phe, Met, Arg, Trp, His, Thr and Lys. TAA = sum of EAA, Tyr, Ser, Glx, Pro, Gly Asx and Ala. BCAA = sum of Ile, Leu and Val. Cys was not included in TAA since it could not be quantified in the plasma samples. Asx and Glx are presented as Asn and Gln are converted to Asp and Glu, respectively, during the acid hydrolysis.**

## Animals

Twenty term, vaginally-delivered piglets (Domestic pigs, species Sus scrofa domesticus) were obtained from an approved local swine farm and transported to the CNRC at 7 days after birth. Piglets were allowed 4 days to acclimate to Children's Nutrition Research Center animal facility housing while being fed a sow's milk formula diet (Litterlife, Merrick's). Piglets were derived from a mixture of Duroc, Hampshire, Yorkshire and Landrace breeds. Piglets were housed individually in stainless steel cages (dimensions 30 cm width x 60 cm height x 60 cm depth) allowing for nose-contact with neighboring cage piglets at 31-32°C with 12 h light (0600-1800 h)/dark cycle. Piglets were provided clean blanket bedding, cage enrichment

and monitored for rectal body temperature and overall clinical health daily by investigators and attending veterinary staff. After acclimation, piglets were surgically implanted with a 20-gauge silicone catheter in the jugular vein and a 6 French polyethylene orogastric feeding tube using aseptic, sterile procedures under general anesthesia with 1-2% isoflurane. The catheters were tunneled subcutaneously, exteriorized dorsally between the shoulders, and attached to tether and harness apparatus fitted on the piglets to allow freedom of movement around the cages. Piglets received sustained release buprenorphine (0.3 mg/mL) at time of surgery for 24-48 h for pain management. The piglets recovered from surgery for 4 d and were fed formula (40 ml/kg; Litterlife, Merrick's) every 4 h and received a jugular infusion of normal saline (2 mL/h) to maintain catheter patency. This rate of formula feeding provided a daily volume of 240 ml and 12.5 g protein per kg body weight. Protein solutions were administered through orogastric catheters, which were placed to ensure that piglets received the full amount of test solution in a time-controlled manner. The study protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, USA) [24].

## Preparation of protein test solutions

All protein test solutions were mixed in batches, using predetermined amounts of water, test protein, and lactose in powdered form to contain a protein level as found in sows' milk [25, 26] 6.25% (w/w) and 1.88 % (w/w) lactose in total. Solutions were stirred until all powder had dissolved. The pH was adjusted to 7.1 by addition of 1 M NaOH. Heat treatment and production of DWPI: 500 mL glass bottles containing 200 mL NWPI were placed in a shaking water bath at 83°C for 20 min, with the water level higher than the level of protein test solution in the bottle. Temperature was monitored and reached a maximum of 80°C after 10 min, the effective heat treatment was therefore 10 min at 80°C. Following heat treatment, bottles were placed in a room temperature water bath for 40 min before placing in a 4°C refrigerator overnight. All test solutions were prepared freshly the day before administration to pigs and stored at 4°C. The morning of the test protein solution feeding, paracetamol was mixed to each test protein solution such that each pig received 60 ml/kg of test protein solution containing 3.75 g protein and 0.02 g/kg paracetamol per unit body weight in the bolus given orogastrically; this ensured a constant dose of protein and paracetamol relative to body weight. The dose of paracetamol was chosen based on previous reports in pigs [27].

## Characterization of protein test solutions

Degree of whey protein denaturation in the protein test solutions was determined by precipitation of denatured protein at pH 4.6 by addition of HCl, followed by centrifugation (15000*g*) and N analysis by Dumas method of the supernatant [28]. The soluble protein proportion in the protein test solutions was determined by Dumas method on the supernatant after centrifugation (15000*g*) at pH 7.1. To assess the extent of Maillardation,

total amino groups were quantified before and after heating using OPA analysis as described previously [29].

## Experimental design



Study design followed an incomplete Latin square model where 20 pigs were randomly assigned to receive two of the three test proteins in random order such that each test protein is replicated (n=13). We used the incomplete Latin Square to limit the feeding to only two of the three test proteins in order to minimize the amount of blood collected from each pig and to complete the study protocol in less than one week given the rapid growth and development of neonatal pigs. In the days between the test protein feeding treatments, pigs were fed sow's milk formula in four feedings per day. During the study, one animal experienced mild diarrhea and after review of the data this animal was dropped out of the analysis. Thus, thirteen animals received PBI (n=13), thirteen animals received NWPI (n=13), and twelve animals received DWPI (n=12), with mean body weight (g)±SD at the time of study of 3336±223, 3393±250, and 3276±322, respectively. On study day 5, following an overnight fast (~8 hr), the piglets were randomly assigned to receive a 60 mL/kg body weight bolus of one of three protein test solutions via the orogastric tube. All staff were blinded to the group assignments until the end of the study and protein test solutions were prepared by a single person at each test period. A baseline blood sample was taken before the bolus and additional blood samples were taken at 15, 30, 45, 60, 90, 120, 150, 180, and 240 min after protein solution administration. Blood samples were collected in EDTA tubes and immediately placed on ice. Samples were then centrifuged (10 min, 4000*g* at 4°C), plasma was transferred to vials, and stored at -80°C until analysis. On study day 8, the above protocol was repeated, with each piglet receiving a second, different protein test solution. At the completion of blood sampling on day 8, piglets were euthanized with an intravenous injection of (1 ml/4.5 kg) a commercially available euthanasia solution (Beauthanasia; Merck Animal Health, Madison, NJ).

## Plasma sample analysis

Plasma levels of glucose, insulin, paracetamol, GLP-1, and AA were measured for each piglet at each sampling point. Glucose was measured with a glucose oxidase kit (Infinity Glucose Oxidase Liquid Stable Reagent; Thermo Scientific, Middletown, VA). To assess gastric emptying kinetics paracetamol was measured with an enzymatic assay (Paracetamol enzyme assay Kit; Cambridge Life Sciences, Ely, UK). Insulin was measured using a radioimmunoassay (Porcine Insulin RIA; Millipore, Darmstadt, Germany). GLP-1 was measured with an ELISA (Glucagon-Like Peptide-1 (Active) ELISA; Millipore, Darmstadt, Germany). Individual plasma free AA concentrations were determined by reverse-phase HPLC, without prior acid hydrolysis, of their phenylisothiocyanate derivatives, as described previously [30]. In this method cysteine and cystine cannot be quantified accurately.

## Data analysis

BCAA was calculated as a sum of isoleucine (Ile), leucine (Leu) and valine (Val). Total essential amino acids (EAA) was calculated as a sum of 10 AA, which are the BCAA mentioned above and arginine (Arg), histidine (His), phenylalanine (Phe), lysine (Lys), methionine (Met), threonine (Thr), and tryptophan (Trp). Arg was included because it is considered essential for piglets [31]. TAA was calculated as a sum of 19 proteinogenic AA, which are the EAAs mentioned above and asparagine (Asn), aspartate (Asp), alanine (Ala), glutamate (Glu), glutamine (Gln), glycine (Gly), proline (Pro), serine (Ser), and tyrosine (Tyr).

Plasma concentrations were analyzed by generalized estimating equations (GEE) with exchangeable correlation structure, and robust estimation was used in order to account for repeated measures (IBM SPSS Statistics). Pig was the subject factor and study period is included in both the model comparing treatments with respect to area-under-the-curve (AUC) and the model comparing treatments across time. The AUC was calculated using incremental trapezoidal integration. Pairwise comparison among treatments were made when indicated by a significant treatment effect and treatment comparisons at specific time points done only when indicated by a significant treatment x time interaction.

Plasma paracetamol TAA, EAA, BCAA and individual EAA concentrations were fitted with a model describing a first order absorption of a single dose with linear elimination (equation 1).

(1) $$C(t) = b + \frac{D \times S}{V} \times \frac{k_a}{k_a - k}\left(e^{-kt} - e^{-k_a t}\right)$$

In this equation C(t) [μM] is the concentration of AA or paracetamol as function of postprandial time t [min], D is the administered dose [μmol], V is the total blood volume [L], $k_a$ is the rate constant for absorption [$min^{-1}$], k is the rate constant for elimination [$min^{-1}$]. The standard model does not include effects of splanchnic extraction and systemic protein breakdown. Hence, for AA, D was multiplied with a factor S that accounts for the proportion of the administered dose that is not extracted by the splanchnic mass, plus the effect of systemic protein breakdown inhibition. The value of S was based on the obtained highest coefficient of determination ($R^2$) and lowest %CV in $k_a$ and k after fitting, which was at a value of 0.15. To account for the basal plasma AA concentrations a constant b was included as well in the equation. For V a value of 100 mL/kg bodyweight was used [32]. Solver function in Microsoft Excel was used to calculate the values for $k_a$, k and b that resulted in the lowest sum of squares of residuals, as well as to calculate the concentration maximum ($C_{max}$) and time to reach $C_{max}$ ($t_{Cmax}$) using the equation with the calculated $k_a$, k and b. Calculated fitting parameters were analyzed using general linear model univariate ANOVA with pig and study period included as covariates in the model (IBM SPSS statistics).

Power analysis indicated that, based on our prior experience with these measurements, and between-animal variation of up to 25-30%, our statistical power calculations indicate 12 pigs/group are necessary to detect, with a type I error of 0.05 and a power of 0.80, a difference of 5-10% in various endpoints of amino acid concentrations. As mentioned above in study design, we started with a total of 20 pigs but had one animal drop out of the study and thus the final analysis includes following group sizes PBI (n=13), NWPI (n=13), and DWPI (n=12). Pairwise comparisons among treatments were made with Bonferroni correction. Differences with a probability <0.05 were considered significant and are indicated in tables and figures by symbols. We used symbols to denote significance of pair-wise comparisons to simplify presentation rather than show multiple letters on graphs.



## Results

### Characterization of protein test solutions

Protein in NWPI was 100% soluble, 91% native and 9% denatured, protein in DWPI was also 100% soluble but only 9% was native and 91% denatured. Protein in PBI was 97% soluble and 73% of the protein was soluble at pH 4.6. Since 30% of the protein in PBI is β-casein (**Table 3.1**), which precipitates at pH 4.6, the whey protein fraction is considered to be 100% native. Assessment of degree of Maillardation by total free amino groups analysis showed no difference between NWPI and DWPI (52±1.6 mM and 56±5.4 mM, respectively).

### Postprandial plasma responses

No significant differences were noted in postprandial plasma concentrations of paracetamol, glucose and GLP-1 among the three protein test solutions (**Figure 3.2A, C and D**). Fitting parameters for plasma paracetamol concentration were also not significantly different among the three protein test solutions (**Table 3.3**). Significant differences were noted in postprandial plasma concentrations of insulin, TAA, EAA, BCAA, and most individual AA among administration of the three protein test solutions. The largest differences were present in the first hour after feeding the protein test solutions.

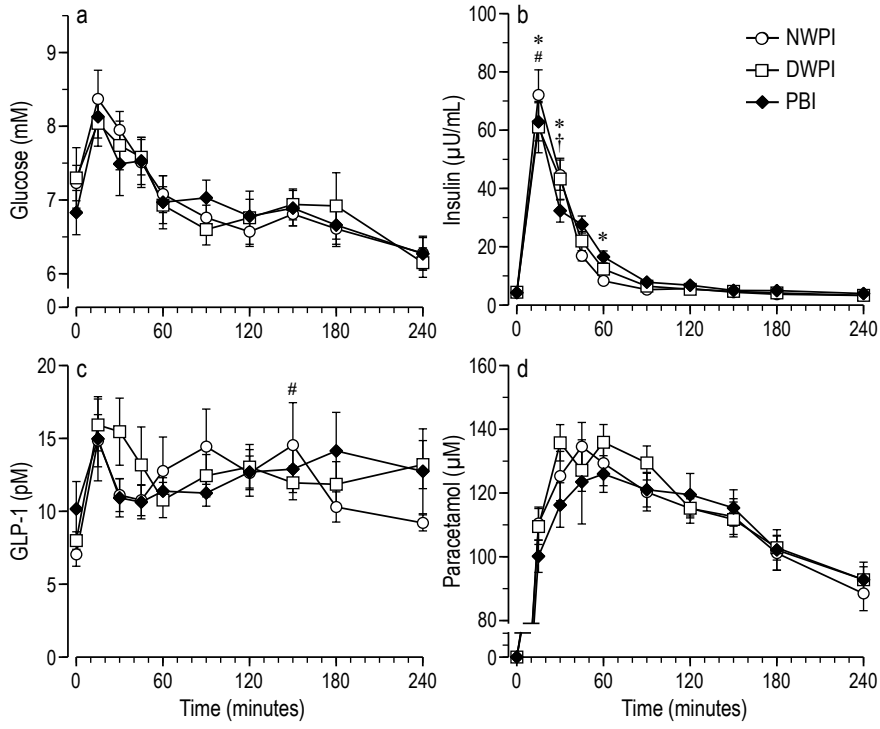


**Figure 3.2: Time course of postprandial plasma concentrations from baseline up to 4 hr post-administration (mean±SEM, n=12-13); a) Glucose, b) Insulin, c) GLP-1, d) Paracetamol; significant effect of time post-administration in all parameters (p<0.01); #significant difference NWPI vs. DWPI, *significant difference PBI vs. NWPI (treatment*time, p<0.05), †significant difference PBI vs. DWPI (treatment*time, p<0.05); GLP-1 = glucagon-like peptide 1, PBI = protein based ingredient, NWPI = native whey protein isolate, DWPI = denatured whey protein isolate.**

## Effect of denaturation

DWPI induced a ~20% lower plasma insulin spike than NWPI at 15 min (**Figure 3.2B**), however, the plasma insulin AUC was not significantly different in DWPI vs. NWPI (**Table 3.2**). Plasma TAA concentrations per time point were not significantly different between NWPI and DWPI (**Figure 3.3A**), neither were the plasma TAA AUC and the plasma TAA fitting parameters (**Table 3.3**). Plasma EAA concentrations per time point were significantly lower (~10%) in DWPI than upon NWPI at 15 and 30 min (**Figure 3.3B**), however, the plasma EAA AUC was not significantly different, neither were the plasma EAA fitting parameters. Plasma BCAA concentrations per time point were significantly lower (13-19%) upon DWPI than upon NWPI from 15 to 60 min (**Figure 3.3C**). However, the plasma BCAA AUC was not significantly different, neither were the plasma BCAA fitting parameters upon DWPI vs. upon NWPI.

**Table 3.2: AUC values for plasma insulin and AA during the 4 hr after feeding.[1]**

| | NWPI | DWPI | PBI |
|---|---|---|---|
| Insulin (mU/mL*min) | 3.13±0.23 | 3.06±0.29 | 3.06±0.19 |
| TAA (mM*min) | 1736.38±52.28[a] | 1692.36±89.02[a] | 1928.16±70.06[b] |
| EAA (mM*min) | 815.45±33.19 | 786.71±59.33 | 875.65±51.35 |
| BCAA (mM*min) | 254.31±9.11[a] | 238.55±17.60[a] | 289.84±13.76[b] |
| Ile (mM*min) | 64.91±2.82 | 63.60±4.07 | 65.67±4.69 |
| Leu (mM*min) | 103.94±5.00 | 97.01±8.24 | 107.41±7.13 |
| Val (mM*min) | 85.79±3.50[a] | 77.41±6.04[a] | 116.92±3.74[b] |
| Arg (mM*min) | 31.31±3.33[a] | 32.25±3.48[a] | 38.36±2.92[b] |
| His (mM*min) | 22.26±2.62[a] | 30.02±3.83 | 33.91±3.04[b] |
| Met (mM*min) | 48.75±2.45[a] | 40.53±2.65[b] | 57.89±2.53[c] |
| Phe (mM*min) | 18.72±1.18[a] | 23.82±2.21[b] | 32.43±1.09[c] |
| Trp (mM*min) | 19.99±1.42[a] | 17.32±1.89 | 16.51±1.24[b] |
| Gln (mM*min) | 150.69±6.08 | 140.81±6.33[a] | 161.13±7.72[b] |
| Glu (mM*min) | 39.17±2.21 | 37.07±2.84[a] | 42.92±2.57[b] |
| Pro (mM*min) | 81.44±2.56[a] | 83.00±3.88[a] | 141.83±5.50[b] |

[1]AUC (mean±SEM, n=12-13). Different letter superscripts indicate significant differences at $p<0.05$. AA = amino acid, AUC = area under the curve, PBI = protein base ingredient, NWPI = native whey protein isolate, DWPI = denatured whey protein isolate.

**Table 3.3: Curve fitting values for plasma AAs and paracetamol after feeding.**[1]

| | NWPI | DWPI | PBI |
|---|---|---|---|
| TAA | | | |
| $k_a$ (*$10^{-3}$) | 1.81±0.186[a] | 1.62±0.232[a] | 3.31±0.276[b] |
| k (*$10^{-3}$) | 27.2±3.6 | 24.5±1.61 | 29.9±4.78 |
| b (μM) | 4418±221.7 | 4265±235.7 | 3770±180.7 |
| $R^2$ | 0.726±0.061 | 0.655±0.058 | 0.802±0.033 |
| $C_{max}$ (μM) | 7657±242[a] | 7450±447[a] | 9010±340[b] |
| $t_{Cmax}$ (min) | 120.4±10.9 | 125.3±6.3[a] | 94.9±8.5[b] |
| EAA | | | |
| $k_a$ (*$10^{-3}$) | 1.87±0.241[a] | 1.93±0.288[a] | 3.58±0.281[b] |
| k (*$10^{-3}$) | 21.4±1.8 | 19.9±1.68 | 25.8±3.95 |
| b (μM) | 1832±178.9[a] | 1544±156.2 | 1358±132.9[b] |
| $R^2$ | 0.728±0.069 | 0.704±0.058 | 0.829±0.037 |
| $C_{max}$ (μM) | 3723.5±150.4 | 3638.3±287.0 | 4255.3±255.8 |
| $t_{Cmax}$ (min) | 138.8±13.6 | 145.1±16.6[a] | 99.8±8.4[b] |
| BCAA | | | |
| $k_a$ (*$10^{-3}$) | 2.72±0.428 | 1.76±0.287[a] | 3.62±0.312[b] |
| k (*$10^{-3}$) | 35.8±6.1 | 25.9±2.49 | 33.8±6.26 |
| b (μM) | 344±62.7 | 350±36.9 | 286±38.4 |
| $R^2$ | 0.761±0.036 | 0.721±0.042 | 0.824±0.034 |
| $C_{max}$ (μM) | 1222.9±52.2 | 1129.7±101.3[a] | 1469.9±82.7[b] |
| $t_{Cmax}$ (min) | 94.0±9.9 | 127.6±16.9 | 91.0±10.4 |
| Paracetamol | | | |
| $k_a$ (*$10^{-3}$) | 3.89±0.182 | 3.90±0.207 | 3.31±0.180 |
| k (*$10^{-3}$) | 49.4±1.9 | 48.4±2.56 | 44.2±2.10 |
| $R^2$ | 0.872±0.023 | 0.849±0.054 | 0.863±0.019 |
| $C_{max}$ (μM) | 140.3±5.2 | 143.7±7.4 | 134.8±6.3 |
| $t_{Cmax}$ (min) | 56.8±2.0 | 58.4±3.2 | 64.9±2.7 |

[1](mean±SEM, n=12-13). Different letter superscripts indicate significant differences at $p<0.05$. b = fasting basal concentration, BCAA = branched chain amino acid, $C_{max}$ = peak maximum concentration, DWPI = denatured whey protein isolate, EAA = essential amino acid, k = rate constant for elimination, $k_a$ = rate constant for absorption, NWPI = native whey protein isolate, PBI = protein based ingredient, $R^2$ = coefficient of determination, TAA = total amino acids, $t_{Cmax}$ = time at peak concentration.

In the DWPI group, plasma Ile at 30 and 45 min, Leu from 0 to 60 min, Val from 30 to 60 min, Lys from 0 up to 45 min, Met from 90 up to 180 min and Trp from 15 up to 60 min was lower than in NWPI (**Figure 3.4**). However only the plasma Met AUC was lower in DWPI vs. NWPI. In contrast, in the DWPI group, His from 90 to 180 min and Phe from 60 to 120 min was higher than in NWPI. However, only the plasma Phe AUC was significantly higher in DWPI vs. NWPI. Plasma Arg and Thr concentrations in the DWPI group were not different from NWPI. The only significantly different fitting parameter found for plasma individual EAA was the rate constant for absorption $k_a$ for Val which was a 1.7-fold higher in DWPI vs. NWPI (**Supplemental Table S3.1**).

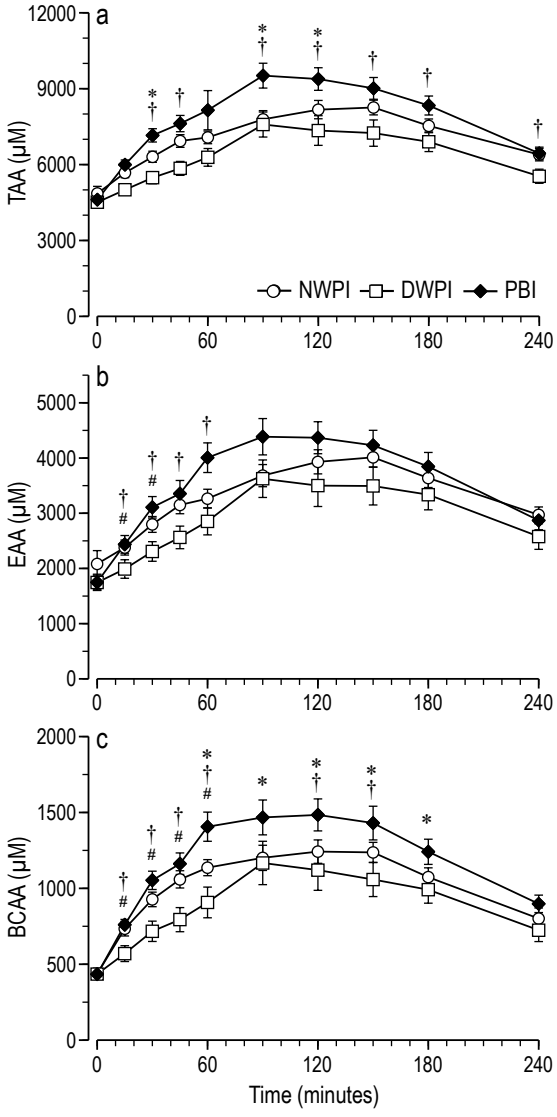


**Figure 3.3: Time course of postprandial plasma summed AA concentrations from baseline up to 4 hr post-administration (mean±SEM, n=12-13); a) TAA b) EAA and c) BCAA; significant effect of time post-administration in all parameters (p<0.01); significant effect of treatment (p<0.05) for BCAA; #significant difference NWPI vs. DWPI (treatment*time, p<0.05); *significant difference PBI vs. NWPI (treatment*time, p<0.05); †significant difference PBI vs. DWPI (treatment*time, p<0.05); BCAA = branched chain amino acid, EAA = essential amino acids, TAA = total amino acids, PBI = protein based ingredient, NWPI = native whey protein isolate, DWPI = denatured whey protein isolate.**

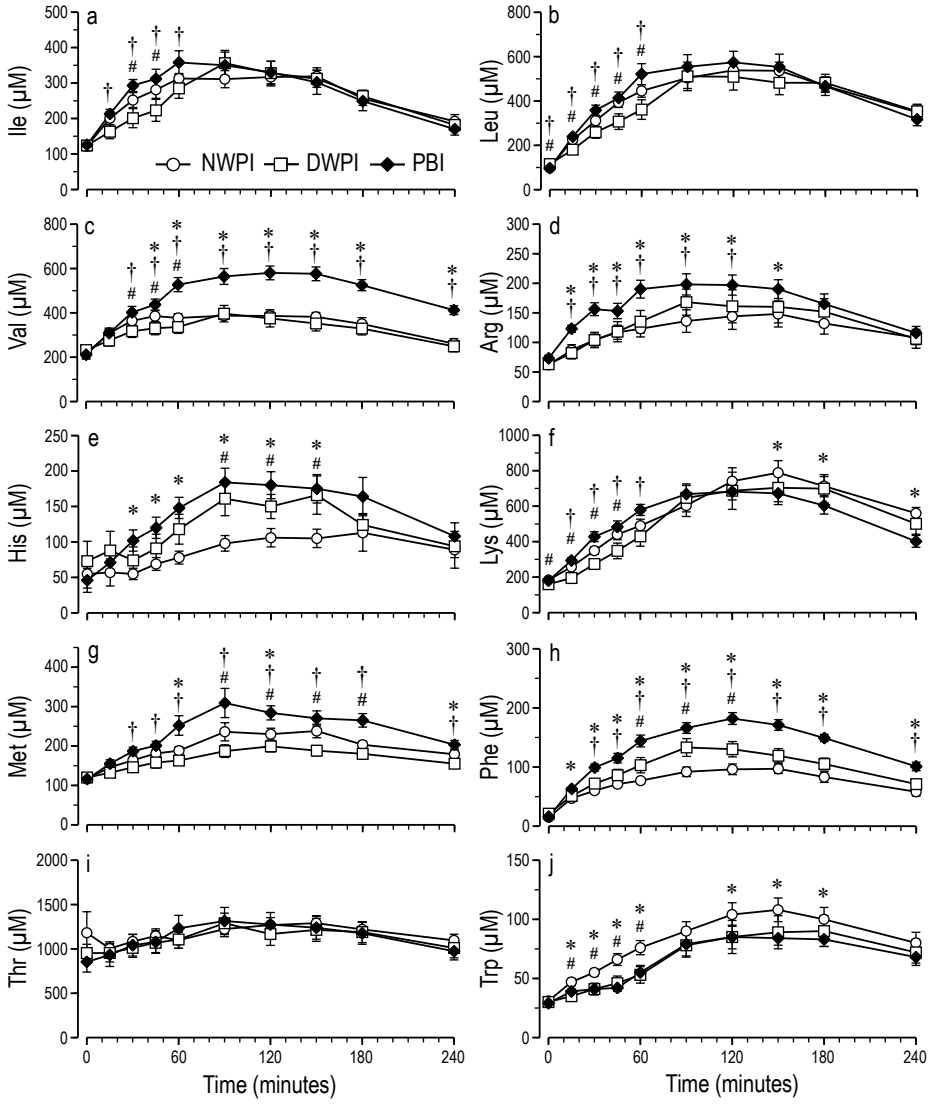


**Figure 3.4: Time course of postprandial plasma concentrations of individual essential amino acids from baseline up to 4 hr post-administration (mean±SEM, n=12-13); a) Ile b) Leu c) Val d) Arg e) His f) Lys g) Met h) Phe i) Thr j) Trp; significant effect of time post-administration in all parameters ($p<0.01$); significant effect of treatment ($p<0.05$) for Val, Arg, His, Met, Trp, Phe; $^{\#}$significant difference NWPI vs. DWPI; $^{*}$significant difference PBI vs. NWPI (treatment*time, $p<0.05$); $^{\dagger}$significant difference PBI vs. DWPI (treatment*time, $p<0.05$); PBI = protein based ingredient, NWPI = native whey protein isolate, DWPI = denatured whey protein isolate.**

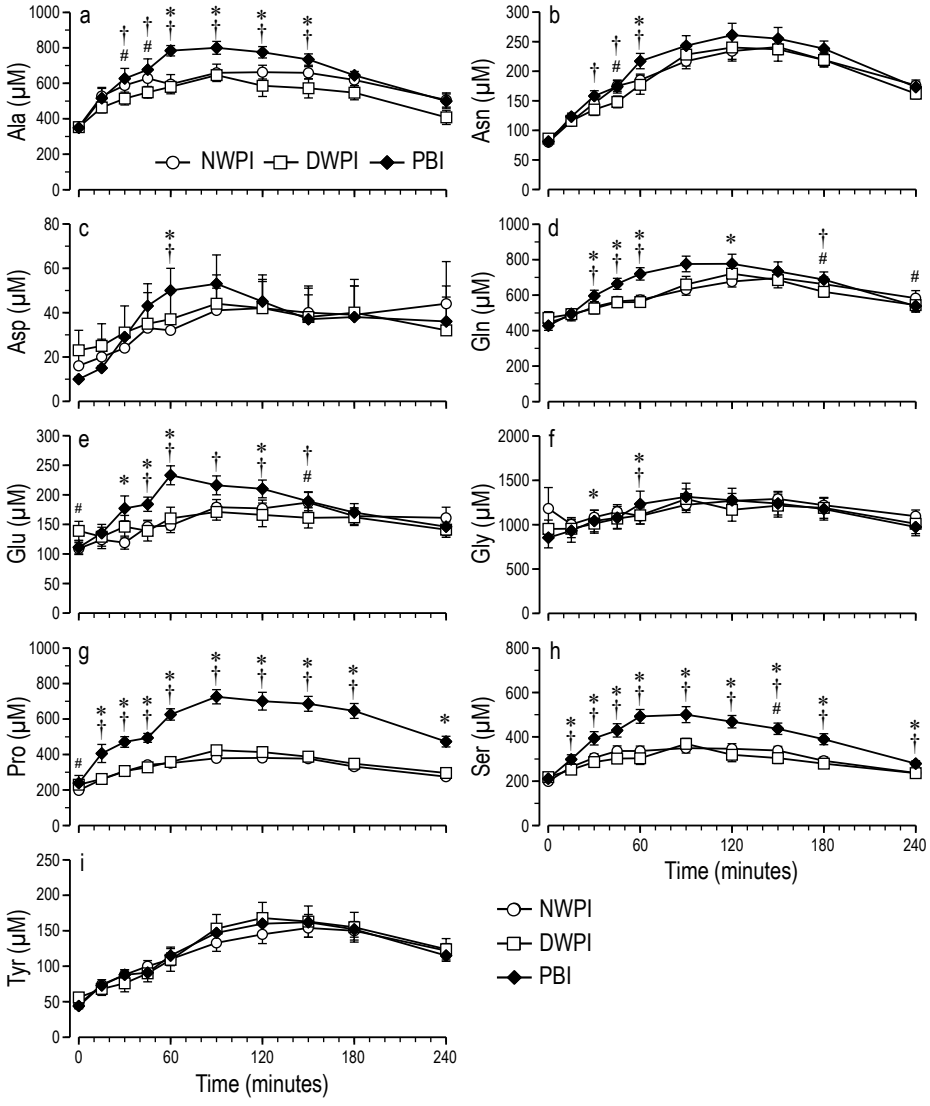


**Figure 3.5: Time course of postprandial plasma concentrations of individual non-essential amino acids from baseline up to 4 hr post-administration (mean±SEM, n=12-13); a) Ala, b) Asn, c) Asp, d) Gln, e) Glu, f) Gly, g) Pro, h) Ser, i) Tyr; significant effect of time post-administration in all parameters ($p<0.01$); significant effect of treatment ($p<0.05$) for Ala, Ser, Pro, Gln, Glu; $^{\#}$significant difference NWPI vs. DWPI (treatment*time, $p<0.05$); $^{*}$significant difference PBI vs. NWPI (treatment*time, $p<0.05$); $^{\dagger}$significant difference PBI vs. DWPI (treatment*time, $p<0.05$); PBI = protein based ingredient, NWPI = native whey protein isolate, DWPI = denatured whey protein isolate.**

In the DWPI group, plasma at Ala 30 and 45 min and Asn at 45 min was lower than in NWPI (**Figure 3.5**). No significant differences in Asp, Glu, Gln, Gly, Pro, Ser and Tyr plasma concentrations were found between DWPI and NWPI. Plasma Orn and Tau were also higher in PBI vs DWPI (**Supplemental figure S3.1**).

## Effect of protein composition

PBI induced a lower plasma insulin concentration than NWPI at 15 and 30 min, while it induced higher insulin concentration at 60 min. The plasma insulin AUC was not significantly different.

Plasma TAA concentrations were significantly higher in PBI than NWPI at 30, 90 and 120 min, the plasma TAA AUC was significantly higher in PBI than NWPI as well. The TAA content (g/100g protein) of PBI was ~1.3% higher than of WPI (**Figure 3.1**). The fitting parameter $k_a$ for TAA was almost a twofold higher and also the calculated TAA $C_{max}$ was also higher in PBI than NWPI. Plasma EAA concentrations and AUCs in PBI were higher, but not significantly different from NWPI (**Figure 3.3B**). The EAA content (g/100g protein) of PBI was ~4% lower than that of NWPI. Concomitantly the fitting parameter $k_a$ for EAA was almost a twofold higher in PBI than NWPI. Plasma BCAA concentrations were higher in PBI vs. NWPI from 60 to 180 min, the plasma BCAA AUC was approximately 14% higher in PBI vs. NWPI. The BCAA content (g/100g protein) of PBI was ~8% lower than that of NWPI. There were no significant differences found in the fitting parameters of plasma BCAA concentration in PBI vs. NWPI.

In the PBI group, plasma Val from 45 to 240 min, Arg from 15 to 150 min, His from 45 to 150 min, Met at 60, 90 and 240 min and Phe from 15 to 240 min was higher than for NWPI. The plasma Val, Arg His, Met and Phe AUCs were also higher in PBI vs. NWPI, respectively 38, 22, 52, 19 and 73%. PBI Val, Arg, His, Met and Phe content (g/100g protein) was higher than that of NWPI. The fitting parameter $k_a$ was higher only for His and Lys upon PBI compared to NWPI. Plasma $C_{max}$ was calculated to be higher for Val, Arg and Phe in PBI vs. NWPI. In addition, a higher k and lower $t_{Cmax}$ for Lys was found in PBI vs. NWPI. In contrast, in PBI, plasma Trp from 15 to 60 min and Lys from 150 to 240 min vs. NWPI, also the plasma Trp AUC was 21% lower in PBI vs. NWPI. Plasma Leu and Thr concentration and AUC were not different between PBI vs. NWPI. PBI Leu content (g/100g protein) was less than that of NWPI. Concomitantly fitting parameter $k_a$ of Leu was higher in PBI vs. NWPI. In PBI, plasma Ser from 15 to 240 min, Gln and Glu from 30 to 60 and at 120 min, Pro from 15 to 240 min, Gly at 30 and 60 min, Asp and Asn at 60 min and Ala from 60 up to 150 min were higher than NWPI. However, only the plasma Pro AUC was significantly higher (74%) in PBI vs. NWPI. No significant differences in Tyr plasma concentration between PBI and NWPI were found.

Differences in postprandial plasma responses between PBI and DWPI generally followed the same pattern as between PBI and NWPI. Plasma insulin concentration was lower at 30 min (**Figure 3.2B**), but plasma insulin AUC was not significantly different in PBI vs. DWPI. Plasma TAA concentrations from 30 to 240 min and AUC were higher in PBI than DWPI, with the exception at 60 min (**Figure 3.3A**). The fitting parameter $k_a$ for TAA was almost a twofold higher, the calculated $C_{max}$ was ~21 higher in PBI than DWPI. Plasma EAA concentrations in PBI were higher than DWPI from 15 to 60 min, however the plasma EAA AUC was not significantly

different. The fitting parameter $k_a$ for EAA was almost a twofold higher in PBI vs. DWPI. Plasma BCAA concentrations in PBI were also higher vs. DWPI from 15 to 150 with the exception of 90 min (**Figure 3.3C**), the plasma BCAA AUC was approximately 22% higher in PBI vs. DWPI. The fitting parameter $k_a$ for BCAA was a twofold higher, in addition a 30% higher plasma $C_{max}$ was calculated from the fitted curves in PBI than DWPI. In the PBI group, plasma Ile from 45 to 60, Leu from 0 to 60 min, Val from 45 to 240 min, Arg from 15 to 120 min, Lys from 15 to 60 min Met from 45 to 240 min and Phe from 45 to 240 was higher than in DWPI. The plasma Val, Arg, Met and Phe AUCs were also higher in PBI vs. DWPI, respectively 51, 19, 42 and 36%. The fitting parameter $k_a$ was found higher only for Leu and Lys in PBI compared to DWPI. Plasma Val, Met and Phe $C_{max}$ were calculated to be higher in PBI vs. DWPI.



## Discussion

Increased concentrations of protein in infant formula vs. human milk have been linked to overweight and obesity risk in children. The early protein hypothesis that has been postulated to explain these differences in growth rate is mechanistically linked to high insulin and IGF-1 levels that are released due to increased plasma AA concentrations. In this study, our aim was to investigate whether changing milk protein denaturation or composition would affect postprandial plasma AA and insulin concentrations. We found that the difference in protein composition significantly impacted postprandial plasma responses (PBI vs. NWPI). While PBI resulted in a lower plasma insulin spike within 15 min of feeding, the plasma concentrations of most AA and BCAA were higher with PBI compared to NWPI. The plasma insulin and GLP-1 plasma AUC were not different between the three protein test solutions. In contrast, the plasma AUC of BCAA was ~14% higher in PBI even though the BCAA content of PBI is ~8% lower than WPI. Since BCAA, especially Leu, and also Arg, are known to promote insulin secretion [33], our finding of lower plasma insulin peak in PBI was not expected. The largest increase in plasma concentration among the BCAA in PBI was for Val, which is a weak insulin secretagogue compared to Leu and Ile [34, 35]. Unexpectedly, we found little effect of denaturation (DWPI vs. NWPI) which runs counter to our hypothesis that denatured proteins, as compared to native proteins, lead to faster absorption of digestion products and increased plasma AA concentrations. This finding is consistent with Barbe et al [36] that observed no significant differences in plasma leucine between unheated and heated (90°C for 10 min) milk protein (containing 80% casein) in mini pigs.

During multiple time points in the postprandial period, the plasma concentrations of several AA were consistently higher with the PBI vs. NWPI/DWPI solutions resulting in higher plasma AUC for these AA. We did not expect this result given that the EAA content of NWPI/DWPI test solutions were higher than PBI. Moreover, by curve fitting we found that the rate constant for absorption ($k_a$) was higher and the $t_{Cmax}$ was earlier for EAA in the PBI group. The

total BCAA content of NWPI/DWPI was slightly higher than PBI, yet Val was more enriched in PBI. Given these differences in BCAA composition, we found that the time course of plasma BCAA levels and plasma AUC were both greater in PBI, especially for Val. This was reflected by the higher $k_a$ of BCAA and Leu for PBI vs. DWPI. We suggest that the observed differences in plasma postprandial AA kinetics, especially BCAA, between the test protein groups are therefore likely related to protein digestion and absorption kinetics in addition to AA composition alone.

The key compositional difference between PBI vs. NWPI/DWPI was that PBI contained β-casein. The fast and slow protein concept as postulated by Boirie is based on the observations that postprandial plasma AA peak height appears to be protein type dependent [17]. For example, whey protein ingestion results in a higher postprandial and earlier AA peak than casein protein ingestion. This slower postprandial AA peak with casein vs. whey protein has been attributed to the slower gastric emptying rate due to the distinct physicochemical behaviour (i.e. clotting) of the proteins under the acidic gastric conditions [18]. *In vitro* investigation of the gastric clotting behaviour using a method described earlier [37] showed that PBI, in contrast to similar preparations containing α-, β-, and κ-caseins, did not form particles larger than 1 mm (data not shown). This indicates that for gastric clotting to form particles that could be contained by gastric pyloric sieving (i.e. larger than 1 mm [38]) a mixture of caseins may be needed. In our current study, the presence of β-casein in the PBI did not alter gastric emptying as assessed by plasma paracetamol appearance, suggesting that indeed a "slowing" effect of β-casein on gastric emptying did not occur. Paracetamol is a water-soluble marker and may therefore not reflect emptying of solidified material. However, a slowing of gastric emptying would not explain the higher plasma AA concentrations as peaks were higher in PBI vs. NWPI. Therefore, it is more likely to be due to the rates of digestion and absorption of AAs in the small intestine.

Previously a complex 13 compartmental model was built to study postprandial nitrogen metabolism. This model demonstrated that intestinal absorption kinetics are affected by protein quantity and quality intake and this in turn impacts first-pass splanchnic catabolism and peripheral availability of dietary amino acids [39]. In this study we used a pharmacokinetic model to fit postprandial plasma amino acid concentrations. Thus, because we did not isotopically label the amino acids in the test proteins, we cannot discriminate between amino acids appearing in the plasma derived from the test protein compared to whole body tissue proteolysis. However, with the adaptations to the model (i.e. inclusion of a term for basal concentrations, and a determined mean fraction of uptake), we were able to gain insights in the relation between protein (and AA) composition on postprandial plasma responses by determining the rate constants for absorption ($k_a$). The fraction of absorption (S) that gave the best fit of our experimental data was 0.15, meaning the sum of splanchnic extraction and inhibition of systemic protein breakdown is estimated to be 85%,

this is in line with what has been found experimentally. Others have experimentally shown that in neonatal piglets the splanchnic extraction of BCAA could amount 44% [40], while we have previously found that the portal-drained viscera can account for use of up to 90% of diet AA [41].



One potential limitation of this study is that the piglets were not fed a complete meal. The test solutions contained only protein and lactose. We aimed to investigate the effects of the protein with as few potential confounders as possible, to understand the mechanism. However, feeding these same proteins as part of a complete meal (i.e. including lipids and higher levels of lactose) or in larger, single bolus feedings may impact the results and warrants further study.

In summary, we conclude that a protein milk composition including β-casein (PBI) did not significantly delay gastric emptying or show slower digestion kinetics compared to a whey protein isolate. The PBI protein mixture did result in a lower postprandial insulin peak, but higher postprandial concentrations of BCAA and several individual AA compared to NWPI/DWPI. The differences observed between PBI and NWPI can be partly explained by the difference in AA composition, but we suggest that differences in protein digestion and absorption kinetics are also involved. Furthermore, we conclude that under the used conditions almost complete whey protein denaturation (91% denatured vs. 91% native) does not result in faster digestion and absorption as reflected in postprandial plasma responses. Curve fitting using pharmacokinetic models can be helpful in gaining insights in the mechanism underlying differences in postprandial responses of protein test solutions with different protein and AA composition by discriminating effects of AA content (D) and kinetics of digestion and absorption ($k_a$).

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# Supplementary material

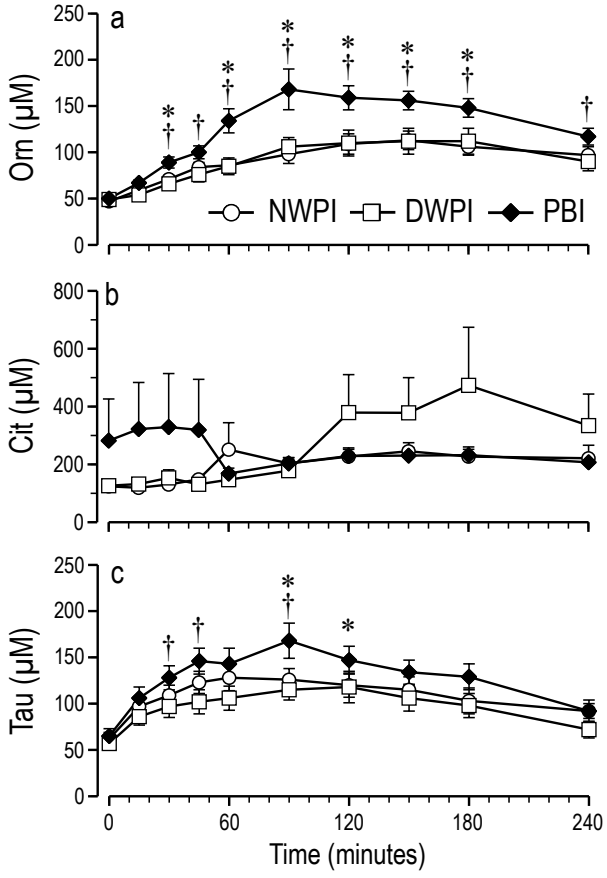
**Supplemental Table S3.1: Curve fitting values for individual plasma AAs after feeding.[1]**

| | | NWPI | DWPI | PBI | Sig. |
|---|---|---|---|---|---|
| Ile | | | | | |
| | $k_a$ (*$10^{-3}$) | 3.83±0.823 | 2.66±0.415 | 4.97±0.734 | |
| | k (*$10^{-3}$) | 40.5±6.3 | 28.7±3.52 | 37.0±5.52 | |
| | b (μM) | 88±20.0 | 7414.1 | 79±10.6 | |
| | $R^2$ | 0.724±0.049 | 0.677±0.046 | 0.779±0.067 | |
| | $C_{max}$ (μM) | 328.3±12.2 | 310.8±26.2 | 357.4±27.8 | |
| | $t_{Cmax}$ (min) | 79.8±9.3 | 108.1±13.5 | 71.2±8.6 | |
| Leu | | | | | |
| | $k_a$ (*$10^{-3}$) | 2.12±0.302 | 1.46±0.236 | 3.30±0.308 | *, † |
| | k (*$10^{-3}$) | 27.4±4.7 | 20.4±2.33 | 34.9±7.00 | |
| | b (μM) | 62±16.4 | 65±14.9 | 39±9.3 | |
| | $R^2$ | 0.816±0.031 | 0.781±0.039 | 0.820±0.026 | |
| | $C_{max}$ (μM) | 516.7±30.3 | 479.0±46.5 | 556.8±37.8 | |
| | $t_{Cmax}$ (min) | 123.7±14.0 | 166.8±26.1 | 94.3±11.1 | † |
| Val | | | | | |
| | $k_a$ (*$10^{-3}$) | 4.19±0.477 | 2.52±0.527 | 2.95±0.244 | # |
| | k (*$10^{-3}$) | 68.4±13.3 | 50.7±9.20 | 23.3±2.39 | * |
| | b (μM) | 188±24.9 | 198±18.1 | 171±20.5 | |
| | $R^2$ | 0.696±0.045 | 0.531±0.075 | 0.850±0.034 | † |
| | $C_{max}$ (μM) | 406.9±18.0 | 368.5±34.3 | 564.8±26.5 | *, † |
| | $t_{Cmax}$ (min) | 60.8±10.1 | 89.3±18.6 | 111.4±9.7 | * |
| Arg | | | | | |
| | $k_a$ (*$10^{-3}$) | 28.2±0.415 | 3.06±0.506 | 4.42±0.527 | |
| | k (*$10^{-3}$) | 33.9±7.6 | 22.5±3.65 | 24.4±2.28 | |
| | b (μM) | 54±7.0 | 47±4.8 | 59±6.0 | |
| | $R^2$ | 0.708±0.044 | 0.686±0.068 | 0.762±0.076 | |
| | $C_{max}$ (μM) | 141.2±18.6 | 157.3±23.8 | 196.6±14.5 | * |
| | $t_{Cmax}$ (min) | 105.0±11.5 | 128.3±20.2 | 90.0±6.4 | |
| His | | | | | |
| | $k_a$ (*$10^{-3}$) | 1.65±0.354 | 2.36±0.515 | 3.52±0.347 | * |
| | k (*$10^{-3}$) | 23.3±3.8 | 20.8±3.55 | 19.8±2.92 | |
| | b (μM) | 34±12.0 | 52±22.5 | 31±16.2 | |
| | $R^2$ | 0.643±0.105 | 0.645±0.097 | 0.760±0.040 | |
| | $C_{max}$ (μM) | 163.5±67.8 | 144.1±18.7 | 176.7±19.1 | |
| | $t_{Cmax}$ (min) | 892.6±759.6 | 138.7±26.2 | 132.1±21.2 | |
| Lys | | | | | |
| | $k_a$ (*$10^{-3}$) | 2.18±0.336 | 2.13±0.394 | 4.15±0.348 | *, † |
| | k (*$10^{-3}$) | 11.8±1.2 | 11.8±1.09 | 23.9±5.54 | *, † |
| | b (μM) | 119±18.6 | 69±15.4 | 106±18.0 | |
| | $R^2$ | 0.856±0.021 | 0.810±0.039 | 0.822±0.028 | |
| | $C_{max}$ (μM) | 729.7±47.5 | 673.2±80.3 | 663.7±36.8 | |
| | $t_{Cmax}$ (min) | 218.7±33.8 | 215.8±37.1 | 105.5±9.0 | † |

**Supplemental Table S3.1 (continued): Curve fitting values for individual plasma AAs after feeding.[1]**

| | NWPI | DWPI | PBI | Sig. |
|---|---|---|---|---|
| Met | | | | |
| $k_a$ (*$10^{-3}$) | 2.64±0.412 | 2.39±0.587 | 4.22±0.601 | |
| k (*$10^{-3}$) | 17.8±1.7 | 55.2±30.40 | 18.2±2.68 | |
| b (µM) | 104±5.7 | 108±11.8 | 91±10.0 | |
| $R^2$ | 0.757±0.051 | 0.572±0.086 | 0.832±0.044 | † |
| $C_{max}$ (µM) | 242.0±21.9 | 187.2±12.9 | 282.6±17.7 | † |
| $t_{Cmax}$ (min) | 283.2±160.5 | 135.7±26.9 | 118.0±9.6 | |
| Phe | | | | |
| $k_a$ (*$10^{-3}$) | 2.10±0.194 | 2.40±0.295 | 2.82±0.177 | |
| k (*$10^{-3}$) | 44.4±14.4 | 24.7±2.05 | 23.0±1.72 | |
| b (µM) | 11.44±3.12 | 9.10±2.80 | 6.22±3.55 | |
| $R^2$ | 0.770±0.035 | 0.831±0.036 | 0.840±0.034 | |
| $C_{max}$ (µM) | 91.1±6.3 | 120.0±11.5 | 164.9±6.7 | *, † |
| $t_{Cmax}$ (min) | 105.2±14.6 | 121.2±19.4 | 108.3±5.7 | |
| Thr | | | | |
| $k_a$ (*$10^{-3}$) | 2.35±0.498 | 3.32±0.642 | 4.49±0.585 | |
| k (*$10^{-3}$) | 18.6±2.6 | 21.2±3.89 | 29.2±7.35 | |
| b (µM) | 963±83.3 | 853±104.3 | 755±109.0 | |
| $R^2$ | 0.554±0.090 | 0.520±0.085 | 0.694±0.059 | |
| $C_{max}$ (µM) | 1262.3±81.8 | 1219.3±109.3 | 1262.2±136.4 | |
| $t_{Cmax}$ (min) | 169.8±32.6 | 147.8±29.1 | 94.6±9.5 | |
| Trp | | | | |
| $k_a$ (*$10^{-3}$) | 1.59±0.199 | 1.03±0.196 | 1.22±0.246 | |
| k (*$10^{-3}$) | 16.0±2.0 | 11.9±1.71 | 11.9±1.98 | |
| b (µM) | 25±3.2 | 21±2.6 | 21±1.7 | |
| $R^2$ | 0.822±0.034 | 0.811±0.042 | 0.771±0.038 | |
| $C_{max}$ (µM) | 102.3±8.4 | 159.4±77.1 | 128.5±67.4 | |
| $t_{Cmax}$ (min) | 213.4±42.6 | 12399.1±11329.7 | 31256.8±14819.0 | |

[1](mean±SEM, n=12-13). #significant difference NWPI vs. DWPI ($p<0.05$), *significant difference PBI vs. NWPI ($p<0.05$), †significant difference PBI vs. DWPI ($p<0.05$); b = fasting basal concentration, BCAA = branched chain amino acid, $C_{max}$ = peak maximum concentration, DWPI = denatured whey protein isolate, EAA = essential amino acid, k = rate constant for elimination, $k_a$ = rate constant for absorption, NWPI = native whey protein isolate, PBI = protein base ingredient, $R^2$ = coefficient of determination, TAA = total amino acids, $t_{Cmax}$ = time at peak concentration.

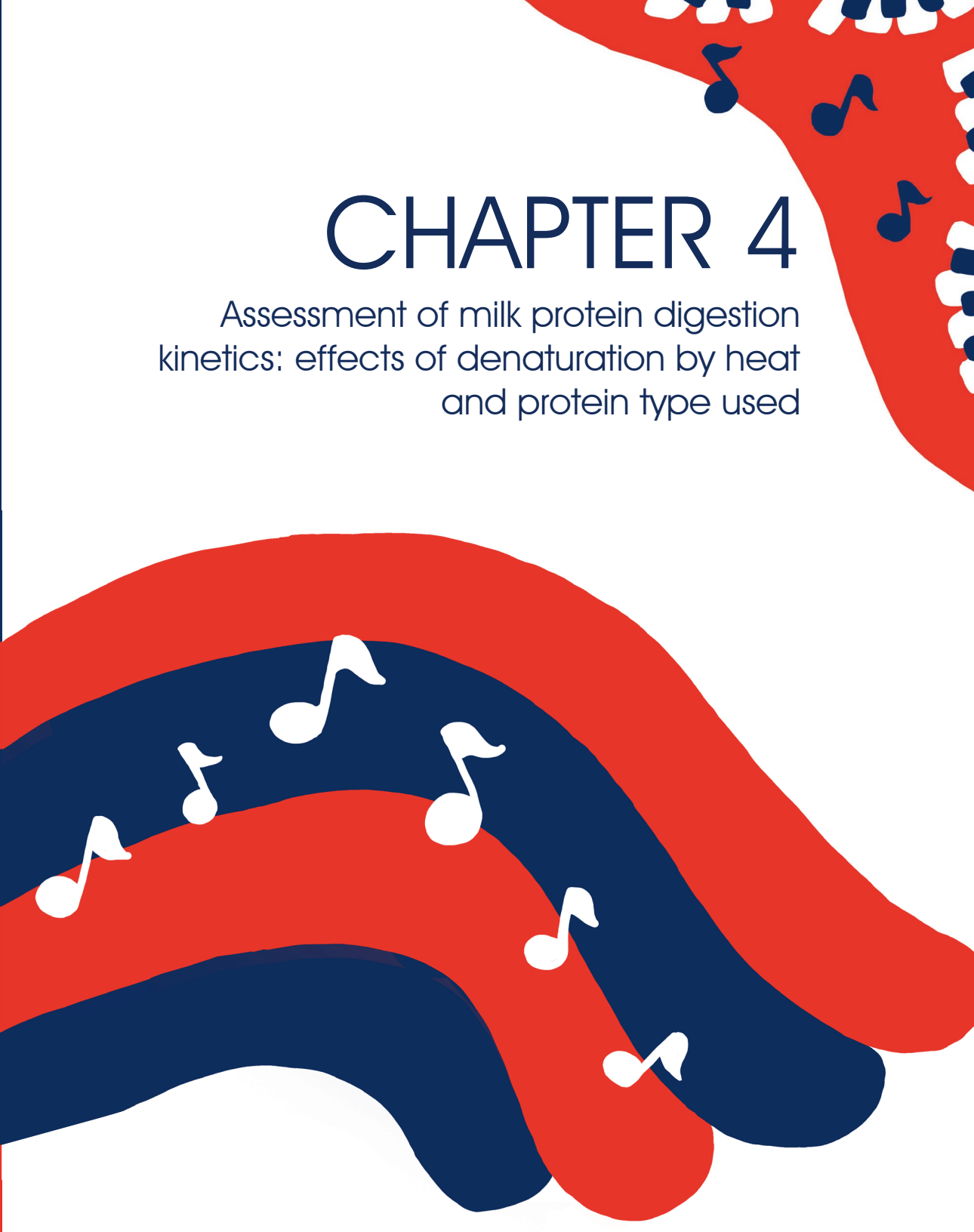



**Supplemental Figure S3.1: Time course of postprandial plasma concentrations of individual non-essential amino acids from baseline up to 4 hr post-administration (mean±SEM, n=12-13); a) Orn, b) Cit, c) Tau; significant effect of time post-administration in all parameters (p<0.01); significant effect of treatment (p<0.05) for Orn and Tau; *significant difference PBI vs. NWPI (treatment*time, p<0.05); †significant difference PBI vs. DWPI (treatment*time, p<0.05); PBI = protein based ingredient, NWPI = native whey protein isolate, DWPI = denatured whey protein isolate.**


4

# CHAPTER 4

## Assessment of milk protein digestion kinetics: effects of denaturation by heat and protein type used



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## Abstract

Knowledge about how molecular properties of proteins affect their digestion kinetics is crucial to understand protein postprandial plasma amino acid (AA) responses. Previously it was found that a native whey protein isolate (NWPI) and heat denatured whey protein isolate (DWPI) elicit comparable postprandial plasma AA peak concentrations in neonatal piglets, while a protein base ingredient for infant formula (PBI, a β-casein-native whey protein mixture) caused a 39% higher peak AA concentration than NWPI. Here, NWPI (91% native), DWPI (91% denatured) and PBI hydrolysis was investigated in a semi-dynamic *in vitro* digestion model (SIM). NWPI and DWPI hydrolysis were also compared in a dynamic digestion model with dialysis (TIM-1) to exclude potential product inhibition effects. In both models, the degree of hydrolysis (DH), loss of intact protein, and release of absorbable products (SIM: <0.5 kDa peptides and free AA, TIM-1: bioaccessible AA) were monitored. Additionally, in SIM, intermediate product amounts and their characteristics were determined. DWPI showed considerably faster intact protein loss, but similar DH and absorbable product release kinetics compared with NWPI in both models. Furthermore, more, relatively large, intermediate products were released from DWPI than from NWPI. PBI showed increased intact protein loss, similar DH, and absorbable product release kinetics, but more, relatively small, intermediate products than NWPI. In conclusion, both whey protein denaturation and β-casein inclusion increased the rate of intact protein loss without affecting absorbable product release during *in vitro* digestion. Our results suggest that intermediate digestion product characteristics are important in relation to postprandial AA responses.

## Introduction

The postprandial increase in plasma amino acid (AA) concentrations is a strong anabolic signal to the body [1]. The postprandial plasma AA concentrations are determined by protein digestion and absorption kinetics in the gastrointestinal tract [2]. Understanding how molecular properties of ingested proteins determine digestion and absorption kinetics is crucial to explain postprandial AA concentrations found in plasma.



Protein digestion kinetics are determined by the gastric passage rate and the protein hydrolysis rate in the gastric and intestinal phases. Absorption kinetics are determined by the intestinal epithelial absorption efficiency of released digestion products. Protein digestion in the gastrointestinal tract is a multiscale process including the enzymatic hydrolysis of intact protein and intermediate products (i.e. peptides) by gastric, pancreatic, and intestinal brush border proteolytic enzymes to ultimately yield products that can be absorbed by the small intestinal epithelium (i.e. AA, di-, and tripeptides) [3].

We previously reported that in neonatal piglets postprandial plasma AA concentrations of milk protein mixtures were not affected by protein denaturation [4]. Feeding a heat-denatured whey protein isolate (DWPI) did not affect postprandial AA plasma concentrations compared to native whey protein isolate (NWPI). Furthermore, the protein composition of milk protein mixtures did affect postprandial plasma AA concentrations without influencing gastric emptying rate. A non-clotting milk protein mixture of β-casein and native whey (PBI) rendered a 39% higher incremental postprandial total AA plasma peak concentration compared to native whey protein (NWPI) alone. Assuming these observations are related to molecular properties of the proteins affecting their digestion, *in vitro* analysis of protein digestion will help to explain the *in vivo* observations.

Both milk protein heat processing and type have been shown to affect protein digestion in ways that in first instance seem conflicting. For example, heat processing of whey protein solutions (90°C for 20 min) resulted in a faster loss of intact proteins during *in vitro* digestion [5], an effect linked to conformational changes, i.e. the unfolding of native protein induced by the exposure to heat (>70°C) [6]. In contrast, heat treatment minimally affects the release of small products of protein digestion *in vitro* [7, 8]. Also, although whey protein elicits higher postprandial plasma AA concentrations *in vivo* than casein [2], *in vitro* a faster intact protein loss is observed for caseins than for whey protein [9]. The *in vivo* findings can be explained by the faster gastric emptying of whey proteins as they remain soluble under gastric conditions, whereas caseins typically form clots which delays gastric passage [2, 10]. A faster *in vitro* disappearance of intact protein may not necessarily lead to a faster release of absorbable digestion products.

Protein hydrolysis is typically described by the degree of hydrolysis (DH), which is defined as the proportion of initially present peptide bonds that have been cleaved. Key in protein hydrolysis is the accessibility of the protein's cleavage sites to the enzyme, as also recognized in the Linderstrøm-Lang theory [11] which discerns two fundamentally different mechanisms of hydrolysis: "one-by-one" and "zipper" [12]. In the one-by-one mechanism, hydrolysis of intact protein is slow compared to hydrolysis of released intermediate peptides. Consequently, proteins are broken down 'one-by-one', resulting in slow loss of remaining intact protein as a function of DH. In the zipper mechanism, which is described for denatured proteins, the hydrolysis of the intact protein as a function of DH is fast and leads to a lot of intermediate peptides. Currently, little is known about protein hydrolysis mechanisms (one-by-one/zipper) in the gastrointestinal tract and how this may affect the release of absorbable products and eventually the appearance of AA in the plasma.

We aimed to evaluate *in vitro* the effect of a difference in milk protein processing and type on protein hydrolysis kinetics and mechanism. We hypothesized that: 1) both whey protein denaturation by heat as well as changing protein composition by including β-casein, increases the rate of intact protein loss, and 2) changing the protein composition (by including β-casein), but not whey protein denaturation, yields a faster absorbable product release. Therefore, intact protein, intermediate peptides and absorbable product amounts were quantified during digestion in a semi-dynamic *in vitro* model of the gastrointestinal tract (SIM). Additionally, as SIM is a closed system in which digestion product accumulation could potentially lead to product inhibition [13, 14], the effect of whey protein denaturation on protein hydrolysis was also investigated in an *in vitro* model of the gastrointestinal tract that included removal of digestion products by means of dialysis (TIM-1).

# Materials and Methods

## Materials

Whey protein isolate powder (WPI; BiPro™; protein content 92% (w/w) [4]; **Table 4.1**) was obtained from Davisco Foods International Inc. (Le Sueur, MN, USA). A protein base ingredient for infant formula powder (PBI; protein content 72.7% (w/w), lactose content 21.9% (w/w) [4]) was prepared on pilot factory scale using cold membrane filtration of fresh cow's milk as described elsewhere [15]. Simulated digestive fluids were made using: α-amylase (Aspergillus oryzae, Sigma A9857, 150 units/mg protein [16], Sigma-Aldrich Chemie N.V., Zwijndrecht, Netherlands), pepsin (Porcine, Sigma P7012, 2,500 units/mg protein [17]), lipase (Rhizopus oryzae, Amano DF 15, 177 FIP units/mg [18], Amano Enzyme Europe Limited, Oxfordshire, United Kingdom), bile extract (Porcine, Sigma B8631) and pancreatin (Porcine, 4xUSP unit activity [18], Sigma P1750). The composition of digestive fluids is described in **Table 2a and 2b**. Fluids containing enzymes or bile were prepared

freshly. Pancreatin solutions were centrifuged (12,000g for 20 min at 4°C) and supernatant was used. Protease inhibitor used was Bowman-Birk inhibitor (BBI) (Sigma T9777), all other chemicals were analytical grade and obtained from Sigma or Merck.

**Table 4.1: Protein composition of milk protein samples (g/100 g protein).**[1]

| | WPI | PBI |
|---|---|---|
| α-lactalbumin | 18.1±2.2 | 12.7±1.3 |
| β-lactoglobulin | 68.0±1.3 | 44.8±3.2 |
| $\alpha_{s1}$-casein | n.d. | n.d. |
| β-casein | n.d. | 30.0±2.3 |
| κ-casein | n.d. | n.d. |
| Others (a.o. IGs and BSA) [2] | 14.0 | 12.1 |

[1]Means±SEM, n=3. Data from [4] wherein the protein composition was analyzed using reversed phase high pressure liquid chromatography (RP-HPLC). [2]Bovine serum albumin (BSA) and immunoglobulins (IGs) could not be quantified with the used RP-HPLC method, SDS-Page analysis showed IgG and BSA presence.

## Milk protein solutions

Milk protein solutions (MPS) were prepared freshly, of which the composition is described in **Table 4.1**. Milk protein powder was dissolved in demineralized water at 62.5 g protein per liter. To WPI 18.8 g lactose per liter was added to match the PBI lactose content. Finally, to prevent gelation during heating, MPS pH was adjusted to 7.1 by addition of 1 M NaOH. Half of the WPI/lactose solution was used as is and is referred to as the native whey protein isolate solution. The other half was heated to denature whey protein at 80°C for 10 min effectively, resulting in a denatured whey protein isolate solution. Following heating, solutions were cooled to room temperature (RT) and were kept at RT in a water bath for at least 40 min before testing. Soluble protein and denatured whey protein contents of MPS were determined. All milk proteins except native whey proteins precipitate at pH 4.6 [19, 20]. Therefore, MPS were centrifuged (15,000 *g* for 30 min) as is (unadjusted pH of 7.1), and after acidification to 4.6 by 1 M HCl addition. Crude protein (nitrogen (N) x6.25) content of the supernatants was quantified according to Dumas. The denatured whey protein amount was calculated by subtracting the amount of soluble protein at pH 4.6 from the total whey protein amount. To confirm heat processing did not cause Maillard induced glycation, free amino groups in the milk protein solutions were quantified using OPA as described below.

## Semi dynamic *in vitro* simulation of infant gastrointestinal tract (SIM)

The SIM is based on a computer controlled parallel fed batch system by Dasgip (Eppendorf) (**Figure 4.1A**) [21, 22]. A miniaturized model was used with 100 mL bioreactors (Dasgip Mini Spinner Type DS0100B). Ratios of MPS to simulated digestive fluid were chosen to simulate the ingestion of a 200 mL meal by an infant. Start volume of the bioreactors was 35 mL MPS and all volumes were adjusted proportionally to this volume.

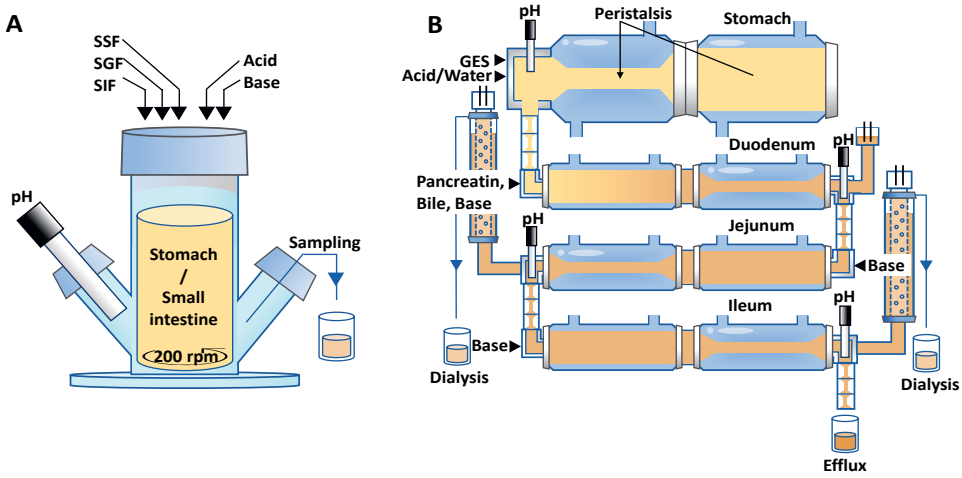


**Figure 4.1: Schematic representation of the *in vitro* models used to simulate infant gastrointestinal conditions. A) Semi-dynamic *in vitro* model of the gastrointestinal tract (SIM). B) Dynamic model of the gastrointestinal tract (TIM-1), adapted from [13]. SGF = simulated gastric fluid, SIF = simulated intestinal fluid, SSF = simulated saliva fluid, GES = gastric enzyme solution.**

The composition of simulated digestive juices is described in **Table 4.2a and 4.2b** and was based on the infant conditions used in reported TIM-1 studies [23-25]. MPS to simulated digestive fluid ratios and enzyme activity at the end of the digestion phases resembled recommendations for static *in vitro* infant digestion model from INFOGEST [26]. Prior to the digestion experiment, bioreactors were filled with 37 mL MPS. Bioreactor temperature was maintained at 37°C using a water bath. After the temperature of the MPS reached 37°C, a 2.0 mL sample was taken from the bioreactors and the gastric phase of 120 min was started by single shots of SSF and SGF, followed by continuous SGF addition. During the gastric phase, the pH was gradually lowered from 7.1 to 4.3 by addition of 1 M HCl following a set curve following the average of *in vivo* observations from literature [27-36]. After the gastric phase, the pH was increased to 6.5 in 10 min by addition of 1 M $NaHCO_3$. The subsequent intestinal phase of 180 min was started with a single shot of SIF followed by continuous SIF addition. During the intestinal phase, the pH was gradually increased to 7.2 by addition of 1 M $NaHCO_3$. Digesta samples (2.0 mL) were taken at 10, 30, 60, 90, 120 min gastric digestion and 2, 6, 10, 15, 20, 30, 45, 60, 90, 120, and 180 min intestinal digestion. Digesta samples were directly diluted 1:1 with sample buffer and snap frozen in liquid nitrogen. Sample buffer pH and concentration were chosen to inhibit enzyme activity during storage. Gastric digesta sample buffer was 0.1 M phosphate buffer pH 7, intestinal digesta sample buffer was 0.1 M phosphate buffer pH 5.5, containing 0.58 g/L BBI. Blank runs, using phosphate buffered saline as food to replace MPS, were performed to determine the contribution of the added simulated digestive fluids to the total amounts of amino groups, peptides and amino acids.

**Table 4.2a: Fluid volume, flow and composition of the gastric phase of SIM and TIM-1.[1]**

| Fluid | SIM | TIM-1 |
|---|---|---|
| MPS *(mL)* | 35 | 200 |
| SSF *(mL)* | 5.8 | 35 |
| α-amylase *(kU/L)*[2] | 180 | 180 |
| NaCl, KCl, $CaCl_2.2\ H_2O$, $NaHCO_3$ *(g/L)* | 6.2, 2.2, 0.3, 1.2 | 6.2, 2.2, 0.3, 1.2 |
| SGF | | |
| Pepsin *(kU/L)*[3] | 230 | 460 |
| Lipase *(kU/L)*[4] | 8.85 | 17.7 |
| NaCl, KCl, $CaCl_2.2\ H_2O$, Na-acetate *(g/L)* | 3.1, 1.1, 0.15, 0.82 | 6.2, 2.2, 0.3, 1.64 |
| Start volume *(mL)* | 1.75 | 5 |
| Flow *(µL/min)* | 87.5 | 250 |
| Water | | |
| Start volume *(mL)* | - | 5 |
| Flow *(µL/min)* | - | $250 - x_a$ |
| 1 M HCl (pH titration stomach) *(mL/min)* | $x_a$ | $x_a$ |
| Total start *(mL/200 mL MPS)* | 43 | 45 |
| Total flow *(mL/min/200 mL MPS)* | $0.5 + x_a$ | 0.5 |

[1]MPS = milk protein solution, SSF = simulated saliva fluid, SGF = simulated gastric fluid, $x_a$ = flow of acid needed to titrate pH to setpoint. [2]Units as defined in [16]. [3]Units as defined in [17]. [4]FIP units, as defined in [18].

**Table 4.2b: Fluid volume, flow and composition of the intestinal phase of SIM and TIM-1.[1]**

| Fluid | SIM | TIM-1 |
|---|---|---|
| SIF | | |
| Pancreatin *(g/L, kU Trypsin/L)*[2] | 12.5, 32 | - |
| Bile extract *(g/L)* | 5 | - |
| NaCl, KCl, $CaCl_2.2\ H_2O$ *(g/L)* | 2.5, 0.3, 0.15 | - |
| Start *(mL)* | 10.5 | - |
| Flow *(mL/min)* | 0.175 | - |
| Pancreatin solution *(g/L, kU Trypsin/L)*[2] | - | 50, 128 |
| Start volume duodenum *(mL)* | - | 7.5 |
| Flow *(mL/min)* | - | 0.25 |
| Bile extract solution *(g/L)* | - | 10 |
| Start volume duodenum *(mL)* | - | 15 |
| Flow *(mL/min)* | - | 0.5 |
| Salt solution | | |
| NaCl, KCl, $CaCl_2.2\ H_2O$ *(g/L)* | - | 10, 0.6, 0.3 |
| Start volume duodenum *(mL)*[3] | - | 37.5 |
| Flow *(mL/min)* | - | $0.25 - x_b$ |
| 1M $NaHCO_3$ (pH titration duodenum) *(mL/min)*[4] | $x_b$ | $x_b$ |
| Total start volume *(mL/200 mL MPS)* | 60 | 60 |
| Total flow *(mL/min/200 mL MPS)* | $1.0 + x_b$ | 1.0 |

[1]MPS = milk protein solution, SIF = simulated intestinal fluid, $x_b$ = flow of base needed to titrate pH to setpoint. [2]TAME units as defined in [51]. [3]In TIM-1 at experiment start also the jejunum and ileum compartments are filled with intestinal salt solution. [4]In TIM-1 also the jejunum and ileum compartments are pH controlled by titration with 1M $NaHCO_3$.

## Dynamic *in vitro* simulation of infant gastrointestinal tract (TIM-1)

Like the SIM, the TIM-1 model features dynamic pH control and continuous fresh simulated digestive fluid addition (**Figure 4.1B**). In contrast to SIM, digestion products are removed in TIM-1 to prevent product inhibition. The TIM-1 model has separate stomach and intestine compartments, with controlled fluid transport mimicking gastrointestinal transit including gastric emptying [14]. The intestinal part is divided in three consecutive compartments simulating the duodenum, jejunum and ileum. Enzyme to substrate ratios (E/S) are dynamic in TIM-1: continuous digestive fluid addition results in an E/S increase, while fluid transport can also result in an E/S decrease due to an increase in substrate concentration. The jejunum and ileum compartments are connected to hollow fiber dialysis membranes that allow removal of water-solubilized molecules (a.o. protein digestion products). In this study membranes with a 1 kDa molecular weight cut-off were used (UFP-1-C-5, GE healthcare GMBH, Solingen Germany). The fraction of the intake that can pass the membrane during digestion is typically referred to as bioaccessible, i.e., the fraction that is available for absorption by the small intestinal epithelium after digestion. Ingestion volume was 200 mL of MPS plus start volumes of SSF and SGF (**Table 2a**). Simulated digestive fluid addition in the TIM-1 stomach compartment consisted of two separate streams, one for SGF and one for 1.0 M HCl and water, each comprising 50% of the total flow. Hence SGF composition was two times more concentrated than that used in SIM (**Table 2a**). Simulated digestive fluid addition in the TIM-1 duodenum compartment consisted of three separate streams, one for bile solution, one for pancreatin solution and one for 1.0 M $NaHCO_3$ and salt solution, comprising 50, 25 and 25%, respectively, of the total flow (**Table 2b**). Gastric emptying half time was set at 60 min. Ileal efflux was minimized by setting ileal half empty time to 1000 min. Gastric pH curve set points were equal to those used in the SIM, the pH set points for duodenum, jejunum and ileum were 6.5, 7.0 and 7.2, respectively. To determine the bioaccessible fraction, dialysate fluid was collected and sampled at t=30, 60, 90, 120, 180, 240, and 300 min. Luminal samples (1.0 mL) were taken from the stomach at 30, 60 and 90 min and from the duodenum at 15, 30, 45, 60, 90, 120, and 180 min. Luminal sample protein concentration was determined using Dumas method. Blank runs, where 200 mL phosphate buffered saline was used as food, were performed to determine the contribution of the added simulated digestive fluids to the amounts of amino groups, peptides and amino acids. After 300 min of simulated digestion the gastric and intestinal contents and ileal efflux were collected and sampled for analysis.

## o-Phthalaldehyde method (OPA)

Free amino groups were quantified using OPA. The OPA reagent was prepared as described previously [37]. Samples were diluted to 5 g protein /L in a 20 g SDS /L solution, stirred for 20 min and stored at 4°C overnight. The samples were then diluted to 2 g/L with Millipore water. Aliquots (5 µL) were added to 300 µL of the OPA reagent solution and equilibrated for 10 min. The presence of alkyl-iso-indols formed by the reaction of free amino groups

with OPA was measured by the absorbance at 340 nm. To calculate the amount of free $NH_2$ groups, a calibration curve was measured using leucine as a reference compound. Total amount of amino groups of NWPI, DWPI and PBI solutions were determined by OPA after hydrolysis in 6 M HCl at 110°C for 22 h. The degree of hydrolysis (DH) at a time point (t) was calculated using equation 1:

$$\mathrm{DH(t)}\ [\%] = \frac{[\mathrm{NH_2}]_t \times \mathrm{df} - [\mathrm{NH_2}]_0}{[\mathrm{NH_2}]_{\mathrm{AH}} - [\mathrm{NH_2}]_0} \times 100 \qquad (1)$$

wherein: $[NH_2]_t$ is the concentration of free amino groups present in the t=x digesta sample, df = dilution factor during digestion, $[NH_2]_0$ is the concentration of free amino groups present in the t=0 sample, and $[NH_2]_{AH}$ is the concentration of free amino groups present in the acid hydrolyzed t=0 sample of the respective MPS,

## Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

Intact protein and HMW peptides in MPS and digesta samples were analyzed by SDS-PAGE under reducing conditions using NuPAGE 4-12% Bis-Tris Midi protein precast gels (WG1402A Invitrogen, Thermo Fisher scientific, Landsmeer, the Netherlands). Samples were diluted to standardize protein concentration to 0.23 g/L using demineralized water. Subsequently 100 µL lithium dodecyl sulfate sample buffer (NuPAGE™ lithium dodecyl sulfate sample buffer (4 X), NP0007 Invitrogen, Thermo Fisher scientific) and 40 µL sample reducing agent containing 500 mM dithiothreitol (NuPAGE™ sample reducing agent (10x), NP0009, Invitrogen, Thermo Fisher scientific) were added to 260 µL diluted sample. Subsequently, samples were heated for 10 min at 70°C. Each lane was loaded with 20 µL heated sample, i.e., 3 µg protein. Gels were fixed in 40% (v/v) methanol, 10% (v/v) acetic acid, rinsed and stained with SimplyBlue SafeStain (LC6060 Invitrogen, Thermo Fisher scientific). Mark12™ Unstained Standard (LC5677 Invitrogen, Thermo Fisher scientific) was used as molecular-weight marker. Gels were imaged using a BIO-RAD Gel Doc XR imager, and band intensity was quantified using Quantity One software. The relationship between protein quantity and band intensity was calibrated using five concentrations of PBI in demineralized water, showing linearity from 1 to 5 µg total protein per lane. The proportion of intact milk protein remaining at a time point (t) during SIM digestion was calculated by expressing the sum of intensity of all bands at t (corresponding to bands present at t=0) as a proportion of the intensity of all bands at t=0 (% of $P_i(0)$). HMW peptide appearance was determined by expressing the sum of appearing band intensity (in the area 5-13 kDa) as a proportion of the total intensity of all bands at t=0 (% of $P_i(0)$). For luminal samples of TIM-1 $P_i(0)$ was multiplied with the fraction of the total milk protein present in the given compartment, thus is expressed as % of the total milk protein present stomach ($P_s(t)$) or duodenum ($P_d(t)$).

## Ultra high-performance liquid chromatography - fluorescence (UPLC-FLR)

UPLC-FLR analysis was performed as described earlier [21]. Briefly, free amino acids were quantified in milk protein solutions and digesta after precipitation of proteins and large peptides with 3.5% (w/v) perchloric acid and filtration. Total amino acids were quantified in MPS and TIM-1 dialysate after hydrolysis in 6 M HCl at 110°C for 22 h prior to injection. The concentration of each amino acid in the sample was determined by UPLC using a pre-column derivatization with OPA and fluorometric detection. Results are reported as weight percentage of initial crude protein content in the respective milk protein solution (% of $P_c(0)$).

## High-performance size exclusion chromatography (HP-SEC)

Peptides were quantified using size exclusion chromatography (SEC) as described earlier [21]. The HPLC system (Shimadzu, 's-Hertogenbosch, the Netherlands) was equipped with a Superdex Peptide 10/300 column (17-5176-01 GE Healthcare, München, Germany) with a 10 kDa HMW cut off. The eluent was 25% (v/v) acetonitrile, 16% (v/v) trifluoracetic acid and detection was by done by absorption at 200 nm. The relationship between elution time and molecular weight was established using ten standards; Cytochrome C from bovine heart (12,327 Da), Aprotinin from bovine lung (6,500 Da), Adrenocorticotropic hormone from porcine pituitary (4,567 Da), Insulin A-chain oxidized ammonium salt from bovine pancreas (2,532 Da), Angiotensinogen 1-14 Renin substrate porcine (1,759 Da), Bradykinin acetate salt (1,060 Da), Bradykinin Fragment 1-7 (757 Da), Bradykinin fragment 1-5 (573 Da), Ala-Ala-Ala-Ala-Ala (373 Da), and Gly-Leu (188 Da). The correlation coefficient of the linear fit between elution time between 9.2 and 19.2 min and 10 log molecular weight was 0.980. The chromatogram of a tryptophane standard showed this amino acid started to elute at 23 min. Therefore, chromatograms were integrated from 11.4 min (5 kDa) to 23 min to exclude free amino acids that absorb at 200 nm. Peptide quantity was calculated using estimation of peptide amino acid composition and peptide molar extinctions coefficients using the methodology and reported extinction coefficients at 214 nm by Kuipers and Gruppen [38]. The average extinction coefficient of the peptides in the integrated area $\overline{\varepsilon(\mathrm{x})}$ was calculated using equation (2) adapted from [39],

$$\overline{\varepsilon(\mathrm{x})} = \varepsilon_b \times \left(\frac{\mathrm{Mw}(\bar{\mathrm{x}})}{\mathrm{Mw}(\overline{\mathrm{AA}})} - 1\right) + \varepsilon\overline{(\mathrm{AA})} \times \frac{\mathrm{Mw}(\mathrm{x})}{\mathrm{Mw}\overline{(\mathrm{AA})}} \qquad (2)$$

where $\varepsilon_b$ is the extinction coefficient of a peptide bond, which equals to 923 $M^{-1}cm^{-1}$, $\mathrm{Mw}(\bar{\mathrm{x}})$ is the mean peptide molecular weight in the integrated area, $\mathrm{Mw}(\overline{\mathrm{AA}})$ is the average amino acid molecular weight in the mean peptide, $\varepsilon\overline{(\mathrm{AA})}$ is the weighted average extinction coefficient of the amino acids in the mean peptide. Peptides were clustered in two fractions, LMW <0.5 kDa and MMW from 0.5-5 kDa. Peptides <0.5 kDa were considered di- and tripeptides. MMW and LMW peptide quantity is expressed as weight percentage of initial crude protein content in the respective MPS (% of $P_c(0)$).

## Data analysis

Data obtained from the MPS were corrected for the level found in blank runs where appropriate. Data from SIM experiments are shown as mean±standard error of the mean (n=3). Parameters measured in the intestinal phase from SIM were fitted using first order reaction kinetics using equation 3:

$$y(t) = m \times (1 - e^{-kt}) + b \qquad (3)$$

wherein y(t) is the value of the parameter at time t, m is the maximal value that can be reached, k is the rate constant and b is the value at 120 min gastric digestion. The solver function in Microsoft Excel was used to calculate the values for k, m and b that resulted in the lowest sum of squares of residuals. Statistical significance of differences was analyzed by ANOVA with Games Howell post hoc test. Differences with a *p* value lower than 0.05 were considered statistically significant. Data from TIM-1 experiments are shown as mean±range (n=2).

# Results

## Characterization of milk protein solutions (MPS)

Crude protein (N x 6.25) in native whey protein isolate (NWPI) was 100% soluble at pH 7.1 and for 91% at pH 4.6, so 9% was denatured. Heating WPI to prepare denatured whey protein isolate (DWPI) did not influence crude protein solubility at pH 7.1 but decreased solubility at pH 4.6 to 9%, therefore in total 91% was denatured. The amount of total free amino groups, as measured by OPA method, was not affected by heating, indicating that no glycation had occurred. Crude protein in protein base ingredient for infant formula (PBI) was 97% soluble at pH 7.1 and for 73% soluble at pH 4.6. Since PBI comprises 30% β-casein (**Table 4.1**), which precipitates at pH 4.6 [40], the whey protein fraction was considered to be entirely native.

SDS-page analysis confirmed that all MPS contained α-lactalbumin, β-lactoglobulin, bovine serum albumin and immunoglobulin G, only PBI contained β-casein (**Figure 4.2**, t=0). Undigested MPS were devoid of HMW peptides (5-13 kDa) and free AA as shown by SDS-page and UPLC-FLR analysis, respectively. HP-SEC analysis showed that all MPS contained less than 1% of $P_c(0)$ LMW peptides (<0.5 kDa), still PBI content was significantly higher than NWPI. Also, PBI MMW peptide (0.5-5 kDa) content was significantly higher than that of NWPI (9.20±0.47 and 4.79±1.07% of $P_c(0)$, respectively). DWPI MMW peptide content was similar to NWPI (3.69±0.90% of $P_c(0)$) **(Supplementary Table S4.1)**.

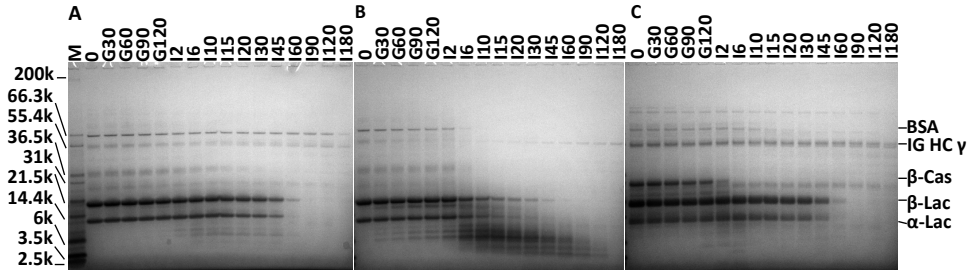


**Figure 4.2: Typical SDS-page gels loaded with SIM digesta samples. A) native whey protein isolate (NWPI), B) denatured whey protein isolate (DWPI), C) protein base ingredient for infant formula (PBI). Lanes are marked with type of sample with prefix indicating the digestion phase (G = gastric, I = intestinal) and number reflecting time in min. M = Molecular weight marker. BSA = Bovine serum albumin; IG HC γ = Immunoglobulin G heavy chain; β-Cas = beta-casein; β-Lac = beta-lactoglobulin; α-Lac = alpha-lactalbumin. SIM = semi-dynamic *in vitro* model of the gastrointestinal tract. Annotation based on [15, 52].**

## Gastrointestinal protein hydrolysis kinetics as affected by whey protein denaturation and protein type used: SIM experiment

*Peptide bond hydrolysis*

DH increased little during the gastric phase for all MPS to <0.3% at 120 min (**Supplementary Table S4.1**). In the intestinal phase, DH increased comparably for all three MPS, reaching ~25% at 180 min (**Figure 4.3**). Although at a few individual timepoints DH differed significantly between NWPI and DWPI, and between NWPI and PBI, DH rate constant ($k_{DH}$) and maximum ($m_{DH}$) were neither different between NWPI and DWPI, nor between NWPI and PBI (**Table 4.3**).

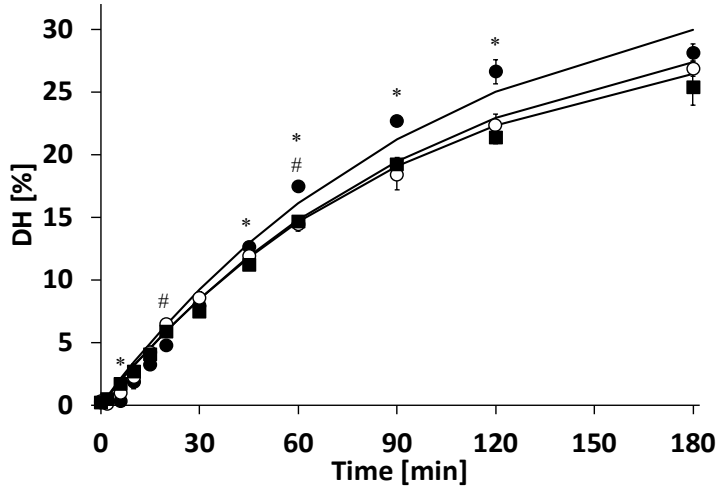


**Figure 4.3:** Degree of protein hydrolysis in SIM intestinal digesta as a function of time. ●) Native whey protein isolate (NWPI), ○) denatured whey protein isolate (DWPI), ■) protein base ingredient for infant formula (PBI); Mean±SEM (n=3); t=0 indicates both gastric phase end and intestinal phase start; Lines are fitted data using first order reaction kinetics; #significant difference NWPI vs. DWPI ($p<0.05$); *significant difference PBI vs. NWPI ($p<0.05$); DH = degree of hydrolysis. SIM = semi-dynamic *in vitro* model of the gastrointestinal tract.

**Table 4.3: Curve fitting values for SIM intestinal phase.**[1]

| | NWPI | DWPI | PBI |
|---|---|---|---|
| DH | | | |
| $k_{DH}$ (*$10^3$) *($min^{-1}$)* | 9.84±0.50 | 9.94±1.48 | 10.6±1.81 |
| $b_{DH}$ *(%)* | 0.00±0.00 | 0.003±0.003 | 0.021±0.021 |
| $m_{DH}$ *(%)* | 36.1±0.82 | 32.9±2.56 | 31.0±2.97 |
| $R^2_{DH}$ | 0.979±0.005 | 0.992±0.004 | 0.995±0.001 |
| LMW | | | |
| $k_{LMW}$ (*$10^3$) *($min^{-1}$)* | 9.86±0.75 | 9.23±0.91 | 13.76±1.38 |
| $b_{LMW}$ *(% of $P_c(0)$)* | 0.00±0.00 | 0.093±0.047 | 0.478±0.451 |
| $m_{LMW}$ *(% of $P_c(0)$)* | 73.4±2.41 | 66.4±4.27 | 54.7±3.62* |
| $R^2_{LMW}$ | 0.983±0.001 | 0.997±0.001# | 0.992±0.004 |
| Free AA | | | |
| $k_{FAA}$ (*$10^3$) *($min^{-1}$)* | 10.3±1.14 | 10.6±1.35 | 11.2±1.29 |
| $b_{FAA}$ *(% of $P_c(0)$)* | 0.00±0.00 | 0.317±0.168 | 0.007±0.007 |
| $m_{FAA}$ *(% of $P_c(0)$)* | 34.0±2.1 | 29.4±2.12 | 26.8±2.46 |
| $R^2_{FAA}$ | 0.986±0.001 | 0.997±0.001# | 0.993±0.002 |

[1]Means±SEM, n=3. k = rate constant, b = value at start of intestinal phase, m = maximal value, $R^2$ = coefficient of determination. Suffix DH, LMW and FAA denote respective parameter. LMW = low molecular weight peptides, AA = amino acids, DH = degree of hydrolysis. NWPI = native whey protein isolate, DWPI = denatured whey protein isolate, PBI = protein base ingredient. % of $P_c(0)$ = weight percentage of crude protein amount at t=0. *significant difference PBI vs. NWPI ($p<0.05$); #significant difference NWPI vs. DWPI ($p<0.05$).

*Hydrolysis of intact protein*

Intact protein hydrolysis predominantly occurred in the intestinal phase, as indicated by fading of most of the protein bands (**Figure 4.2 and 4.4**). Several bands appeared below α-lactalbumin, indicating high molecular weight peptides were released (**Figure 4.2B**). Intestinal intact protein loss in DWPI digesta was significantly faster than in NWPI digesta, and reached completion after 60 min, whereas at that timepoint only 70% degradation was observed for NWPI (**Figure 4.4B**).

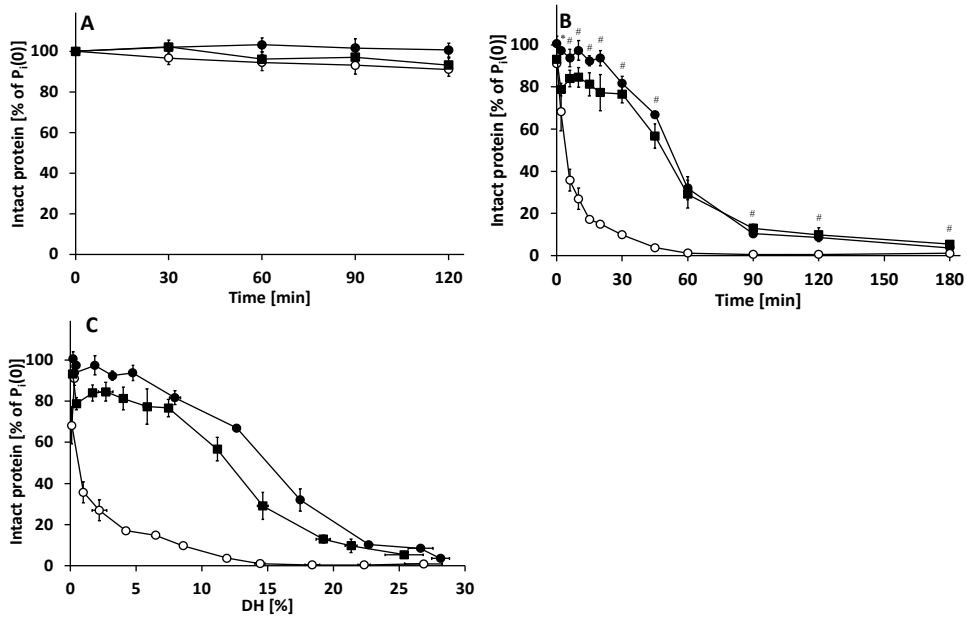


**Figure 4.4: Intact protein hydrolysis in SIM intestinal digesta as determined by SDS-page and densiometric analysis of bands. ●) Native whey protein isolate (NWPI), ○) denatured whey protein isolate (DWPI), ■) protein base ingredient for infant formula (PBI). Mean±SEM (n=3). Total intact milk protein expressed as % of intact milk protein amount at t=0 ($P_i(0)$) in the A) gastric phase in time, B) intestinal phase as a function of time and C) as a function of DH in the intestinal phase; #significant difference NWPI vs. DWPI (P <0.05); *significant difference PBI vs. NWPI (P <0.05). SIM = semi-dynamic *in vitro* model of the gastrointestinal tract.**

Intact protein as a function of DH showed increased enzyme activity towards intact proteins relative to intermediate peptides in DWPI than NWPI, as shown by the complete disappearance of intact protein at a lower DH for DWPI (**Figure 4.4C**). Intact β-casein from PBI was more readily hydrolyzed than intact native whey proteins (**Figure 4.2C**); at 2 min of intestinal digestion only 10.7±1.2% of the initial β-casein band intensity (at 0 min) remained. Consequently, the total intact milk protein loss was faster in PBI than that in NWPI (**Figure 4.4A and 4.4B**), although the effect size was smaller than with DWPI. Intact protein as a function of DH showed higher enzyme activity towards intact proteins relative to intermediate peptides of PBI compared to NWPI (**Figure 4C**).

*Absorbable products of digestion (LMW peptides and FAA)*

Gastric digestion released very low amounts of LMW peptides and FAA <0.2% of $P_c(0)$) for all MPS (**Supplementary Table S4.1**). In intestinal digesta, the time course of both LMW peptide and FAA release were similar for all MPS, reaching on average ~50 and 25% of $P_c(0)$, respectively, at 180 min (**Figure 4.5A and 4.5B**). DWPI digesta LMW and FAA content was significantly lower than that of NWPI during the 2nd hour of intestinal digestion, however the rate constant and maximum of both LMW peptide and FAA release ($k_{LMW}$ and $k_{FAA}$ / $\mathbf{m_{LMW}}$ and $\mathbf{m_{FAA}}$) were similar for NWPI and DWPI (**Table 4.3**).

PBI digesta LMW peptide amount was significantly higher than that of NWPI in the beginning of the intestinal phase (10-15 min), while from 90-180 min this was the opposite. Similarly, free AA levels in PBI intestinal digesta were significantly lower than those in NWPI from 90-180 min. The rate constants of both LMW peptide and FAA release ($k_{LMW}$ and $k_{FAA}$) from NWPI and PBI were similar (**Table 4.3**). However, the maximal concentration LMW peptides ($m_{LMW}$) was significantly lower for PBI than NWPI, while the FAA maximum ($m_{FAA}$) was similar for NWPI and PBI. Absorbable product amounts (i.e., LMW peptides and FAA) as a function of DH exhibited a strong linear correlation for all milk protein solutions ($r \geq 0.996$; **Figure 4.5C and 4.5D**).



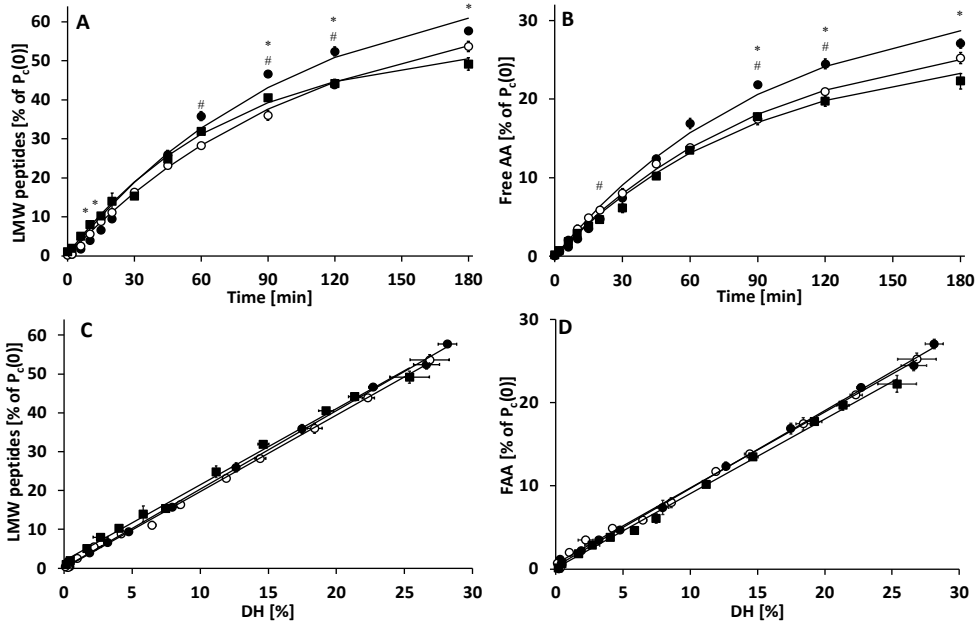


**Figure 4.5: Release of absorbable products in SIM intestinal digesta; Low molecular weight peptides (LMW, <0.5 kDa) as determined by HP-SEC and free AA as determined by UPLC-FLR expressed as weight percentage of the crude protein amount at t=0 ($P_c(0)$) as function of time (A,B) or as function of the degree of hydrolysis (C,D). ●) Native whey protein isolate (NWPI), ○) denatured whey protein isolate (DWPI), ■) protein base ingredient for infant formula (PBI). Lines in A and B are fitted data using first order reaction kinetics. Lines in C and D are linear regression lines. #significant difference NWPI vs. DWPI (p<0.05); *significant difference PBI vs. NWPI (p<0.05). SIM = semi-dynamic *in vitro* model of the gastrointestinal tract.**

*Intermediate digestion products (MMW and HMW peptides)*

Gastric digestion resulted in a small rise (<5% of $P_c(0)$) in MMW peptide (0.5-5 kDa) content for all milk protein solutions. Gastric HMW peptide (5-13 kDa) release was significantly higher for DWPI than for NWPI (9.05±1.56 vs 2.63±0.75% of $P_c(0)$ respectively) (**Supplementary Table S4.1**). DWPI and NWPI digesta MMW peptide content was similar in the early intestinal phase. However, from 45-90 min, DWPI digesta MMW peptide content was significantly lower than that of NWPI (**Figure 4.6A**). DWPI intestinal digesta HMW peptide content

increased sharply to a peak at 10 min on 66% of $P_i(0)$ and was consistently higher than that of NWPI up to 45 min (**Figure 4.6B**). The DWPI intestinal HMW peptide peak of 66% of $P_i(0)$ occurred when only 2% of peptide bonds were cleaved (**Supplementary Figure S4.1**). PBI and NWPI Intestinal digesta MMW content both peaked after 60 min at ~60% of $P_c(0)$ (**Figure 4.6A**). However, from 6-45 min PBI intestinal digesta MMW content was consistently higher than that of NWPI. In contrast to MMW, intestinal digesta HMW peptide content stayed below 10% of $P_i(0)$ for both protein solutions but was always lower for PBI than NWPI (**Figure 4.6B**).

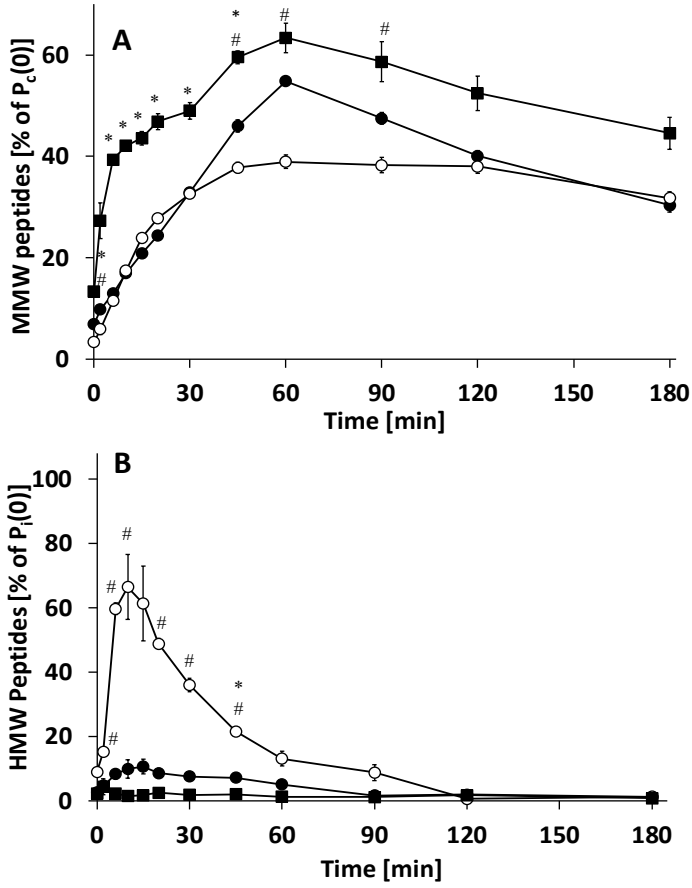


**Figure 4.6: Intermediate digestion products in SIM intestinal digesta. A) Medium molecular weight peptides (MMW, 0.5-5 kDa) expressed as weight % of the crude protein ($P_c(0)$), as determined by HP-SEC. B) High molecular weight peptides (HMW, 5 – 13 kDa) expressed as % of the intact milk protein content at t=0 ($P_i(0)$), as determined by SDS-page and densiometric analysis. ●) Native whey protein isolate (NWPI), ○) denatured whey protein isolate (DWPI), ■) protein base ingredient for infant formula (PBI). Mean±SEM (n=3). #significant difference NWPI vs. DWPI (p<0.05); *significant difference PBI vs. NWPI (p<0.05). SIM = semi-dynamic *in vitro* model of the gastrointestinal tract.**

## Gastrointestinal protein hydrolysis kinetics as affected by whey protein denaturation: TIM-1 experiment

Gastric emptying of protein resulted in a duodenal crude protein (Nx6.25) concentration as measured by Dumas method that followed a bell-shaped curve that peaked at 60 min, closely resembling the concentration as calculated using experimentally used volumes, flows and gastric emptying curve (**Supplementary Figure S4.2**). DH of duodenal digesta first increased from 0 to 15 min, then decreased until 60 min, after which it continued to increase (**Figure 4.7**). The dip in DH at 60 min coincided with the crude protein concentration peak (**Supplementary Figure S4.2**). The time course of duodenal DH for both NWPI and DWPI was similar, although at t=180 min NWPI duodenal DH was higher than DWPI, at which time point most protein had passed to jejunum and ileum, and only ~17% was still present in the duodenum.



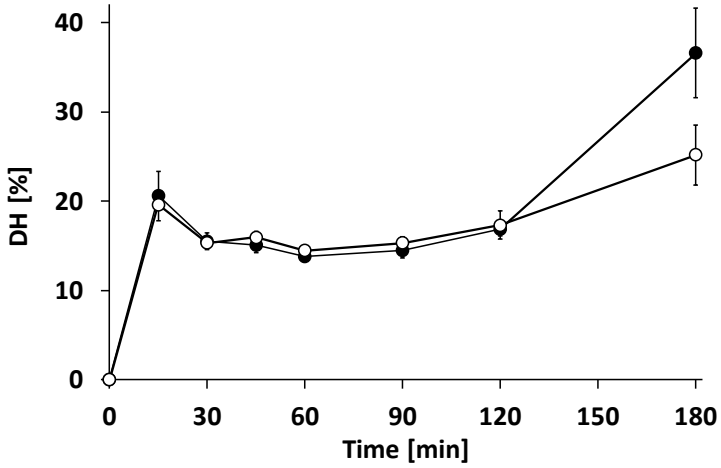


**Figure 4.7: Average degree of hydrolysis in TIM-1 duodenum compartment as determined using OPA. ●) Native whey protein isolate (NWPI), ○) denatured whey protein isolate (DWPI). Displayed data are means±range (n=2).**

In the stomach compartment of TIM-1 the time course of intact protein loss was similar in NWPI and DWPI; after 90 min about 85% of protein present was still intact (**Figure 4.8A**), at this time ~75% of the MPS was already emptied from the stomach into the duodenum compartment. The proportion of intact protein present in the duodenum ($P_d(t)$) peaked at 60 min, but at a 3x higher level for NWPI (**Figure 4.8B**). Intact protein maximum coincided with the duodenal crude protein concentration peak (**Supplementary Figure S4.2**).

Duodenal digesta intact protein plotted against DH showed that at similar DH less intact protein was present in DWPI than in NWPI (**Figure 4.8C**).

The time course of cumulative bioaccessible fraction (free AA and AA bound in peptides <1 kDa, jejunum and ileum combined) showed a similar increase for both DWPI and NWPI

(**Figure 4.9**). After 300 min the total bioaccessible amount was ~80% of the recovered AA for both NWPI and DWPI. The total recovery of AA (free plus in peptide form) was 77.0±5.46%.

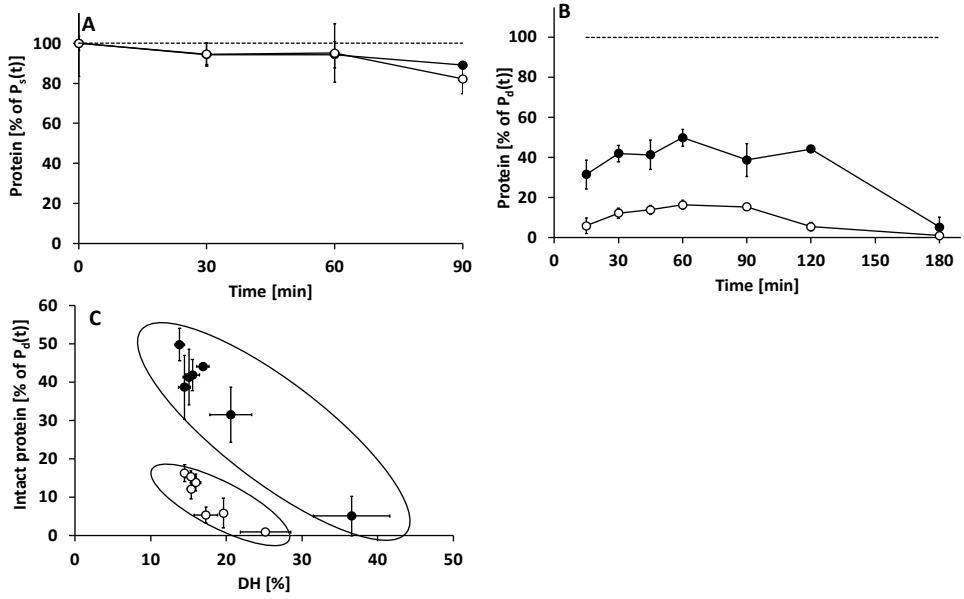


**Figure 4.8: Intact milk protein in TIM-1 lumen as determined by densiometric analysis. ●) Native whey protein isolate (NWPI), ○) denatured whey protein isolate (DWPI). Expressed as % of the total milk protein present (P(t)) in the A) stomach as function of time, and B) duodenum as function of time: and C) duodenum as function of DH. Dotted lines in A and B represent the total milk protein present in the compartment. Displayed are means±range (n=2). Suffix S and D denote stomach and duodenum respectively.**

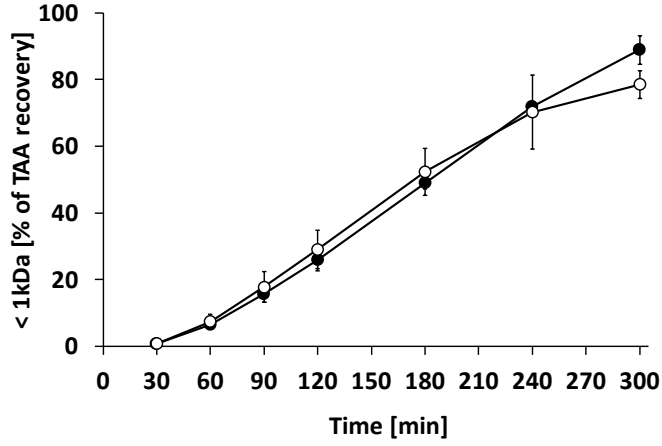


**Figure 4.9: Cumulative bioaccessible fraction (free AA and bound AA in dialysate <1kD, jejunum and ileum combined) as a function of time in TIM-1 as measured by UPLC after acid hydrolysis. Expressed as percentage of total amino acid recovery. ●) Native whey protein isolate (NWPI), ○) denatured whey protein isolate (DWPI). Displayed are means±range (n=2). TAA = total amino acid.**

## Discussion

In the current study we evaluated the effect of a difference in milk protein denaturation and type on protein hydrolysis kinetics during *in vitro* digestion. We hypothesized that denaturation of whey protein by heat as well as changing protein composition by including β-casein, increases rate of intact protein loss, and that changing the protein composition, but not protein denaturation would yield a faster absorbable product release. We based this hypothesis on our previously reported findings that in neonatal piglets the postprandial plasma AA concentrations of milk protein mixtures were affected by protein composition, but not denaturation [4]. **Table 4.4** summarizes the results of the current study.



**Table 4.4. Results summary.**[1]

| | NWPI | DWPI | PBI |
|---|---|---|---|
| Brief sample description | Native whey | Denatured whey | Native whey + β-casein |
| DH increase rate | = | = | = |
| Intact protein loss rate | low | high | medium |
| HMW release | low | high | low |
| MMW release | medium | low | high |
| LMW release (absorbable) | = | = | = |
| FAA release (absorbable) | = | = | = |

[1] Overall protein digestion results summarized. DH = degree of hydrolysis, FAA = free amino acids, HMW = high molecular weight peptides, MMW = medium molecular weight peptides, LMW = low molecular weight peptides. NWPI = native whey protein isolate, DWPI = denatured whey protein isolate, PBI = protein base ingredient. = indicates similar results between three groups.

Heat denaturation affected intact protein loss kinetics to a larger extent than protein composition did (**Figure 4.4**). Intact protein from a whey solution containing 91% denatured proteins (DWPI) was hydrolyzed considerably faster than a whey solution containing 91% native proteins (NWPI). Peptide bond cleavage (as assessed by DH (**Figure 4.3 and Table 4.3**)) and absorbable product release kinetics (FAA and LMW peptides in SIM (**Figure 4.5, Table 4.3**), and bioaccessible AA in TIM-1 (**Figure 4.7**)) were not affected by denaturation, but intermediate digestion product composition was affected by denaturation: less MMW and more HMW peptides were released (**Figure 4.6**). A change in protein type, i.e., from native whey protein (NWPI) to a milk protein mixture containing β-casein and native whey protein (PBI), increased the rate of intact protein loss (**Figure 4.4**), but did not affect peptide bond cleavage (i.e., DH) (**Figure 4.3**) and absorbable product release (FAA and LMW peptides) (**Figure 4.5 and Table 4.3**). Intermediate digestion product composition was also affected by protein type: more MMW and less HMW peptides were present in intestinal digesta of the PBI than NWPI (**Figure 4.6**). The TIM-1 results confirm results obtained in SIM; therefore, product inhibition did not play a dominant role in the SIM experiments.

## Gastrointestinal protein hydrolysis kinetics as affected by heat denaturation of whey protein

Heating milk protein can lead to protein aggregation and precipitation as well as glycation when reducing carbohydrates (lactose) are present, which could decrease susceptibility to enzymatic hydrolysis [12, 41]. In the current study a relatively mild heat treatment was applied, which did not result in a reduction of protein solubility or glycation. Therefore, the observed effects of heating WPI are likely mostly due to denaturation (unfolding) and aggregation. The increased intact protein hydrolysis rate during digestion as a result of heat-induced whey protein denaturation was also shown before [8, 42-44]. In line with our hypothesis, we found that denaturation of whey protein did not affect absorbable product release (FAA and LMW peptides) during digestion in SIM (**Figure 4.5 and Table 4.3**) or the accumulation of bioaccessible fraction (dialysate <1 kDa) in TIM-1 (**Figure 4.9**). This is in accordance with observations that heating (121°C, 8-20 min) does not affect release of FAA and LMW (and MMW) (in the form of TCA (12%) soluble N) from infant formula protein [8], or absorbable products (dialysate N <1 kDa) from whey protein [7], during static *in vitro* gastrointestinal digestion. Interestingly, we found that denaturation considerably increases the amount of HMW peptides during intestinal digestion (**Figure 4.6**). An increased rate of intact protein loss combined with increased appearance of peptides in the HMW molecular weight range in DWPI than NWPI was also found by others in a dynamic *in vitro* gastrointestinal digestion model [5]. In our study at 2% DH, 66% of DWPI initial protein was present as HMW, confirming that intact protein cleavage was preferred over intermediate product cleavage after denaturation. A change in intermediate product composition could potentially infer a change in presented epitopes and immunological activity.

## Gastrointestinal protein hydrolysis kinetics as affected by protein type used

Intact β-casein is expected to be hydrolyzed rapidly during *in vitro* digestion compared with native whey proteins, as this was shown by others [9, 42]. Indeed, we observed a high rate of intact β-casein loss (**Figure 4.2C**), which was reflected in total intact protein hydrolysis rate that was higher for PBI compared to NWPI in the early small intestinal phase. On the one hand, β-casein, unlike native whey proteins, does not exhibit a globular structure but has a more open and mobile conformation [45]. Globular structure could limit cleavage sites' accessibility to enzymes [12], hence absence thereof could relatively increase cleavage sites' accessibility to enzymes. On the other hand, β-casein self-assembles into micelles [46] in contrast to whey proteins, which could potentially limit cleavage site accessibility to enzymes as well. Furthermore, β-casein has ~30% fewer potential cleavage sites for digestive enzymes (pepsin, trypsin and chymotrypsin combined) compared to whey proteins (3.3 mMol/g vs 4.7 mMol/g for both α-Lac and β-Lac) (PeptideCutter, www.expasy.org). Apparently, the impact of these primary and quaternary structural features on intact protein hydrolysis is less than the absence of a compact tertiary structure. Contrary to our hypothesis, it was found that the rate of absorbable product release (FAA and LMW peptides) of PBI were not

increased compared to NWPI (**Figure 4.5 and Table 4.3**). Moreover, curve fitting indicated the maximum amount of LMW peptides that can be released ($m_{LMW}$) to be lower from PBI than NWPI, rather than higher. We based our absorbable product release hypothesis on the observation that in neonatal piglets postprandial plasma AA concentrations were higher from PBI than NWPI [4]. Apparently, other factors next to absorbable product release (as we defined it for this study), like brush border enzyme hydrolysis and absorption efficiency, are playing a large role in actual absorbable product formation *in vivo*.



## Gastrointestinal protein hydrolysis mechanism as affected by changes applied to milk protein and its importance in absorbable product release

A limited number of *in vitro* protein digestion reports [47, 48] mention Linderstrøm-Lang's theory that describes two fundamentally different initial protein hydrolysis mechanisms ("one-by one" vs "zipper") [11]. In the current study, in NWPI hydrolysis the enzyme affinity towards intact proteins was very low compared to its affinity towards intermediate peptides, assuming a "one-by-one" like mechanism (**Figure 4.4C**). In SIM, enzymes showed an increased affinity towards intact proteins vs intermediate peptides for DWPI compared with NWPI (**Figure 4.4C**). For the TIM-1 duodenum a continuous relation between DH and proportion of intact protein could not be plotted due to in- and efflux of protein in this compartment. However, also in the TIM-1 duodenum at similar DH, more intact protein was hydrolyzed from DWPI than NWPI (**Figure 4.8C**). The DWPI hydrolysis mechanism is therefore more "zipper" like. The considerably higher HMW intermediate digestion product release in SIM from DWPI compared to NWPI (**Figure 4.6B**) supports the assumed mechanism type. Intact PBI hydrolysis showed an initial fast drop with increasing DH, attributed to intact β-casein hydrolysis, but the following slow decline was almost parallel to NWPI. Therefore, PBI protein hydrolysis mechanism initially is "zipper", but thereafter "one-by-one". The release of considerably more MMW intermediate digestion products from PBI than NWPI confirms the different mechanisms followed. Both changes applied, i.e. in protein heat exposure and type, are essentially a reduction in total protein tertiary structure (by structure alteration or by dilution, respectively) and as such are bound to change the hydrolysis mechanism to a more zipper-like type due to an increased probability of intact protein cleavage versus intermediate product cleavage. Additionally, the products of intact protein cleavage in DWPI (HMW peptides) may be resistant to cleavage when they are part of soluble aggregates as a result of heat induced intermolecular disulphide bond formation. Our results can thus be explained by Linderstrøm-Lang`s theory on protein hydrolysis. We found strong correlations between DH and absorbable product release in the intestinal phase of SIM for all protein solutions tested. This correlation implies that absorbable product release was independent of the mechanism of protein hydrolysis. The TIM-1 experiment confirms this; although in the TIM-1 duodenum the hydrolysis mechanism was different, a similar accumulation of bioaccessible products (free AA and AA bound in peptides <1 kDa) in the dialysate from jejunum and ileum for NWPI and DWPI was found (**Figure 4.9**). Hydrolysis

mechanism independent absorbable product release during *in vitro* digestion suggests the importance of the pancreatic exopeptidases (carboxypeptidase A and B) that act in concert with the endopeptidases trypsin, chymotrypsin and elastase. Taken together our data can be explained by Linderstrøm-Lang`s theory. However, there is no link to absorbable product release, and it might thus be less relevant for explaining postprandial plasma amino acid concentrations.

## Potential limitations

One potential limitation of the current study might be that the same protein concentration was used as was tested in neonatal piglets, while the *in vitro* digestion models (SIM and TIM-1) were set to simulate human infant conditions. Infants consume milk with ~13 g/L protein, while the neonatal piglets consumed 62.5 g/L (similar to sows` milk) [4], hence in the current *in vitro* study the enzyme : substrate ratio was lower than what is to be expected *in vivo* in human infants. Although this limits the translatability to the *in vivo* situation, it does allow to investigate the effects in more detail.

## The nature of absorbable products in in vitro digestion models

We found previously in neonatal piglets that including β-casein in milk protein composition increased incremental postprandial plasma AA peak by 39% compared with whey protein alone [4]. We expected therefore the absorbable product release during *in vitro* protein digestion to be increased in the same manner in the current study. This was not the case; absorbable product release (LMW (<0.5 kDa) peptides and free AA) was similar for NWPI and PBI: the summed area under the curve of FAA and LMW as a function of time (**Figure 4.5A and B**) for the first 45 min intestinal phase was only 1.5% higher for PBI than for NWPI. It needs to be considered that our *in vitro* models do not include intestinal brush border enzymes, which have been shown to cleave also MMW peptides [49, 50]. Therefore, we probably underestimate the importance of MMW in the release of absorbable products of protein digestion. In the first 45 min of intestinal phase PBI digesta, MMW content was considerably higher than that of NWPI digesta (twice as high at 20 min). Moreover, the summed area under the curve of FAA, LMW and MMW as a function of time (**Figure 4.5A, 5B and 6A**) in this period was 36% higher for PBI than NWPI. Taking MMW peptides into account in absorbable product release, thus greatly improved the *in vitro* - *in vivo* correlation.

## Conclusions

We conclude that both milk protein type and denaturation by heat increased the rate of intact protein loss and changed the mechanism of initial protein hydrolysis without affecting free amino acid and LMW peptide (<0.5 kDa) release during *in vitro* digestion. Our findings can be partially explained by the Linderstrøm-Lang`s theory of intact protein hydrolysis mechanisms. However, the theory does not help explain absorbable product release and

might thus be less relevant for explaining postprandial plasma amino acid concentrations. Our results suggest that the nature of intermediate digestion products, especially MMW peptide levels, is important in relation to postprandial AA responses.

## Conflicts of interest



EA, GGMT and IBR are employees of Danone Nutricia Research.

## Acknowledgments

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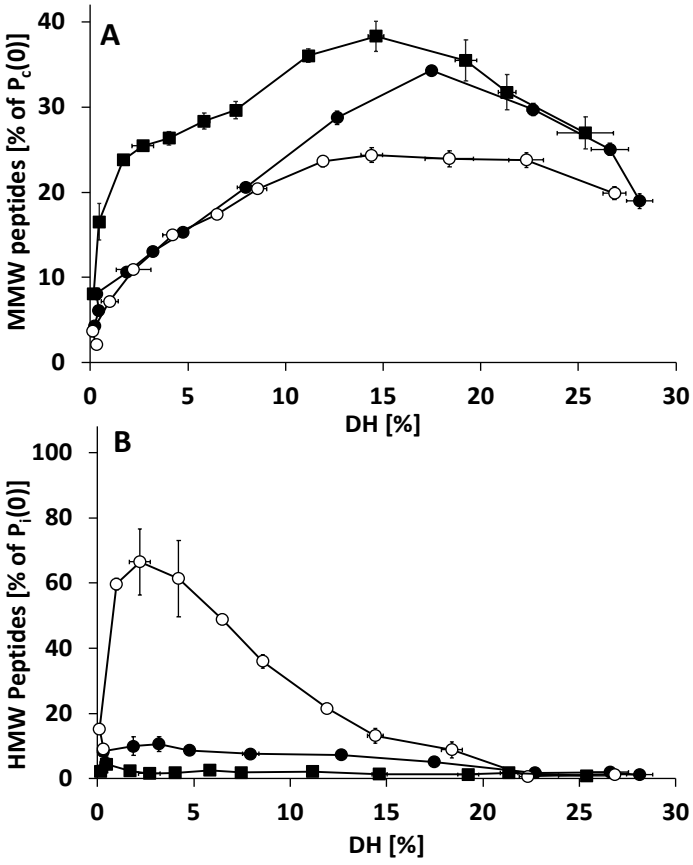
# Supplementary material

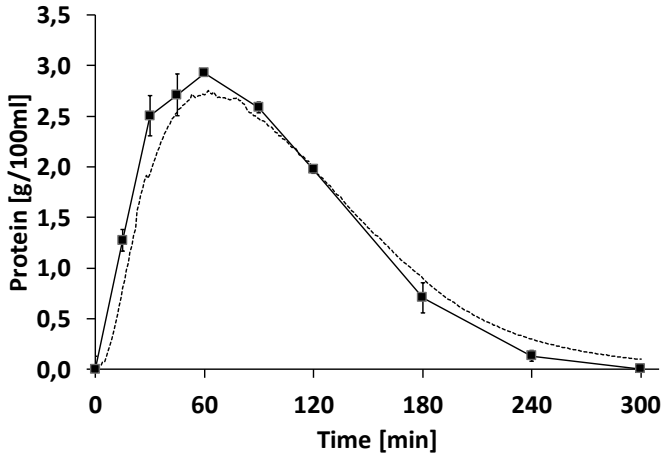
**Supplementary table S4.1: Degree of protein hydrolysis, free amino acid and peptide content of undigested milk protein solutions and after gastric digestion in SIM.[1]**

| | NWPI | | DWPI | | PBI | |
|---|---|---|---|---|---|---|
| | **MPS** | **G120** | **MPS** | **G120** | **MPS** | **G120** |
| DH *(%)* | 0 | 0.22±0.04 | 0 | 0.31±0.07 | 0 | 0.17±0.01 |
| LMW *(% of $P_c(0)$)* | 0.13±0.03 | 0.24±0.02 | 0.09±0.03 | 0.17±0.06 | 0.91±0.05$^{*}$ | 1.01±0.04$^{*}$ |
| FAA *(% of $P_c(0)$)* | 0 | 0.06±0.03 | 0 | 0.06±0.03 | 0 | 0.13±0.02 |
| MMW *(% of $P_c(0)$)* | 4.79±1.07 | 6.91±0.14 | 3.69±0.90 | 3.40±0.35 | 9.20±0.47$^{*}$ | 13.37±0.57$^{*}$ |
| HMW *(% of $P_c(0)$)* | 0 | 2.63±0.75 | 0 | 9.05±1.56$^{\#}$ | 0 | 2.29±0.28 |

[1]Means±SEM, n=3. SIM = semi-dynamic *in vitro* model of the gastrointestinal tract. MPS = milk protein solution. G120 = end of gastric digestion (t=120 min). DH = degree of hydrolysis, LMW = low molecular weight peptides, FAA = free amino acids, MMW = medium molecular weight peptides, HMW= high molecular weight peptides. NWPI = native whey protein isolate, DWPI = denatured whey protein isolate, PBI = protein base ingredient. % of $P_c(0)$ = weight percentage of crude protein amount at t=0. $^{*}$significant difference PBI vs. NWPI ($p<0.05$); $^{\#}$significant difference NWPI vs. DWPI ($p<0.05$).




**Supplementary Figure S4.1: Intermediate digestion products in the intestinal phase of SIM as function of digesta degree of hydrolysis. ●) Native whey protein isolate (NWPI), ○) denatured whey protein isolate (DWPI), ■) protein base ingredient for infant formula (PBI). A) Medium molecular weight peptides (MMW, 0.5-5 kDa) as determined by HP-SEC expressed as weight % of the crude protein ($P_c(0)$). B) High molecular weight peptides (HMW, 5-13 kDa) expressed as % of the intact milk protein content at t=0 ($P_i(0)$) as determined by SDS-page and densiometric analysis. Mean±SEM (n=3).**

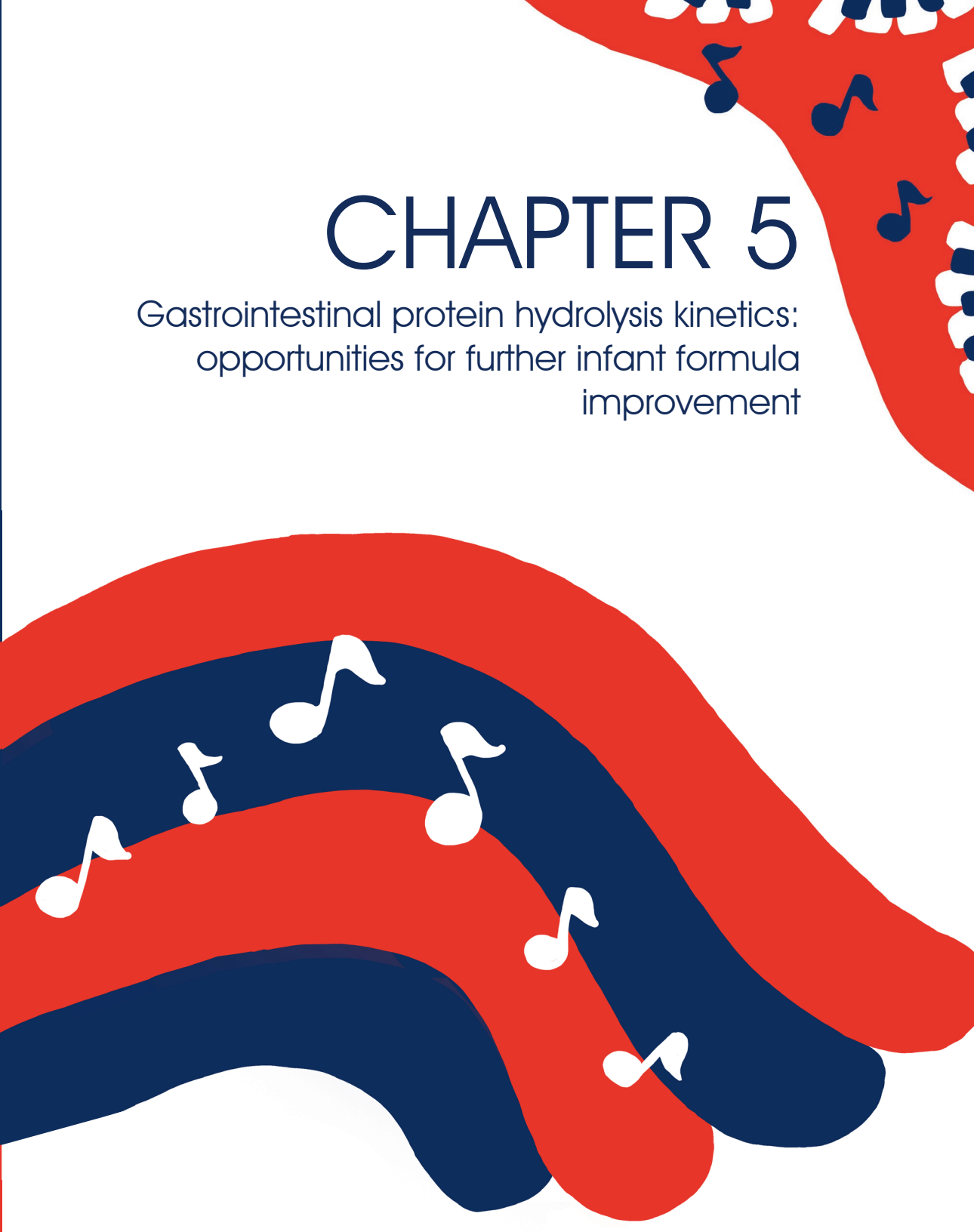



**Supplementary Figure S4.2: Milk protein concentration in TIM-1 duodenum, Dotted line calculated based on mean logged actual gastric emptying curve, ■) measured using dumas and corrected for blank run protein levels. Displayed are means±standard deviation.**


5

# CHAPTER 5

## Gastrointestinal protein hydrolysis kinetics: opportunities for further infant formula improvement



*Abrahamse, E., Thomassen, G.G.M., Renes, I.B., Wierenga, P.A. and Hettinga, K.A. Submitted version.*
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## Abstract

The postprandial plasma essential amino acid (AA) peak concentrations of infant formula (IF) are higher than those of human milk (HM) in infants. In addition, several HM proteins have been recovered intact in infant stool and appeared digestion resistant *in vitro*. We therefore hypothesized that gastrointestinal protein hydrolysis of IF is faster than HM and leads to accelerated absorbable digestion product release. HM and IF protein hydrolysis kinetics were compared in a two-step semi-dynamic *in vitro* infant digestion model, and the time course of protein degree of hydrolysis (DH), loss of intact protein and release of free AA and peptides was evaluated. Gastric DH increase was similar for IF and HM, but the rate of intestinal DH increase was 1.6 times higher for IF than HM. Intact protein loss in IF was higher than HM from 120 min gastric phase until 60 min intestinal phase. Intestinal phase total digestion product (free AA + peptides <5 kDa) concentrations increased ~2.5 times faster in IF than HM. IF gastrointestinal protein hydrolysis and absorbable product release is faster than HM, possibly due to the presence of digestion resistant proteins in HM. This might present an opportunity to further improve IF bringing it closer to HM.

## Introduction

Human milk (HM) is the gold standard of infant nutrition and delivers nutrients to the infant to ensure the best possible growth and development [1]. When HM is not available, infant formula (IF) is an alternative. HM presents protein to the infant that is different in a.o. composition, processing and matrix from IF, which can impact gastrointestinal digestion [2-4]. Since gastrointestinal protein digestion is a key determinant of systemic amino acid (AA) delivery rate and amount [5], efforts to support comparable protein digestion between HM and IF are warranted.



Protein is an essential nutrient as it is the only dietary source of (essential) amino acids (E) AAs. In the gastrointestinal tract, protein is digested to ultimately yield free AA, di-, and tripeptides, which can be absorbed by the small intestinal epithelium [6]. The kinetics of gastrointestinal protein digestion are determined by the rate of gastric emptying and protein hydrolysis by gastric, pancreatic, and intestinal brush border proteases and peptidases.

The postprandial (pp) plasma EAA peak concentration of humanized cow's milk (HCM) protein based IF was found to be 18% higher than that of iso-proteinaceous human milk (HM) in preterm infants [7]. This higher plasma peak could be due to differences in protein digestion and absorption kinetics in infants, as was shown for pp AA concentrations in adults [5]. Several studies have shown that IF gastric emptying rate is similar or slower than that of HM [8]. Together, this suggests that the release rate of absorbable protein digestion products upon hydrolysis of IF protein might be higher than that of HM protein.

The compositional humanization of cow's milk (CM) protein in modern IF involves a change in casein to whey ratio (c/w) from 80:20 to 40:60 to mimic the ratio as found in mature HM [9]. However, casein and whey protein compositions differ between CM and HM. For example, the most abundant whey protein in CM is β-lactoglobulin, a protein which is absent in HM. In contrast, lactoferrin only constitutes 2% of the whey composition of CM, which in HM is the second most abundant whey protein [2]. As the protein hydrolysis rate depends on the protein type, these protein compositional differences may result in differences in the protein hydrolysis rate [10, 11].

It has long been known that HM contains proteins which, in addition to EAA delivery, have functions in digestion and nutrient absorption, show anti-microbial activity and are key in the development of the gastrointestinal and immune system. Many of these functional proteins depend on their intact structure to exert their function in the gastrointestinal tract [9]. As such, some are relatively resistant to digestion, for example lactoferrin which can be retrieved intact in low amounts (6-10% of intake) in the stool of breastfed infants [12]. In addition, human milk contains inhibitors of gastrointestinal proteases [13], possibly

to protect the functional proteins from breakdown, which may also affect overall protein hydrolysis kinetics.

Additionally, milk protein processing and product matrix, which are very different between HM and IF have both shown contradictory effects on protein hydrolysis rate and pp AA concentrations [4, 14]. Still, higher plasma EAA concentrations have been observed after IF ingestion [7]. We hypothesize that the release rate of absorbable products of protein digestion of IF protein is higher than HM. The aim of this study is to compare the time course of protein hydrolysis of HM and IF in a two-step semi-dynamic *in vitro* model of the infant gastrointestinal tract (SIM). The *in vitro* model does not contain brush border enzymes that are responsible for releasing absorbable products from intermediate peptides. Therefore, the time course of release of not only free AA (FAA), di- and tripeptides but also intermediate digestion products (medium molecular weight peptides (MMW, <5kDa)) are evaluated. Additionally, the time course of the degree of protein hydrolysis (DH) and loss of intact protein are assessed to get insights in the potential underlying and driving mechanisms.

# Materials and Methods

## Materials

The composition of infantile digestive fluids was as follows. Simulated saliva fluid (SSF) consisted of: 0.6 g/L α-amylase (Aspergillus oryzae, Sigma A9857, 150 units/mg protein [15], Sigma-Aldrich Chemie N.V., Zwijndrecht, Netherlands), 6.2 g/L NaCl, 2.2 g/L KCl, 0.3 g/L $CaCl_2 \cdot 2H_2O$ and 1.2 g/L $NaHCO_3$ in distilled water, adjusted to pH 6.3. Simulated gastric fluid (SGF) consisted of: 125 mg/L lipase (Rhizopus oryzae, Amano DF 15, 177 FIP units/mg [16], Amano Enzyme Europe Limited, Oxfordshire, United Kingdom), 50 mg/L pepsin (Porcine, Sigma P7012, 2,500 units/mg protein [17]), 3.1 g/L NaCl, 1.1 g/L KCl, 0.15 g/L $CaCl_2 \cdot 2H_2O$, 0.82 g/L Na-acetate, in distilled water, adjusted to pH 5.8. Simulated intestinal fluid (SIF) consisted of: 5.0 g/L bile extract (Porcine, Sigma B8631), supernatant of centrifuged (12,000*g* for 20 min at 4°C) pancreatin (Porcine, 4xUSP unit activity [16], Sigma P1750) 12.5 g/L, 2.5 g/L NaCl, 0.3 g/L KCl, 0.15 g/L $CaCl_2 \cdot 2H_2O$ adjusted to pH 7.0. Simulated digestive fluids were prepared freshly. Protease inhibitor used was Bowman-Birk inhibitor (BBI) (Sigma T9777), all other chemicals were of analytical grade and obtained from Sigma or Merck.

## Sample description

HM was obtained from 8 donors in the Netherlands from 2015 to 2019, after written informed consent. Donors indicated having surplus milk that was not needed to feed their infant. In total 17 mature HM donations were made, on average on day 157 after delivery (range 60-251). After collection, milk was stored directly at -80°C. On the day before testing in late 2019, donated milk was thawed by placing at 4°C, and subsequently pooled. Infant

formula (IF) suitable for infants up to 6 months of age was bought in a local supermarket. IF reconstitution rate (11.85% (w/v)) was different from recommendations on pack (13.60% (w/v)) to match HM pool protein equivalent content. Infant milk protein equivalent ($P_{eq}$) was defined to consist of "true protein" as defined by Lonnerdal [9], and FAA, and was calculated using equation 1:

$$P_{eq}(0)\ [g/L] = ([N] - [NPN]) \times 6.25 + [AA] \quad (1)$$



wherein: [N] is the concentration of nitrogen present in the infant milk as quantified using Dumas method, [NPN] is the concentration of non-protein nitrogen (NPN, N soluble in 12% (w/v) trichloroacetic acid, 6.25 is the protein conversion factor, and [AA] is the concentration of free AA in infant milk quantified by UPLC as described below. FAA were included in calculation of protein equivalent because they are excluded from the "true protein" concentration, as they are soluble in 12% trichloroacetic acid, while they are a source of AA to the infant. $P_{eq}(0)$ as we define it does not include all LMW and MMW peptides present in HM and IF because it is unknown whether these peptides are all soluble in 12% trichloroacetic acid (i.e., it is unknown if they all contribute to NPN). Infant milk nutritional composition is given in **Table 5.1**.

**Table 5.1: Nutritional composition of the test products.**[1]

| g/L | HM | IF |
|---|---|---|
| N | 1.72±0.01[4] | 1.66±0.03 |
| NPN | 0.28±0.10[4] | 0.16±0.02 |
| True protein[2] | 9.00±0.68[4] | 9.38±0.35 |
| Free AA | 0.35±0.02[4] | 0.02±0.01 |
| Protein equivalent[3] | 9.35±0.69[4] | 9.40±0.47 |
| Fat | 34.00±1.05[5] | 29.60[6] |
| Carbohydrates | 59.50±1.79[5] | 63.60[6] |

[1]Infant milk nutritional composition; N and NPN (Non protein nitrogen, 12% trichloroacetic acid soluble N) as determined by Dumas, FAA as determined by UPLC. (Means±sd). [2]True protein = ((N-NPN)*6.25) as recommended by Lonnerdal [9]. [3]Protein equivalent ($P_{eq}$) = sum of true protein and free amino acids. [4]Data from pooled human milk. [5]Weighted means of data obtained using MIRIS human milk analyzer on individual donations. [6]Data on pack for 13.60% (w/v) reconstitution rate converted to used 11.85% (w/v) reconstitution rate.

## Semi dynamic *in vitro* simulation of infant gastrointestinal tract (SIM)

HM and IF were digested *in vitro* using the SIM. The SIM is based on a computer controlled parallel fed batch system by Dasgip equipped with 100 mL bioreactors (Eppendorf, Dasgip Mini Spinner Type DS0100B) (**Figure 5.1**) [18, 19]. Ratios of milk to simulated digestive fluid were chosen to simulate the ingestion of a 200 mL meal by a 0–6-month-old infant. Start volume of the bioreactors was 35 mL milk and all volumes were adjusted proportionally to this volume. Milk to simulated digestive fluid ratios and enzyme activity at the end of

the digestion phases resembled recommendations for static *in vitro* infant digestion model from INFOGEST [20]. Prior to the digestion experiment, bioreactors were filled with 37 mL infant milk. Bioreactor temperature was maintained at 37°C using a water bath. After the temperature of the milk reached 37°C, a 2.0 mL sample was taken from the bioreactors and the gastric phase of 120 min was started by a single shot of SSF and SGF, followed by continuous SGF addition.

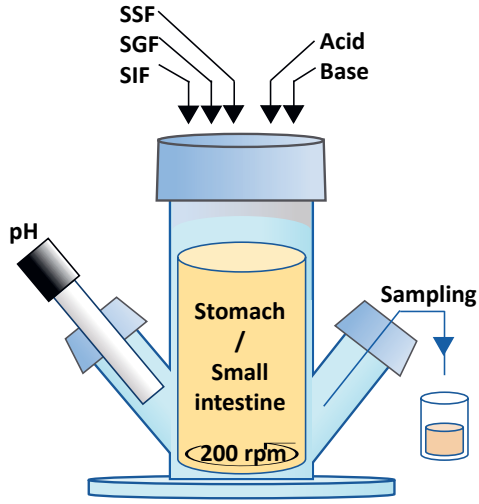


**Figure 5.1: Schematic representation of SIM *in vitro* model of the infant gastrointestinal tract. SSF = simulated saliva fluid, SGF = simulated gastric fluid, SIF = Simulated intestinal fluid.**

During the gastric phase the pH was gradually lowered following a set curve based on *in vivo* observations by addition of 1 M HCl to closely mimic the dynamic postprandial infant gastric pH (**Figure 5.2A**). After the gastric phase the pH was increased to 6.5 in 10 min by addition of 1 M $NaHCO_3$. The subsequent intestinal phase of 180 min was started by a single shot of SIF followed by continuous SIF addition. During the intestinal phase the pH was gradually increased to 7.2 at 180 min by addition of 1 M $NaHCO_3$ (**Figure 5.2B**).

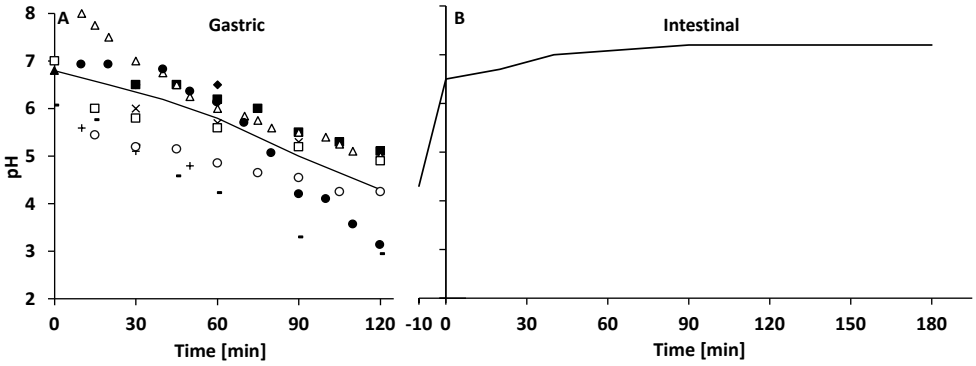




**Figure 5.2: SIM pH curves. A) Pre-set gastric pH curve (straight line) and *in vivo* reference values from: ■ [37], ◆ [38], ▲ [39], × [40], ○ [41], · [42], + [43], △ [44], ● [45], and □ [46]. B) Pre-set intestinal pH curve based on values used by Blanquet et al. in TIM-1 [47]. Pre-set gastric pH curve is polynomial (3rd order) trendline of mean in vivo pH per timepoint.**

Digesta samples (2.0 mL) were taken at 10, 30, 60, 90, and 120 min of gastric phase and 2, 6, 10, 20, 30, 60, 120, and 180 min of intestinal phase. Gastric and intestinal digesta were visually homogeneous and no large lumps were present that could hamper pipetting, which indicates digesta samples were representative. Digesta samples were directly diluted 1:1 with sample buffer (0.1 M phosphate buffer) and snap frozen in liquid nitrogen. Sample buffer pH and content were chosen to inhibit enzymatic activity during storage. Gastric sample buffer was pH 7, intestinal sample buffer was pH 5.5 and contained 0.58 g/L BBI. Blank runs, using phosphate buffered saline to replace milk, were performed to determine the contribution of the added simulated digestive fluids to the total concentrations of amino groups, peptides and AA.

## o-Phthalaldehyde method (OPA)

To determine the degree of hydrolysis (DH) free amino acid groups were quantified using the OPA method. The OPA reagent was prepared as described previously [21]. Samples were diluted to 5 g protein /L in a 20 g SDS /L solution, stirred for 20 min and stored at 4°C overnight. The samples were then diluted to 2 g protein /L with Millipore water. Aliquots (5 µL) were added to 300 µL of the OPA reagent solution and equilibrated for 10 min. The presence of alkyl-iso-indols formed by the reaction of free amino groups with OPA was measured by the absorbance at 340 nm. To calculate the concentration of free $NH_2$ groups, a calibration curve was measured using leucine as a reference compound. Total concentration of amino groups of HM and IF were determined by OPA after hydrolysis in 6 M HCl at 110°C for 22 h. The degree of hydrolysis (DH) at a time point (t=y) was calculated using equation 2:

$$\mathrm{DH(t)}\ [\%] = \frac{[\mathrm{NH_2}]_\mathrm{t} \times \mathrm{df} - [\mathrm{NH_2}]_0}{[\mathrm{NH_2}]_\mathrm{AH} - [\mathrm{NH_2}]_0} \times 100 \qquad (2)$$

wherein: $[NH_2]_t$ is the concentration of free amino groups present in the t=y digesta sample, df = dilution factor during digestion, $[NH_2]_0$ is the concentration of free amino groups present in the t=0 sample, and $[NH_2]_{AH}$ is the concentration of free amino groups present in the acid hydrolysed t=0 sample of the infant milk. The total infant milk peptide bond concentration is calculated by subtracting $[NH_2]_0$ from $[NH_2]_{AH}$ DH increase per phase (gastric or intestinal) is calculated by subtracting corresponding DH(0) from DH(t).

## Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

Intact protein in HM, IF, and digesta samples was analyzed by reducing SDS-PAGE using NuPAGE 4-12% Bis-Tris Midi protein precast gels (WG1402A Invitrogen, Thermo Fisher scientific, Landsmeer, the Netherlands). Samples were diluted to standardize protein concentration to 0.23 g/L using demineralized water. Subsequently 10 µL lithium dodecyl sulfate sample buffer (NuPAGE™ lithium dodecyl sulfate sample buffer (4 X), NP0007 Invitrogen, Thermo Fisher scientific) and 4 µL sample reducing agent containing 500 mM dithiothreitol (NuPAGE™ sample reducing agent (10x), NP0009, Invitrogen, Thermo Fisher scientific) was added to 26 µL diluted sample. Subsequently samples were heated for 10 min at 70°C. Each lane was loaded with 20 µL heated sample, i.e. 6.6 µg protein. Gels were run using SDS containing running buffer (XT MES running buffer, 1610789, Bio-Rad, Veenendaal, the Netherlands). Gels were fixed in 40% (v/v) methanol, 10% (v/v) acetic acid, rinsed and stained with SimplyBlue SafeStain (LC6060 Invitrogen, Thermo Fisher scientific). PageRuler™ Plus prestained protein ladder (PI26620, Fischer Scientific) was used as molecular-weight marker. Gels were imaged using a BIO-RAD Gel Doc XR imager and band intensity was quantified using Quantity One software. The relationship between protein quantity and band intensity was calibrated using five concentrations of a whey protein concentrate in demineralized water, showing linearity from 1 to 5 ug total protein per lane. The proportion of total intact milk protein remaining at a time point (t=y) during SIM digestion was calculated by expressing the sum of intensity of all bands at t=y (corresponding to bands present at t=0) as a proportion of the intensity of all bands at t=0 (% of $P_i(0)$). The same procedure was followed for individual proteins (x) present as single bands at t=0 and t=y and is expressed as (% of $P_{xi}(0)$).

## Ultra high-performance liquid chromatography - fluorescence (UPLC-FLR)

Quantification of free AA using UPLC-FLR was performed as described earlier [18]. Briefly: infant milk and digesta samples were prepared for elution by precipitation of proteins and large peptides with 3.5% (w/v) $HClO_4$ and filtration. The concentration of each AA in the sample was determined by UPLC using a pre-column derivatization with OPA and fluorometric detection. AA quantity in digesta samples is expressed as weight percentage of respective infant milk protein equivalent (% of $P_{eq}(0)$, **Table 5.1**). Cysteine, methionine and proline could not be quantified under the conditions used.

## High-performance size exclusion chromatography (HP-SEC)

Peptides were separated by size and then quantified using size exclusion chromatography (SEC) as described earlier [18]. The HPLC system (Shimadzu, 's-Hertogenbosch, the Netherlands) was equipped with a Superdex Peptide 10/300 column (17-5176-01 GE Healthcare, München, Germany) with a 10 kDa HMW cut off. Samples were centrifugated for 5 min (12,000 *g*), after which 20 µL supernatant was injected onto the column. The eluent was 25% (v/v) acetonitrile, 16% (v/v) trifluoracetic acid and detection was by done by absorption at 200 nm. The relationship between elution time and molecular weight was established using ten standards; Cytochrome C from bovine heart (12,327 Da), Aprotinin from bovine lung (6,500 Da), Adrenocorticotropic hormone from porcine pituitary (4,567 Da), Insulin A-chain oxidized ammonium salt from bovine pancreas (2,532 Da), Angiotensinogen 1-14 Renin substrate porcine (1,759 Da), Bradykinin acetate salt (1,060 Da), Bradykinin Fragment 1-7 (757 Da), Bradykinin Fragment 1-5 (573 Da), Ala-Ala-Ala-Ala-Ala (373 Da), and Gly-Leu (188 Da). The correlation coefficient of the linear fit between elution time between 9.2 and 19.2 min and 10 log molecular weight was 0.980. The chromatogram of a tryptophane standard showed that this amino acid started to elute at 23.0 min. Therefore, chromatograms were integrated from 11.4 min (5 kDa) to 23.0 min to exclude free amino acids that absorb at 200 nm. Peptide quantity was calculated using estimation of peptide amino acid composition and peptide molar extinctions coefficients using the methodology and reported extinction coefficients by Kuipers and Gruppen [22]. The average extinction coefficient of the peptides in the integrated area $\overline{\varepsilon(\mathrm{x})}$ was calculated using equation (3), adapted from [23],

$$\overline{\varepsilon(\mathrm{x})} = \varepsilon_b \times \left( \frac{\mathrm{Mw}(\bar{\mathrm{x}})}{\mathrm{Mw}(\overline{\mathrm{AA}})} - 1 \right) + \varepsilon\overline{(\mathrm{AA})} \times \frac{\mathrm{Mw}(\mathrm{x})}{\mathrm{Mw}(\overline{\mathrm{AA}})} \qquad (3)$$

wherein: $\varepsilon_b$ is the extinction coefficient of a peptide bond, which equals to 923 $M^{-1}cm^{-1}$, $\mathrm{Mw}(\bar{\mathrm{x}})$ is the mean peptide molecular weight in the integrated area, $\mathrm{Mw}(\overline{\mathrm{AA}})$ is the average amino acid molecular weight in the mean peptide, $\varepsilon\overline{(\mathrm{AA})}$ is the weighted average extinction coefficient of the amino acids in the mean peptide. Peptides were clustered in two fractions, LMW <0.5 kDa and MMW from 0.5-5 kDa. Peptides <0.5 kDa were considered di- and tri- peptides. LMW and MMW peptide quantity in digesta samples is expressed as weight percentage of respective infant milk protein equivalent (% of $P_{eq}(0)$).

## Data analysis

Data obtained from HM and IF were corrected for the level found in blank runs where appropriate. Data is shown as mean±standard error of the mean (n=3) unless specified otherwise. The concentration of total digestion products (TP) was calculated as the sum of FAA, LMW and MMW concentrations. Parameters (DH, FAA, LMW and TP) measured in the intestinal phase were fitted using first order reaction kinetics using equation 4:

$$z(t) = m \times (1 - e^{-kt}) + b \qquad (4)$$

wherein: z(t) is the value of the parameter at time t, m is the maximal increase in value that can be reached, k is the rate constant and b is the value at 120 min gastric phase. The solver function in Microsoft Excel was used to calculate the values for k, m and b that resulted in the lowest sum of squares of residuals. Results of k, m and b are reported with suffix to denote the respective parameter (DH, FAA, LMW and TP). Statistical significance of differences was analyzed by the Kruskal Wallis test in SPSS 19. Differences with a p value below 0.05 were considered statistically significant.

# Results

## Characterization of human milk and infant formula

Nitrogen (N) and non-protein N (NPN) concentrations were respectively ~0.06 g/L and ~0.12 g/L higher in HM than in IF, leading to a lower true protein concentration in HM than IF (**Table 5.1**). In contrast, HM total FAA concentration, as determined using UPLC-FLR, was ~14 times that of IF. Approximately 70% (w/w) of HM FAA consisted of glutamine and glutamic acid. The protein equivalent ($P_{eq}(0)$, sum of true protein and FAAs) was similar for HM and IF and consisted for 96.26 and 99.79% of true protein, respectively, with the remainder being FAA. Low molecular weight peptide (LMW, <0.5 kDa) concentrations in HM, as determined using HPSEC, were ~3 times higher in HM than in IF (9.27±1.65 and 2.95±1.05% of $P_{eq}(0)$, respectively). However, medium molecular weight peptide (MMW, 0.5–5 kDa) concentrations were similar (11.40±6.32 and 8.29±1.60% of $P_{eq}(0)$ for HM and IF, respectively) (see next paragraph for more details). The sum of FAA, LMW and MMW peptide concentrations, (during digestion referred to as total digestion products (TP)), was similar in HM and IF. If it is assumed that LMW and MMW peptides as determined using HPSEC are not intact protein and are not soluble in TCA (thus included in "true protein"), then this would mean that the intact protein concentration is 75.59 and 88.55% of $P_{eq}(0)$ for HM and IF, respectively. Similar total peptide bond concentrations were found in HM and IF, being 85.2±4.9 and 88.8±2.6 mM, respectively, as determined using OPA. Protein composition analysis using SDS-page, as shown in **Table 5.2**, revealed that the six most abundant proteins in HM together constitute ~96% of $P_i(0)$, in order of abundance: α-lactalbumin, lactoferrin, β-casein, serum albumin, free secretory component (SC), and κ-casein. In IF six proteins were detected (adding up to 100%); in order of abundance: β-lactoglobulin, β-casein, κ-casein, α-lactalbumin, α-casein and serum albumin.

**Table 5.2: Infant milk protein composition as determined by SDS-page and densitometry.[1]**

| % of total protein band intensity | HM | IF |
|---|---|---|
| Minor whey proteins | 4.50±0.31 | - |
| Lactoferrin | 22.54±1.29 | - |
| Serum albumin | 10.70±0.31 | 4.91±0.38 |
| Secretory component of Ig | 11.06±2.00 | - |
| α-casein | - | 11.61±0.23 |
| β-casein | 17.81±1.34 | 20.35±0.68 |
| κ-casein | 8.60±1.04 | 19.36±0.49 |
| β-lactoglobulin | - | 31.18±0.31 |
| α-lactalbumin | 24.78±1.15 | 12.59±0.51 |

[1]Means±SEM (n=3). IF contains bovine form of the protein, human milk contains human form.



## Gastrointestinal protein hydrolysis in SIM

*Gastric digestion*

In the gastric phase (0-120 min), milk protein degree of hydrolysis (DH) increased to ~6.0% at 120 min for both IF and HM (**Figure 5.3**). Gastric protein hydrolysis caused extensive intact protein loss, which at 120 min was higher for IF than HM: 40.7±3.0 and 52.3±6.0 % of $P_i(0)$ remained intact, respectively (**Figure 5.4 and 5.5**).

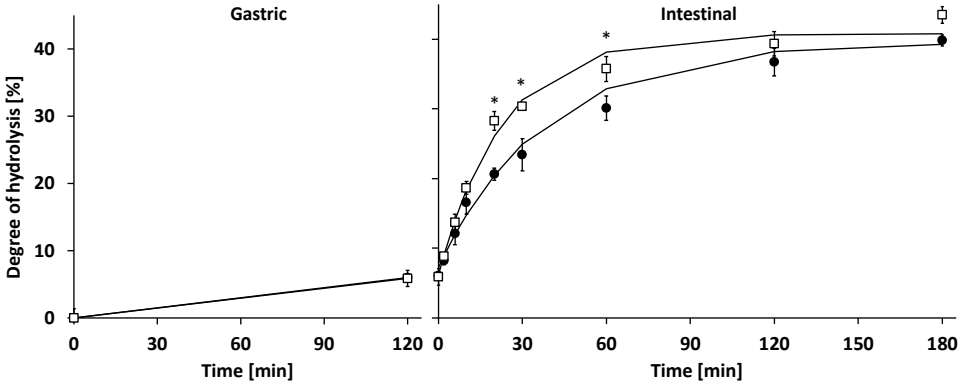


**Figure 5.3: Protein degree of hydrolysis in gastric and intestinal digesta in time. ●) Human milk (HM), □) Infant formula (IF); Lines in the intestinal phase are fitted data using first order reaction kinetics; Mean±SEM (n=3). *significant difference (p<0.05).**

Intact protein loss at 120 min for all caseins was high in both HM and IF: up to 20% remained intact (**Figure 5.6**). Intact whey protein was digested considerably less than casein; in both HM and IF whey proteins were still maximally up to 65% intact at 120 min, except for intact serum albumin in IF, of which only 18% remained intact (**Figure 5.6B**). Gastric protein

hydrolysis was accompanied by less than 3% of $P_{eq}(0)$ FAA release for both HM and IF (**Figure 5.7A**). The increase in LMW peptides was similar in HM and IF: 12.6±3.08 and 13.1±2.27% of $P_{eq}(0)$, respectively (**Figure 5.7B**). Substantially more MMW peptides were released from IF than HM: 39.9±2.75 vs 24.4±7.75% of $P_{eq}(0)$, respectively (**Figure 5.7C**). The concentration of total digestion products (sum of FAA, LMW and MMW) at the end of the gastric phase was similar at ~60% of $P_{eq}(0)$, although the increase was larger in IF due a lower concentration at t=0 (**Figure 5.7D**).

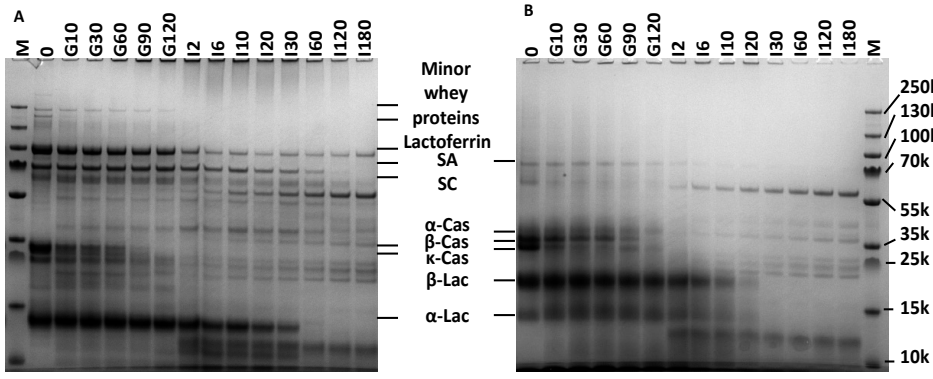


**Figure 5.4: Typical SDS-page gels loaded with undigested milk and digesta samples. A) human milk (HM), B) infant formula (IF). Lanes are marked with type of sample with prefix indicating the digestion phase (G = gastric, I = intestinal) and number reflecting time in min. M = Molecular weight marker. SA = serum albumin, SC = secretory component, α-Cas = alpha-casein, β-Cas = beta-casein, κ-Cas = kappa-casein, β-Lac = beta-lactoglobulin; α-Lac = alpha-lactalbumin. Annotation based on [2, 11].**

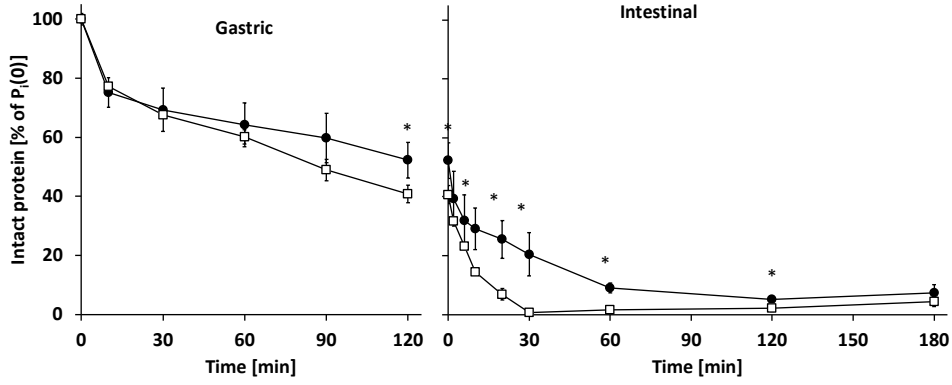


**Figure 5.5: Total intact protein hydrolysis in gastric and intestinal digesta in time, as determined by SDS-page and densiometric analysis of bands. ●) Human milk (HM), □) infant formula (IF); Total intact protein expressed as % of total intact milk protein amount at t = 0 ($P_i(0)$). Mean±SEM (n=3). *significant difference ($p<0.05$).**

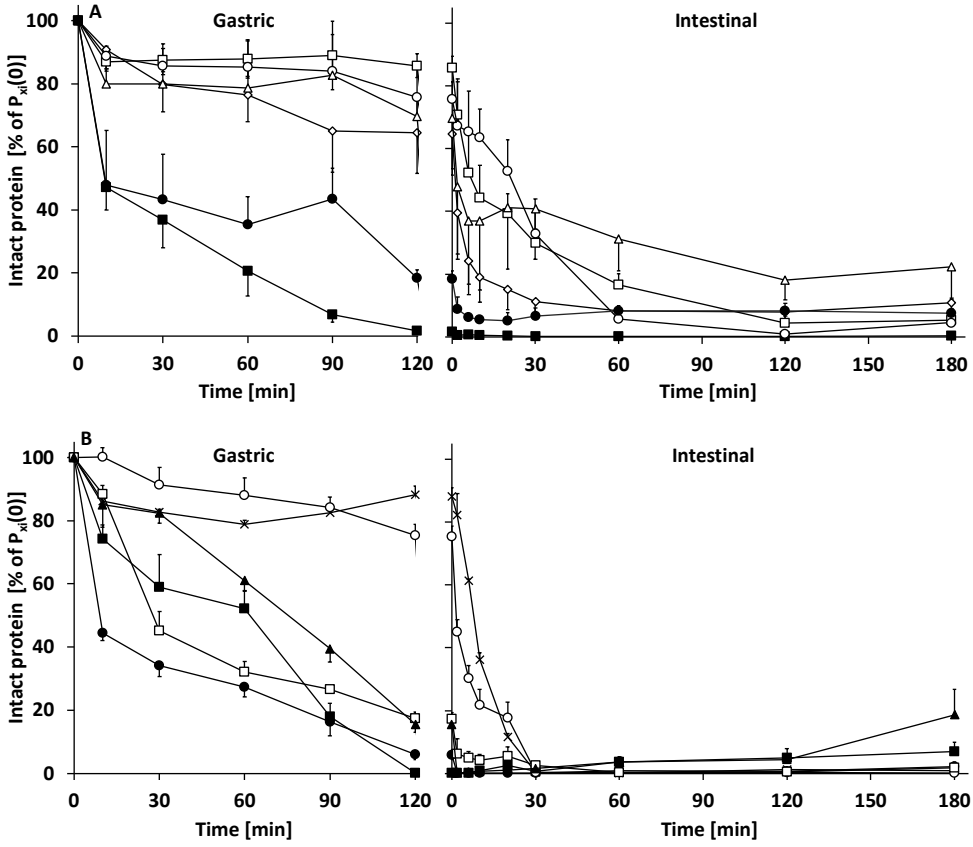


**Figure 5.6: Individual intact protein hydrolysis of the six most abundant proteins in gastric and intestinal digesta in time, as determined by SDS-page and densiometric analysis of bands. A) human milk (HM), B) infant formula (IF); ◇) Lactoferrin, □) Serum albumin, ○) α-lactalbumin, △) Secretory component, ×) β-lactoglobulin, ▲) α-casein, ■) β-casein and ●) κ-casein. Intact protein expressed as % of respective intact milk protein amount at t = 0 ($P_{xi}(0)$). Mean + or - SEM (n=3).**

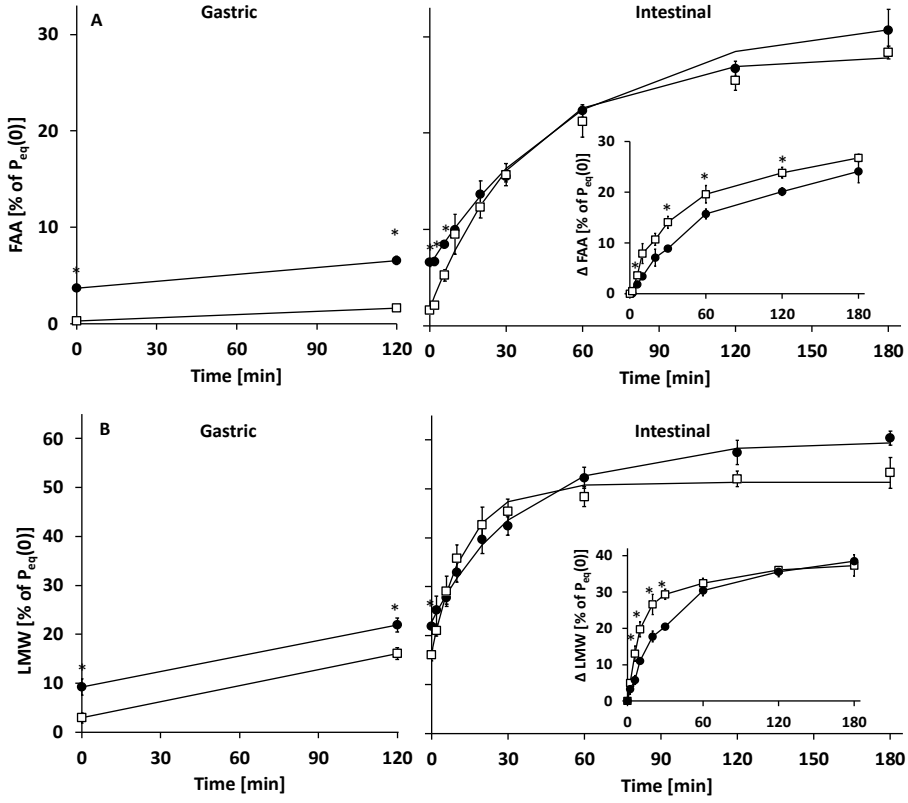


**Figure 5.7A and B: Protein digestion products of human milk (HM, ●) and infant formula (IF, □) in time, expressed as weight percentage of protein equivalent in the respective infant milk (% of $P_{eq}$(0)). A) free amino acids (FAA), B) Low molecular weight peptides (LMW, <0.5 kDa), As determined by UPLC (A), as determined by HP-SEC (B). Lines in intestinal phase are fitted data using first order reaction kinetics. Inserts show respective protein digestion products time course vs t=0 intestinal phase; Mean±SEM (n=3). *significant difference (p<0.05).**

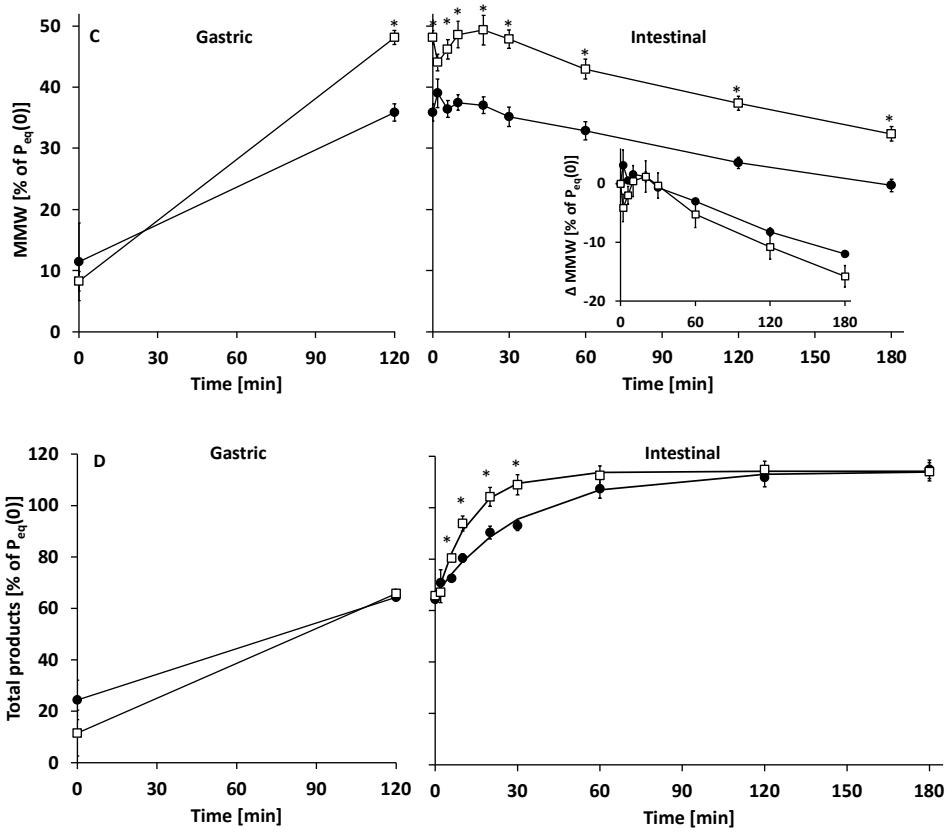


**Figure 5.7C and D: Protein digestion products of human milk (HM, ●) and infant formula (IF, □) in time, expressed as weight percentage of protein equivalent in the respective infant milk (% of $P_{eq}$(0)). C) Medium molecular weight peptides (MMW, 0.5-5 kDa) and D) Total protein digestion products (TP, sum of FAA, LMW and MMW). As determined by by HP-SEC (C). Lines in D intestinal phase are fitted data using first order reaction kinetics. Inserts in C shows respective protein digestion products time course vs t=0 intestinal phase; Mean±SEM (n=3). *significant difference (p<0.05).**

*Intestinal digestion*

Intestinal DH increase was faster for IF than HM; $k_{DH}$ of IF was 1.6 times higher than of HM (**Table 5.3**) and IF DH from 20-60 min was also higher than HM DH (**Figure 5.3**). However, from 120 min onwards, DH was similar again for IF and HM. Additionally, $m_{DH}$ was also similar for IF and HM, indicating that the same DH plateau was being reached, albeit at a slower rate for HM. Intestinal total intact protein loss of IF was considerably faster than that of HM. After 30 min, IF intact protein loss was complete (intact protein=0% $P_i$(0)), while HM intestinal digesta still contained 20.4±6.4% of $P_i$(0) intact protein at 30 min (**Figure 5.5**). At this digestion time point, ~11% lactoferrin, ~30% α-lactalbumin and serum albumin, and ~40% free secretory component in HM was still intact (**Figure 5.6A**).

**Table 5.3: Curve fitting values of infant milk protein hydrolysis in SIM intestinal phase.[1]**

| | HM | IF |
|---|---|---|
| DH | | |
| $k_{DH}$ (*$10^3$) *(min$^{-1}$)* | 26.28±6.27[a] | 42.92±2.70[b] |
| $b_{DH}$ *(%)* | 7.26±0.28 | 6.20±0.96 |
| $m_{DH}$ *(%)* | 32.31±2.31 | 34.61±1.35 |
| $R^2_{DH}$ | 0.977±0.001 | 0.974±0.001 |
| Free AA | | |
| $k_{FAA}$ (*$10^3$) *(min$^{-1}$)* | 16.44±2.90 | 26.24±5.23 |
| $b_{FAA}$ *(% of $P_{eq}$(0))* | 6.24±0.23[a] | 1.76±0.42[b] |
| $m_{FAA}$ *(% of $P_{eq}$(0))* | 25.82±3.21 | 26.27±1.21 |
| $R^2_{FAA}$ | 0.988±0.007 | 0.982±0.012 |
| LMW | | |
| $k_{LMW}$ (*$10^3$) *(min$^{-1}$)* | 27.84±1.42[a] | 72.32±1.22[b] |
| $b_{LMW}$ *(% of $P_{eq}$(0))* | 22.77±1.81[a] | 16.52±0.78[b] |
| $m_{LMW}$ *(% of $P_{eq}$(0))* | 36.84±1.78 | 34.76±1.77 |
| $R^2_{LMW}$ | 0.988±0.003 | 0.984±0.005 |
| Total products | | |
| $k_{TP}$ (*$10^3$) *(min$^{-1}$)* | 31.94±2.04[a] | 79.65±9.51[b] |
| $b_{TP}$ *(% of $P_{eq}$(0))* | 65.88±2.32 | 62.98±0.93 |
| $m_{TP}$ *(% of $P_{eq}$(0))* | 48.17±2.39 | 51.21±0.93 |
| $R^2_{TP}$ | 0.981±0.008 | 0.979±0.005 |

[1]Means±SEM. Means having different letters are significantly different $p<0.05$. k = rate constant, b = value at start of intestinal phase, m = maximal increase in value, $R^2$ = coefficient of determination. Suffix DH, FAA, LMW and TP denote respective parameter. AA = amino acids, DH = degree of hydrolysis, LMW = low molecular weight peptides, $P_{eq}$(0) = protein equivalent content of the respective infant milk. TP = Total digestion products (sum of free AA, LMW and MMW). HM = human milk, IF = infant formula.

SDS-page of intestinal digesta samples showed several bands (a.o. at 25, 35 and 60 kDa) increasing in density over time (**Figure 5.4**). These bands may represent pancreatic enzymes present in SIF (i.e., trypsin, chymotrypsin/elastase and triglyceride lipase/α-amylase respectively, based on their molecular weight) or breakdown products of larger milk proteins. Intestinal protein hydrolysis was accompanied by a similar FAA release rate ($k_{FAA}$) for both HM and IF (**Table 5.3).** As a consequence of the higher FAA concentration in undigested HM, FAA concentrations up to 6 min intestinal phase and $b_{FAA}$ were both higher for HM than IF (**Figure 5.7A and Table 5.3**). However, the concentration of released FAA was higher at several timepoints in IF than HM (**Figure 5.7A insert**). Undigested HM contained higher LMW peptide concentrations than IF, and gastric release of LMW peptides was similar, resulting in higher intestinal phase starting LMW concentration ($b_{LMW}$) for HM (**Figure 5.7B and Table 5.3**). IF intestinal LMW release rate was 2.6 times that of HM ($k_{LMW}$ in **Table 5.3**). The concentration of released LMW was also higher from 6-30 min in IF than HM (**Figure 5.7B insert**). During the intestinal phase, IF digesta MMW concentration was consistently

higher than that of HM digesta (**Figure 5.7C**) However, the MMW concentration decrease in time was similar for both HM and IF (**Figure 5.7C insert**). The biggest difference was at 30 min, where IF digesta MMW concentration was 1.4 times that of HM. The concentration of TP (sum of FAA, LMW and MMW) in the intestinal phase increased ~2.5 times faster in IF than HM ($k_{TP}$ in **Table 5.3**). The concentration of released TP was also higher from 6-30 min in IF than HM (**Figure 5.7D**). The end concentration of TP in the intestinal phase was similar at ~114% of $P_{eq}(0)$ (>100% reflecting the contribution of LMW and MMW peptides present in undigested milk which were not included in $P_{eq}(0)$, and the addition of water molecules in the hydrolysis process, which contributes ~6.6% $P_{eq}(0)$ at the end of digestion).



## Discussion

In the current study we compared *in vitro* the time course of protein hydrolysis of HM with that of a HCM based IF, using gastrointestinal conditions mimicking those in infants. We hypothesized that the absorbable digestion product release rate of IF would be higher than that of HM. This hypothesis was based on the observation by Moro *et al.*, that postprandial plasma EAA peak concentrations of IF were higher than those of HM [7], and the observations that several HM proteins were more hydrolysis-resistant than IF proteins [2, 9, 12]. In line with our hypothesis, it was observed that the concentration of TP (sum of FAA, LMW and MMW) in the intestinal phase increased ~2.5 times faster in IF than HM (**Figure 5.7D and Table 5.3**)), mostly due to the differences in MMW concentrations: The intestinal MMW peptide peak concentration was 1.4 times higher for IF than HM (**Figure 5.7C).** Additionally, the rate of intestinal DH increase was 1.6 times higher for IF than HM (**Table 5.3**). Intact protein loss was higher in IF than in HM from 120 min gastric phase until 60 min intestinal phase (Figure 5).

In contrast to our observations that IF has a higher digestion product release rate than HM, Maathuis *et al*. reported a lower protein digestion rate from HCM protein based IF than HM [24]. In the reported study, the digestion rate was assessed as the accumulation of N in intestinal dialysate of tiny-TIM-1, a dynamic model of the gastrointestinal tract that includes gastric emptying and digestion product removal by means of dialysis. A possible explanation for the different observation compared to the current study could be that HM contains considerably higher concentrations of (non-AA) NPN than IF (20 vs 6 % of total N, respectively) [24]. Non-AA NPN consists of small molecules, such as urea, that are easily dialysed and might therefore contribute to the perceived higher accumulation rate of digestion products, while non-AA NPN does not constitute actual absorbable AA containing fragments.

In the current study a similar DH increase during the gastric phase was accompanied by a higher intact protein loss for IF than HM, suggesting that per protein molecule more cleavages occurred in HM than IF (**Figure 5.3 and 5.5**). At the same time, a similar increase in FAA and LMW peptides was observed for HM and IF, but a higher increase in MMW peptides for IF, suggesting that in HM the proportion of gastric digestion products larger than MMW was higher than in IF. One possible explanation could be the smaller average AA chain length of proteins in undigested IF compared to undigested HM. The probability of multiple cleavages within one molecule increases with AA chain length, as does the probability that cleavage products are bigger than 5 kDa (the upper limit of MMW). Indeed, the protein weighted mean molecular weight based on SDS-page analysis was ~28 kDa vs ~54 kDa for IF and HM, respectively.

A higher intestinal DH increase rate for IF than HM was accompanied by a faster loss of intact protein and an increased release rate of LMW peptides and TP. The DH and TP release at the end of intestinal digestion were similar for IF and HM. Apparently, in the intestinal phase the remaining peptide bonds in HM contain cleavage sites that are less accessible than those of IF protein. This could be a result of differences in protein composition, processing and/or matrix between HM and IF.

Intact caseins were more susceptible to gastric hydrolysis than intact whey proteins, in both HM and IF (**Figure 5.6**), which is in accordance with previous reports [2, 10, 11]. During the gastric phase, intact serum albumin in IF was already degraded by 82%, relative to 14% for HM. Particularly intact HM serum albumin, α-lactalbumin and free secretory component were resistant to intestinal hydrolysis. Intact α-lactalbumin in HM was degraded slower in the intestinal phase than in IF (**Figure 5.6**).This could be partly due to denaturation of α-lactalbumin in IF, which is described to be induced by IF industrial processing [14]. The higher hydrolysis resistance of human milk whey proteins than the cow's milk counterparts was also previously observed [2].

Although CM based IF protein composition was "humanized" with respect to the casein/whey ratio, protein composition still greatly differs between IF and HM. As mentioned, HM contains higher levels of functional whey proteins with functions other than nutritional, like immunomodulatory, that are different than IF. Interestingly, these are also shown to be relatively resistant to hydrolysis. For example, lactoferrin, secretory immunoglobulin A and lysozyme have been retrieved intact in small amounts (<10% of intake) in the stool of breastfed infants [12]. Although these proteins can also be produced by the infant's intestine, it has been suggested that the main part has a dietary origin as stool of breastfed infants contains higher levels than that of IF-fed infants [25]. In line with our observations (Figure 6), other *in vitro* digestion studies have shown serum albumin, lactoferrin and immunoglobulins to be the most digestion resistant proteins in (mature) HM after infant

*in vitro* digestion [26]. The higher resistance to hydrolysis of those proteins could be due to post-translational (protective) modifications, such as the high degree of glycosylation observed for human vs bovine lactoferrin, which hampers hydrolysis by trypsin [27].

Protease inhibitors present in HM, such $\alpha_1$-antitrypsin and $\alpha_1$-antichymotrypsin [13], may inhibit peptide bond cleavage by the major intestinal proteases. However, concentrations of protease inhibitors in HM show no negative correlation with the overall level of proteolysis during *in vitro* digestion [26]. Furthermore, the magnitude of their effect on overall protein hydrolysis kinetics is still unknown.



The manufacturing of IF includes several (heat) processing steps, while HM is typically consumed without any prior treatment. Heat processing has been shown to decrease its susceptibility to hydrolysis due to aggregation and precipitation, as well as due to glycation [14, 28], but also to increase whey proteins' to hydrolysis due to denaturation [10, 29]. Heat induced protein glycation is unlikely to be responsible for a higher DH increase rate of IF, as this likely has the opposite effect and slows down hydrolysis [14]. Heat processing-induced whey protein denaturation is reported to make whey proteins susceptible to hydrolysis by pepsin, and to induce gastric intact protein loss and DH increase [30]. However, for intestinal digestion, where native whey protein can already be hydrolyzed, an increased rate of intact protein loss is observed at a similar DH increase rate [31]. Whey protein denaturation more likely leads to a change in the hydrolysis mechanism in the intestinal phase by increasing the probability to cleave intact protein over intermediate peptides as described by Adler-Nissen [32]. Therefore, heat induced protein denaturation is unlikely to be responsible for the observed increased intestinal DH increase rate of IF compared to HM.

The milk matrix in which proteins resides in IF is also different from HM. Typically, in IF the emulsion is comprised of very small lipid droplets (mode diameter <0.5 um) coated with protein, while the HM lipid emulsion structure is comprised of much (>10 times) larger milk fat globules coated with phospholipid membranes. This results in a higher physical association between lipids and proteins in IF compared to HM. However, typical IF emulsions contain only ~24 mg surface protein per g fat [33], for the currently tested IF this would mean ~13% of the protein is acting as emulsifier and is thus present on the interface. Moreover, increased physical association might lessen accessibility for the digestive enzymes. Indeed, others have shown that homogenization of raw CM leads to slower loss of intact whey proteins during *in vitro* intestinal digestion [34]. Therefore, the difference in emulsion characteristics between IF and HM do not explain the faster on protein hydrolysis.

The abundant proteins, which were found in this study by SDS-page determination, were in line with previous reports [2, 11]. However, the HM protein casein to whey ratio (c/w) of ~27:73 in this study, is lower than the expected 40:60 or 50:50 [9]. Contrarily, the c/w of

~51:49 in IF was higher than the expected 40:60. These differences vs expectations might be due to individual donor variations in the case of HM, or the specifics of industrial IF production related selection of (protein) ingredients. We cannot exclude that this skewed protein composition towards casein richness of IF vs HM contributed to the found faster protein hydrolysis in IF.

Regarding the correlation of *in vitro* absorbable product release with *in vivo* postprandial amino acid responses, it needs to be considered that our *in vitro* model does not include intestinal brush border enzymes, which have been shown to cleave MMW peptides [35, 36]. One approach could therefore be to view TP release *in vitro* to resemble absorbable product release *in vivo.* It appears that our TP release data correlate with the available *in vivo* data. Moro *et al.* reported a 1.18 times higher pp EAA peak of HCM protein based IF than iso-proteinaceous HM in preterm infants [7]. We congruently observed that in the period where the difference between IF and HM digesta MMW concentrations was maximal, i.e., the first 10-30 min of the intestinal phase, IF digesta contained 1.15-1.17 times more TP. Therefore, TP release assessed *in vitro* may be a useful proxy for *in vivo* absorbable product release.

Of the three differences in milk characteristics discussed between HM and IF (i.e., protein composition, heat processing and product matrix), the first is the most plausible explanation for the observed differences in hydrolysis kinetics and TP release. Hence, to further improve infant formula, and to bring IF protein digestion rate closer to that of HM, changing IF protein composition to include more slowly digestible proteins might be an interesting approach to investigate.

## Conclusions

We conclude that the total gastrointestinal digestion product release rate of humanized cow's milk based IF is higher than that of HM. Total digestion product release rate assessed *in vitro* may be useful as a proxy for *in vivo* absorbable product release rate. Our results suggest that differences in protein composition are at least partially responsible for the observed differences in release rate. These findings may present an opportunity for further improvement of IF to bring it closer to HM.

## Author Contributions

Conceptualization, E.A.; methodology, E.A.; software, E.A., G.G.M.T; formal analysis, E.A., G.G.M.T.; resources, I.B.R.; data curation, E.A., P.A.W; writing—original draft preparation, E.A.; writing—review and editing, P.A.W, I.B.R, K.H; visualization, E.A., G.G.M.T.; supervision,

K.H., I.B.R.; project administration, E.A., K.H., I.B.R.; funding acquisition, E.A., I.B.R. All authors have read and agreed to the published version of the manuscript.

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## Informed consent statement

Informed consent was obtained from all subjects involved in the study.

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## Conflicts of Interest

E.A., G.G.M.T. and I.B.R. are employees of Danone Nutricia Research.

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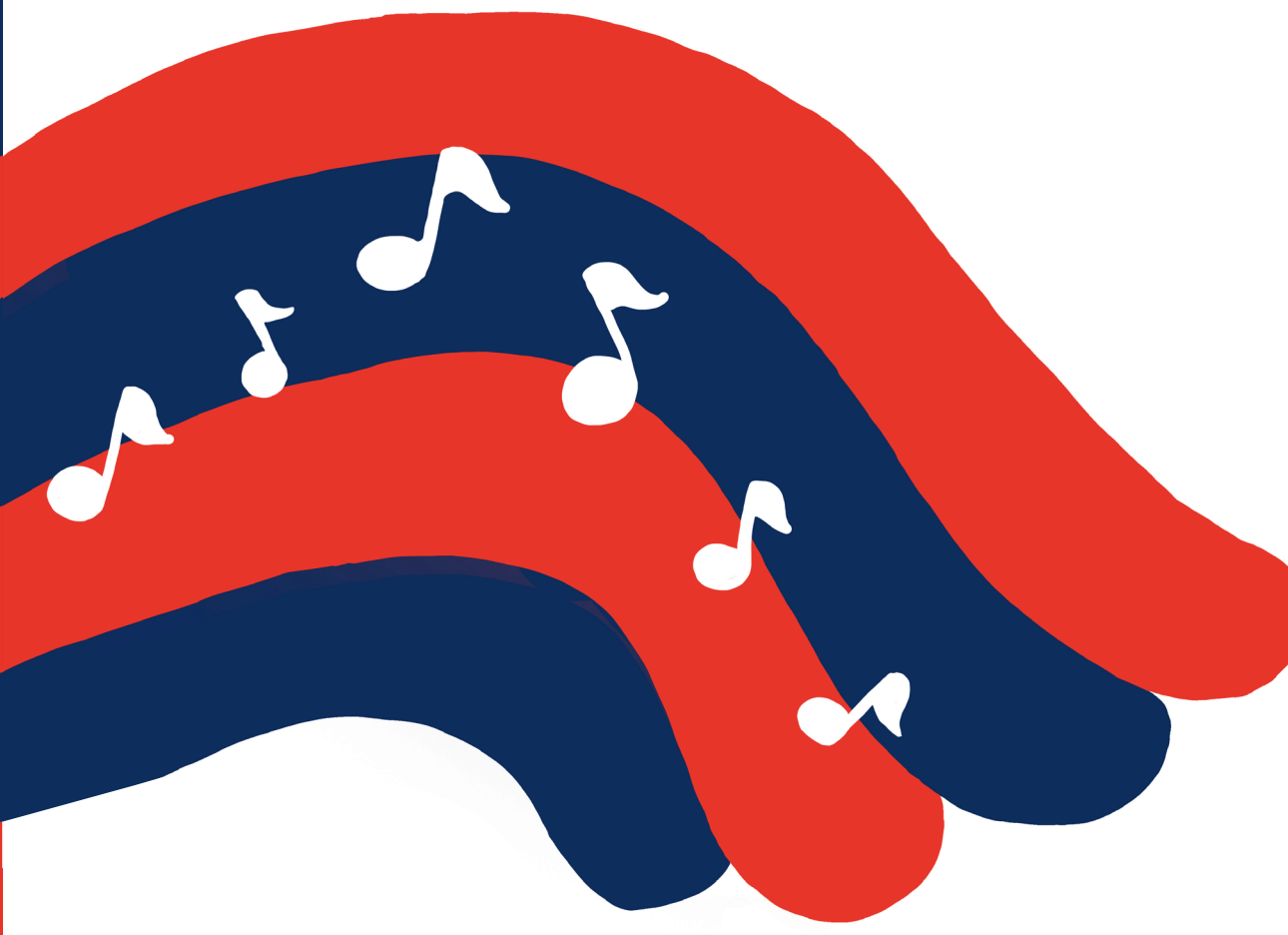
6

# CHAPTER 6

## Fermented infant formula increases ileal protein digestibility and reduces ileal proteolytic activity compared to standard and hydrolyzed infant formula in piglets



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## Abstract

An infant formula containing milk fermented by the bacteria *Bifidobacterium breve* and *Streptococcus thermophilus* (Lactofidus) has been reported to alleviate functional digestive symptoms in infants. It was hypothesized that improved protein digestibility of the fermented infant formula could contribute to this effect. The aim of this study is to evaluate the protein digestibility of a specific fermented (FF), a standard (SF), and an extensively hydrolyzed (HF) protein formula. Four-week-old piglets (n=7) were fitted with a T-cannula at the terminal ileum and received each formula in a Latin square design. Respectively FF, SF and HF contained: 117, 93 and 119 g crude protein per kg powder, a casein/whey ratio of 60/40, 50/50 and 0/100 %/%, and 15, 54 and 56 gr fiber per kg powder. Ileal digesta were collected and analyzed for amino acids and proteolytic activity. FF had a significantly higher apparent ileal crude protein digestibility (92.1±1.0%) compared to SF and HF (84.4±1.0% and 83.9±0.9% respectively). FF had also a significantly higher dry matter digestibility compared to SF and HF. The ileal crude protein flow of FF was significantly lower compared to that of SF and HF. The ileal flow of FF total proteolytic activity was significantly lower than that of SF, but not significantly different from that of HF (412±163 vs. 1530±163 and 703±156 kU/8h, respectively). The fermented formula had in piglets a significantly higher apparent ileal crude protein digestibility compared to the standard and the hydrolyzed formula, and displayed lower ileal proteolytic activity compared to standard formula. Both effects may contribute to the alleviation of common digestive symptoms reported in infants fed fermented infant milk formula.

## Introduction

Adequate protein digestion and absorption are essential to meet the amino acid (AA) requirements of infants for growth, maintenance, and development. The digestive system and its digestive capacity are not fully mature and further develop during infancy [1, 2]. For instance, the postprandial gastric pH of infants is higher than that of adults [3]. As efficient protein denaturation and the enzymatic activity of the gastric protease pepsin depend critically on the stomach's low intraluminal pH [3-5], the higher postprandial pH in infants could, therefore, hamper dietary protein digestion and absorption.



In case of incomplete digestion, proteins will reach the colon where their fermentation may generate potentially harmful compounds (*e.g.* ammonia, phenols, indols, nitrosamines), gases (hydrogen sulphide) and microbial proteases [6, 7]. The latter ones have been linked for example to colonic pain and impaired barrier function [8, 9].

Several studies have shown that production processes can impact infant milk protein digestibility and, ultimately, the metabolic status of the infant [10, 11]. Additionally, several studies have reported beneficial gastrointestinal effects of fermented or acidified infant formulas [12-15]. As such, it has been reported that a starch thickened formula containing a specific milk ferment of *Bifidobacterium breve* and *Streptococcus thermophilus* reduced flatulence intensity and alleviated gut discomfort in infants [16]. The fermentation process acidifies the product, which may for example improve protein digestibility via an enhanced gastric pepsin activity. Hence, we hypothesized an improved protein digestibility of this fermented formula.

Another approach to improve protein absorption is prescription of products containing only extensively hydrolyzed protein, which may be done when infants are suffering from malabsorption related chronic diarrhea [17]. These products are usually based on extensively hydrolyzed whey protein and about 85% of the peptides have a molecular weight lower than 1500 Da and could therefore be considered as pre-digested. Hence, we expected that an extensively hydrolyzed protein-based formula would result in the highest possible protein digestibility.

The objective of the current study was thus to investigate protein digestibility of a fermented infant formula in comparison to a standard, and an extensively hydrolyzed infant formula in young piglets. For this purpose, the piglets were fitted with an ileal cannula as a model to study dietary protein digestion in infants. Infant and piglet anatomy and digestive physiology have been reported to be highly comparable [18, 19].

# Methods

## Piglets

Eight age- and weight-matched (Landrace x York) male piglets (mean weight 4.9±0.16 kg) were selected 14 days after birth from three different litters. Six piglets were required for the study, and two additional piglets were included in the procedures in case of dropouts. The piglets were housed in two groups of four at the facilities of the Animal Sciences Group in Lelystad in a temperature-controlled room at 28°C. At three weeks of age, all eight piglets were fitted with a T-cannula at the terminal ileum after overnight fasting. After the procedure, the piglets were housed individually and allowed to recover for 10 days before the start of the digestion experiment. One piglet died shortly after insertion of the cannula due to an obstruction of the gut. Six piglets were included in the digestion experiment, of which one piglet developed leakage of the cannula. This piglet had to be replaced by the last piglet that was not included initially in the digestion experiment. This piglet received two out of three formulas. The piglet that had to be replaced completed one formula.

The digestive system of three-week-old piglets has been described as an approximation of the digestive system of three-month-old infants [20]. The piglets used in this study were for technical reasons older than this, i.e., 30-40 days. It has been demonstrated that 30-40 day-old piglets fed a milk-based formula are a good model to study the suckling piglet, which resembles the digestive system of infants that are younger than six months [21]. The ileal cannula is a necessity to enable the determination of ileal digestibility. Ileal digestibility is a more relevant parameter than fecal digestibility, because amino acids that appear in the colon are most probably lost for body protein synthesis. In addition, it allows direct measurement of protein flow into the colon [22].

All experimental procedures were reviewed and approved by the independent ethics committee Wageningen University (No. 2009126, Wageningen, The Netherlands). The health status of the piglets was monitored at least on a daily basis by visual and manual inspection.

## Formulas and feeding

During the digestion study, the piglets were fed with one of three different, commercially available powdered formulas: the formula containing milk fermented by the bacteria *Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065 (Lactofidus, fermented formula (FF)), a standard milk formula (Nutrilon standard-2) (SF), and an extensively hydrolyzed milk formula (Nutrilon Pepti-2) (HF), based on 100% hydrolyzed whey protein. Formula compositions are given in **Supplemental Table S6.1**. All formulas were reconstituted using demineralized water at 37°C, resulting in 21-22% DM.

In contrast to FF and SF, the HF contained 1 g/L vanilla extract in order to improve the taste and acceptance of the formula. The pH of each reconstituted formula was different; SF: 6.7, FF: 5.6, and HF: 6.4. The piglets were fed from a feeding container, which allowed recording of the ingested amount. Before the onset of the digestion study (day 31), the piglets received four times daily at 7:00, 9:30, 14:00 and 16:00 hours ad libitum a mixed formula with 20 % DM composed of equal amounts of all three formulas. Voluntary food intake was recorded during six days before the start of the digestion studies in order to assess the required average amount of formula per piglet. The total amount of provided formula was increased by 100 g liquid formula per day in order to compensate for changes in dietary requirements due to the growth of the piglets. During the digestion studies the formula was provided to the piglets equally divided over the four feeding times. During the sampling of ileal digesta (day 31-36), the piglets received each individual formula for 2 days in a Latin square design, to ensure that the order of feeds received by the piglets is equally distributed (**Figure 6.1**). Chromium oxide ($Cr_2O_3$, 0.25 g/kg DM), an indigestible marker, was added to the formulas in order to calculate nutrient digestibility.



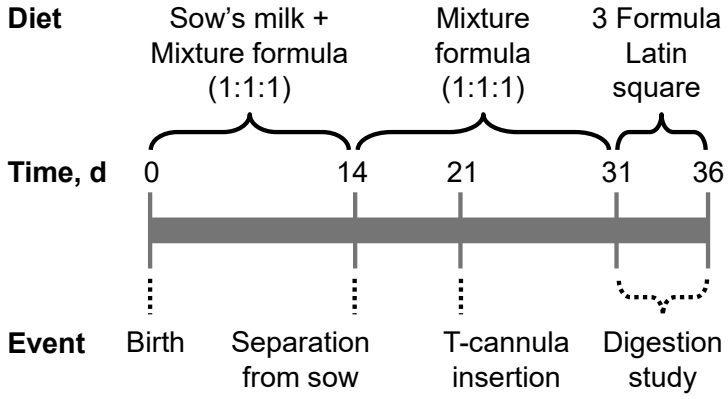


**Figure 6.1: Study design.**

## Sample collection

During the digestion study, all ileal digesta were collected from the T-cannula of each piglet individually on the second day they received a specific formula, i.e., day 32, 34, and 36, from 9.00 to 17.00 h. The digesta were collected in small bags that were emptied immediately and stored on ice. Within two hours, weight and pH of all digesta samples were determined. Samples were stored at -20°C until further processing. For enzymatic activity measurements samples (2 mL) were cleared by centrifugation (13,000*g*, 10 min, 4°C). Aliquots of supernatants were stored at -80°C. All digesta samples were individually freeze-dried and homogenized by grounding.

## Chemical analyses

The chemical analyses of freeze-dried digesta samples and formula powders for DM, $Cr_2O_3$, and crude protein (CP) were performed at CCL Nutricontrol (Veghel, The Netherlands). DM was determined at 80°C using gravimetry. $Cr_2O_3$ was analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). CP was analyzed using the Kjedahl method (total nitrogen x 6.25).

AAs were analyzed after hydrolysis in 6 M HCl. Hydrolysis was performed under vacuum to preserve methionine. Quantification of AA was done using ultra fast liquid chromatography (UFLC) using a pre-column derivatization and fluorometric detection. Fluorenylmethoxycarbonyl (FMOC) was used for proline, and o-phtaldialdehyde/3-mercaptopropionic acid was used for derivatization of all other AAs. Tryptophan was not determined.

## Analysis of proteolytic enzyme activities

The activity of pancreatic enzymes was analyzed in the collected ileal digesta. Total proteolytic activity was determined fluorometrically with a standard assay kit according to the manufacturer's instructions (E6638, Invitrogen, Carlsbad, Ca, USA). Porcine pancreatin (4x USP, P1750, Sigma, Zwijndrecht, The Netherlands) was used as a reference. Proteolytic activity was further characterized using the specific serine protease inhibitor AEBSF (10 mM). Specific trypsin activity was determined with 0.25 mM $N_\alpha$-Benzoyl-L-Arginine Ethyl Esther (BAAE) (B4500, Sigma, Zwijndrecht, The Netherlands) as a substrate. Absorbance change was monitored at 253 nm for 10 min at 25°C. Bovine trypsin (T9201, Sigma, Zwijndrecht, The Netherlands) was used as a standard. Specific chymotrypsin activity was determined with 1.18 mM $N_\alpha$-Benzoyl-L-Tyrosine Ethyl Esther (BTEE) (B6125, Sigma, Zwijndrecht, The Netherlands) as a substrate. Absorbance change was monitored at 256 nm for 10 min at 25°C. Bovine chymotrypsin (C3142, Sigma, Zwijndrecht, The Netherlands) was used as standard. Specific elastase activity was analyzed with 4.4 mM SucAla3-PNA (S4760, Sigma, Zwijndrecht, The Netherlands) as substrate and porcine elastase (E7885, Sigma, Zwijndrecht, The Netherlands) as standard. Absorbance change was monitored at 410 nm for 10 min at 25°C. Enzyme activity was calculated and expressed in 3 ways: as units per gram wet digesta, as units per 8h, and as units per gram protein intake.

## Calculations

Equations used for calculation of flow and digestibility of X (X = AAs, CP, etc.)

$$\text{Ileal flow of X [g/8h]} = \text{X}_{\text{digesta}}\text{[g/8h]} \times \frac{\text{Cr}_2\text{O}_3 \text{ diet [g/kg DM]}}{\text{Cr}_2\text{O}_3 \text{digesta [g/kg DM]}}$$

$$\text{Apparent ileal X digestibility [\%]} = \frac{\text{X intake [g/d]} - \text{X ileal flow [g/8h]}}{\text{X intake [g/d]}} \times 100\%$$

### Statistical analysis

Statistical analyses were performed using IBM SPSS 19 software. Variables were checked for Gaussian distribution with the Shapiro–Wilk test. Levene's test for equality of variance was used to estimate homogeneity of variances. Effect of treatment was tested using univariate ANOVA. (GLM procedure, type III including intercept, design; diet piglet period) with LSD post-hoc test. $p<0.05$ was set as threshold for significance. The statistics are based on n=7 piglets, yielding n=6 observations (see also piglets paragraph in methods section). Values in text are means±SEMs.



## Results

### Formula intake

The mean weight of the piglets at the end of the experiment was 9.8±0.3 kg. DM intake (DMI) of FF was significantly lower compared to that of SF, while DMI of HF was intermediate (384±4.2 vs. 399±4.2 and 388±4.0 g/day respectively). The intervention formulas varied in composition, consequently the intake of individual dietary components varied per formula. The mean intake of protein was significantly higher in the FF compared to the SF fed group. The protein intake in the HF fed group was also significantly higher compared to the SF fed group, (FF: 45.0±0.36, SF: 37.0±0.36, HF: 46.2±0.34 g/day). The intake of fiber, however, was significantly lower in the piglets fed FF (5.7±0.7 g/day) compared to the piglets fed SF (21.6±0.7 g/day) or HF (21.7±0.6 g/day).

### Ileal digesta

The total ileal digesta, calculated by using ileal flow as determined with the indigestible marker, was significantly lower in the FF group than in the other two groups. The total ileal digesta in the HF group was also significantly lower than in the SF group. **(Table 6.1 and Figure 6.2A and B)**.

**Table 6.1: Properties of ileal digesta of piglets equipped with an ileal T-cannula after being fed for two days with a specific fermented, a standard, or an extensively hydrolyzed protein formula. [1]**

| | FF | SF | HF |
|---|---|---|---|
| pH | 7.97±0.06 | 7.93±0.06 | 7.86±0.06 |
| Osmolality, mOsmol/kg | 304±2.0$^{b}$ | 304±2.0$^{b}$ | 311±2.0$^{a}$ |
| Dry matter, g/kg DMI | 38±6.9$^{c}$ | 134±6.9$^{a}$ | 107±6.6$^{b}$ |
| Crude protein, g/kg DMI | 27.1±7.63$^{b}$ | 64.1±7.63$^{a}$ | 69.2±7.33$^{a}$ |
| Crude protein, g/g PI | 0.08±0.01$^{b}$ | 0.16±0.01$^{a}$ | 0.16±0.01$^{a}$ |

[1]Values are means±SEM, n=6. Labelled means in a row without a common letter differ, $p<0.05$. DMI, dry matter intake; FF = fermented formula; HF = hydrolyzed formula; PI = protein intake; SF = standard formula.

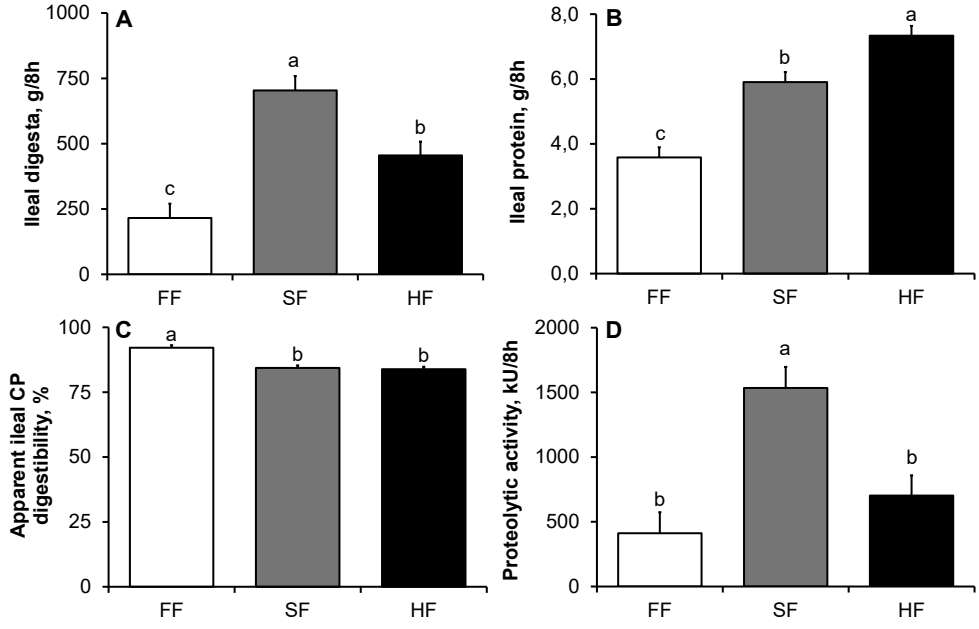


**Figure 6.2: Protein digestion of a specific fermented, a standard, and an extensively hydrolyzed protein formula. Ileal digesta was collected for 8 hours via a T-cannula at the terminal ileum in infant formula fed piglets. A) total ileal digesta, B) ileal protein, C) apparent ileal crude protein digestibility, and D) total ileal protease activity. Values are means±SEM, n=6. Labelled bars without a common letter differ, p<0.05. CP = crude protein; FF = fermented formula; HF= hydrolyzed formula; SF= standard formula.**

The total corrected DM and protein amount per kg DMI at the end of the ileum in the FF group was the significantly lower than the two other groups. The crude protein flow per gram protein intake in the piglets fed FF was half of that of the piglets fed the other two formulas. This indicates a significant reduction in DM and protein flow into the colon in FF fed piglets compared to piglet fed the other two formulas.

The pH of the ileal digesta was identical for all formulas, also ileal digesta osmolality of piglets fed FF and SF was within comparable ranges, although it was significantly higher in HF fed piglets compared to FF fed piglets.

## Ileal protein and AA digestibility

The apparent digestibility of CP and total AA were also calculated using ileal flow as determined with the indigestible marker. The CP and total AA digestibility of FF was significantly higher than that the other formulas **(Table 6.2 and Figure 6.2C)**. The same was applicable for the digestibility of all individual AAs, except lysine, which was only higher than that found for SF. The glycine digestibility was low in all formulas and differed most notably between the formulas. The CP and total AA digestibility of SF and HF were similar; however, several individual AA digestibility of SF were significantly higher than those of HF.

### Ileal enzymatic activities

The activities of pancreatic enzymes in ileal digesta were analyzed. The total proteolytic activity in ileal digesta was reduced by 93±6% after treatment with the specific serine protease inhibitor AEBSF (data not shown). This indicates that almost all ileal activity is derived from serine proteases, i.e., digestive enzymes. Total proteolytic **(Table 6.3 and Figure 6.2D)**, and specific trypsin, chymotrypsin, and elastase activity (U/8h) were significantly higher in ileal digesta of piglets fed SF compared to piglets fed FF, as well as piglets fed HF, with exception of chymotrypsin activity (U/8h) which was not significantly different between SF and HF fed piglets. The different enzymatic activity levels found in the digesta of FF fed piglets (U/8h) were comparable to that found in HF fed piglets, with the exception of elastase which was significantly higher in the HF fed group. All the different enzymatic activity levels found in the digesta, expressed per gram protein intake (PI), were significantly lower in piglets fed FF compared to piglets fed SF. The enzymatic activity levels per g protein intake found in the digesta of FF fed piglets were comparable to those of HF fed piglets.



## Discussion

In the current study, we could confirm that significantly lower amounts of protein were detected in ileal digesta of FF fed piglets than in the ileal digesta of the piglets fed SF or HF. Total colonic protein delivery was thus lower in FF fed piglets compared to pigs fed the other two formulas. Enhanced colonic protein fermentation has been suggested to be associated with gastrointestinal discomfort [6-9]. In this context, beneficial effects of the specific fermented infant formula that was investigated in this study on e.g. overall gastrointestinal discomfort and flatulence have been reported a clinical trial in infants [16].

The lower colonic protein delivery, which was observed here in piglets, was also accompanied by a lower flow of proteolytic activity. This suggests that less detectable protease activity, most likely originating from pancreatic digestive enzymes, still yielded efficient protein digestion.

The hypothesis that extensively hydrolyzed protein-based formula has a higher CP or AA digestibility compared to SF had to be rejected. We found comparable CP and AA digestibility as well as CP flow per gram protein intake between HF and SF fed piglets. The total proteolytic and trypsin activity flow per gram protein intake, however, was lower in the HF fed group compared to the SF fed group. So, per gram of protein intake HF was also “easier to digest” than SF, though this did not result in a lower amount of protein delivered to the colon. Dietary peptides, i.e. in the form of protein hydrolysates, have been reported to increase the secretion of endogenous proteins in pigs, as well as in humans, and other species [23-25].

In addition to the differences in serine protease levels found at the end of the ileum, which suggest different rates of pancreatic secretion, we also found significantly different glycine digestibility for the different formulas. Glycine is present in high amounts in bile, as part of the conjugated bile salts. During the sample preparation for HPLC the acid hydrolysis removes the glycine from the bile salts, leaving bile acids and glycine. The lower glycine digestibility of SF and HF might therefore point towards higher endogenous (bile) secretion.

One aspect of the difference in protein composition between the formulas is the casein/whey ratio. Casein and whey have been demonstrated to be digested at different rates, possibly through impacting gastric emptying. However, the total digestibility of both is considered very high [26]. Therefore, it is unlikely that differences in casein/whey ratio are responsible for the observed differences in digestibility.

It must be considered that the complete formulas which were used in this study differ also in aspects not related to protein, such as dietary fiber quantity and quality. Some specific fibers, such as pectin or grain hulls, have been reported to increase ileal nitrogen losses, i.e. due to increases in digesta viscosity, their water holding capacity, or mechanical abrasiveness [27-30]. The fermentation during production of the fermented formula yields transgalacto-oligosaccharides, whereas the standard and hydrolyzed formulas contain supplemented short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides. Based on their relatively short chain length and physicochemical properties, it must be considered that these oligosaccharides are unlikely to yield one of the aforementioned effects [31-33]. In addition, despite equal intake of identical sources of fermentable fiber, ileal total proteolytic enzyme activity was significantly lower in HF compared to SF fed piglets. The ileal total proteolytic enzyme activity was furthermore similar between FF and HF, despite differences in dietary fiber intake. The observed effect of fermented formula on ileal total protease activity can thus not be fully explained by differences in fiber quality and quantity between the formulas.

Although the effect of proteases and undigested protein on colonic physiology could not be investigated in this study due to the cannulation of the piglets, ample evidence from literature points towards the negative impact of both. Endogenous proteins, including proteases, have a low bioavailability in the small intestine, but can be easily metabolized by the gut's microbiota. The endogenous proteins that flow into the colon are broken down by microbiota, producing metabolites and gasses like ammonia and hydrogen sulphide. Exposure of the colon to these metabolites is linked to gut discomfort via a direct negative effect on epithelial proliferation, metabolism, gas-induced flatulence and bloating [7]. In this way, the reduced release of endogenous proteins could be linked to beneficial effects of the fermented formula.

Additionally, serine proteases (i.e., trypsin, chymotrypsin and elastase), mast cells (i.e., tryptase), as well as microbial proteases can directly activate protease activated receptors (PARs), such as PAR-2. PAR-2 activation has been shown to increase pain perception, reduce epithelial barrier function, and may trigger and sustain inflammation [9, 34]. We speculate that the reduced colonic delivery of endogenous proteases and reduced stimulation of microbial protease production from undigested protein could be linked to the beneficial effects of the fermented formula. This is an interesting lead which will require further investigation.

In summary, the fermented formula has a higher apparent protein digestibility compared to a standard formula or hydrolyzed formula. It must be considered that the formulas differed in characteristics other than the fermentation process, which will have to be investigated in future studies. The obtained results point, however, towards a mechanistic pathway that involves a different physiological response to the fermented formula. This response might involve a diminished secretion of endogenous proteins, a related reduction in exposure of the gastrointestinal tract to undigested proteins, including (serine) proteases. We hypothesize that this reduced exposure may contribute to the observed beneficial effects of fermented formula on gastrointestinal discomfort. The basic mechanism of higher apparent protein digestibility of the fermented formula yet remains to be elucidated.



## Acknowledgements

E.A., S.H., H.B., M.A., J.K., and T.L. designed the research; E.A. and S.H. conducted the research, E.A, S.H and I.R. analyzed the data; S.H., E.A. I.R. and T.L. wrote the paper; T.L. had primary responsibility for the final content. All authors read and approved the final manuscript. The authors thank Sietse-Jan Koopmans, Ruud Dekker, and Jan van der Meulen from the Animal Sciences Group Lelystad for execution of the piglet trial, Yu Lu, and Gerrit de Vrij for their analytical support and Bert van de Heijning for critical review of the manuscript.

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# Supplementary material

**Supplemental Table S6.1: Composition of powdered infant formulae fed to piglets.[1]**

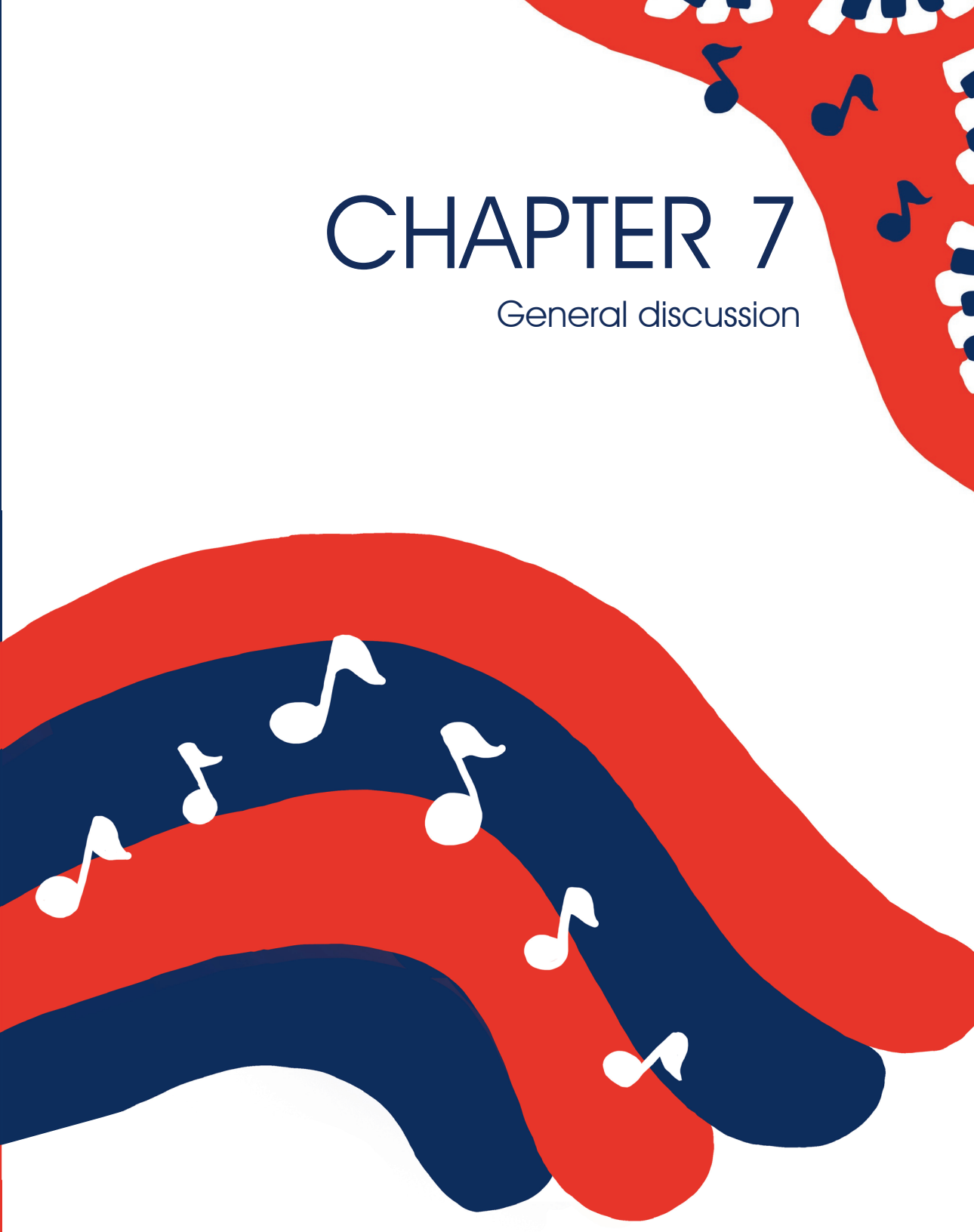
| | FF | SF | HF |
|---|---|---|---|
| Energy, kcal/kg | 4930 | 4630 | 4650 |
| Crude protein, g/kg | 117 | 93 | 119 |
| Casein/whey, %/% | 60/40 | 50/50 | 0/100 |
| Total AA, g/kg[2] | 107 | 88 | 118 |
| Alanine | 4.2 | 3.6 | 6.3 |
| Arginine | 3.4 | 2.7 | 2.8 |
| Aspartic acid | 9.9 | 8.5 | 13.4 |
| Glutamic acid | 23.4 | 18.0 | 21.4 |
| Glycine | 2.3 | 1.8 | 2.2 |
| Histidine | 2.5 | 1.9 | 2.0 |
| Isoleucine | 5.3 | 4.5 | 7.6 |
| Leucine | 10.5 | 8.9 | 12.3 |
| Lysine | 5.9 | 6.6 | 11.1 |
| Methionine | 2.2 | 1.9 | 2.3 |
| Phenylalanine | 4.8 | 3.7 | 3.8 |
| Proline | 9.7 | 7.5 | 8.0 |
| Serine | 6.6 | 5.1 | 6.0 |
| Threonine | 5.5 | 4.7 | 8.6 |
| Tyrosine | 4.3 | 3.3 | 3.0 |
| Valine | 6.3 | 5.0 | 6.8 |
| Minerals, mg/kg | | | |
| Ca | 4500 | 4350 | 4470 |
| Mg | 340 | 510 | 400 |
| Fe | 70 | 55 | 70 |
| Zn | 35 | 36 | 35 |
| Cu[3] | 2.8 | 3.9 | 2.8 |
| Fat, g/kg[4] | 230 | 215 | 218 |
| Carbohydrates, g/kg[4] | 593 | 582 | 561 |
| Fibre, g/kg[4] | 15 | 54 | 56 |

[1]AA= amino acid; FF= fermented formula; HF= hydrolyzed formula (100 % unfermented and extensively hydrolyzed whey); SF= standard formula. [2]Sum of 16 individual AAs. [3]Below detection limit (=5 mg/kg). [4]Values as indicated on package.


7

# CHAPTER 7

## General discussion



## Aims and key findings

Currently in protein quality definitions the kinetics of amino acid (AA) availability, reflected in plasma AA peak concentrations after ingestion, are not considered, while these are an important determinant of the use of the AAs as building blocks for maintenance and growth [1, 2]. Another clinical outcome related to dietary protein intake is the amount of protein reaching the colon, which can negatively influence gut heath [3]. Key determinants of these outcomes lie in physiological, physical, and chemical processes that occur in the stomach and small intestine, which include gastric emptying rate, protein hydrolysis kinetics and total protein digestibility. These processes in turn are influenced by the characteristics of the ingested food, such as protein composition, product heat processing and product matrix. The overall research question addressed in this thesis was: How do these food characteristics impact overall protein digestion, and how is this reflected in postprandial plasma (pp) AA peak concentration and colonic protein flow? The specific aims of this thesis were to:

**1)** Gain insights in the relative importance of gastric emptying and protein hydrolysis kinetics in determining the postprandial AA peak.
**2)** Differentiate the individual effects of the mentioned food characteristics on gastrointestinal processes.
**3)** Assess and improve correlation between *in vitro* and *in vivo* studies.

A better understanding will increase the ability to formulate products with an improved protein quality tailored to the specific needs of vulnerable populations as for instance infants or elderly people.

**Chapter 2** showed that addition of extra carbohydrates and lipids lowered the pp AA concentrations of whey protein and casein in healthy elderly, and that changes in gastric emptying and protein hydrolysis were responsible, as shown in a semi-dynamic *in vitro* model of the gastrointestinal tract (SIM) using elderly conditions. **Chapter 3** demonstrated that changing native whey protein composition by inclusion of non-clotting casein increased pp AA concentrations in neonatal piglets, but whey protein heat induced denaturation did not, and that both interventions did not affect gastric emptying. **Chapter 4** showed that the kinetics and mechanisms of protein hydrolysis and digestion product release were affected by milk protein composition and denaturation in the SIM and the TNO *in vitro* model of the gastrointestinal tract (TIM-1) using infant conditions. In addition, it was found that the correlation between *in vitro* digestion measures and *in vivo* pp AA concentrations was improved when intermediate digestion products were taken into consideration. **Chapter 5** showed increased rates of peptide bond hydrolysis and digestion product release of cow's milk based infant formula (IF) compared to human milk (HM) in the SIM using infant conditions, and suggested protein compositional differences as key determinant. **Chapter**

**6** demonstrated that in piglets, IF ileal protein digestibility and colonic protein flow were affected by IF matrix.

## Relative importance of gastric emptying and protein hydrolysis kinetics in determining the postprandial AA peak

Systemic plasma AA concentrations are a net-result of influx of AAs from the intestine through overall protein digestion kinetics, systemic protein turnover and metabolic demands. Theoretically, the two key determinants of overall protein digestion kinetics are the gastric emptying rate (GER) and protein hydrolysis rates in the stomach and small intestine [4-6]. Currently, the body of evidence that GER is important is large, while less is known about the contribution of protein hydrolysis rates.



**Chapter 2** confirmed that the GER has a large impact on pp AA peak concentrations, as both the addition of calories and exchanging whey protein for clotting casein decreased the pp AA peak concentrations in healthy elderly subjects (**Figure 2.2**). Moreover, the total release of free AA (FAA) during *in vitro* hydrolysis was also decreased by addition of calories and exchanging whey protein for clotting casein (**Figure 2.5**), indicating it was an additional contribution to lowering pp AA peak concentration. As the chosen interventions influenced both GER and hydrolysis kinetics, it was difficult to judge the relative importance of the two determinants. In **chapter 3** it was therefore attempted to investigate the influence of food characteristics on pp AA without influencing GER, this time in neonatal piglets. The pp paracetamol concentration, used as a marker for GER, was not affected by whey protein denaturation, nor by inclusion of β-casein in a native whey protein solution (**Figure 3.2D**), indicating that GER of at least the liquid phase was the same in all groups. *In vitro* analysis of the β-casein-whey mixture (protein base ingredient (PBI) for IF) showed that, unlike the typical micellar casein behaviour under gastric conditions, this preparation did not form any clots >1 mm and thus would not undergo pyloric sieving. Therefore, the pp paracetamol concentration acted as a marker for the GER of the whole meal. Still, significantly different pp AA peak concentrations were observed (**Figure 3.3**). The pp AA peak concentration of PBI was 18% higher than that of native whey protein isolate (NWPI), suggesting other factors than GER were determinants of the pp AA peak concentrations. Pharmacokinetic modelling results pointed towards protein hydrolysis and absorption kinetics as key determinants; PBI rate constant for absorption ($k_a$) was 1.83 times as high as NWPI (**Table 3.3**). In **chapter 4** gastrointestinal hydrolysis of the milk protein solutions NWPI and PBI was investigated *in vitro*. Here it was shown that although the protein degree of hydrolysis (DH) increase rates were similar, in the first 30 min of intestinal digestion the total concentration of FAA and peptides <5 kDa (i.e., total digestion products, TP) of PBI was consistently and considerably

(1.3-2.9 times) higher than that of NWPI (**Figure 4.5A, 4.5B and 4.6A**, combined in **Figure 7.1**).

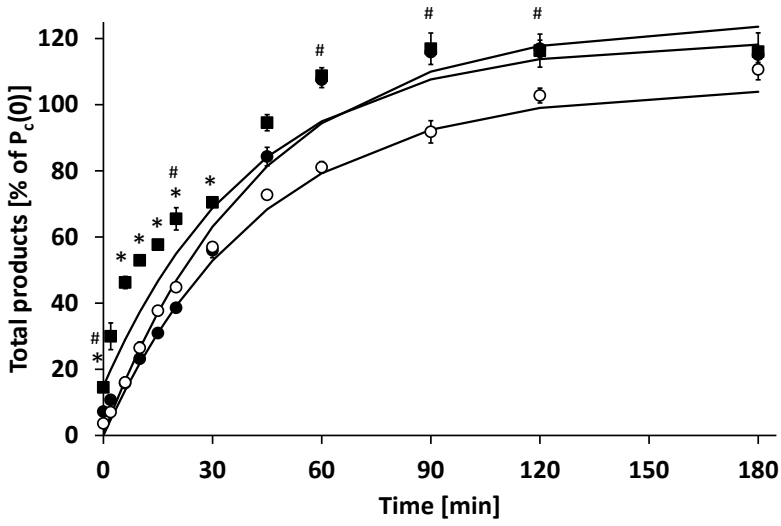



**Figure 7.1: Milk protein solution intestinal total digestion product release in semi-dynamic *in vitro* model of the gastrointestinal tract (SIM). ●) Native whey protein isolate (NWPI), ○) denatured whey protein isolate (DWPI), ■) protein base ingredient for infant formula (PBI). Lines were fitted data using first order reaction kinetics. Mean±SEM (n=3). #Significant difference NWPI vs. DWPI ($p<0.05$); *significant difference PBI vs. NWPI ($p<0.05$). ANOVA-LSD.**

Together this strongly suggests that indeed differences in rate of TP release were responsible for the observed differences in pp AA peak concentrations in the neonatal piglets. In **chapter 5** it was shown that IF gastrointestinal peptide bond hydrolysis and TP release rates were higher than those of HM (**Figure 5.3 and 5.7**). Clinical investigations have previously shown that the pp essential AA (EAA) peak concentrations of IF are higher than those of HM in pre-term infants (**Figure 1.1**) [7], and that the GER of IF is similar or slower than that of HM [8], together suggesting that in infancy protein hydrolysis kinetics are dominant over GER in determining pp AA peak concentrations. In conclusion, so far protein hydrolysis kinetics have been overlooked and deserve more emphasis in future studies that try to explain or predict pp AA peak concentrations.

## Individual effects of food characteristics on gastrointestinal processes

Postprandial plasma AA peak concentrations and gastrointestinal processes are known to be influenced by food characteristics, such as protein composition, heat processing and product matrix. Protein composition includes the variety of structures (primary, secondary, tertiary and quaternary) present. Heat processing is involved in the industrial production of

milk protein-based product. Product matrix includes the presence of other nutrients and components, as well as the products` structural organization.

## Protein composition

Protein composition has been shown to impact pp AA concentrations [10], as a result of which the concept of "fast/slow" protein by Boirie was implemented to indicate the rate of pp AA appearance [5]. The main physiological mechanism underlying the fast/slow protein concept is the GER differences between whey protein and casein as a result of their distinct physicochemical behaviour under gastric conditions [5]. In this concept fast proteins have a high GER and elicit a high pp AA peak concentration, while slow proteins have a low GER and as a result elicit a lower pp AA peak concentration. The difference in physicochemical behaviour is solidification (clotting); solid particles of >1 mm are likely retained in the stomach *in vivo* due to pyloric sieving [11]. The results of **chapter 2** were in line with the fast/slow concept, as only the casein based nutritional supplements (CNS) formed clots under elderly gastric conditions, whereas the whey protein based nutritional supplements (WPNS) remained liquid (**Figure 2.4**). Furthermore, the pp AA peaks of the CNS were lower than those of the WPNS (**Figure 2.2**). However, **chapter 3** showed that intact caseins are not always slowly emptied proteins. Firstly, it was found that inclusion of β-casein in a native whey protein solution (PBI) did not lead to formation of particles >1 mm under gastric conditions *in vitro* (data not shown). Secondly, in neonatal piglets GER of PBI was not decreased compared to native whey protein (NWPI) alone, as indicated by the similar pp paracetamol concentrations (**Figure 3.2D**). Interestingly, the pp AA peak of PBI was higher than that of NWPI (**Figure 3.3**), suggesting that the protein hydrolysis rate is increased due to the inclusion of β-casein in a native whey protein solution in PBI.

**Chapter 2** also showed increased appearance of peptides <1 kDa and FAA in the CNS compared to the WPNS early in the intestinal phase of *in vitro* digestion (**Figure 2.5**). In this set of experiments, it could not be excluded that suboptimal intestinal pH control was responsible for this effect. The SIM was allowed 10 min for pH neutralization between the gastric and intestinal phases, but the base strength was not high enough and as a result the CNS needed 20 min and the WPNS 30 min to reach the set pH of 6.5. The experiment was repeated with increased base strength. Once more the results showed higher concentrations of peptides <1 kDa and FAA in the early intestinal phase of the CNS compared to the WPNS (data not shown), indicating that hydrolysis of caseins is fast compared to whey proteins. In **chapter 4** it was observed that including β-casein in PBI compared to NWPI minimally affected the kinetics of peptide bond hydrolysis (**Figure 4.3**). However, as mentioned earlier, the release of TP was profoundly increased in PBI, especially a larger amount of medium molecular weight peptides (MMW, 0.5-5 kDa) were released (**Figure 4.6A**). TP release in PBI was consistently (1.3-2.9 times) higher than in NWPI in the first 30 min of intestinal digestion (**Figure 7.1**). From this it follows that the rate of TP release of β-casein is higher than that of

whey proteins, confirming that β-casein is actually a fast protein. The findings that caseins are hydrolyzed fast compared to whey proteins can be attributed to the absence of a dense tertiary structure in caseins in contrast to whey proteins.

**Chapter 5** shows that IF protein hydrolysis is faster than that of HM, i.e. both DH increase and TP release rate were higher in IF than HM (**Figure 5.3 and 5.7**). These increased rates in IF are attributed to protein compositional differences compared to HM, whereas the differences in matrix and processing are likely not responsible. Two aspects of the protein compositional differences between HM and IF are: 1) the presence of protease inhibitors in HM, and 2) the difference in protein composition within casein and whey fractions, inferring overall compositional differences even if IF protein is humanized (i.e. its casein to whey ratio is changed to 40/60 to reflect the average composition of HM) [12, 13]. Two additional sets of experiments were conducted to investigate the relative contributions of these two protein compositional aspects.

In the first set of experiments, the effect of protease inhibitors as found in HM on gastrointestinal protein hydrolysis was investigated in the SIM. The concentrations of protease inhibitors; α1-antitrypsin (A1AT) and α1-antichymotrypsin (AACT), were quantified in the HM pool sample used in **chapter 5** using ELISA and were found to be 0.04±0.002 g/L and 0.01±0.001 g/L, respectively. These concentrations are within the range of what is typically found in mature HM [14]. IF was supplemented with 0.04 g pure human serum A1AT /L only (IFAT) or in combination with 0.01 g pure human serum AACT /L (IFATACT) to simulate the protease inhibitors found in the HM pool sample. The *in vitro* protein hydrolysis of IFAT and IFATACT was compared to unsupplemented IF in the SIM. The results showed that intestinal DH and TP increase were minimally affected by the presence of the protease inhibitors (**Figure 7.2**). Although at t=45 min a slightly lower DH was found in IFAT and IFATACT than in IF, at all other timepoints and in all fitting parameters of both DH and TP release no differences were found. This indicates that the presence of protease inhibitors in HM is not a key determinant of the slower protein hydrolysis compared to IF.

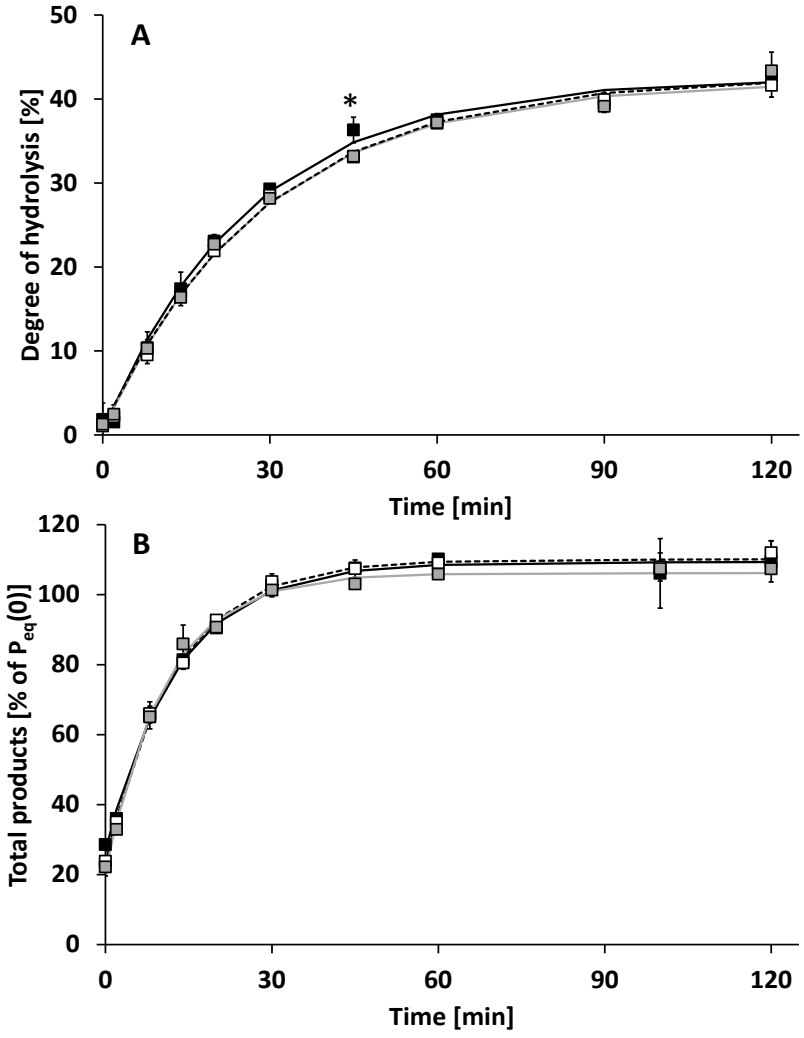



**Figure 7.2: The effect of protease inhibitors on intestinal protein hydrolysis of infant formula in the semi-dynamic *in vitro* model of the gastrointestinal tract (SIM). A) Degree of hydrolysis, B) Total digestion products (<5 kDa). Lines in A and B are fitted data using first order reaction kinetics. Means±SEM (n=3). ■) Infant formula (IF), □) Infant formula with 0.04 g/L human $\alpha_1$-antitrypsin (IFAT), ■) Infant formula with 0.04 g/L human α1-antitrypsin and 0.01 g/L human $\alpha_1$-antichymotrypsin (IFATACT). *IF significantly different from IFAT and IFATACT. ANOVA-LSD p<0.05.**

In the second set of experiments, the effect of differences in whey protein composition on protein hydrolysis was investigated in SIM. The whey protein compositions were established by mixing pure individual bovine whey proteins. As mentioned earlier, the protein composition of both the casein and the whey fractions are very different in human and bovine milk [12]. However, pure bovine caseins are not all commercially available. Moreover, we observed in **chapter 4 and 5** that compared to whey proteins, the hydrolysis of caseins leads to faster digestion product release. Additionally, HM GER is similar or higher

than that of IF, which could be linked to less casein clotting in HM, suggesting caseins are not a key driver of the observed pp AA differences between HM and IF. Therefore, whey protein fractions were focused on. Both human and bovine whey consist of numerous proteins. To keep the experimental approach simple, only the four most abundant proteins were used; α-lactalbumin (Ala), β-lactoglobulin (Blg), lactoferrin (Lf), and serum albumin (SA) [12]. Using these proteins in pure form, cows` milk whey (CW) was simulated, and in two steps this composition was brought closer to the whey protein composition of HM. In the first step, by increasing Ala and decreasing Blg to make Ala enriched cows` whey (ACW), and in the second step by replacing the remainder of Blg with Lf to make humanized cows` whey (HCW), **Table 7.1** describes the exact protein composition of the three protein mixtures. The whey protein mixtures were digested at 14 g total protein/L as typically found in IF.

**Table 7.1: Whey protein mixtures composed of pure bovine proteins.**[1]

| Protein [%(w/w)] | CW | ACW | HCW |
|---|---|---|---|
| α-lactalbumin | 24 | 47.8 | 47.8 |
| β-lactoglobulin | 66 | 42.2 | 0 |
| Lactoferrin | 0 | 0 | 42.2 |
| Serum albumin | 10 | 10 | 10 |

[1] CW = cows` whey, ACW = Ala enriched cows` whey, HCW = humanized cows` whey.

The results showed that enrichment of CW with Ala (ACW) did not influence protein hydrolysis, as the intestinal DH and TP release quantity and kinetics were similar as CW (**Figure 7.3**). However, HCW intestinal DH and TP release were considerably (~20-30%) lower than that of CW and ACW at several points during the first 45 min. Furthermore, the rate constant of intestinal DH increase kinetics was ~30% lower for HCW than CW. Moreover, the HCW rate constant of intestinal TP release kinetics tended to be lower (~55%) than CW and ACW rate constants (p=0.074 and 0.057, respectively).

This suggests that the whey protein compositional differences between HM and IF may be a key determinant of the slower DH increase and TP release of HM compared to IF. Specifically, the abundance of Lf in HM compared to IF may be a major underlying factor. Other *in vitro* digestion studies have shown Lf to be one of the most digestion resistant proteins in HM [15]. Also, native bovine Lf and bovine Lf digestion derived peptides were found to be relatively resistant to infant digestion *in vitro* [16, 17]. Human Lf is shown to be more resistant to hydrolysis than bovine Lf, an effect attributed to post-translational modifications, with the degree of glycosylation in human Lf being high compared to bovine Lf, which hampers hydrolysis by trypsin [18]. In conclusion, protein composition is a major determinant of both gastric emptying and protein hydrolysis kinetics and is therefore a key means to further optimize pp AA of nutritional solutions to meet the specific requirements of infants and elderly.

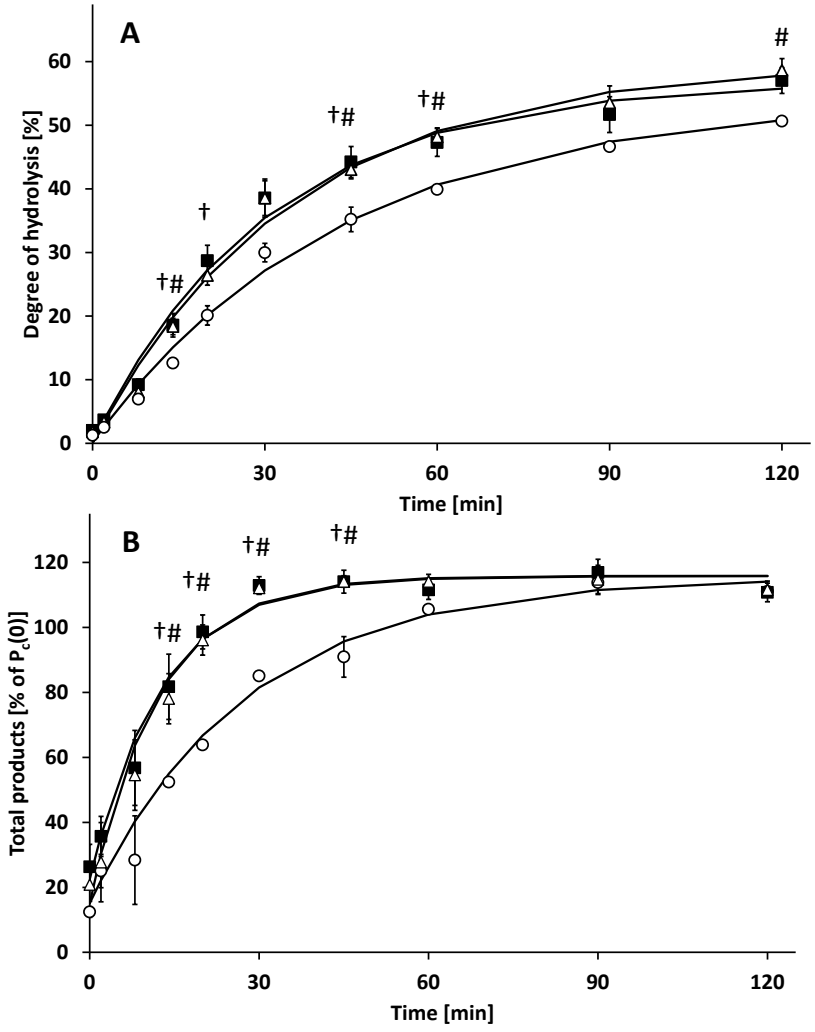



**Figure 7.3: Intestinal protein hydrolysis of whey protein mixtures in SIM. ■) Cows` whey (CW), △) α-lactalbumin enriched cows` whey (ACW), ○) Humanized cows` whey (HCW). A) Degree of hydrolysis, B) Total digestion products (<5 kDa). Lines in A and B are fitted data using first order reaction kinetics. Means±SEM (n=3). †HCW significantly different from CW, #HCW significantly different from ACW. ANOVA-LSD p<0.05. SIM = semi-dynamic *in vitro* model of the gastrointestinal tract.**

## Protein heat processing

Heat treatment can impact protein structurally, which may influence physiological outcomes after ingestion. Key examples are protein denaturation and aggregation and AA chemical modification [9]. During the industrial manufacturing of products for infants and elderly the main structural modifications that occur are protein denaturation and aggregation due to the relatively low heat load that is applied, other modifications include glycation during production and shelf life of powdered products.

**Chapter 3** showed that, contrary to our hypothesis, in neonatal piglets denaturation of native whey protein isolate slightly lowered pp concentrations of EAA and branched chain AA (BCAA) at several timepoints in the first 60 min compared to NWPI (**Figure 3.3**). These effects were too small to be reflected in the pharmacokinetic modelling parameters. No differences in pp paracetamol concentrations between denatured whey protein isolate (DWPI) and NWPI were observed, showing that the GER was not affected by the denaturation of protein and is not underlying the observed differences in pp AA concentrations. In **chapter 4**, whey protein denaturation was found to increase susceptibility of intact protein to hydrolysis, without affecting the DH increase, in SIM intestinal phase, thus changing the mechanism of intestinal hydrolysis (**Figure 4.3 and 4.4**). Release of FAA and LMW peptides (<0.5 kDa) was minimally increased by denaturation (**Figure 4.5**). However, considerable decreases in MMW and increases in high molecular weight peptide (5-13 kDa, HMW) concentrations were induced by whey protein denaturation (**Figure 4.6**). The release of TP, reflecting the combined changes in FAA, LMW and MMW, showed a slight decrease as result of denaturation (**Figure 7.1**). This correlates with the slight decrease observed in pp EAA/BCAA concentrations in neonatal piglets (**Figure 3.3**), suggesting that protein hydrolysis kinetics were a key determinant. In conclusion, the main effect of heat induced whey protein denaturation is a change in nature of intermediate digestion products towards larger peptides. This could potentially infer a change in presented epitopes and immunological activity or other bioactivity relevant for infant health. It would therefore be of interest to further investigate the molecular composition of these peptide fractions by identification using a peptidomics approach combined with biological assays to test actual bioactivity.

## Protein product matrix

Milk protein is usually consumed as part of a mixed meal that is typically an emulsion or suspension, wherein milk proteins are part of the structural organization. The presence of, and association with, other components can impact the gastrointestinal digestion of protein. In this thesis protein product matrix was varied in two ways, each in a dedicated study; 1) addition of extra lipids and carbohydrates to nutritional supplements for elderly, and 2) fermentation of infant formula.

**Chapter 2** shows that the effect of addition of extra lipids and carbohydrates decreased the pp AA concentrations of both CNS and WPNS. A larger caloric load is well known to slow gastric emptying [19, 20], because the stomach doses emptying at about 2.25 kCal per minute on average [21], which can explain the decrease in pp AA peak concentrations. Interestingly, in **chapter 2** the pp AA peak lowering effect of additional calories was found to be smaller for CNS than WPNS (**Figure 2.2**). Using *in vitro* experiments, it was found that the high caloric CNS (C320) generated smaller clots during gastric digestion than the low caloric CNS (C150) (**Figure 2.4**). This indicates that the clot particles were rendered more soft and less resistant to mechanical breakdown by shear. *In vivo* this could lead to an increased GER

because less antral grinding would be needed, and thus counteract the GER lowering effect of additional calories. Additionally, softer clots would allow a higher diffusion of pepsin and a resulting higher gastric hydrolysis of protein [22]. The observed higher release of peptides and FAA in the early intestinal phase in C320 vs C150 corroborates this theory (**Figure 2.5**). Softer clots in C320 vs C150 can be explained by inclusion of more lipids in the clot by encapsulation of lipid droplets and hydrophobic interactions between the proteins and lipids [23]. Since the WPNS remained liquid in the stomach, these effects do not apply and only the GER lowering effect of increased caloric load is observed. Thus, the smaller decrease of pp AA peak concentration as a result of addition of extra lipids and carbohydrates in CNS can be explained by the interaction of the lipids with casein clotting and consequently gastric emptying and protein hydrolysis, which does not happen in the case of WPNS.



In **chapter 6** the apparent ileal crude protein and total AA digestibility of a fermented IF (FF) was higher than that of a standard IF (SF) and that of an extensively hydrolyzed protein based IF (HF) in piglets (**Figure 6.2 and Table 6.2**). At the same time, a lower ileal protease activity and lower glycine digestibility in FF was found, indicating pancreatic and biliary secretions were lower in FF followed by HF and SF (**Table 6.2 and 6.3**). From this it is unclear if the altered physiological response to the FF in the piglets was the driving force of the altered ileal digestibility. To investigate this further, *in vitro* ileal protein digestibility of the three formulae was studied in TIM-1. In this study the enzyme to substrate ratio was equal for all three formulae as the protein intake was standardized at 3.2 g in 200 mL. The results showed that the *in vitro* ileal protein digestibility, measured as TAA in <1 kDa dialysate, of FF was higher than that of SF, but similar to HF (**Figure 7.4**).

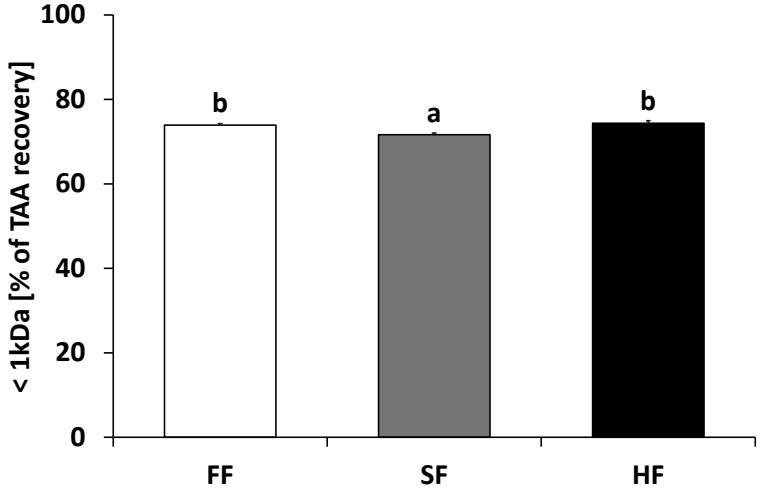



**Figure 7.4: Infant formula Ileal total AA digestibility in TIM-1. FF = fermented formula, SF = standard formula, HF = hydrolyzed protein formula. Means±SEM (n=3). Bars not sharing a letter are significantly different. ANOVA-LSD p<0.05. TIM-1 = TNO *in vitro* model of the gastrointestinal tract.**

This indicates that, although there was a different physiological response to the fermented formula, this was likely not the key driving force of different protein digestibility. The lactic acid bacteria used in the fermentation of FF are known to have little to no proteolytic activity; the protein is therefore expected to remain structurally unchanged. More research is required to elucidate the mechanism of improved protein digestibility. A potential angle to investigate could be the role of the (inanimate) microorganisms and/or their components. Interestingly, others have found that co-ingestion of (probiotic) bacteria with a protein meal improves the AA availability in healthy adults [26], demonstrating the potential contribution of microorganisms and/or their components to protein digestion. The apparent reduced pancreatic/biliary secretions observed *in vivo* could be a result of bioactive peptides coming from the microorganisms. Peptides are known to increase the secretion of endogenous proteins [24, 25], the propensity to do so might be AA sequence specific. In conclusion, the protein product matrix's main effects are on gastric emptying via physical and physiological feedback mechanisms, but protein hydrolysis can also be affected.

## Correlation between *in vitro* and *in vivo* studies

*In vitro* gastrointestinal protein hydrolysis can be analyzed on three main levels: 1) the loss of intact protein, 2) the increase in degree of hydrolysis and 3) the release of digestion products. In the studies described in this thesis all three levels were considered. It appeared that the latter of the three correlated the best with *in vivo* pp AA peak concentrations. Still, the main challenge of *in vitro* protein digestion approaches is to appropriately mimic the *in vivo* release of absorbable protein digestion products. The products of protein digestion that are absorbed by the intestinal epithelium *in vivo* are FAA and di- and tripeptides [27, 28]. *In vitro* models typically lack the peptidases from the intestinal brush border membrane (BBM) because of difficulty to obtain and apply relevant preparations. BBM peptidases are responsible for the last step in hydrolysis of intermediate digestion products to form absorbable digestion products. This last hydrolysis step thus needs to be simulated using specific methodology. A variety of different methods have been used, such as; precipitation [29, 30], dialysis/membrane filtration [31-33] (**Chapter 4 and 7**), and partial integration of HP-SEC chromatograms (**Chapter 2, 4, 5 and 7**). Obviously, it is crucial for all methods to set an appropriate (molecular weight) cut-off. Currently, there is no clear consensus on the set cut-off in published works, which range from 1–5 kDa (~8–44 residues). Practical considerations, scarce data on the actual substrates of BBM peptidases, and correlation of the *in vitro* digestion outcomes with *in vivo* data, underlie the choices made. As discussed in **Chapter 1**, it has long been assumed that the substrates of BBM peptidases are oligopeptides up to ~1 kDa [34], however more recent studies indicate peptides up to 3.5 kDa can be cleaved by BBM enzymes [35, 36]. As these reports supply indirect evidence, additional studies are required that systematically assess the upper limit of BBM peptidase substrate

peptide length to inform the cut-off choice. In this thesis initially 1 kDa was chosen as cut-off (**Chapter 2**, and TIM-1 studies in **chapter 4 and 7**) based on the long-standing assumption that only these peptides can be further broken down by BBM peptidases. Later, in an attempt to stratify into (directly) absorbable digestion products and intermediate digestion products that require further BBM enzyme processing before they can be absorbed, we separated two fractions: LMW peptides (<0.5 kDa) and MMW peptides (0.5-5 kDa), respectively. Although this approach should be further refined through information based on new upper limit definition, it was clear from the analysis in **chapters 3, 4 and 5** that the consideration to include MMW in the *in vitro* total digestion product fraction (on top of FAA and LMW) greatly enhanced the correlation with pp AA peak concentrations determined *in vivo*. In conclusion, *in vitro* protein digestion product release including intermediate peptides correlates with *in vivo* postprandial AA peak concentrations. However, there is an opportunity for improvement in conducting further studies using BBM peptidases to determine the most appropriate molecular weight cut-off.



## Conclusions

It can be concluded that not only gastric emptying rate, but also protein hydrolysis kinetics determine the pp AA peak concentration. However, protein hydrolysis kinetics have been generally overlooked and deserve more emphasis in future studies that try to explain or predict pp AA peak concentrations. Regarding the individual effects of food characteristics, it can be concluded that; firstly, protein composition is a major determinant of both gastric emptying and protein hydrolysis kinetics and is therefore a key means to further optimize pp AA of nutritional solutions to meet the specific requirements of infants and elderly. Secondly, heat induced protein denaturation only limitedly affects gastric emptying, protein hydrolysis kinetics, and pp AA peak concentrations. Lastly, protein product matrix's main effects are on gastric emptying via physical and physiological feedback mechanisms, but protein hydrolysis can also be affected. Concerning the correlation between *in vitro* and *in vivo* studies; a key challenge of *in vitro* protein digestion approaches remains to appropriately mimic the *in vivo* release of absorbable protein digestion products. *In vitro* protein digestion product release that includes intermediate peptides correlates with *in vivo* postprandial AA peak concentrations better than does absorbable digestion product release only.

## Future directions for products for elderly and infants

The improved understanding and new insights of the relationships between food characteristics, gastrointestinal processes and their systemic and colonic consequences

obtained in this thesis allow the following recommendations for improvement of milk products to optimize protein quality specifically tailored to the needs of elderly and infants.

Nutritional supplements for frail elderly that aim to increase muscle protein synthesis could consist of a low caloric, non-clotting milk protein solution, that will have a high GER and therewith will maximize pp AA peak concentrations. In addition, non-clotting proteins that are rapidly hydrolyzed to protein digestion products <5 kDa, for example β-casein, can be included to further maximize pp AA peak concentrations.

Milk formula for infants that aims to be close to human breast milk with regard to pp AA peak concentrations, and minimize colonic protein flow, could consist of an increased proportion of slow but completely digested proteins. A higher proportion of whey proteins over caseins will lead to a slower release of protein digestion products <5 kDa during gastrointestinal digestion and could therewith lower IF pp AA peak concentrations. Specifically, an enrichment in Lf could magnify the pp AA lowering effect, because Lf is hydrolyzed to protein digestion products <5 kDa more slowly than other whey proteins.

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# SUMMARY

# Summary

Protein is an important part of human nutrition; it delivers essential amino acids (AA) and nitrogen-containing molecules, which are required for maintenance and growth. The AA requirements are described in age-dependent reference AA composition patterns. Current protein quality definitions, consider how well the source AA composition matches the reference and its total digestibility in the gastrointestinal tract. However, the kinetics of availability, reflected in plasma AA peak concentrations after ingestion, are usually not considered, while this is an important determinant of the use of the AA building blocks for tissue protein synthesis or oxidation. Another clinical outcome related to dietary protein intake is the amount of protein reaching the colon, which can negatively influence gut heath. **Chapter 1** describes in detail the key determinants of postprandial plasma (pp) AA peak concentrations and colonic protein flow; the physiological, physical, and chemical processes that occur in the stomach and small intestine, including gastric emptying rate, protein hydrolysis kinetics and total protein digestibility. In addition, the current state of knowledge on the influence of characteristics of the ingested food, such as protein composition, product processing and product matrix on the mentioned gastrointestinal processes is provided. The relationships between gastrointestinal processes and product characteristics and their interplay are not well understood. Therefore, the overall research question addressed in this thesis was: How do these food characteristics impact overall protein digestion, and how is this reflected in postprandial plasma (pp) AA peak concentration and colonic protein flow?

The specific aims of this thesis were to:

1) Gain insights in the relative importance of gastric emptying and protein hydrolysis kinetics in determining the postprandial AA peak.
2) Differentiate the individual effects of the mentioned food characteristics on gastrointestinal processes.
3) Assess and improve correlation between *in vitro* and *in vivo* studies

The work of this thesis combined *in vitro* and *in vivo* digestion studies on milk proteins in humans and piglets to advance the ability to formulate products with an improved protein quality tailored to the specific needs of vulnerable populations as for instance infants or elderly people.

The requirement of leucine and essential AA (EAA) to stimulate muscle protein synthesis increases with age. To target muscle anabolism, it is suggested that higher pp concentrations of leucine and EAA are needed in elderly people. In **chapter 2** the matrix effect of increased energy density on the pp AA concentrations of whey protein and casein was investigated in healthy elderly. Furthermore, to better understand the relative importance of gastric emptying and protein hydrolysis, *in vitro* investigations in a newly developed semi-dynamic

model of the gastrointestinal tract (SIM) simulating elderly conditions were undertaken. Four iso-nitrogenous protein (21g) supplements were studied containing leucine-enriched whey protein with 150/320 kcal (W150/W320) or casein protein with 150/320 kcal (C150/C320); all products contained carbohydrates (10 or 32g) and fat (3 or 12g). The pp leucine peak concentration was twofold higher for W150 vs. C150 (521±15 vs. 260±15 µmol/L), higher for W320 vs. C320 (406±15 vs. 228±15 µmol/L), and higher for low-caloric vs. high-caloric products. Similar effects were observed for the peak concentrations of EAA and total AA (TAA). *In vitro* gastric coagulation was observed only for the casein protein supplements. Intestinal digestion for 90 min resulted in higher levels of free TAA, EAA, and leucine for W150 vs. C150, for W150 vs. W320, and for C150 vs. C320. Addition of extra carbohydrates and lipids lowered the pp AA concentrations of whey protein and casein in healthy elderly. These differences appear to be mediated by the gastrointestinal behaviour of these products.



Multiple studies have indicated that formula-fed infants show a different growth trajectory compared to breast-fed infants. The observed growth rates are suggested to be linked to higher postprandial levels of branched chain AA (BCAA) and insulin related to differences in protein quality. In **chapter 3** the effect of whey protein denaturation, as well as the effect of changing native milk protein composition by incorporation of non-clotting casein, on pp AA, insulin, GLP-1 and glucose concentrations in neonatal piglets was studied. Furthermore, gastric emptying measurement and pharmacokinetic modelling of pp AA data was performed to gain insights in the role of protein hydrolysis. Neonatal piglets were bolus fed two of three milk protein solutions: native whey protein isolate (NWPI), denatured whey protein isolate (DWPI), or protein base ingredient (PBI) for infant formula (IF) comprising whey and β-casein. DWPI (91% denatured protein) vs NWPI (91% native protein) showed lower essential AA (EAA) (~10%) and BCAA (13-19%) concentrations in the first 30-60 min. However, TAA concentration per time-point and AUC, as well as EAA and BCAA AUC were not different. PBI induced a ~30% lower postprandial insulin spike than NWPI, yet pp TAA concentration at several time-points and AUC was higher in PBI than NWPI. The TAA rate constant for absorption ($k_a$) was twofold higher in PBI than NWPI. pp EAA concentrations and AUCs in PBI and NWPI were not different. Changing native whey protein composition by inclusion of non-clotting casein increased pp AA concentrations in neonatal piglets, but whey protein heat induced denaturation did not. Both interventions did not affect gastric emptying. The differences between PBI and NWPI were partly explained by the difference in AA composition, but more likely differences in protein hydrolysis and absorption kinetics.

Knowledge about how molecular properties of proteins affect their digestion kinetics is crucial to understand protein pp AA responses.

Therefore, to further elucidate mechanisms of the observations in chapter 3, in **chapter 4** protein hydrolysis and absorbable product release kinetics and mechanisms as affected

by milk protein composition or denaturation were investigated in SIM and a dynamic *in vitro* model of the gastrointestinal tract (TIM-1) simulating infant conditions. In addition, directions for improved correlations between *in vitro* digestion measures and *in vivo* pp AA concentrations was sought. In both models, the degree of hydrolysis (DH), loss of intact protein, and release of absorbable products (SIM: <0.5 kDa peptides and free AA, TIM-1: bioaccessible AA) were monitored. Additionally, in SIM, intermediate product amounts and their characteristics were determined. DWPI showed considerably faster intact protein loss, but similar DH and absorbable product release kinetics compared with NWPI in both models. Furthermore, more, relatively large, intermediate products were released from DWPI than from NWPI. PBI showed increased intact protein loss, similar DH, and absorbable product release kinetics, but more, relatively small, intermediate products than NWPI. Thus, both whey protein denaturation and β-casein inclusion increased the rate of intact protein loss without affecting absorbable product release during *in vitro* digestion. Our results suggest that intermediate digestion product characteristics are important in relation to pp AA responses, as it was found that the correlation between *in vitro* digestion measures and *in vivo* pp AA concentrations was greatly improved when intermediate digestion products were taken into consideration.

**Chapter 5** provided detailed insights in the differences in kinetics of protein hydrolysis and absorbable product release of human milk (HM) and cow's milk based IF in SIM simulating infant conditions. It has been shown that the pp EAA peak concentrations of IF are higher than those of HM in infants. In addition, several HM proteins have been recovered intact in infant stool and appeared digestion resistant *in vitro*. It was therefore hypothesized that gastrointestinal protein hydrolysis of IF is faster than HM and leads to accelerated absorbable digestion product release. The time course of DH, loss of intact protein and release of free AA and peptides was evaluated. Gastric DH increase was similar for IF and HM, but the rate of intestinal DH increase was 1.6 times higher for IF than HM. Intact protein loss in IF was higher than HM from 120 min gastric phase until 60 min intestinal phase. Intestinal phase total digestion product (free AA + peptides <5 kDa) concentrations increased ~2.5 times faster in IF than HM. IF gastrointestinal protein hydrolysis and absorbable product release is faster than HM, possibly due to the presence of digestion resistant proteins in HM. This might present an opportunity to further improve IF bringing it closer to HM.

In **chapter 6** IF protein digestibility and colonic protein flow as affected by formula protein composition and matrix was investigated in ileal cannulated piglets. An IF containing milk fermented by the bacteria *Bifidobacterium breve* and *Streptococcus thermophilus* has been reported to alleviate functional digestive symptoms in infants. It was hypothesized that improved protein digestibility of the fermented infant formula could contribute to this effect. The aim of this study was to evaluate the protein digestibility of a specific fermented (FF), a standard (SF), and an extensively hydrolyzed protein based (HF) formula. Four-week-old

piglets (n=7) were fitted with a T-cannula at the terminal ileum and received each formula in a Latin square design. Ileal digesta were collected and analyzed for AA and proteolytic activity. FF had a significantly higher apparent ileal crude protein digestibility (92.1±1.0%) compared to SF and HF (84.4±1.0% and 83.9±0.9% respectively). The ileal crude protein flow of FF was significantly lower compared to that of SF and HF. The ileal flow of FF total proteolytic activity was significantly lower than that of SF, but not significantly different from that of HF (412±163 vs. 1530±163 and 703±156 kU/8h, respectively). Fermented formula had in piglets a significantly higher apparent ileal crude protein digestibility compared to the standard and the hydrolyzed formula, and displayed lower ileal proteolytic activity compared to standard formula, possibly via a mechanistic pathway that involves a different physiological response. Both effects may contribute to the alleviation of common digestive symptoms reported in infants fed fermented infant milk formula.

In **chapter 7** all observations were discussed, and future directions in research and nutritional solutions for infants and elderly people are proposed. It was concluded that not only gastric emptying rate, but also protein hydrolysis kinetics determine the pp AA peak concentration. However, protein hydrolysis kinetics have been generally overlooked and deserve more emphasis in future studies that try to explain or predict pp AA peak concentrations. Regarding the individual effects of food characteristics, firstly, it was concluded that protein composition is a major determinant of both gastric emptying and protein hydrolysis kinetics and is therefore a key means to further optimize pp AA peak concentration of nutritional solutions to meet the specific requirements of infants and elderly. Secondly, heat induced protein denaturation only limitedly affects gastric emptying, protein hydrolysis kinetics, and pp AA concentrations. However, the impact of heat induced denaturation on intermediate digestion product characteristics is large, which could potentially infer a change in presented epitopes and immunological activity or other bioactivity relevant for infant health. Interesting further investigations could therefore include the analysis of the molecular composition and bioactivity of these peptide fractions. Lastly, protein product matrix's main effects are on gastric emptying via physical and physiological feedback mechanisms, but protein hydrolysis can also be affected. Concerning the correlation of *in vitro* and *in vivo*, the main challenge of *in vitro* protein digestion approaches remains to appropriately mimic the *in vivo* release of absorbable protein digestion products. *In vitro* protein digestion product release that includes intermediate peptides (i.e., TP, <5 kDa) correlates with *in vivo* postprandial AA peak concentrations better than does absorbable digestion product (FAA + di- and tripeptides) release only. However, further studies using BBM peptidases to determine the most appropriate molecular weight cut-off for the intermediate peptides are required. Lastly, recommendations for improvement of milk products to optimize protein quality tailored to the needs of elderly and infants were made that involve specific changes in protein composition.

# ABBREVIATIONS

# Abbreviations

| | |
|---|---|
| AA | Amino acid |
| ACW | α-lactalbumin enriched cow`s whey |
| AGE | Advanced glycation end-products |
| Ala | α-lactalbumin |
| AUC | Area under the curve |
| BCAA | Branched chain amino acid |
| BBM | Brush border membrane |
| Blg | β-lactoglobulin |
| C150 | Casein based nutritional supplement low caloric |
| C320 | Casein based nutritional supplement high caloric |
| CNS | Casein based nutritional supplements |
| CS | Cleavage sites |
| CM | Cow`s milk |
| CP | Crude protein |
| CW | Cow`s whey |
| DH | Degree of hydrolysis |
| DIAAS | Digestible indispensable amino acid score |
| DMI | Dry matter intake |
| DWPI | Denatured whey protein isolate |
| EAA | Essential amino acid |
| EMM | Estimated marginal mean |
| FAA | Free amino acids |
| FF | Fermented infant formula |
| GER | Gastric emptying rate |
| GI | Gastrointestinal |
| GIT | Gastrointestinal tract |
| HF | Hydrolyzed protein based infant formula |
| HCM | Humanized cow`s milk |
| HCW | Humanized cow`s whey |
| HM | Human Milk |
| HMW | High molecular weight peptides (5-13 kDa) |
| iAUC | Incremental area under the curve |
| IF | Infant formula |
| Ig | Immunoglobulins |
| Lf | Lactoferrin |
| Lz | Lysozyme |
| LMW | Low molecular weight peptides (<0.5 kDa) |
| MMW | Medium molecular weight peptides (0.5-5 kDa) |

| | |
|---|---|
| MPS | Milk protein solutions |
| N | Nitrogen |
| NPN | Non protein nitrogen |
| NWPI | Native whey protein isolate |
| PBI | Protein base ingredient (for infant formula) |
| PDCAAS | Protein digestibility corrected amino acid score |
| pp | Postprandial plasma |
| SA | Serum albumin |
| SF | Standard infant formula |
| SGF | Simulated gastric fluid |
| SIF | Simulated intestinal fluid |
| SIM | Semi-dynamic *in vitro* model of the gastrointestinal tract |
| SSF | Simulated saliva fluid |
| TIM-1 | TNO dynamic *in vitro* model of the gastrointestinal tract |
| TAA | Total amino acids |
| TP | Total digestion products (<5 kDa) |
| UHT | Ultra-high temperature |
| WPI | Whey protein isolate |
| WPNS | Whey protein based nutritional supplements |
| W150 | Whey protein based nutritional supplement low caloric |
| W320 | Whey protein based nutritional supplement high caloric |

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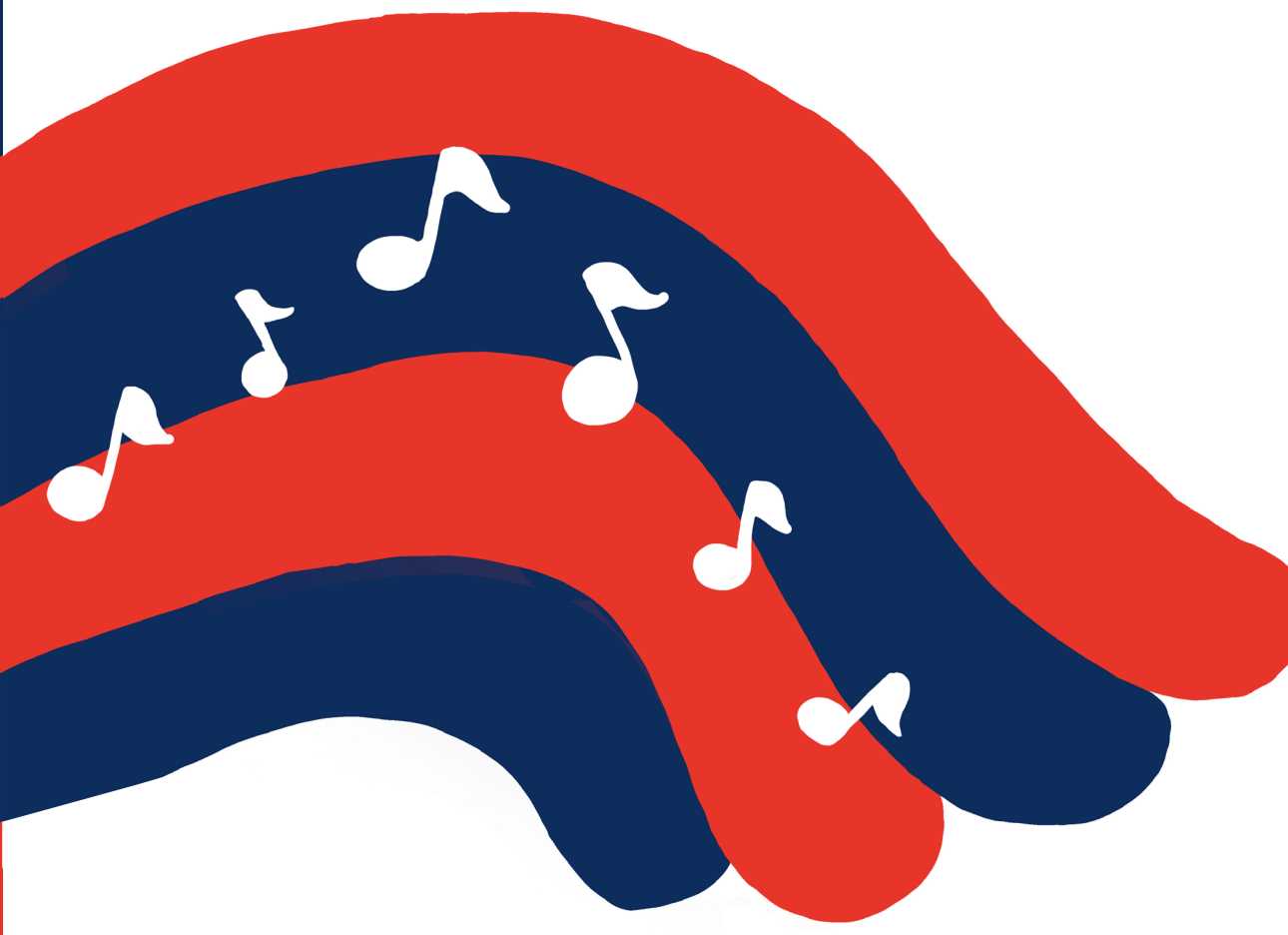
# ABOUT THE AUTHOR



## Curriculum vitae

Evan Abrahamse was born on March 18th, 1976, in Amersfoort, the Netherlands. After graduating from high school, he started studying Chemistry at Utrecht University in 1995. After two years he switched to the Hogeschool van Utrecht and obtained his ing. in biochemistry in 2001. His internship was performed at Numico Research in Wageningen and involved intestinal epithelial permeability and transport studies that aimed to prevent or restore a leaky gut. His thesis internship was also done at this lab, which formed his first opportunity to study digestion processes, as here the gastrointestinal survival of bioactive oligo-saccharides was investigated. In 2001 he was hired as assistant scientist at Numico Research focusing on digestion and working with the TIM-1 model. In the following years his research focused on the digestion of carbohydrates to tailor medical nutrition products to the specific needs of diabetic patients, and the gastrointestinal behaviour of thickened infant formulae specifically designed to prevent gastroesophageal regurgitation. In 2006 he joined the Gut Biology and Microbiology platform of Prof. Dr Jan Knol and the following year he became an employee of Danone when this company acquired Royal Numico. With this merger the scope of research was broadened to also include the gastrointestinal survival of probiotic bacteria. In 2009 he was promoted to scientist. The main research topics during this period were the impact of protein composition on gastric physico-chemical behaviour and gastric emptying of medical nutrition tube feeds, and the impact of fat architecture on lipid digestion in infancy. This research has yielded 14 patents so far. In 2015, after qualifying exams in advanced food chemistry and nutritional physiology he started his PhD at the laboratory of Food Chemistry at Wageningen University & Research under the supervision of Prof. Dr Harry Gruppen, with Dr Peter Wierenga and Dr Bert van de Heijning. The final year of the PhD project was at the Food Quality and Design group at Wageningen University & Research under the supervision of Dr Kasper Hettinga with Dr Ingrid Renes. The obtained results of this project are presented in this thesis.

# List of publications

## This Thesis

**E. Abrahamse**, S. Huybers, M. S. Alles, I. B. Renes, J. Knol, H. Bouritius and T. Ludwig, Fermented infant formula increases ileal protein digestibility and reduces ileal proteolytic activity compared with standard and hydrolyzed infant formulas in piglets, The Journal of Nutrition, 2015, 145, 1423-1428.

Y. C. Luiking, **E. Abrahamse**, T. Ludwig, Y. Boirie and S. Verlaan, Protein type and caloric density of protein supplements modulate postprandial amino acid profile through changes in gastrointestinal behaviour: A randomized trial, Clinical Nutrition, 2016, 35, 48-58.

R. J. Welch-Jernigan, **E. Abrahamse**, B. Stoll, O. B. Smith, P. A. Wierenga, B. J. M. van de Heijning, I. B. Renes and D. G. Burrin, Postprandial amino acid kinetics of milk protein mixtures are affected by composition, but not denaturation, in neonatal piglets, Current Developments in Nutrition, 2018, 3.

**E. Abrahamse**, G. G. M. Thomassen, I. B. Renes, P. A. Wierenga and K. A. Hettinga, Gastrointestinal protein hydrolysis kinetics: opportunities for further infant formula improvement, Nutrients, 2022, 14, 1512.

**E. Abrahamse**, G. G. M. Thomassen, I. B. Renes, P. A. Wierenga and K. A. Hettinga, Assessment of milk protein digestion kinetics: effects of denaturation by heat and protein type used, Food & Function, 2022, DOI:10.1039/D2FO00542E.



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J. Luttikhold, K. van Norren, M. Minor, N. Buijs, C. C. M. van den Braak, T. Ludwig, **E. Abrahamse**, H. Rijna and P. A. M. van Leeuwen, The effect of fibers on coagulation of casein-based enteral nutrition in an artificial gastric digestion model, Food & Function, 2014, 5, 1866-1871.

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M. Navis, V. Muncan, P. T. Sangild, L. Møller Willumsen, P. J. Koelink, M. E. Wildenberg, **E. Abrahamse**, T. Thymann, R. M. van Elburg and I. B. Renes, Beneficial effect of mildly pasteurized whey protein on intestinal integrity and innate defense in preterm and near-term piglets, Nutrients, 2020, 12, 1125.

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M. M. L. Grundy, **E. Abrahamse**, A. Almgren, M. Alminger, A. Andres, R. M. C. Ariëns, S. Bastiaan-Net, C. Bourlieu-Lacanal, A. Brodkorb, M. R. Bronze, I. Comi, L. Couëdelo, D. Dupont, A. Durand, S. N. El, T. Grauwet, C. Heerup, A. Heredia, M. R. Infantes Garcia, C. Jungnickel, I. E. Kłosowska-Chomiczewska, M. Létisse, A. Macierzanka, A. R. Mackie, D. J. McClements, O. Menard, A. Meynier, M.-C. Michalski, A.-I. Mulet-Cabero, A. Mullertz, F. M. Payeras Perelló, I. Peinado, M. Robert, S. Secouard, A. T. Serra, S. D. Silva, G. Thomassen, C. Tullberg, I. Undeland, C. Vaysse, G. E. Vegarud, S. H. E. Verkempinck, M. Viau, M. Zahir, R. Zhang and F. Carrière, INFOGEST inter-laboratory recommendations for assaying gastric and pancreatic lipases activities prior to in vitro digestion studies, Journal of Functional Foods, 2021, 82, 104497.

# Overview of completed training activities

## Discipline specific activities

| | Organization | Year |
|---|---|---|
| 2nd International Conference of Food Digestion, Madrid, ES | INFOGEST | 2013 |
| Lorentz workshop Biophysics, Biochemistry and Physiology of Fat Digestion, Leiden, NL | ECIS | 2013 |
| 3rd International Conference of Food Digestion, Wageningen, NL | INFOGEST | 2014 |
| 12th Euro Fed Lipid Congress, Montpellier, FR | EFL | 2014 |
| 4th International Conference of Food Digestion, Napoli, IT | INFOGEST | 2015 |
| TNO TIM symposium and user group meeting, Porto, PT | TNO | 2015 |
| 48th European Society for Paediatric Gastroenterology, Hepatology and Nutrition annual meeting, Amsterdam, NL | ESPGHAN | 2015 |
| 9th Nizo Dairy Conference, milk protein functionality, Papendal, NL | NIZO | 2015 |
| 5th EAAP International Symposium on Energy and Protein Metabolism and Nutrition, Krakow, PL | EAAP | 2016 |
| 5th International Conference of Food Digestion, Rennes, FR | INFOGEST | 2017 |
| TNO TIM symposium and user group meeting, Amsterdam, NL | TNO Triskelion | 2017 |
| INFOGEST WG4 workshop, Uppsala, SE | INFOGEST | 2017 |
| Nicolas Appert Food symposium, Wageningen, NL | Nicolas Appert | 2017 |
| 10th Nizo Dairy Conference, milk protein functionality, Papendal, NL | NIZO | 2017 |
| 17th Food Colloids Conference: Application of Soft Matter Concepts, Leeds, UK | University of Leeds | 2018 |
| Infogest Symposium and Workshop, Leeds, UK | INFOGEST | 2018 |
| 6th International Conference of Food Digestion, Granada, ES | INFOGEST | 2019 |
| INFOGEST WG4 workshop, Granada, ES | INFOGEST | 2019 |
| Virtual 1st Global Plant-Based Foods & Proteins Research Conference 2020, online | Bridge2Food | 2020 |
| Virtual International Conference on Food Digestion 2021, online | INFOGEST | 2021 |
| 6th World Congress for Paediatric Gastroenterology, Hepatology and Nutrition annual meeting, online | ESPGHAN | 2021 |

## General Courses

| | Organization | Year |
|---|---|---|
| Engage and Influence | Danone Nutricia Research | 2013 |
| Cross-Functional Leadership | Danone Nutricia Research | 2014 |
| Project Management soft skills | Danone Nutricia Research | 2015 |
| Experimental Design and Statistical Analysis (3R`s in Animal experiments) | FRAME | 2016 |
| Philosophy and Ethics in Food science and technology | WGS | 2016 |
| Writing Grant Proposals | WGS | 2017 |

## Other activities

| | Organization | Year |
|---|---|---|
| Preparation of research proposal | FCH | 2015 |
| PhD study tour to Japan | FCH | 2016 |
| Weekly group meetings at WUR and Danone Nutricia Research | WUR & Danone Nutricia Research | 2015-2021 |

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