THE EFFECT OF MILK PROCESSING ON PROTEIN DIGESTION AND AMINO ACID ABSORPTION IN THE GASTROINTESTINAL TRACT OF PIGS AS A MODEL HUMAN

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ABSTRACT

Globally, milk is processed using heat and homogenisation to improve food safety and extend shelf life. These common processing techniques can alter the native structures present in milk, including protein structures. However, the impact of these processing-induced changes on the digestion of milk protein and subsequent absorption of amino acids in the human body is not yet fully understood.

The overall objective of this research was to understand how heat treatment and homogenisation affect milk protein coagulation and digestion in the stomach, and to investigate how changes to gastric coagulation (curd formation) influence amino acid (AA) absorption in the small intestine and AA concentrations in blood circulation. Due to the limited accessibility of the human gastrointestinal tract, pigs were used as a model of the human.

An initial study using raw bovine (cow), caprine (goat), and ovine (sheep) milk established the role of gastric curd formation in small intestinal AA absorption in piglets at a single postprandial time point. Specifically, differences in the retention of AA in the gastric curd were responsible for differences in the small intestinal AA absorption across milk of different species.

A separate study using bovine milk as a milk model was then conducted to determine the effect of heat treatment and homogenisation on the kinetics of milk protein digestion and small intestinal AA absorption. The selected processing treatments were pasteurisation, ultra-high temperature treatment (UHT), and homogenisation. Raw milk was included as a comparator. In the stomach, heat treatment and homogenisation altered the strength and structure of the curd formed during gastric digestion, which in turn affected both milk protein hydrolysis and the rate of AA entering the small intestine. Differences in the release of digested protein and AA into the small intestine were reflected in the kinetics of AA absorption of the processed milk types. For example, UHT milk had both a faster rate of AA entering the small intestine and a faster rate of AA absorption. Processing also altered the appearance of some AA in blood circulation; however, these differences were not directly reflective of the differences observed in their small intestinal absorption kinetics.

In conclusion, this PhD research demonstrated that the rate of small intestinal AA absorption was modulated by gastric curd formation, indicating that milk processing could be used as a strategy to modulate protein digestion and AA absorption in the gastrointestinal tract.

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Natalie Ahlborn

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LIST OF ABBREVIATIONS

AA	Amino acid
AIA	Acid-insoluble ash
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BW	Body weight
BCAA	Branched-chain amino acid
Cryo-SEM	Cryo-scanning electron microscopy
Cr_2O_3	Chromium (III) oxide
DIAAS	Digestible indispensable amino acid score
DM	Dry matter
DSI	Distal small intestine
EDTA	Ethylene diamine tetra acetic acid
EAA	Essential amino acid
FA	Fatty acid
GIT	Gastrointestinal tract
HPLC	High-performance liquid chromatography
LCMS	Liquid chromatography-mass spectrometry
LNAA	Long neutral amino acid
MBIE	Ministry of Business, Innovation and Employment (New Zealand)
MFGM	Milk fat globule membrane
MUAEC	Massey University Animal Ethics Committee

NEAA	Non-essential amino acid
NRC	National Research Council (Washington, USA)
NS	Not significant
NZ3M	New Zealand Milk Means More
ODS	Output Delivery System
NRC	National Research Council
PH	Pasteurised homogenised
PLFL	Protein-lipid-free lactose
PNH	Pasteurised non-homogenised
PSI	Proximal small intestine
RDI	Recommended daily intake
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SCFA	Short-chain fatty acid
SEM	Standard error of the mean
TAA	Total amino acid
TEM	Transmission electron microscopy
TiO_2	Titanium dioxide
UHT	Ultra-high temperature homogenised
USDA	United States Department of Agriculture

RESEARCH OUTPUTS

Journal articles

1. Ahlborn, N. G., Montoya, C. A., Hodgkinson, S. M., Dave, A., Ye, A., Samuelsson, L. M., Roy, N. C., & McNabb, W. C. (2023). Heat treatment and homogenization of bovine milk loosened gastric curd structure and increased gastric emptying in growing pigs. *Food Hydrocolloids, 137,* 108380.

2. Ahlborn, N. G., Montoya, C. A., Roy, D., Stroebinger, N., Ye, A., Samuelsson, L. M., Roy, N. C., Moughan, P. J. & McNabb, W. C. (2023). Differences in small intestinal apparent amino acid digestibility of raw sheep, cow, and goat milk are explained by gastric amino acid retention in piglets as an infant model. *Frontiers in Nutrition 10:1226638 (Special Collection: Non-cow milk for infants and older adults).*

3. Ahlborn, N. G., Montoya, C. A., Ye, A., Samuelsson, L. M., Roy, N. C., & McNabb, W. C. (2024). Heat treatment and homogenisation influence the gastric protein digestion of bovine milk protein in the growing pig as an adult human model. *The Journal of Nutrition. In press, available online. DOI: 10.1016/j.tjnut.2024.04.035*.

4. Ahlborn, N. G., Montoya, C. A., Ye, A., Samuelsson, L. M., Roy, N. C., & McNabb, W. C. Heat treatment and homogenisation of bovine milk influenced the true small intestinal absorption kinetics of and blood plasma concentration of amino

acids in the growing pig as an adult human model. *Manuscript in preparation, to be submitted to The Journal of Nutrition (American Society of Nutrition).*

5. Ahlborn, N. G., Montoya, C. A., Ye, A., Samuelsson, L. M., Roy, N. C., & McNabb, W. C. The influence of processing on the digestion and absorption of nutrients from whole bovine milk – a review. *Manuscript in preparation, to be submitted to Trends in Food Science and Technology.*

Conference abstracts and presentations

1. The influence of processing on bovine milk protein and lipid digestion and absorption. Riddet Institute Student Colloquium, 7th – 9th April 2021, Wellington, New Zealand (*oral presentation*).

2. Milk and our tummies – how do they team up? Dairy Science and Technology Symposium, 21st – 25th June 2021, Aarhus University, Denmark (*oral presentation, online*).

3. Heat it, homogenise it, eat it: what does commercial milk processing mean for digestion? Dairy Industry Workshop, 17th – 18th August 2022, Palmerston North, New Zealand (*oral presentation*).

4. Commercial milk processing influences the digestion of bovine milk protein. Riddet Institute Colloquium, 14th – 16th November 2022, Napier, New Zealand (*oral presentation*).

5. Bovine milk processing could be used to modulate the kinetics of amino acids entering the small intestine. International Symposium of Dietary Protein for Human Health, 14th – 16th September 2023, Utrecht, The Netherlands *(oral presentation).*

Media outputs

1. Animation: Heat treatment and homogenisation of bovine milk loosened gastric curd structure and increased gastric emptying in growing pigs. Ahlborn, N. G., Montoya, C. A., Hodgkinson, S. M., Dave, A., Ye, A., Samuelsson, L. M., Roy, N. C., & McNabb, W. C. (2023). *Riddet website, https://riddet.ac.nz/news-events-heading/news-articles?view=article&id=487&catid=8*
2. Animation: Differences in small intestinal apparent amino acid digestibility of raw sheep, cow, and goat milk are explained by gastric amino acid retention in piglets as an infant model. Ahlborn, N. G., Montoya, C. A., Roy, D., Stroebinger, N., Ye, A., Samuelsson, L. M., Roy, N. C., McNabb, W. C. & Moughan, P. J. *In progress.*

Scholarships and awards

1. Riddet Institute CoRE PhD research scholarship, November 2019 – July 2023.
2. FoodHQ IFAMA Scholarship, 2020. Selected as part of New Zealand's team to compete in the IFAMA International Student Case Competition, Rotterdam, Netherlands. Included attendance at the 2020 IFAMA conference and pre-conference agri-food tour. *Event cancelled due to Covid-19.*

3. Selected for New Zealand's MBIE 'Young Researchers' cohort for attendance at the Global Young Scientists Symposium, 2020 *(online due to Covid-19).*

4. 1st place with team 'The Melt' - QING Innovation Track – 2021 Competition for Climate Adaptation in Food Systems, hosted by Wageningen University of Research, Netherlands. Included talk at pre-COP26 (2021 United Nations Climate Change Conference) 'Youth4Climate' event *(online due to Covid-19).*

5. 2nd place - Riddet Institute Student Oral Presentation competition, Riddet Student Colloquium, Napier, New Zealand, 2021.

6. Riddet Institute Postgraduate Student Travel Award for attendance and presentation at the International Symposium of Dietary Protein for Human Health, Utrecht, the Netherlands, 2023.

CHAPTER 1.
INTRODUCTION

Since the early domestication of milk-producing animals, milk has remained an integral part of the human diet and an important source of nutrition for many cultures world-wide. The continued universality of milk consumption is partially driven by its nutritional value, in terms of both quality and quantity. However, much of the milk consumed today is heat treated and/or homogenised, which can alter the native structures of nutrients present in milk. For example, heat treatment partially denatures whey proteins, resulting in the formation of protein aggregates (Anema & Li, 2003), whereas homogenisation restructures the milk fat globule, resulting in the adsorption of proteins to the new fat droplet surface (Lee & Sherbon, 2002; Ye et al., 2004).

Once consumed, milk coagulates in the stomach forming a cheese-like solid (curd), which is mainly comprised of protein and lipid (Roy et al., 2021). Various characteristics of the curd, such as structure and strength, are influenced by processing-induced changes to milk nutrient structures (Li et al., 2022; Mulet-Cabero et al., 2019; Ye et al., 2017; Ye et al., 2019). Further, processing has been shown to increase gastric hydrolysis of milk protein *in*

vitro (Mulet-Cabero et al., 2019; Ye et al., 2016), which could be expected to influence the degree of digestion of protein released into the small intestine. However, a comprehensive understanding of the gastric protein digestion and gastric emptying of processed milk has been limited to mostly *in vitro* studies.

Studies in pigs have demonstrated that the gastric release of total nitrogen can influence small intestinal absorption (Gaudichon et al., 1994; Montoya et al., 2018). Therefore, it could be expected that the altered gastric curd formation and digestion reported for different milk types may have implications for the gastric release of protein into the small intestine, with subsequent effects on small intestinal amino acid (AA) absorption. However, detailed literature discussing the *in vivo* digestion of processed milk beyond the gastric stage is also lacking.

Despite the nutritional importance of the absorption process, the small intestinal absorption of AA from milk is rarely reported. This knowledge gap may be partially due to limitations associated with various small intestinal models: *in vitro* digestion models do not sufficiently emulate the processes occurring in the human, *in vivo* studies using animal models are complex, expensive, and variable in terms of result translation to humans, and clinical studies often require ileostomates, who are difficult to recruit. In addition, to understand the digestion of milk protein and absorption of AA (and some peptides), it is essential to determine the effects of each dynamic stage of the digestive process leading up to absorption, which further adds to the scale of study required.

In the context of nutritional research, the pig is generally accepted as one of the best models for the human at various life stages, due to the close anatomical and physiological similarity

of the gastrointestinal tract (Miller & Ullrey, 1987; Moughan et al., 1992; Odle et al., 2014; Roura et al., 2016; Rowan et al., 1994). A further advantage of the pig, as opposed to other animal models, is that it can be accustomed to the consumption of 'human meals' throughout the day, which allows for the investigation of specific digestive responses related to the consumption of a fixed volume of milk. The use of the pig also allows for simultaneous sample collection from various sites (e.g., gastrointestinal digesta, blood, and tissue samples), while providing sample volumes sufficient for multiple analyses. Of particular importance for absorption studies is the strong correlation of pig and human ileal digestibility values across a variety of food types (Hodgkinson et al., 2022), which allows for direct translation of ileal absorption as determined in pigs onto humans. Thus, the pig model can provide valuable insight into the kinetics of processed milk digestion and resulting AA absorption in the human, which are otherwise difficult to measure clinically.

Understanding the influence of common processing techniques on milk protein digestion and AA absorption will inform the development of products with enhanced nutritional outcomes. Such knowledge is especially relevant for the optimised delivery of nutrients to support physiological functions of populations with modified protein requirements, such as the ill or the elderly.

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CHAPTER 2.

LITERATURE REVIEW: THE EFFECT OF MILK PROCESSING ON THE DIGESTION AND ABSORPTION OF BOVINE MILK NUTRIENTS IN THE HUMAN GASTROINTESTINAL TRACT

This chapter is in preparation for submission as a manuscript to Trends in Food Science and Technology (impact factor 16.002): Ahlborn, N. G., Montoya, C. A., Ye, A., Samuelsson, L. M., Roy, N. C., & McNabb, W. C. The influence of processing on the digestion of protein and absorption of amino acids from whole bovine milk – a review.

2.1. Introduction

Evidence recording the continued human consumption of milk from various ruminant sources dates back as far as 400 BC, indicating that milk has long been a key source of nutrition for human beings. As a result of large production volumes and widespread availability, bovine milk is now the most consumed milk worldwide.

From a protein perspective, milk is an excellent source of nutrition, both in terms of quantity and quality. For example, milk protein (36 g/L) contains all essential amino acids (EAA) in a readily bioavailable form, which are required for various metabolic processes in the human body, such as protein synthesis. However, despite the long history and globality of bovine milk consumption, the human digestion of whole milk protein only began gaining attention in the mid-1900s. More recently, research efforts have focussed on understanding the behaviour of whole milk, milk products and milk components in the stomach. In particular, the elucidation of the impact of common commercial processes on the native structures of bovine milk protein and lipid has motivated researchers to investigate the influence of processing-induced structural changes on the digestion and absorption of these nutrients in the human gastrointestinal tract. Despite this attention, and the importance of milk for human nutrition, knowledge of the digestion of processed milk beyond the gastric stage is lacking.

As a highly valuable protein source, the digestion of processed bovine milk and subsequent absorption of nutrients are important to understand. Therefore, to determine gaps in the current literature and to place this PhD dissertation research within the existing knowledge field, this chapter reviews the relevant literature published to date. While this review is centred on the digestion of bovine milk protein and absorption of amino acids (AA),

interactions between milk protein and lipid under processing conditions and in the gastric environment make a major contribution to the digestive outcomes of milk protein. Therefore, the composition and the gastric digestion of, and the effect of processing on, the lipid fraction of milk are also reviewed.

The research questions resulting from the knowledge gaps identified in the literature review are described at the end of this chapter. The research questions are followed by the research aim, the overall hypothesis, and the subsequent research approach designed to answer the research questions.

2.2. Milk composition and nutrient structure

2.2.1. Proteins

Milk is one of the most readily available sources of protein, offering close to 32 g/L of protein in the case of bovine milk (Pereira, 2014). The total protein in milk can be mostly split into insoluble and soluble fractions, known as casein and whey protein, respectively. In bovine milk, the ratio of casein and whey protein is approximately 4:1 (Haug et al., 2007) (Table 2.1). However, this ratio varies across species (e.g., 6:1 for ovine milk and 3:1 for caprine milk) (Park et al., 2007; Roy et al., 2020a).

Caseins were originally defined by Jenness et al. (1956) in the paper 'Nomenclature of Proteins of Bovine Milk' as "phosphoproteins that precipitate from raw skim milk in response to acidification to pH 4.6 at 20°C". Further research demonstrated that milk caseins can be divided into four categories: αs_1-, αs_2-, β-, and κ-caseins, based on their primary structures.

Table 2.1. Macronutrient composition and structural characteristics of bovine milk[1]

Protein (g/L)	30 – 35
Total casein protein	24.6 – 28
αs_1-casein	8 – 10.7
αs_2-casein	2.8 – 3.4
β-casein	8.6 – 9.3
κ-casein	2.3 – 3.3
Total whey protein	5.5 – 7.0
β-lactoglobulin	3.2 – 3.3
α-lactalbumin	1.2 – 1.3
Immunoglobulins	0.3 – 0.7
Serum albumin	0.1 – 0.2
Lactoferrin	0.02 – 0.75
Fat (g/L)	36
Lactose (g/L)	47
Calcium (g/L)	1.22
Energy (kcal/L)	690
Casein micelle diameter (μm)	0.18
Fat globule diameter (μm)	2.8 – 4.6
Density (g/cm^3)	1.02 – 1.04

[1] Adapted from Claeys et al. (2014); Farrell Jr et al. (2004); Fox and Brodkorb (2008); Goulding et al. (2019); Hurley and Theil (2013); Jenness (1980); Jenness et al. (1956); Park (2009); Roy et al. (2020b); Selvaggi et al. (2014).

In bovine milk, the αs_1-casein fraction makes up 38 - 40% of the total casein, αs_2-casein makes up to 10%, β-casein 35 - 45%, and κ-casein the remaining 5 - 12% (Farrell Jr et al., 2004; Goulding et al., 2019). A further casein, γ-casein, is also identifiable in milk, although γ-casein is a peptide fragment derived from β-casein by the indigenous enzyme plasmin (Eigel et al., 1984).

The phosphorylation of caseins has major significance for their functional and nutritional properties, including hydration, solubility, and heat stability, as well as the binding of calcium, allowing caseins to exist in milk in a soluble form (De Kruif et al., 2012). As a result of a high proline content, casein proteins do not have stable secondary or tertiary structures, which results in a flexible, open structure exposing the majority of the hydrophobic residues (Horne, 1986). As such, the surface of the casein proteins is strongly hydrophobic, resulting in a tendency to associate in aggregates of 10 – 20 casein molecules (Horne, 2020). This behaviour plays a role in the formation of casein micelles, stabilising the milk caseins (Holt, 1992).

Current knowledge suggests that in milk, the αs_1-, αs_2-, and β-caseins bind with calcium phosphate to form relatively stable micelles (Fox & Brodkorb, 2008), with most of the κ-casein in milk incorporated near the surface of these micelles, forming a protective coating (Dalgleish, 1990; De Kruif et al., 2012). The κ-casein appears to create a layer around the micelle with macro-peptides protruding in a ‘hairy’ coating, providing steric stabilisation, which prevents aggregation of the micelles (Figure 2.1) (Fox & Brodkorb, 2008; Horne, 1986). It is thought that individual proteins can cross this layer, particularly as the matrix changes temperature, which has implications for processing and digestion (Anema & Li, 2003a; Dalgleish, 1990; Dalgleish & Corredig, 2012; Edwards & Jameson, 2014).

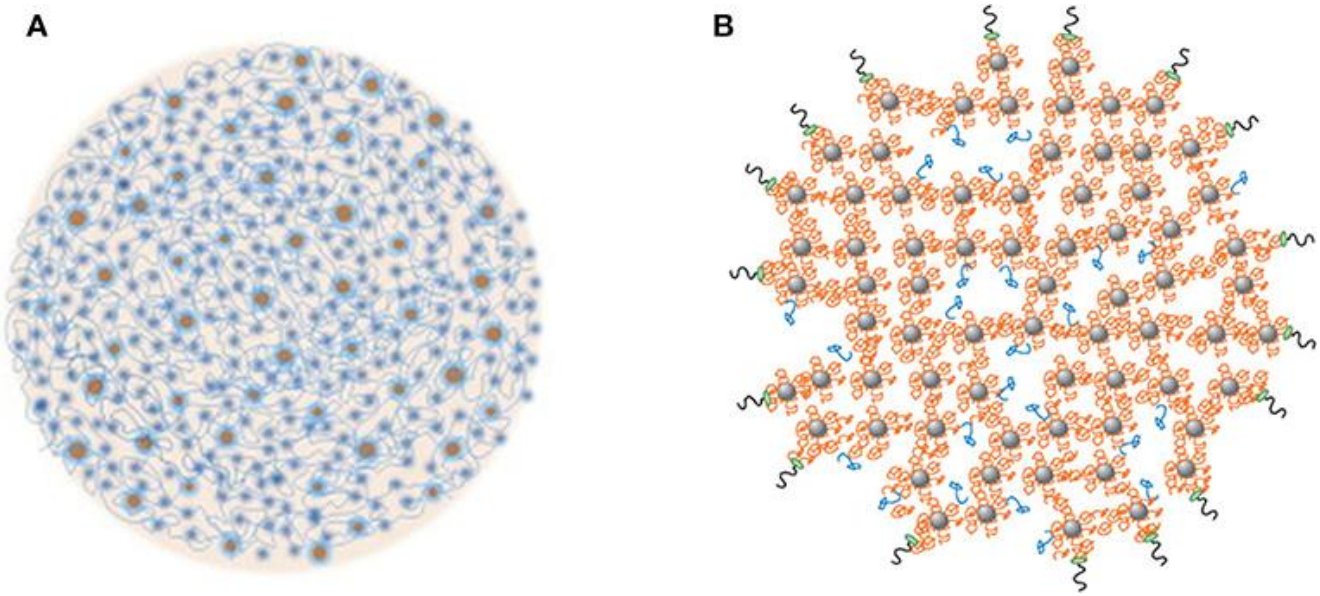

Figure 2.1. Proposed casein micelle models.
A: The model suggested by De Kruif et al. (2012); **B:** The model suggested by Dalgleish and Corredig (2012). Both models are derived from Holt and Horne's 1996 nanocluster model, showing α_s- and β-casein residues (A: blue; B: orange) bound to calcium phosphate nanoclusters (A: orange; B: grey), stabilised by a κ-casein 'shell' (A: blue loose strands; B: black 'hairs'). In the model proposed by Dalgleish and Corredig (2012), some β-casein molecules (blue) are also shown hydrophobically bonded to other caseins. Image reproduction permission granted by Elsevier (A) and Annual Reviews (B) for inclusion into the thesis only.

The casein micelle structure is maintained by hydrophobic effects, electrostatic interactions, and bound colloidal calcium phosphate (Holt et al., 2013). Subsequently, a large portion of the calcium in milk is associated with casein micelles, where the incorporation into the κ-casein-stabilised micelle is responsible for the high level of solubilised calcium in milk (Fox & Brodkorb, 2008).

Around 20% of the total protein in bovine milk is whey protein, which can be fractionated into the two principal proteins, β-lactoglobulin (60 - 70%) and α-lactalbumin (20 - 28%), as well as the minor proteins immunoglobulins (10%), bovine serum albumin (3%), and lactoferrin (0.1 - 2%) (Edwards & Jameson, 2014; Farrell Jr et al., 2004; McSweeney & Fox, 2013). Unlike caseins, whey proteins are highly structured and have a rigid and globular structure (López-Fandiño, 2006).

The rigid structure and stable secondary and tertiary structures of whey proteins means that they do not aggregate in their native form. As a result, whey proteins exist in milk in monomers, dimers, or small quaternary structures. β-lactoglobulin contains two intramolecular disulfide bonds, and cysteine as a free thiol group. In contrast, α-lactalbumin contains four intramolecular disulphide bonds, lacks cysteine and phosphate, and binds calcium in a specific pocket (McKenzie & White, 1991). These properties have implications for heat stability.

Of the minor whey proteins, immunoglobulin (1% - 2% of total protein) and lactoferrin (up to 2.5% of total protein) are both biologically active. The main immunoglobulin in bovine milk is IgG1 (73%), with IgG2 (2.5%), IgA (18%), and IgM (6.5%) making up the rest of the immunoglobulin protein fraction (Hurley & Theil, 2013). In milk, immunoglobulins

act immunogenically to protect the gastrointestinal mucosa against pathogens, although, in colostrum, the immunoglobulins also exist to transfer passive immunity to the neonate.

Lactoferrin acts as an antioxidant in the gastrointestinal lumen (Mulder et al., 2008) and also improves the bioavailability of iron (Walzem et al., 2002). In contrast, the function of bovine serum albumin is unclear, although it may have a role in lipid synthesis due to its propensity for fatty acid (FA) binding (Morrisett et al., 1975).

The presence of a further proteinaceous fraction in milk, the proteose peptone, comprising entirely of peptides, has been partially accredited to the action of indigenous plasmin (O' Mahony et al., 2013). However, some portion of this peptide fraction is indigenous to the milk (Fox & Kelly, 2003). In addition to the casein and whey fractions and the proteose peptone, around 3 - 5% of the total nitrogen in bovine milk consists of non-protein nitrogen (NPN) (i.e., urea, creatine, uric acid, and AA such as taurine) (Goulding et al., 2019).

Due to their low concentration in bovine milk, immunoglobulins, lactoferrin, bovine serum albumin, proteose peptone, and NPN fractions are thought to have minimal contribution to the overall physicochemical properties of milk (Goulding et al., 2019) and thus are not discussed in further detail in this review.

2.2.2. Amino acids

In the context of protein nutrition, the superior value of milk is related to both the quantity and quality of the protein present. While bovine milk contains all EAA, it also provides excellent levels of non-essential AA (NEAA) (Lucey et al., 2017) (Table 2.2).

Table 2.2. Amino acid composition of bovine milk[1]

Essential amino acids, mg/100 mL milk (mg/g protein)	
Histidine	100 (29.3)
Isoleucine	140 (41.0)
Leucine	290 (85.0)
Lysine	270 (79.1)
Methionine	60 (17.6)
Phenylalanine	160 (46.9)
Threonine	150 (44.0)
Tryptophan	50 (14.7)
Valine	160 (46.9)
Non-essential amino acids, mg/100 mL milk (mg/g protein)	
Alanine	100 (29.3)
Arginine	110 (32.2)
Aspartate	260 (76.2)
Cysteine	20 (5.9)
Glycine	60 (17.6)
Glutamate	770 (225.7)
Proline	320 (93.8)
Serine	160 (46.9)
Tyrosine	150 (44.0)
Total essential amino acids	1380 (404.5)
Total non-essential amino acids	1950 (571.6)
Total amino acids	3330

[1] Adapted from Claeys et al. (2014), Jenness (1980), and Jenness et al. (1956).

Further, as an animal protein source, milk protein is readily bioavailable and highly digestible (Gaudichon et al., 1994), which means the AA content of milk can be almost fully utilised for various roles within the human body, including as neurotransmitters or in protein synthesis (Wu, 2009).

To measure protein quality, the digestible indispensable AA score (DIAAS) coefficients are generally used, and protein sources with a DIAAS coefficient over 0.75 are considered sufficient for the provision of all AA for the six-month- to three-year-old growing child, as the population with the highest protein demand (FAO, 2011). The DIAAS coefficients of skim milk, milk protein concentrate, and whey protein concentrate are reported as greater than 1.00 (Maathuis et al., 2017; Mathai et al., 2017)), indicating that milk protein can supply the AA requirements of humans with the highest AA demand. Further, in terms of meeting global nutritional requirements, a model which considers both quantity and bioavailability of AA from 98 common dietary foods has indicated that bovine milk is the main dietary item contributing to the delivery of lysine (Smith et al., 2022). The same modelling also suggested that bovine milk is the second greatest provider of the EAA threonine and leucine, and the third greatest provider of histidine, methionine, and tryptophan (Smith et al., 2022). These findings indicate that milk is an essential source of nutrition.

2.2.3. Lipids

Lipids in milk are secreted in globules and coated by the milk fat globule membrane (MFGM). The MFGM comprises proteins, phospholipids, glycoproteins, enzymes, and trace elements. Within the globule in bovine milk, 98% of the lipids exist as

triacylglycerols, with the remaining 2% accounted for by diacylglycerol, cholesterol, phospholipids, and free FA. Fat-soluble vitamins, flavour compounds, hydrocarbons, and ether lipids are also present in minor amounts (Lindmark Månsson, 2008).

Milk fat makes up around 4.2% of bovine milk and consists of approximately 66% saturated fats, 30% monounsaturated fats, and 4% polyunsaturated fats (Lucey et al., 2017) (Table 2.3). The saturated fats are comprised of palmitic (30%), myristic (11%), and stearic (12%) acids. Short-chain FA (SCFA) and medium-chain FA are around 11%, which are mainly butyric and caproic acids (4.4% and 2.4%, respectively).

Only 13 FA are present in concentrations of more than 1% of the total FA, yet up to 400 FA species have been identified in bovine milk lipid (Lucey et al., 2017). FA up to C16:0 are synthesised in the mammary gland, while longer chains and monounsaturated FA are derived from plasma lipids. Multiple factors, such as species, breed, season, lactation stage, and diet, influence the amount and composition of lipids (Jensen et al., 1991).

Table 2.3. Typical fatty acid (FA) and cholesterol content of bovine milk[1]

Fatty acids, g/100 mL milk (% total FA)	
Saturated FA	2.0 - 2.6 (55.7 - 72.8)
Monounsaturated FA	0.8 - 1.1 (22.7 - 30.3)
Polyunsaturated FA	0.08 - 0.22 (2.4 - 6.3)
Linoleic acid	0.04 - 0.11 (1.2 - 3.0)
α-Linolenic acid	0.01 - 0.06 (0.3 - 1.8)
Conjugated linoleic acids	0.007 - 0.09 (0.2 - 2.4)
Cholesterol	0.01 (0.3)

[1] Values adapted from Park et al. (2007), Balthazar et al. (2017), Claeys et al. (2014) and Medhammar et al. (2012).

2.2.4. Vitamins and minerals

Regarding micronutrient content, bovine milk is a rich source of vitamins and minerals, with naturally high levels of calcium, phosphorus, magnesium, zinc, manganese, copper, and B vitamins (Table 2.4) (Nohr & Biesalski, 2009; Park et al., 2007). Of particular significance is milk's contribution of calcium and the vitamins B_2, B_5, and B_{12} (riboflavin, pantothenic acid, and cobalamin, respectively) to the adult human diet, where modelling has demonstrated the essential role milk plays in providing adequate dietary levels of these vitamins and minerals to the growing global population (Smith et al., 2022).

Table 2.4. Vitamin and mineral composition of bovine milk[1]

Vitamin content, µg/100 mL milk	
Thiamin (B_1)	28 – 90
Riboflavin (B_2)	160 – 429
Niacin (B_3)	300 – 500
Pantothenic acid (B_5)	350 – 408
Pyridoxine (B_6)	30 – 70
Biotin (B_9)	2 – 4
Folic acid (B_9)	1 – 18
Cobalamin (B_{12})	0.27 – 0.7
Ascorbic acid (vitamin C)	300 – 2300
Vitamin A (RE)	17 – 50
Cholecalciferol (vitamin D3)	0.3
α-Tocopherol (vitamin E)	20 - 184
Mineral content, mg/100 mL milk	
Calcium	112 – 123
Phosphorus	59 – 119
Potassium	106 – 163
Magnesium	7 – 12
Sodium	58
Iron	0.03 – 0.1
Zinc	0.3 – 0.55
Copper	0.01 – 0.08

[1] Adapted from Claeys et al. (2014), Gulati et al. (2018), Medhammar et al. (2012), Toscano et al. (2023), and Zhang et al. (2022). RE, retinol equivalents.

2.3. The impact of processing on bovine milk proteins and lipids

For food safety and standardisation purposes, milk is usually heat treated and homogenised prior to human consumption. To prepare bovine milk for consumers, the main commercial treatments are typically pasteurisation (72 °C for 15 s), ultra-high temperature (UHT) treatment (140 °C for 4 s) and homogenisation (approximately 16 - 18 MPa, two-stage). The effects of these processes on native bovine milk protein and lipid structures are the focus of the following sections.

2.3.1. Heat treatment

2.3.1.1. Whey protein denaturation

The effect of heating varies between milk proteins due to variations in their structures and assemblies. The main effect of heating on bovine milk is related to the instability of whey protein under heating conditions. When subjected to temperatures greater than 70 °C, the whey protein begins to denature, and a portion of the denatured protein tends to self-assemble into aggregates via disulfide bonds (McSwiney et al., 1994). A further portion of the denatured whey protein also associates with denatured MFGM protein (Ye, Singh, Oldfield, & Anema, 2004) and micellar κ-casein (Anema & Li, 2003a). In the latter process, denatured β-lactoglobulin and α-lactalbumin form complexes which interact with κ-casein via thiol-sulphide interactions, leading to their association with casein micelles (Corredig & Dalgleish, 1999; Gezimati et al., 1997; Singh, 2007). A representation of this process is shown in Figure 2.2.

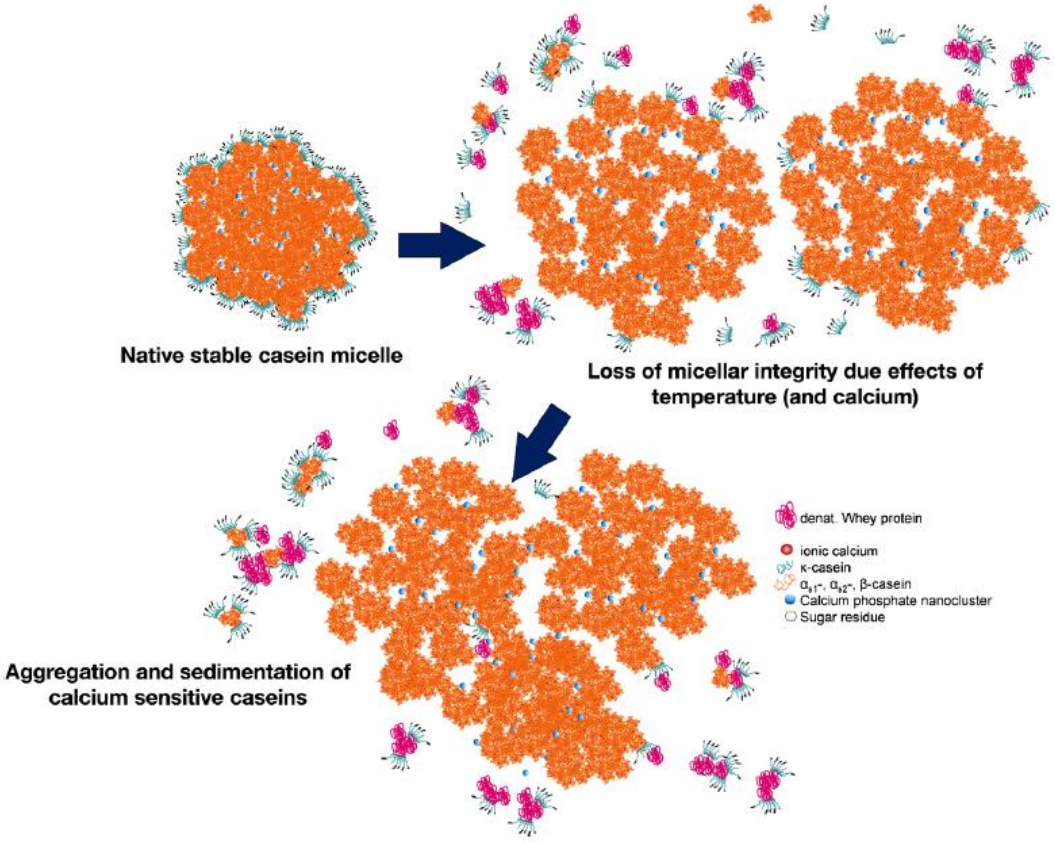

Figure 2.2. Diagram illustrating the heat-induced formation of whey protein and casein complexes via partial denaturation of whey protein and association with κ-casein (Dumpler et al., 2017).
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The extent of denaturation is relative to the severity of the heating conditions. As such, under standard pasteurisation conditions (72 °C for 15 s), around 80% of bovine whey protein remains in its native form (Rynne et al., 2004). In contrast, approximately 30% of the whey protein in traditionally treated UHT milk (140 °C for 4 s) remains native (McMahon et al., 1993).

2.3.1.2. Changes to casein micelle characteristics

Casein proteins are less affected by heating than whey proteins, which is thought to be due to their lack of stable secondary and tertiary structures (Goulding et al., 2019). Notwithstanding the association of whey proteins, the arrangement of casein micelles, as in milk, is stable at high temperatures (< 140°C). However, some colloidal calcium phosphate bound to micelles may be solubilised during processing, releasing individual caseins. This finding was demonstrated by Anema and Klostermeyer (1997), who found that raising milk temperature above 110 °C increased the amount of soluble casein present. Similarly, Singh (2007) also reported that heat treatment results in a less tight casein micelle with a smaller structure and more unbound (soluble) caseins.

Regarding casein released, κ-casein is the main protein dissociated from micelles at temperatures between 20 °C and 90 °C. However, at temperatures below 80 °C α_s- and β-casein also comprise a portion of the soluble casein (Dalgleish, 1990). An induced reduction in pH coupled with an increase in temperature up to 80 °C also causes some degree of micellar dissociation, as the colloidal calcium phosphate solubilises, releasing individual caseins (De Kruif et al., 2012; Tunick et al., 2016).

The free κ-casein is associated with denatured β-lactoglobulin in the serum phase (Deeth, 2019). The distribution of denatured whey protein with either micelle-bound or free κ-casein is mainly determined by the extent of heating, the pH, and the rate of temperature change applied during heat treatment (Anema & Li, 2003b; Corredig & Dalgleish, 1996a, 1996b).

2.3.1.3. Maillard reaction

A further heat-induced modification of the native milk protein structures occurs through the Maillard reaction, resulting in the lactosylation of some protein. At temperatures over 100 °C, lactose, a reducing milk sugar, reacts with the primary amines of lysine. This reaction can reduce lysine bioavailability and change milk colour and flavour (Van Boekel, 1998). Protein cross-linking via disulphide bonding with several Maillard reaction products can also occur, decreasing the bioavailability of some EAA, including lysine (Friedman et al., 1981).

2.3.1.4. Other heat-induced changes to milk proteins

Other heat-induced modifications include deamidation, dephosphorylation, and the non-Maillard-related cross-linking of proteins (Holland et al., 2011). The latter effects only occur to a small degree in UHT processing, where the extent of structural alteration is determined by the extent of heat treatment (time and temperature) (Holland et al., 2011).

2.3.2. Homogenisation

2.3.2.1. Changes to milk fat globule characteristics

Homogenisation is typically used after pasteurisation to standardise the distribution and size of fat globules throughout the milk by disrupting the MFGM (Figure 2.3). This disruption reduces fat globule size to around 1 μm and creates a new oil-water interface, which a portion of the caseins in milk adsorbs to, stabilising the new smaller fat globules (MacIerzanka et al., 2012; Sarkar et al., 2009). The combination of heat and homogenisation incorporates whey proteins into the restructured MFGM, which has been reported using sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2.4) (Garcia et al., 2014; Lee & Sherbon, 2002; Ye, Singh, Taylor, & Anema, 2004). The restructured globules show increasing levels of milk protein adsorption as the homogenisation pressure increases, which creates a larger surface area (Lee & Sherbon, 2002). Increasing heating temperature and time also increases the amount of whey protein adsorption (Ye, Singh, Taylor, & Anema, 2004). The specific protein composition of the stabilised milk fat globule also varies with homogenisation and heat treatment conditions utilised (Sharma et al., 1996; Ye, Singh, Taylor, & Anema, 2004).

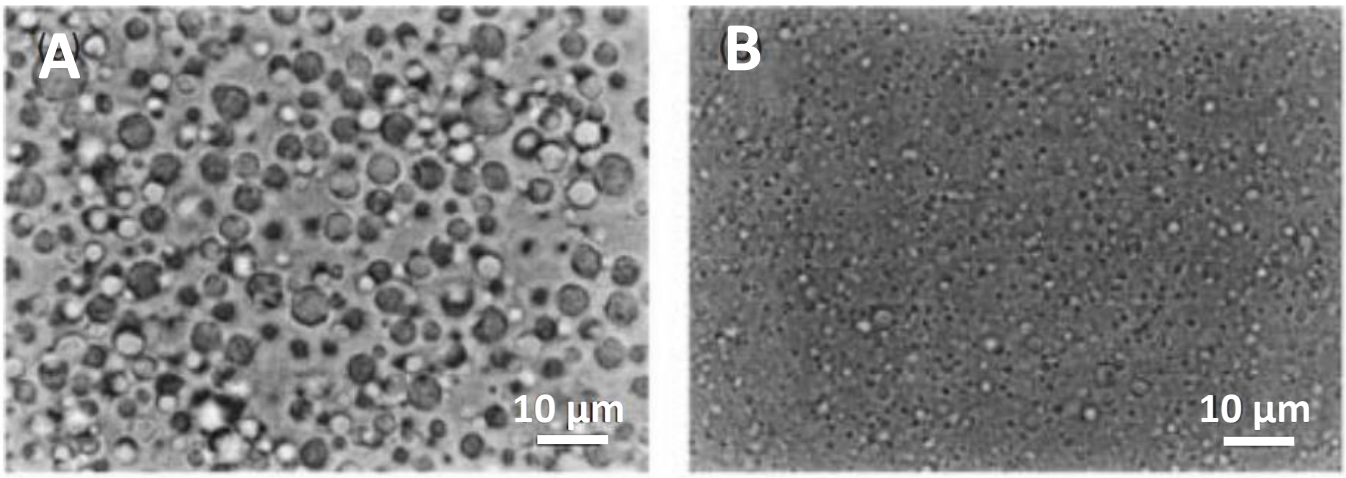

Figure 2.3: Light microscopy images of raw bovine milk before homogenisation (A) and after homogenisation at 18 MPa (B) (Hayes & Kelly, 2003).
Image reproduction permission granted by Cambridge University Press for thesis inclusion only.

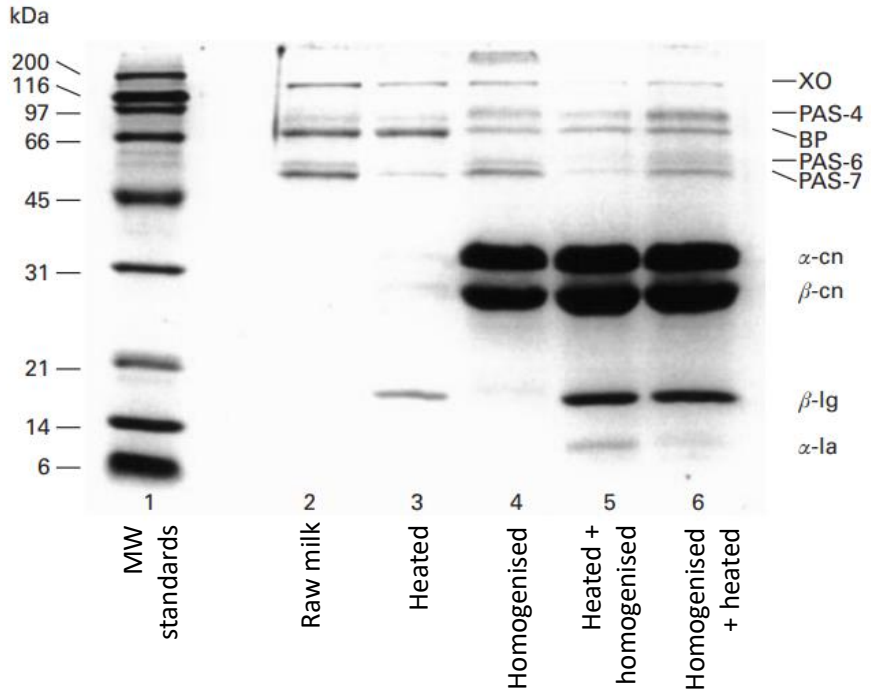

Figure 2.4: Proteins identified in the milk fat globule membrane of raw, heated (80°C), homogenised (16 MPa, two-stage), and heated-homogenised bovine milk (Lee & Sherbon, 2002).
Milk in lane 5 was first heated, then homogenised, whereas milk in lane 6 was first homogenised, then heated. MW, molecular weight; XO, xanthine oxidase; PAS-4, -6, and -7, membrane glycoproteins; BP, butyrophilin. Image reproduction permission granted by Cambridge University Press for thesis inclusion only.

2.4. Digestion and absorption of bovine milk proteins and lipids in the gastrointestinal tract

Although some minor digestion begins in the mouth by lingual lipase, the majority of milk digestion begins in the stomach, where muscular peristaltic contractions mix the milk with acidic gastric juice and lingual and gastric enzymes, forming a chyme. Pepsin begins hydrolysing protein, releasing peptides and AA, whereas lipids are hydrolysed by lingual and gastric lipases, releasing FA. Particles smaller than ~ 2 μm are continuously emptied into the small intestine. However, the gastric emptying of chyme depends on its caloric content and rheological properties (Marciani et al., 2001). Peristaltic contractions transit the chyme along the small intestine, defined by three main segments: the duodenum, jejunum, and ileum. During small intestinal transit, the hydrolysis of protein, lipid, and peptides continues under the action of chymosin, trypsin, elastase, and lipase released from the pancreas. Digested products (AA and/or peptides (di- or tripeptides), monoglycerides, FA, and glycerol) diffuse through the mucus layer lining the epithelial wall, are further hydrolysed by brush border enzymes secreted by the microvilli, and then passively or actively absorbed by enterocytes in the intestinal epithelial layer (Kiela & Ghishan, 2016; Wang et al., 2013). Some passive diffusion of SCFA into enterocytes also occurs. Numerous neural and hormone-mediated feedback mechanisms regulate the release of chemical and enzymatic secretions throughout the gastrointestinal tract, as well as gastric motility and meal transit (Boland, 2016).

Once dietary protein digestion products are absorbed, they are subjected to metabolism in the splanchnic bed (stomach, intestinal, liver, pancreatic, and spleen tissue) before entering the systemic blood circulation (Stoll & Burrin, 2006). Depending on their size and polarity,

dietary lipid digestion products are transported into the portal vein or lymph fluid and released into the circulation. Nutrients which remain unabsorbed at the end of the small intestine (i.e., the terminal ileum) transit into the large intestine, where they are partially utilised by the resident microbiota as substrates for metabolism. Figure 2.5 provides a visual summary of human gastrointestinal digestion and absorption.

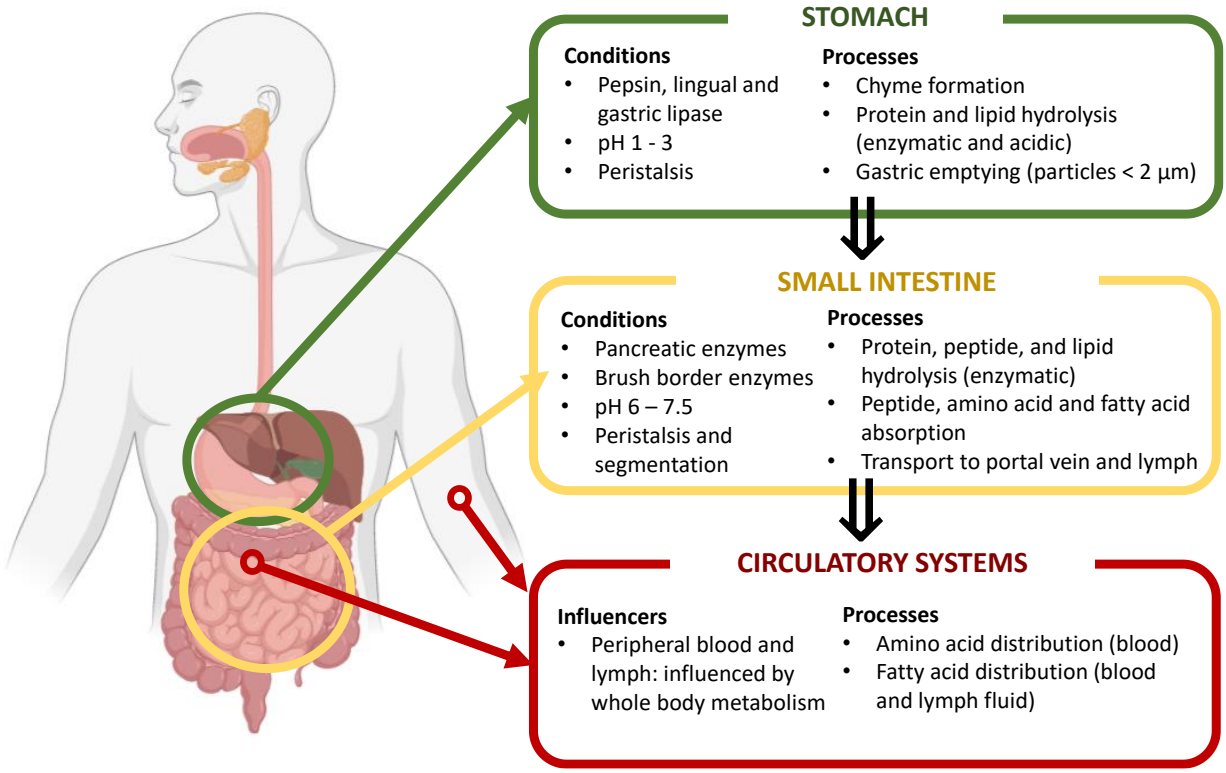

Figure 2.5: A graphical summary of digestion and absorption in the human gastrointestinal tract relevant to milk digestion.
Figure created with Biorender.com.

One major factor which impacts the digestion of milk is the structural differences in protein (casein vs whey; native vs processed) and their resulting different behaviours in the stomach and small intestine. Thus, the following sections discuss the gastric digestion of milk protein and lipid and the small intestinal absorption of milk AA and FA. The influence of processing on milk protein digestion, lipid digestion and AA and FA absorption is also reviewed.

Various aspects of digestion and absorption can be difficult to assess in human nutritional research, which is often carried out using human digestion models. The more common of these models are also evaluated in the following sections.

2.4.1. Human digestion and absorption models

Human gastrointestinal digestion and absorption are dynamic, complex, feedback-mediated processes. The most accurate approach for investigation of human digestion and absorption is through the use of clinical studies. However, the determination of most aspects of postprandial gastric digestion and small intestinal absorption requires sample collection directly from the gastrointestinal tract and is thus invasive and ethically burdensome. In addition, the high level of inter-individual variation for a set primary outcome between humans means that the effect size required to have an adequately powered study often demands many participants, for which recruitment can be challenging. As a result, nutritional research is often carried out using *in vitro, in vivo,* and *ex vivo* human models. These approaches tend to be less labour-intensive, less expensive, more rapid and have greater repeatability than clinical studies. However, each human model also has specific limitations, and as such, the physiological relevance of each model varies.

2.4.1.1. *In vivo* animal models

In terms of relevance to human gastrointestinal digestion and absorption, the non-human primate and the pig are considered two of the gold standard models. While non-human primates are physiologically closer to humans than pigs, they are prohibitively expensive to acquire and maintain, and limited numbers are available for study (Havel et al., 2017). As a result, non-human primates are rarely used for nutritional research.

As the porcine gastrointestinal physiology and functionality are similar to that of the human, the pig is often used as the next most realistic human gastrointestinal model (Guilloteau et al., 2010; Miller & Ullrey, 1987; Moughan et al., 1992; Odle et al., 2014; Roura et al., 2016; Sciascia et al., 2016). In addition, pigs are omnivores and adapt readily to meal feeding (Moughan et al., 1992; Rowan et al., 1994). As a result of the size of the pig, the volume of sample collected from each gastrointestinal location is generally sufficient for multiple concurrent analyses, which allows for a more thorough understanding of the interrelated digestive and/or absorptive processes occurring in each location.

Nevertheless, the pig model also has limitations. Depending on the age (i.e., piglet vs growing pig vs mature pig) and breed of the pig used, the physical size and rapid growth can result in challenges accommodating large numbers of animals for long periods. In addition, as with clinical studies, pig experiments are also time-consuming, logistically challenging, labour-intensive, and costly.

Rodents (typically rats or mice) are also considered acceptable models for human gastrointestinal digestion and absorption, particularly for protein digestion (Deglaire &

Moughan, 2012). In contrast to pigs, rats are readily available, easily maintainable, affordable human models, and it is comparatively straightforward to accommodate them in large numbers (Miranda & Pelissier, 1983; Rutherfurd & Moughan, 2003). However, while still comparable to humans, the rat's gastrointestinal physiology and functionality are less similar to humans than that of pigs (Deglaire & Moughan, 2012; Hugenholtz & de Vos, 2018). For example, the rat does not have a gallbladder and has a faster gastric transit time than humans relative to its size (Hugenholtz & de Vos, 2018). Further, the rat does not naturally meal-feed, which restricts reliable comparison of the outcomes of single bolus consumption. Their small size also restricts the volume of gastrointestinal content available for sampling, which can limit study outcomes.

While studies using animals are currently the most realistic means for modelling human digestion and absorption, there are ethical concerns around animal models, and the general trend is to reduce the use of animals where possible. To this end, the Three R principles, as proposed by Russell and Burch (1959) in the Principles of Humane Experimental Technique, provide the internationally accepted ethical framework for animal experimentation. The Three R principles are replacement (i.e., substitution with non-sentient alternatives); reduction (i.e., the use of as fewer animals as possible to achieve the required precision); and refinement (i.e., the use of the least severe procedures possible, and the appropriate mitigation of distress and pain).The Three R principles are now included in legislation governing the use of animals in research throughout the world, and are actively promoted within the scientific community (Guillén, 2017; Hubrecht & Carter, 2019).

2.4.1.2. *Ex vivo* models

The rodent gastrointestinal tract is used further in *ex vivo* models, utilising intestinal tissue isolated from euthanised animals. This approach allows for the assessment of various processes, such as the absorption of nutrients into the mucus layer, the transport of nutrients across the epithelial layer, changes in motility, and tissue permeability (Blanquet et al., 2004; Mackie et al., 2020). However, removing the subepithelial muscular layer and nerve circuits limits peristaltic movements, and applying a 'digesta' solution across the whole tissue sample does not replicate the nutrient concentration gradient observed along the small intestine in the human. Further, it can result in saturation of the system (Lundquist & Artursson, 2016). In addition, a lack of circulation means that the use of intestinal tissue is limited by a tissue viability time of 1 – 3 hours (Blanquet et al., 2004; Mackie et al., 2020).

2.4.1.3. *In vitro* models

In vitro models are often used to circumnavigate the need for specialist animal handling facilities and expertise and the ethical concerns associated with *in vivo* animal models. Compared with *in vivo* models, *in vitro* models are also rapid, highly reproducible, and less expensive.

Static *in vitro* models aim to simulate gastrointestinal digestive behaviour using a series of stages carried out in glass vessels, with fixed pH and enzyme concentrations introduced at the beginning of the simulation. These models cannot mimic the mechanical forces (i.e., peristaltic movements) and dynamic conditions (i.e., pH gradients, continuous acid and enzymatic secretions, gastric emptying, digesta flow, absorption) that occur in the human gastrointestinal tract. To allow for comparison between laboratories, the INFOGEST

model was developed, which provides standardised static *in vitro* digestion parameters relevant to healthy adult humans (Brodkorb et al., 2019; Minekus et al., 2014). Despite the limitations of the static model, Egger et al. (2017) and Egger et al. (2019) showed that milk protein hydrolysis determined in the INFOGEST model was comparable with hydrolysis results reported in the adult pig model. These comparisons indicate that the static INFOGEST model can be useful to investigate fundamental aspects of food digestion in specific gastric conditions.

The gradual addition of gastric secretions throughout the simulated digestion period, the introduction of gentle mixing, and a constant emptying of digesta into the small intestinal phase, as described by Mulet-Cabero, Egger, et al. (2020), creates a semi-dynamic *in vitro* model. While this approach brings static digestion models closer to human digestion, aspects such as stomach physiology, peristaltic movements, and the influence of food physicochemical properties on gastric emptying are not addressed.

To this end, several dynamic *in vitro* models have been developed, which, in addition to continuous secretion, typically incorporate a variety of flexible vessels, pumps, rollers, stirrers, and external pressure systems to simulate the dynamic physical and chemical aspects of human gastrointestinal digestion. Complex computer systems often control the dynamic parameters of these models.

The more commonly used dynamic *in vitro* models for gastric digestion are the human gastric simulator (HGS) (Kong & Singh, 2010), the dynamic gastric model (DGM) (Wickham et al., 2012), and the gastric digestion simulator (GDS) (Kozu et al., 2014). Continuous models simulating the behaviour of food in both the stomach and small

intestinal include the TNO gastrointestinal model (TIM) (Minekus, 2015), the *in vitro* dynamic system (DIDGI) (Ménard et al., 2014; Ménard et al., 2015), and the engineered stomach and small intestinal system (ESIN) (Guerra et al., 2016). These models add a filter membrane system to remove small molecules from the simulated duodenal and jejunal sections by dialysis. The TIM-2 (Bellmann et al., 2016), the simulator of the human intestinal microbial system (SHIME) (van de Wiele et al., 2015) and the simulator of the gastrointestinal tract (SIMGI) (Barroso et al., 2015) models also incorporate a large intestinal simulation by the addition of further vessels and microbial fermentation stages.

A major limitation of these models is that they still lack the morphological and anatomical structures (e.g., small intestinal microvilli) of the gastrointestinal tract. Further, dynamic *in vitro* models do not account for the gastrointestinal regulatory processes (i.e., hormonal and nervous system feedback control mechanisms), longitudinal enzymatic gradients, and mucosal cell activity. Small intestinal nutrient absorption and nutrient transport kinetics cannot be assessed.

Another approach for assessing intestinal epithelial cell absorption uses cellular models, including Caco-2 cell lines, cell monolayers, two-dimensional multiple-cell models, and self-assembling three-dimensional organoid models (Barnett et al., 2021; Barnett et al., 2023; Jung & Kim, 2022). Some authors have used cell culture approaches coupled to *in vitro* models to include epithelial cell-nutrient interactions, which can more accurately simulate some aspects of small intestinal absorption secondary to gastric digestion (Déat et al., 2009; Vors et al., 2012). Similarly, *ex vivo* systems paired with *in vitro* models have been shown to produce gastrointestinal protein digestion and AA disappearance kinetic

trends comparable to those reported in the *in vivo* pig model, as demonstrated using isolated milk protein (Mulet-Cabero, Torcello-Gómez, et al., 2020).

2.4.2. Gastric digestion of bovine milk

Under gastric conditions, the casein fraction of milk forms a clot (or curd) in the stomach. The curd consists of a solid mass caused by the coagulation of casein micelles due to the acidic gastric environment and enzymatic hydrolysis of κ-casein by pepsin, destabilising the casein micelles (Tam & Whitaker, 1972; Wang et al., 2018; Yang et al., 2022; Ye, 2021). The mechanical action of the stomach also plays a role in curd formation and leads to the incorporation of milk fat globules into the curd, as demonstrated in an *in vivo* infant model (Roy et al., 2022). While caseins are highly susceptible to enzymatic hydrolysis, their clotting behaviour decreases the overall casein surface area, leading to a reduced rate of diffusion of pepsin into the curd structure (Wang et al., 2018). Based on different profiles of plasma AA appearance in the blood of adult humans given purified preloads of heat-treated caseins, it has been speculated that by limiting the rate of casein hydrolysis by pepsin, the curd disintegration and subsequent gastric empty rate are also reduced (Hall et al., 2003), which could lead to a slower release of caseins and derived peptides and AA into the small intestine.

In contrast to caseins, an *in vitro* study has shown that native whey protein does not coagulate in the gastric environment and remain soluble in the gastric liquid phase (Ye et al., 2016b). This persistent solubility resulted in a rapid disappearance of whey protein in the stomach, as demonstrated in rats (Ye et al., 2019) and in humans using nasogastric intubation (Boutrou et al., 2013; Mahé et al., 1996). Studies using a semiquantitative

analytical approach in simulated gastric fluid have shown that the major whey protein β-lactoglobulin is, in its natural state, largely resistant to hydrolysis by pepsin (Inglingstad et al., 2010; Peram et al., 2013). Further, in rats fed whole bovine skimmed milk, the level of hydrolysis of α-lactalbumin was lower than that of the casein protein (Miranda & Pelissier, 1983). Thus, based on the rapid gastric disappearance combined with the resistance of whey protein to gastric hydrolysis, it could be assumed that most native whey proteins in milk are rapidly released into the small intestine in a less digested state than native casein proteins.

The milk fat globules are relatively stable in a simulated gastric environment, as the MFGM proteins are not susceptible to pepsin hydrolysis, and the phospholipid arrangement is not susceptible to digestion by gastric lipase (Hamosh et al., 1999; Ye et al., 2010). Under dynamic simulated gastric conditions, the milk fat globules are incorporated into the gastric curd and remain entrapped within the protein network formed by the coagulated caseins (Roy et al., 2021; Ye et al., 2016b). The relevance of this finding to *in vivo* digestion was confirmed by Roy et al. (2022), who reported similar results in piglets as an infant model.

2.4.3. The influence of heat treatment and homogenisation on the gastric digestion of bovine milk

The influence of heat treatment and homogenisation on gastric milk coagulation has been well-characterised *in vitro*, and these studies are supported by a small number of *in vivo* studies. It is confirmed that changes to the native protein and lipid structures of bovine milk alter the gastric curd structure *in vitro*. Ye et al. (2016a, 2017) showed that during dynamic simulated digestion, heat-induced casein-whey aggregation can disrupt gastric milk

coagulation by restricting the ability of protein to form a network. Due to this disruption, the curd formed from milk heated over 90 ℃ is softer, with a looser, more open structure than that of pasteurised and raw milk (Figure 2.6) (Mulet-Cabero et al., 2019; Ye et al., 2016a).

In addition, Ye et al. (2017) also showed that homogenisation combined with heat treatment led to better incorporation of the smaller, restructured fat droplets into the gastric curd matrix *in vitro* which further loosens the curd structure, resulting in a less dense mass than that formed by unheated homogenised or raw milk during digestion (Gallier et al., 2013a; Ye et al., 2017) (Figure 2.7).

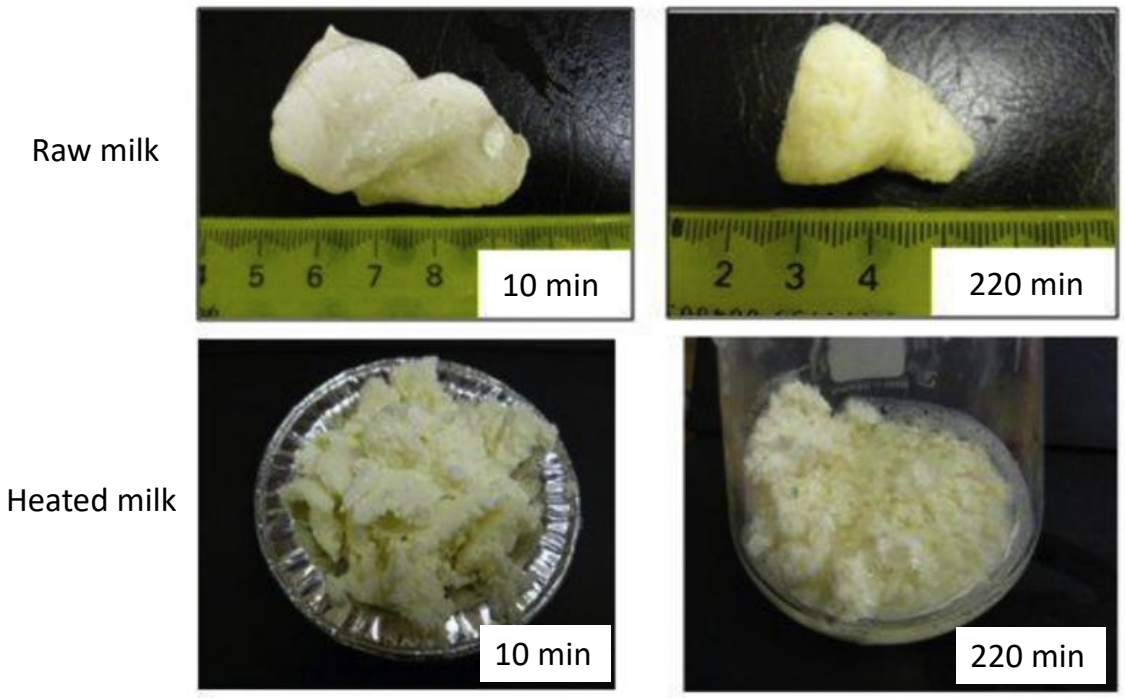

Figure 2.6: Curd formed during *in vitro* gastric digestion of raw and heated (90 ℃) skim milk at two digestion times (Ye et al., 2016b).
Images reproduced with permission from Elsevier for inclusion into the thesis only.

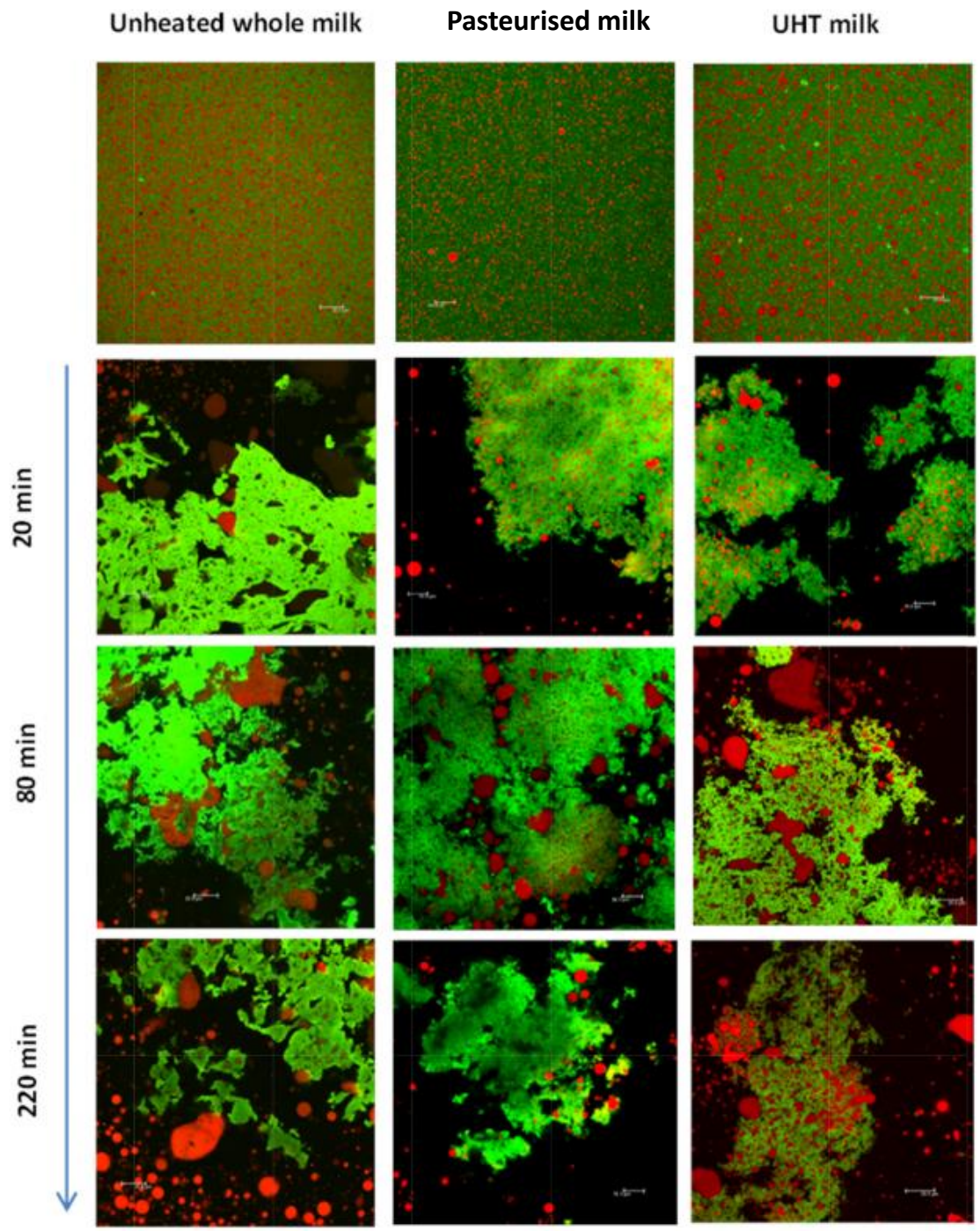

Figure 2.7: Confocal microscopy images of gastric curd formed from unheated homogenised milk, pasteurised and homogenised milk (72 °C for 15 s), and UHT homogenised milk (140 °C for 4 s) during digestion in the rat (Ye et al., 2019). Protein networks are stained green, whereas fat globules are stained red. For all images, scale bars are 20 µm. Image reproduction permission granted by Elsevier for thesis inclusion only.

Regarding gastric curd disintegration, the more open and porous structure formed after heating and homogenisation increases the protein network surface area, creating easier access for pepsin, resulting in a greater rate of casein proteolysis under simulated gastric digestion (Figure 2.8) (Mulet-Cabero et al., 2019; Ye et al., 2017). In addition, the increased rate of proteolysis appeared to be responsible for increased fat globule release from the curd (Ye et al., 2017).

In vivo rat studies have also investigated the influence of milk processing on gastric curd formation and hydrolysis, and the results from these studies supported the observations using *in vitro* digestion methods, especially for intense heat treatment (i.e., 140 ℃ for 4 s) (Miranda & Pelissier, 1983; Wada & Lönnerdal, 2014; Ye et al., 2019).

Although gastric curd structure and disintegration play a significant role in determining the outcomes of gastric digestion, processing-induced changes to whey protein structures also result in increased gastric hydrolysis. Using extracted porcine gastric enzymes, Kitabatake and Kinekawa (1998) showed that the heat-induced denaturation of whey protein renders it more susceptible to enzymatic hydrolysis. Specifically, heat treatment has been shown to change the naturally pepsin-resistant β-lactoglobulin into a more digestible form by exposing enzymatic cleavage sites (Guo et al., 1995). Barbé et al. (2013) postulated that heat-induced whey protein denaturation and subsequently greater gastric hydrolysis were responsible for the greater level of hydrolysed β-lactoglobulin observed in the duodenum of cannulated pigs fed heated milk, compared to unheated milk.

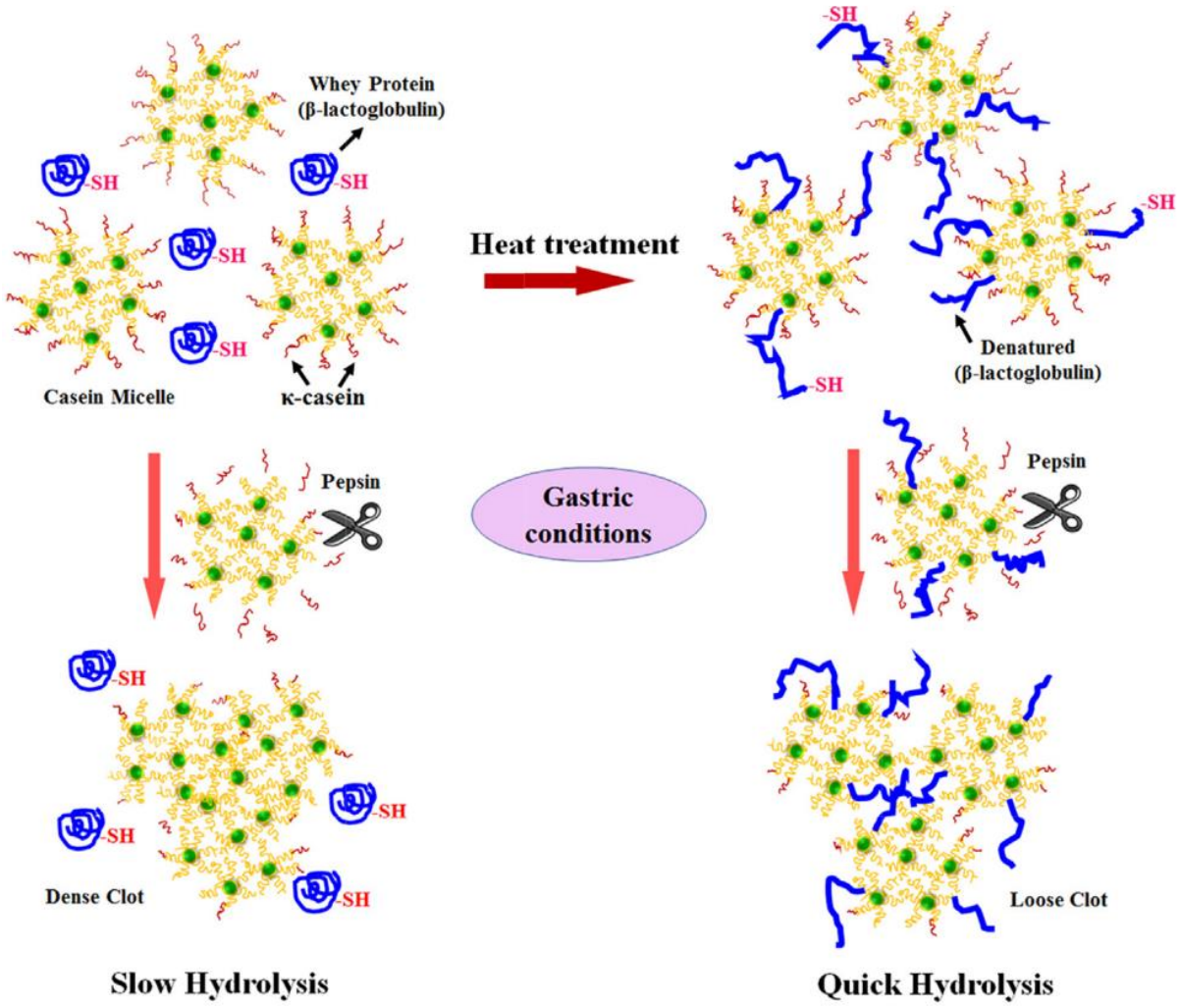

Figure 2.8: A theoretical schematic describing the gastric curd formation of raw and UHT milk and subsequent effects on protein hydrolysis (Ye et al., 2019).
Casein micelles are depicted in yellow with green centres, with κ-casein tails (red). The whey protein β-lactoglobulin (blue), with protruding sulfhydryl (SH) groups, is shown both in its native form in unheated milk or denatured (unfolded) in UHT milk. Image reproduction permission granted by Elsevier for thesis inclusion only.

Further, in heated, homogenised milk, the adsorption of milk protein to the restructured milk fat globule, combined with some heat-induced unfolding of the protein near the interface layer, has been suggested to further increase the susceptibility of the milk protein to pepsin hydrolysis *in vitro* (MacIerzanka et al., 2012; Sarkar et al., 2009). In terms of milk lipid, it is understood that homogenisation may increase the digestion of milk lipid by destroying the MFGM, allowing for an increased rate of hydrolysis by gastric lipase (Gallier et al., 2013a; Gallier et al., 2012; Lee & Sherbon, 2002).

Despite the processing-induced differences in gastric curd disintegration and hydrolysis, the gastric emptying of processed bovine milk protein and lipid is rarely reported. An *in vivo* pig study by Meisel and Hagemeister (1984) reported that heat treatment and homogenisation increased the release of dry matter from the stomach, although curd disintegration was not reported. While this result suggests that processing could alter gastric emptying of protein and/or lipid, this study used one mini-pig, and thus could be considered observational. In another study, Scanff et al. (1991) used two cannulated pre-ruminant calves as a monogastric model to investigate the gastric release of protein and AA from raw and pasteurised milk into the small intestine over a 12 h period. Despite similar gastric emptying of casein and whey protein of pasteurised milk and raw milk, the authors reported that the AA profile of the digesta at the beginning of the duodenum was altered by pasteurisation, suggesting processing could result in differences in the gastric release of AA. However, these results should be interpreted cautiously due to the small sample size and the large variability observed in that study. Further, despite the monogastric properties of the pre-ruminant calf, various physiological and enzymatic characteristics of the bovine gastrointestinal tract vary from that of the human (i.e.,

enzymatic profile, gastric volume, peristaltic movements, gastrointestinal tract orientation) (Huber, 1969).

Despite the processing-induced differences in gastric curd disintegration and hydrolysis, no reliable studies have investigated the gastric emptying of protein or lipid from heat treated and/or homogenised milk into the small intestine. Further, it could be expected that the reported processing-induced changes to gastric milk coagulation, combined with differences in gastric protein hydrolysis, could affect the gastric emptying rate of digested protein and AA from the gastric solid (curd) and liquid fractions. However, the gastric release of digested protein and AA from heat treated and homogenised milk has not yet been investigated.**Small intestinal absorption of amino acids from bovine milk and appearance in peripheral blood**

Of the milk protein, only native β-lactoglobulin enters the small intestine in a relatively intact form due to its resistance to pepsin hydrolysis. However, nasogastric intubation identified more peptides of β-lactoglobulin origin than of α-lactoglobulin origin in the jejunal digesta of humans who consumed casein and whey protein (Boutrou et al., 2013). This finding suggests that β-lactoglobulin is quickly hydrolysed once it reaches the small intestine. Further, protein profiles of the jejunal digesta of pigs fed bovine milk suggested that β-lactoglobulin was completely hydrolysed during duodenal transit (Barbé et al., 2013). Thus, the pepsin resistance characteristic of native β-lactoglobulin does not hinder small intestinal digestion and absorption.

Existing research on the small intestinal absorption and circulatory appearance of AA from milk protein has primarily focussed on isolated protein fractions. Regarding absorption, the casein and whey fractions are known as ‘slow’ and ‘fast’ proteins, respectively. These terms were proposed by Boirie et al. (1997) in analogy to carbohydrate metabolism terminology and reflect the rate at which AA from each fraction appears in the peripheral blood circulation of humans (Figure 2.9). For example, whey protein ingestion results in a sharp increase in peripheral blood AA concentration (Lacroix et al., 2006). It has been proposed that the rapid postprandial appearance of AA after whey protein consumption could result from the rapid gastric release of the soluble major whey protein β-lactoglobulin (Hall et al., 2003), which may have subsequently increased the rate of digestion and absorption (Barbé et al., 2013; He & Giuseppin, 2014).

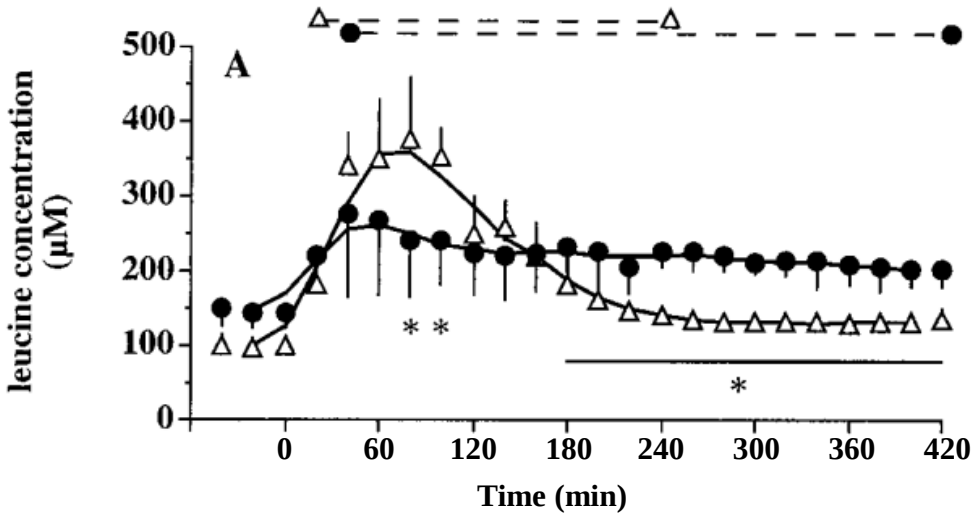

Figure 2.9: Postprandial concentration of leucine in the peripheral blood of humans after consumption of casein (●) or whey (Δ) protein meals (Boirie et al., 1997).
An asterisk (*) indicates differences ($P \leq 0.05$) between the two protein meals. The dashed lines at the top of each panel indicate differences ($P \leq 0.05$) from baseline leucine levels. Image reproduced under the PNAS License to Publish, copyright 1997 National Academy of Sciences.

Contrary to the rapid peak in circulatory AA levels seen as a response to whey protein intake, casein intake results in a lower maximum AA concentration, with elevated peripheral blood AA concentration sustained over a longer period (Dangin et al., 2001; Lacroix et al., 2006). Overall, based on the indirect measures of peripheral blood AA concentrations, it has been proposed that the different gastric emptying rates of casein and whey fractions in milk affect the rate of protein digestion and AA absorption in the small intestine. However, to date, there is no study investigating the link between whole milk gastric protein emptying, small intestinal AA absorption, and circulatory AA appearance in humans.

Based on an *in vitro* study which investigated gastric curd composition and breakdown, Ye et al. (2016a) postulated that the release of milk lipid into the small intestine for lipolysis is dependent on the disintegration behaviour of the curd protein network. Once in the small intestinal luminal environment, milk fat globules are rapidly hydrolysed. However, the composition-related surface properties of the MFGM determine the rate of lipolysis by pancreatic enzymes (Gallier & Singh, 2012; Ye et al., 2011).

When hydrolysed, milk fat globules release polar and non-polar lipids which are absorbed by small intestinal enterocytes and transported into the portal vein and lymphatic system (Dixon, 2010; Iqbal & Hussain, 2009; Michalski, 2009). However, milk fat globules resist lipolysis in the small intestine unless they are first exposed to gastric lipase (Gallier et al., 2012; Gallier et al., 2013b). This is due to the need to accumulate FA at the surface of fat globules to accommodate the binding of pancreatic lipase (Gallier et al., 2013a).

2.4.5. The influence of heat and homogenisation on small intestinal absorption of amino acids from bovine milk and appearance in peripheral blood

Based on studies using other protein sources, it is possible that processing-induced differences in the gastric release of total protein, digested protein, and AA could have implications for the small intestinal absorption of AA and FA. Studies in pigs have highlighted the modulatory role of the gastric release of total nitrogen on small intestinal absorption of AA (Gaudichon et al., 1994; Montoya et al., 2018). Gaudichon et al. (1994) showed that the gastric emptying rate of total nitrogen in whole milk was strongly positively correlated to the absorption rate of total N in the small intestine. More recently, Montoya et al. (2018) demonstrated in pigs fed beef protein that both the rate and the amount of digested protein entering the small intestine can influence AA absorption. In addition, the extent of hydrolysis of protein entering the small intestine has been shown to influence the location of absorption within the small intestine (Montoya et al., 2018), as well as the time needed for their absorption, as a result of the time required for intestinal hydrolysis (Koopman et al., 2009). Thus, it is expected that processing could modulate the small intestinal AA absorption kinetics of milk protein due to differences in gastric curd formation and hydrolysis and the possible subsequent effect on the gastric emptying kinetics of digested protein and AA.

While no studies to date have determined the effect of heat treatment and homogenisation on AA absorption in the small intestine, studies investigating the postprandial peripheral plasma appearance of AA from heated and homogenised milk have suggested relationships between curd formation, AA absorption and plasma AA appearance. A link between gastric

curd characteristics and AA absorption has been suggested by Kaufmann (1984), who found a softer gastric curd and higher blood AA concentration in mini-pigs fed UHT compared to those fed pasteurised milk. The gastric emptying rate was not reported in that study. This finding was later supported by another study by Barbé et al. (2013), in which mini-pigs consumed unheated and heated milk. They found that the stiffer curd formed by unheated skimmed milk delayed gastric emptying in mini-pigs and suggested a link to lower AA concentration in the small intestinal digesta. Lacroix et al. (2008) suggested that a greater increase in postprandial AA appearance in the circulatory system of humans after consuming bovine UHT milk, compared to pasteurised milk was due to the effect of heat treatment on small intestinal absorption. In contrast, a recent study in older adults found no difference between the postprandial peripheral EAA appearance after UHT or pasteurised milk consumption. The same study reported that removal of the milk lipid fraction (skimmed vs whole milk) slowed the appearance of AA (Horstman et al., 2021). However, this study used a direct steam process to UHT treat the milk, which is a milder UHT treatment than the typically used indirect heating method. Direct steam heating results in a lower level of whey protein denaturation compared to indirectly heated UHT milk (Lyster et al., 1971) and thus could be expected to form a firmer curd. This effect has been demonstrated in rennet-coagulated UHT milk, which was processed under varying conditions (Perkin et al., 1973). The firmer curd could be expected to result in slower gastric protein emptying characteristics, which, based on the links suggested by Barbé et al. (2013) and Kaufmann (1984), may slow small intestinal AA absorption and could partially explain the result observed by Horstman et al. (2021).

It is important to note that AA concentrations in the circulatory system are influenced by protein metabolism in the gastrointestinal epithelium and the liver and may not accurately represent postprandial dietary AA absorption. Thus, while published evidence using postprandial circulatory AA appearance suggests that heat-induced changes to milk coagulation behaviour could influence the absorption of milk AA, the impact of heat treatment and homogenisation on AA absorption in the small intestine remains to be investigated.

As evidenced above, the milk matrix is a complex system containing a heterogeneous mix of components with differing structures. However, some studies discussed above have reported the postprandial gastric digestion and peripheral blood AA appearance when human volunteers consumed extracted casein and whey protein as individual ingredients (Boirie et al., 1997; Boulier et al., 2023; Farup et al., 2016; Hall et al., 2003; Koopman et al., 2009; Trommelen et al., 2020). Thus, while studies using milk ingredients make an important contribution to the fundamental understanding of the behaviour of these milk proteins in the human gastrointestinal tract, it is important to note that, due to the role of the whole milk matrix in nutrient interactions during digestion, care must be taken when translating these results to the protein consumed in whole milk.

2.4.6. Large intestinal fermentation

The gastric digestion of milk protein and lipid and the small intestinal absorption of AA and FA are important for the nutritional value of milk, as the absorption of dietary nutrients can be considered complete at the end of the small intestine (Moughan, 2003; Stein et al., 2007). Nutrients which remain unabsorbed at the end of the terminal ileum transit into the

large intestine, where they are partially utilised by the resident microbiota as substrates for metabolism (Wong et al., 2012). The consumption of whole milk, milk protein, and lipid has been shown to alter the composition of the caecal microbiota in the rat model (Bai et al., 2016) and the faecal microbiota in humans (Fernandez-Raudales et al., 2012), which could have implications for the production of nutritionally beneficial fermentation end-products, such as short-chain FA (Ahlborn et al., 2020). Considering that the majority of milk nutrients are mostly digested by the end of the small intestine (Gaudichon et al., 1994), the contribution of the end-products of microbial fermentation to the nutritional outcomes of milk consumption has been suggested in a review to be minimal (Horstman & Huppertz, 2022).

2.5. Concluding remarks

In summary, the impact of processing on bovine milk protein and lipid structures and gastric digestion has been researched using *in vitro* digestion models. However, whether the reported differences in gastric milk coagulation, curd structure and protein hydrolysis persist *in vivo* remains to be determined. Further, in the context of processed bovine milk, there is limited knowledge on the impact of an altered gastric curd structure on emptying digested protein, lipid, FA, and AA into the small intestine. Finally, studies conducted in humans have shown that processing influences the appearance of AA in peripheral blood, and some authors have proposed that this result is due to the modulation of small intestinal AA absorption as a result of processing. However, there is currently no research investigating the small intestinal absorption of AA from processed bovine milk. As a result, the small intestinal absorption of AA from processed bovine milk and their subsequent transport to the circulatory systems are poorly understood.

Research bridging the gap between published evidence of processing-induced structural changes to bovine milk protein and lipid and the impacts of these changes on gastric digestion and small intestinal absorption is essential to advance our understanding of milk's nutritional value and the potential impact of processing on human health.

2.6. Research questions

It is evident from the literature review that various aspects of the digestion of processed bovine milk and resulting nutrient absorption are yet to be elucidated. Based on the knowledge gaps outlined in the literature review, the influence of heat and homogenisation on both the protein and lipid digestion and AA and FA absorption of bovine milk warrants investigation.

However, due to the limited timeframe of the doctoral research and the extensive research on the effects of heat and homogenisation on native milk protein structures, milk protein and AA were selected as the focus of this PhD dissertation. Thus, the overall aim of the research presented in this PhD dissertation was to determine the influence of processing on the digestion of milk protein in the stomach and AA absorption in the small intestine, respectively.

To understand the mechanisms by which milk processing influences protein digestion and small intestinal AA absorption, the digestion of processed milk throughout each dynamic location of the gastrointestinal tract must be investigated in detail. When considering this in the context of the current literature, some of the questions that remain to be answered are:

1. How do processing-induced changes to native milk protein and lipid structures influence gastric curd structure, and how does this affect the gastric emptying of milk protein?

2. What do processing-induced changes to gastric curd structure and gastric emptying mean for milk protein hydrolysis in the stomach, and does this influence the digested protein and AA released from the stomach?

3. In the context of milk, how do processing-induced differences in the release of digested protein and AA from the stomach alter the kinetics of small intestinal AA absorption?

Based on existing knowledge, the main hypothesis was that the altered gastric curd formation and digestion reported for different milk types could have implications for the gastric release of protein and AA into the small intestine, with subsequent effects on small intestinal AA absorption, which could link to the appearance of AA in blood. It is important to note that in the context of AA, disappearance from the small intestine is assumed to equate to absorption (Holmes et al., 1974). Thus, throughout this thesis the outcomes of AA disappearance are reported as absorption.

2.7. Dissertation structure

To test the hypothesis and answer the research questions posed by the literature review, data from two pig studies were used to determine how gastric curd formation and emptying modulate the small intestinal AA absorption of milk in a human model. The research approach described in this section is visually summarised in Figure 2.10.

This thesis research began by exploring the effect of gastric milk coagulation on the apparent small intestinal absorption of AA from raw milk in an *in vivo* piglet model. The gastric curd formation and emptying of protein from raw bovine, caprine, and ovine milk have already been thoroughly characterised in the piglet as an infant model (Roy et al., 2022). Therefore, as an initial study, Chapter 3 aimed to build on previous research by investigating whether the consumption of milk from different species (bovine, caprine, and ovine), as milk with known differences in curd formation, would result in detectable differences in small intestinal AA absorption at a single time point. For this study, raw milk was used to remove the potentially confounding effect of milk processing and to provide a link to past studies. In addition, the piglet, rather than the pig, was used as a human model to align the results with published results.

As this PhD research aimed to understand the effects of processing on milk digestion and nutrient absorption, the next step was to introduce various milk processing techniques. Ideally, the influence of processing on the digestion of bovine, caprine and ovine milk and resulting AA absorption would be simultaneously investigated *in vivo*. However, the combination of species (3), diet (5), and sampling time point (6) would result in a complex, larger-scale study, which would be difficult to complete. In addition, the doctoral research timeframe restricted the scope of the research to milk from one species only. As the effects of heating and homogenisation on the nutrient structures of whole bovine milk have been well characterised, bovine milk was selected as a milk model.

Chapters 4 – 6 comprise data from a study conducted in nine-week-old growing pigs as an adult human model. This study followed the gastric digestion and small intestinal absorption of processed bovine milk over time, which allowed for the determination of the

kinetics of various aspects of digestion and absorption. Based on the availability to the general population, the selected processing techniques were heating (pasteurisation and UHT treatment) and homogenisation. Raw milk was also included as a comparator.

To begin the processed bovine milk protein digestion section of the research, Chapter 4 investigated the gastric curd formation and kinetics of macronutrient emptying of each bovine milk type. The impact of heat treatment and homogenisation on curd strength, curd and liquid macro- and micro-structure, gastric pH, and the gastric emptying of dry matter, total protein, and total lipid was determined in the growing pig.

Chapter 5 determined the effect of heat treatment and homogenisation of bovine milk on the kinetics of apparent gastric degree of protein hydrolysis, the gastric disappearance of individual proteins, and the release of digested protein and AA into the small intestine of the growing pig.

In Chapter 6, the influence of heat treatment and homogenisation on the kinetics of AA absorption of bovine milk was investigated in the growing pig. The true small intestinal AA absorption of the differently processed milk types was determined. The link between gastric emptying and small intestinal AA absorption was also investigated. As a final physiological outcome, the postprandial plasma concentration of AA in the portal vein over time and in the peripheral blood of pigs fed each milk type was compared.

Chapter 7 provided a general discussion of the thesis research findings by considering the results from each chapter in the context of the existing literature. The limitations of the research were discussed, and suggestions for future research based on the overall conclusion of the thesis research were made.

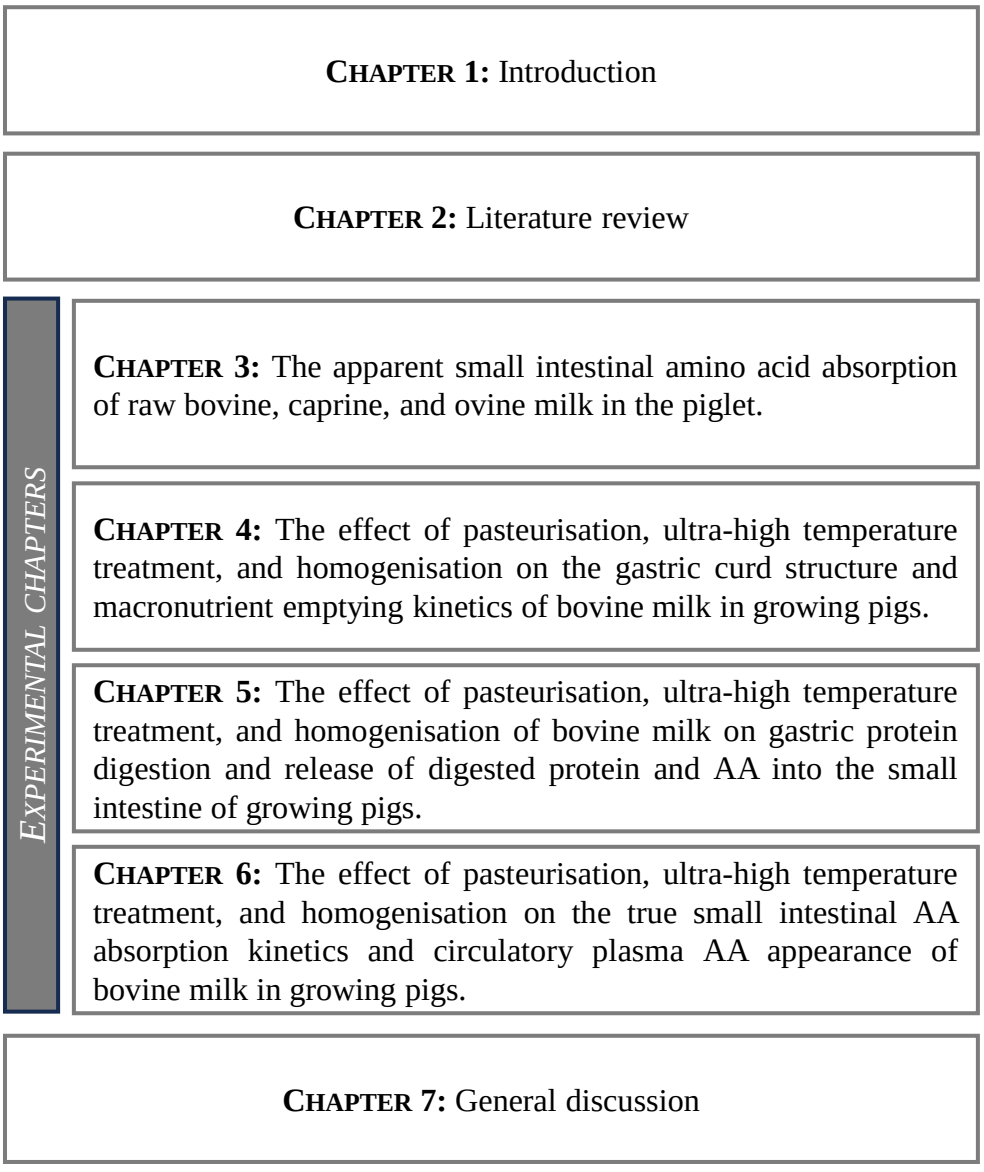

Figure 2.10: A visual summary of the thesis structure and content.

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CHAPTER 3.

DIFFERENCES IN SMALL INTESTINAL APPARENT AMINO ACID ABSORPTION OF RAW SHEEP, COW, AND GOAT MILK ARE EXPLAINED BY GASTRIC AMINO ACID RETENTION IN PIGLETS AS AN INFANT MODEL

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HIGHLIGHTS

- On average, 35% of the dietary amino acids were taken up in the small intestine in the first 210 min post-prandially.
- Two-thirds of the apparent amino acid absorption occurred in the first quarter of the small intestine.
- The apparent absorption of amino acids from ovine milk was, on average, 29% higher than from bovine milk.
- When apparent absorption was corrected for gastric amino acid retention, most differences between piglets fed different milk types disappeared.

Approximately 70% of the work contained in this chapter was contributed by the student.

3.1. Abstract

The rate of gastric emptying of milk from different ruminant species differs, suggesting that the small intestinal absorption of nutrients could also differ across these milk types. However, the influence of milk from different species on the small intestinal amino acid (AA) absorption of milk has not yet been investigated. Thus, this study aimed to determine the small intestinal AA absorption of raw bovine, caprine, and ovine milk in the piglet as an animal model for the infant. Seven-day-old piglets (n = 12) consumed either bovine, caprine, or ovine milk diets for 15 days (n=4 piglets/milk). On day 15, fasted piglets received a single meal of fresh raw milk normalised for protein content and containing the indigestible marker titanium dioxide. The entire gastrointestinal tract contents were collected at 210 min postprandially. Apparent AA absorption (disappearance) in different regions of the small intestine was determined. On average, 35% of the dietary AA were apparently taken up in the small intestine during the first 210 min post-feeding, with 67% of the AA absorption occurring in the first quarter ($P \leq 0.05$) and 33% in the subsequent two quarters. Overall, except for isoleucine, valine, phenylalanine, and tyrosine, the small intestinal apparent absorption of all AA at 210 min postprandially in piglets fed ovine milk was, on average, 29% higher ($P \leq 0.05$) than for those fed bovine milk. Except for lysine, there was no difference in the apparent absorption ($P > 0.05$) of any AA between piglets fed caprine milk or ovine milk. The apparent absorption of alanine was higher ($P \leq 0.05$) in piglets fed caprine milk than those fed bovine milk. When apparent absorption was corrected for gastric AA retention, only small significant differences in the small intestinal apparent absorption of AA were observed across milk types. In conclusion, apparent small

intestinal AA absorption at 210 min postprandially was different for piglets fed bovine, caprine, and ovine milk. When corrected for gastric AA retention, the differences in apparent absorption across species largely disappeared. The apparent AA absorption differed across small intestinal locations.

3.2. Introduction

Globally, bovine milk provides an affordable and accessible source of nutrition. In regions where bovine milk is not readily available, cultural preferences prevail, or self-sufficiency is required, the consumption of non-bovine milk such as ovine, caprine, and camel milk is common (Hilali et al., 2011). In the past decade, Western countries have seen a rise in the consumption of non-bovine milk (Tsakalidou & Papadimitriou, 2016). This trend has been driven by increased demand for speciality foods (Hilali et al., 2011), recognition of the variation in nutrient composition and nutritional benefits (Claeys et al., 2014; Crowley et al., 2017; Verduci et al., 2019), and a perceived (anecdotal) reduction in digestive discomfort after consumption of non-bovine milk when compared with bovine milk (Haenlein, 2001; Jandal, 1996). The compositional differences of non-bovine milk have been of interest in developing infant formulas for specialised infant nutrition as an alternative to widely available bovine milk-based infant formulas (Crowley et al., 2017). Despite this increasing interest in non-bovine milk, there is relatively little knowledge on the digestion of protein and absorption of amino acids (AA) from non-bovine milk, particularly in human infants.

The nutrient composition of bovine, caprine, and ovine milk varies (Park et al., 2007). For example, the protein content of ovine milk is higher than that of caprine and bovine milk. The major milk protein fractions, casein and whey, are also in different proportions in bovine, caprine, and ovine milk (Raynal-Ljutovac et al., 2007). Thus, the AA profiles of each milk type vary (Claeys et al., 2014; Jandal, 1996).

Previous studies have described the impact of species on the gastric digestion of ruminant milk. For example, the solid phase ‘curd’ formed during *in vitro* gastric digestion of raw

ovine and caprine milk has a different protein structure (Li et al., 2022), and the curd is also softer (Roy et al., 2021; Tagliazucchi et al., 2018) than in raw bovine milk. The softer curd formed by the raw caprine and ovine milk results in a faster gastric emptying of protein from the piglet stomach (Roy et al., 2022). These results for raw milk are consistent with *in vitro* results observed for caprine and bovine milk-based infant formulas (Hodgkinson et al., 2018; Maathuis et al., 2017).

During the gastric digestion of milk, a continuous flow of whey proteins, partially digested casein components (peptides), and AA is expected to enter the small intestine, where the proteins are further digested, and the AA are absorbed. Montoya et al. (2018) showed that the digestion and absorption of AA in both the proximal and medial small intestines were positively correlated to the extent of gastric emptying. Similarly, Gaudichon et al. (1994) showed that in mini-pigs fed either yoghurt or milk, the kinetics of dietary nitrogen absorption were controlled by the kinetics of dietary nitrogen flow into the small intestine.

Thus, based on the softer curd structure and faster protein emptying rate of ovine milk and caprine milk compared to bovine milk (Roy et al., 2022), it is hypothesised that there are differences in the amounts of AA taken up throughout the small intestine in piglets consuming caprine and ovine milk, compared to those consuming bovine milk. However, the absorption of AA from raw whole bovine, caprine, and ovine milk from the small intestine has not yet been reported.

This study aimed to determine the apparent absorption of AA from bovine, caprine, and ovine milk in different small intestinal regions of piglets at 210 min postprandially. This time point was selected to align with a similar accompanying study where piglets were fed

the same milk types, and different gastric digestion and emptying parameters were analysed (Roy et al., 2022). Absorption refers to the disappearance of an AA from the digestive tract and is assumed to equate with the uptake of the AA (Holmes et al., 1974). The piglet is a common animal model for the human digestion of milk and infant formula, based on digestive and absorptive similarities from the mouth to the terminal ileum of both species (Guilloteau et al., 2010; Moughan et al., 1992; Odle et al., 2014; Rowan et al., 1994). Because of their significance for various aspects of human health (Shimomura & Kitaura, 2018; Volpi et al., 2003; Wu, 2009), the absorption of physiologically relevant AA groups (essential AA (EAA), branched-chain AA (BCAA), non-essential AA (NEAA), and long neutral AA (LNAA)) was also quantified.

3.3. Materials and methods

3.3.1. Animals, housing, and dietary treatments

This study was approved by the Massey University Animal Ethics Committee (protocol no. MUAEC 18/97). Locally sourced Large White × Landrace entire male piglets (n = 12, 7 days of age, weight 5.17 ± 0.16 kg, mean ± SEM) were selected from seven different sows, and were housed in individual plastic metabolism crates at the Animal Production Unit of Massey University, Palmerston North. The room was temperature-controlled (28 ± 2 °C) and operated under a 16 h:8 h light:dark cycle. There was daily socialisation for 1 h, and toys were provided to enrich the experimental conditions for the animals. After arrival, the piglets underwent a 12-day acclimatisation period to adapt to bottle-feeding (suckling from a rubber teat), feeding frequency, and feed volume. The piglets received reconstituted spray-dried bovine, caprine, or ovine milk during the adaptation period. On day 13, the piglets received the experimental diets (Figure 3.1). The experimental diets were fresh raw

whole bovine milk (Massey University Dairy Farm No. 4, Palmerston North, New Zealand), fresh raw caprine milk (days 13 and 14: Phoenix Goats, Palmerston North, New Zealand; day 15: Dairy Goat Co-op, Hamilton, New Zealand), and fresh raw ovine milk (Neer Enterprises Ltd, Carterton, New Zealand). Further detail is provided by Roy et al. (2022). Raw milk was not provided from the arrival of the animals as sufficient caprine and ovine milk were not available for the entire study.

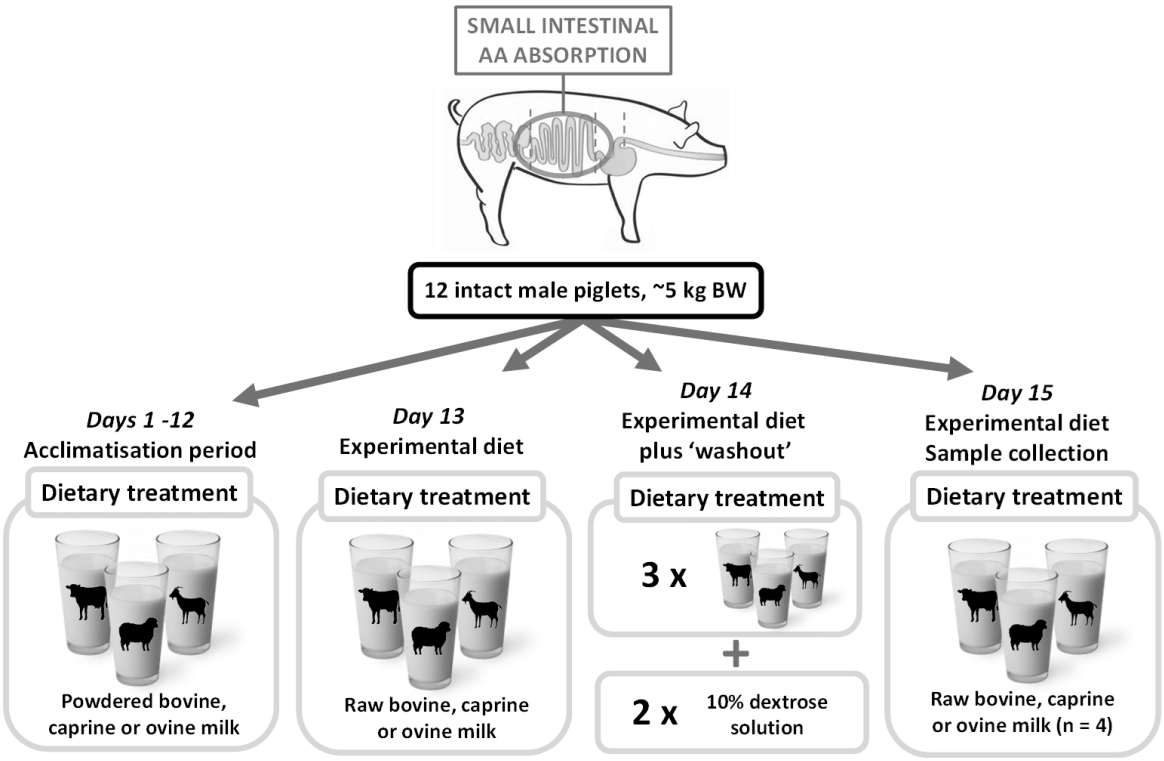

Figure 3.1. Pictorial representation of the piglet study.
On arrival, piglets were randomly allocated to either a bovine, caprine, or ovine milk group. From days 1 – 12, piglets were acclimatised to the housing situation, feed frequency, volume, and bottle-feeding method. During this period, piglets consumed reconstituted powdered bovine, caprine, or ovine milk. From day 13, five feeds of the experimental diet (raw bovine, caprine, or ovine milk) were consumed daily. On day 14 (the day prior to sampling), piglets received three meals of the experimental diets, and the final two meals were a 10% dextrose solution as a milk nutrient 'washout' period. On day 15, piglets consumed one morning milk meal and were euthanised at 210 minutes post-feeding. The contents of the entire gastrointestinal tract were collected in sections. AA, amino acid; BW, bodyweight.

3.3.2. Experimental design

From days 13 – 15, the piglets consumed the experimental diets in 5 daily meals at 3.5-hour intervals. The volume offered to each animal in each meal was calculated to provide 2 g of protein per kg of body weight (BW) (Table 3.1). On day 14, piglets received three fresh whole milk meals, followed by two meals consisting of a 10% dextrose solution (Figure 3.1) to provide an 18 h washout for any residual milk components to leave the gastrointestinal tract prior to the sampling day meal (Roy et al., 2022). On day 15, fasted piglets (21 – 22 days old) were bottle-fed one fresh milk meal, which contained 5.1 – 5.8 mg suspended titanium dioxide (TiO_2)/g dry matter (DM) and 0.34 – 0.55 g polyethylene glycol/g DM as indigestible markers to measure the meal flow throughout the gastrointestinal tract (Nixon & Mawer, 1970; Rutherfurd et al., 2012). The TiO_2 and the polyethylene glycol were included to allow for measurement of meal flow in the solid and liquid phases, respectively. All piglets consumed their meal in 2 to 3 min and were euthanised 210 min post-feeding. Each piglet was anaesthetised 15 min before its euthanasia time with Zoletil 100 (zolazepam and tiletamine, both 50 mg/mL; Zoetis Inc., Parsippany-Troy Hills, NJ, US) reconstituted with 2.5 mL Ketamine and 2.5 mL Xylazine, both 100 mg/mL (Phoenix Pharm NZ). The final solution contained 50 mg/mL of each drug and was administered at a dosage of 0.03 - 0.04 mL of the mixed solution/kg BW by intramuscular injection in the neck. Following sedation, each piglet was intravenously administered a second dose of the cocktail (30 μL/kg BW) to induce deep anaesthesia. Once anaesthetised, the piglets were euthanised by an intra-cardiac injection of sodium pentobarbitone (0.3 mL of Pentobarb 300/kg BW, Provet NZ Pty Ltd).

Table 3.1. Milk, energy and nutrient allowances and amino acid composition of the fresh raw whole bovine, caprine, or ovine milk provided to piglets in each experimental meal during the last three experimental days[1,2].

	Bovine	Caprine	Ovine
		g/kg bodyweight	
Fresh milk (g)	55.3[b]	63.1[a]	31.9[c]
Crude protein	2.0 ± 0.02	2.0 ± 0.04	2.0 ± 0.02
Fat – total	2.2 ± 0.08	2.0 ± 0.06	2.0 ± 0.02
Lactose	2.5 ± 0.03[a]	2.5 ± 0.03[a]	1.3 ± 0.01[b]
Dry matter	7.2 ± 0.06[a]	7.1 ± 0.11[a]	5.6 ± 0.02[b]
Gross energy (kcal/kg BW)	41.9 ± 0.59[a]	38.6 ± 0.45[b]	34.3 ± 0.05[c]
		mg/g protein	
Isoleucine	30.1 ± 0.61[b]	31.3 ± 0.20[b]	37.0 ± 0.40[a]
Leucine	52.4 ± 1.05[c]	56.5 ± 0.25[b]	74.1 ± 0.95[a]
Valine	34.6 ± 0.73[b]	41.6 ± 0.34[a]	42.9 ± 0.39[a]
Total BCAA	*117.0 ± 2.40[c]*	*129.4 ± 0.78[b]*	*154.0 ± 1.73[a]*
Histidine	12.2 ± 0.26[c]	13.2 ± 0.11[b]	18.9 ± 0.25[a]
Lysine	23.8 ± 0.54[b]	25.2 ± 0.14[b]	42.1 ± 0.16[a]
Methionine	12.0 ± 0.17[b]	11.7 ± 0.31[b]	18.2 ± 0.20[a]
Phenylalanine	26.2 ± 0.55[c]	28.7 ± 0.21[b]	34.5 ± 0.55[a]
Threonine	22.0 ± 0.55[c]	27.7 ± 0.29[b]	31.7 ± 0.35[a]
Tryptophan	14.4 ± 0.27	14.8 ± 0.07	15.0 ± 0.16
Total EAA	*227.6 ± 4.58[c]*	*250.7 ± 1.37[b]*	*314.4 ± 2.85[a]*
Alanine	17.0 ± 0.40[b]	16.8 ± 0.14[b]	27.7 ± 0.37[a]
Arginine	17.8 ± 0.40[c]	16.5 ± 0.07[b]	25.6 ± 0.17[a]
Asparagine	36.6 ± 1.42[b]	37.1 ± 0.81[b]	58.3 ± 0.63[a]
Cysteine	6.5 ± 0.14	7.5 ± 0.44	7.1 ± 0.33
Glutamine	127.3 ± 3.34[b]	132.7 ± 1.26[b]	172.4 ± 1.93[a]
Serine	27.9 ± 0.71[b]	27.8 ± 0.23[b]	39.4 ± 0.39[a]
Tyrosine	25.5 ± 0.68[c]	23.0 ± 0.12[b]	35.5 ± 0.17[a]
Total NEAA	*268.0 ± 7.04[b]*	*270.9 ± 2.56[b]*	*383.7 ± 3.04[a]*
Total LNAA	*183.1 ± 3.77[c]*	*195.9 ± 1.04[b]*	*240.9 ± 2.11[a]*

BCAA, branched-chain amino acids; EAA, essential amino acids; NEAA, non-essential amino acids; LNAA, large neutral amino acids.

[1] The milk volumes provided to the piglets were calculated to deliver 2 g of protein per kg of bodyweight.

[2] Values are means ± SEM, n = 3 batches/milk. Means in a row without a common superscript differ ($P \leq 0.05$) in a comparison between milk types.

Following euthanasia, the abdomen was opened. The oesophagus, pylorus, ileal caecal junction, and rectum were clamped. The whole gastrointestinal tract was then dissected. The total gastric contents were collected as previously described by Roy et al. (2022). The small intestine was uncoiled, and one clamp was placed approximately 20 cm before the ileal-caecal junction (terminal ileum). The remaining small intestine was separated into two even sections (proximal and distal small intestine; PSI and DSI, respectively). The whole digesta from each section (PSI, DSI, and terminal ileum) were collected using three flushes of distilled water. The large intestine was also uncoiled, and the digesta of the cecum and colon (proximal and distal) were collected as described by Montoya et al. (2022). The gastric chyme, small intestine, and large intestinal digesta were immediately frozen on dry ice and stored at -20 °C. The samples were then freeze-dried, ground, and sieved (particle size $\leq$ 1 mm). All contents were analysed for TiO_2 content to determine the transit rate of the meal through the gastrointestinal tract. Insufficient sample volume for each gastrointestinal section prevented the analysis of the polyethylene glycol content.

The amounts of AA remaining in the stomach, PSI, DSI, and terminal ileal contents were analysed. The TiO_2 content of each small intestinal location was then used in conjunction with the AA content in each location to determine the apparent AA absorption throughout the small intestine. It was assumed that digesta were equally distributed in each location for PSI and DSI. Thus, it could be expected that dividing the PSI AA content in half results in an AA content representative of the first quarter of the small intestine (i.e., half-way along the PSI, Figure 3.2) at 210 min post-feeding. The AA absorption in the first quarter of the small intestine can thus be calculated. Applying the same assumption to the DSI to calculate the AA content in the first half of the DSI section allows for determination of the

apparent AA absorption in the first three-quarters of the small intestine. The terminal ileal samples represented the apparent absorption over the entire small intestine.

3.3.3. Chemical analyses

During the study, three batches of fresh raw milk were collected and analysed for macronutrient and AA contents. Each raw milk type, gastric solid fraction, gastric liquid fraction, PSI, DSI, terminal ileal, caecal, and colonic contents were analysed for DM (AOAC 990.19 (AOAC, 2006)) and TiO_2 (Short et al., 1996). Each milk type, the gastric solid and liquid fractions, and small intestinal digesta were also analysed for AA content (24 h HCl hydrolysis, *o*-phthaldialdehyde pre-column derivatisation, followed by reverse-phase chromatography (Rutherfurd et al., 2012)). For the milk types, the cysteine and tryptophan contents were analysed by performic acid oxidation and alkaline hydrolysis, respectively.

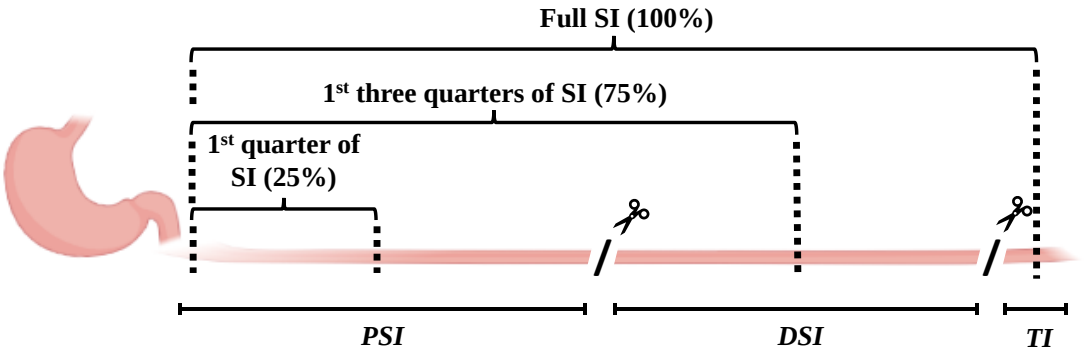

Figure 3.2. Diagram showing the small intestinal sections.
The small intestine was dissected into the proximal and distal small intestine (PSI and DSI), and terminal ileum, and the entire digesta from each section were collected. The amino acid (AA) and titanium oxide (TiO_2) contents of the PSI and DSI digesta were determined, and it was assumed that they represented the values in the middle of each small intestinal location. Thus, the PSI and DSI values are assumed to represent the AA absorption in the first quarter (25%) and the first three-quarters (75%) of the small intestine. The terminal ileal values represent the complete small intestine (100%).

The milk types were analysed in triplicate for the AA content, and the gastrointestinal contents were analysed in duplicate. Sulphur AA and tryptophan were not analysed, as sample volumes were limited. Before the HCl hydrolysis for AA analysis, the gastric samples were defatted using a diethyl ether/petroleum ether extraction (Appendix 1: Figure A1.1).

3.3.4. Calculations

On average, 15, 12, and 13% of the TiO_2 reached the large intestine at 210 min for the bovine, caprine, and ovine milk groups (Table 3.2). However, only three piglets (two fed bovine milk and one fed caprine milk) had enough terminal ileal digesta to analyse both TiO_2 and AA contents. As smaller amounts of digesta are required for AA analysis compared to the TiO_2, only the AA analysis was conducted for the other nine piglets with insufficient terminal ileal sample amounts. The mean TiO_2 content of the terminal ileal digesta of the two piglets fed bovine milk was used to calculate the amount of AA released into the large intestine for the piglets fed bovine milk with small terminal ileal samples. The same calculation was used for piglets fed caprine and ovine milk, except that for ovine milk, the mean of the three piglets with sufficient terminal ileal digesta was used. Thus, AA absorption at the terminal ileum was calculated using mean values across species as detailed below, but the results are presented only as indicative values in the Supplementary material.

Table 3.2. TiO_2 content recovered in the gastrointestinal tract of piglets fed raw whole bovine, caprine, or ovine at 210 min postprandially[1].

	Bovine	Caprine	Ovine
		mg *(% consumed)*	
Consumed	113.8 ± 13.4 (100%)	114.3 ± 14.8 (100%)	122.7 ± 10.5 (100%)
Stomach	54.9 ± 4.8 (49%)	67.0 ± 8.3 (59%)	59.3 ± 12.7 (47%)
Proximal small intestine	11.0 ± 1.0 (10%)	8.0 ± 0.8 (7%)	11.4 ± 1.2 (10%)
Distal small intestine	28.1 ± 4.7 (24%)	24.1 ± 6.9 (21%)	33.1 ± 6.3 (28%)
Terminal ileum[2]	2.3 ± 0.6 (2%)	1.3 ± 0.5 (1%)	2.9 ± 0.8 (2%)
Cecum	1.7 ± 0.3 (2%)	1.5 ± 0.8 (1%)	3.4 ± 1.0 (3%)
Proximal colon	11.5 ± 4.1 (9%)	10.0 ± 2.5 (9%)	11.6 ± 3.2 (10%)
Distal colon	4.1 ± 2.4 (3%)	2.3 ± 1.1 (2%)	1.1 ± 0.3 (1%)
Recovered	113.5 (99.7%)	114.2 (99.9%)	122.8 (100%)

[1] Values are means ± SEM, $n = 4$ piglets. There were no differences ($P > 0.05$) in the amount of TiO_2 recovered from piglets fed each milk type in any location.
[2] The TiO_2 recovered from the terminal ileum of piglets fed each ruminant milk was estimated using the TiO_2 recovered from the terminal ileal digesta of two piglets fed cow milk, and one fed goat milk.

No correction for endogenous AA was made; thus, only apparent absorption values are reported. The amount of TiO_2 in each gastrointestinal location, the amount of AA present prior to the small intestinal location of interest, the amount of AA in the location of interest, and the amount of AA released post the small intestinal location of interest were considered to calculate the apparent AA absorption in each small intestinal location. The amount of TiO_2 that appeared in and was released from each location was calculated as follows (PSI and the first quarter of the small intestine as an example):

$$TiO_2 \text{ content}_{PSI} \text{ (g on DM basis)} = \text{Total content}_{PSI \text{ digesta}} \text{ (g DM)} \times TiO_2 \text{ concentration}_{PSI \text{ digesta}} \text{ (\% DM)} / 100$$

$$TiO_2 \text{ content}_{\text{until PSI}} = TiO_2 \text{ content}_{\text{Stomach}} + TiO_2 \text{ content}_{PSI}$$

$$TiO_2 \text{ content}_{\text{after PSI}} = TiO_2 \text{ content}_{DSI} + TiO_2 \text{ content}_{\text{Terminal ileum}} + TiO_2 \text{ content}_{\text{Large intestine}}$$

The amount of AA present prior (stomach) and in the location of interest (PSI) and released after the location of interest was calculated as follows:

$$\text{AA content}_{\text{Stomach}} \text{ (mg on DM basis)} = \text{AA concentration}_{\text{Stomach}} \text{ (\%)} \times \text{Total content}_{\text{Stomach}} \text{ (g DM)} / 100$$

$$\text{AA content}_{PSI} \text{ (mg on DM basis)} = \text{AA concentration}_{PSI} \text{ (\%)} \times \text{Total content}_{PSI} \text{ (g DM)} / 100$$

$$\text{AA content}_{\text{released after 1st quarter small intestine}} \text{ (mg)} = (\text{AA content}_{PSI} \times TiO_2 \text{ content}_{\text{after PSI}}) / TiO_2 \text{ content}_{PSI \text{ digesta}}$$

The amount of AA present prior and in the location of interest, and released after the location of interest were used to calculate the AA content recovered in each location, followed by the apparent AA absorption (or disappearance), as described by Montoya et al. *(unpublished)* (first quarter of the small intestine as an example):

$$\text{AA unabsorbed}_{\text{1st quarter small intestine}}\ (\text{mg}) = \text{AA content}_{\text{Stomach}} + \text{AA content}_{\text{PSI}} + \text{AA content}_{\text{released after 1st quarter small intestine}}$$

$$\text{Apparent AA absorption (\%)}_{\text{1st quarter}} = (\text{AA content}_{\text{milk}} - \text{AA unabsorbed}_{\text{1st quarter small intestine}})/\text{AA content}_{\text{milk}} \times 100$$

The AA retained in the stomach were subtracted from the dietary AA intake to calculate the apparent absorption of AA available for uptake in the small intestine, as AA uptake only occurs in the small intestine. The calculation of unabsorbed AA described above was adjusted to exclude the AA retained in the stomach, and the apparent absorption of AA available for uptake was calculated as follows:

$$\text{AA content}_{\text{entering small intestine}}\ (\text{mg}) = \text{AA content}_{\text{milk}} - \text{AA content}_{\text{stomach}}$$

$$\text{AA unabsorbed}_{\text{available, 1st quarter small intestine}}\ (\text{mg}) = \text{AA content}_{\text{PSI}} + \text{AA content}_{\text{released after 1st quarter small intestine}}$$

$$\text{Apparent AA absorption (\%)}_{\text{available, 1st quarter small intestine}} = (\text{AA content}_{\text{entering small intestine}} - \text{AA unabsorbed}_{\text{available, 1st quarter small intestine}})/\text{AA content}_{\text{entering small intestine}} \times 100$$

The apparent absorption of physiologically important AA groups (EAA, BCAA, NEAA and LNAA) were calculated using the sum of the AA amounts for each type of AA, followed by the same calculations as the individual AA.

3.3.5. Statistical analyses

The present animal experiment was carried out as part of a larger animal study designed to investigate the gastric curd formation of raw bovine, caprine, and ovine milk as a primary outcome. Based on other studies reporting the kinetic parameters of curd formation in pigs (Gaudichon et al., 1994; Montoya et al., 2018; Tari et al., 2018), four pigs were required to detect a difference in mean values powered over 80% at $P < 0.05$. There were no previous studies reporting apparent small intestinal AA absorption in pigs, which limited the ability to carry out power calculations for this outcome. However, as the main study found differences in curd formation between milk types with a sample size of four pigs (Roy et al., 2018), it was expected that these differences could be translated to differences in apparent small intestinal AA absorption in the small intestine.

For apparent AA absorption, statistical analyses were conducted using the PROC MIXED statement of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA; RRID:SCR_008567). A linear mixed model was used to test the effect of milk species (bovine, caprine, and ovine) and small intestinal location (first quarter, first three-quarters, and whole) and the interaction between milk species and small intestinal location as fixed effects on individual AA absorption and total EAA, BCAA, NEAA and LNAA absorption. The pig was used as a random effect. The most appropriate covariance structure (simple, autoregressive, and unstructured) was selected after fitting the model by the restricted maximum likelihood

method and comparing the models using the log-likelihood ratio test. Once the covariance structure was selected, the interaction term was removed when it was not significant.

The AA composition of milk and the amount of AA present in the stomach at 210 min were compared across milk types using a one-way ANOVA model (PROC ANOVA procedure of SAS). Batches of milk and piglets were used as experimental units. For the residuals of the model, the normal distribution was evaluated using the ODS Graphics, and the homogeneity of variance was evaluated using the repeated statement by fitting models with the restricted maximum likelihood test and comparing them using the log-likelihood ratio test. When the F-value of the analysis of variance was significant ($P \leq 0.05$), least-square means were compared using an adjusted Tukey test.

3.4. Results

The mean TiO_2 recovery across all gastrointestinal sections was 98% (Table 3.2). On average, across all milk types, 52% of the consumed TiO_2 remained in the stomach at 210 min post-feeding. The PSI, DSI, and terminal ileum contained, on average, 9, 24, and 2% of the TiO_2, respectively. A further 13% of the consumed TiO_2 was recovered in the large intestine (caecum, proximal, and distal colon).

Different ($P \leq 0.05$) amounts of AA were observed across raw milk types on a mg AA/g protein basis (Table 3.1). Except for valine, tryptophan, cysteine and proline, ovine milk contained significantly higher ($P \leq 0.05$) amounts of each AA than both bovine and caprine milk. Histidine, leucine, phenylalanine, threonine, arginine, and tyrosine were also present in higher ($P \leq 0.05$) amounts in caprine milk than in bovine milk. There was no difference ($P > 0.05$) in the amount of tryptophan or cysteine between each milk type.

At 210 min post-feeding, a higher ($P \leq 0.05$) ratio (mg retained/mg consumed) of all AA, except for tyrosine, remained in the stomach of piglets fed bovine milk than for those fed ovine milk (Table 3.3). For example, 64% and 35% of the dietary leucine consumed remained in the stomach of piglets fed bovine milk or ovine milk, respectively. In particular, lysine appeared to have remained entirely in the stomach of piglets fed bovine milk at 210 min, whereas piglets fed caprine and ovine milk retained 78% and 47% of lysine, respectively. Except for alanine, arginine, and asparagine, there was no difference ($P > 0.05$) in the gastric retention of AA in piglets fed caprine milk compared to piglets fed bovine milk.

Table 3.3. Gastric retention of amino acids in piglets fed bovine, caprine, or ovine milk at 210 min post-feeding[1].

	Bovine	Caprine	Ovine
	mg retained/mg AA consumed		
Ile	0.61 ± 0.06^{a}	0.42 ± 0.05^{ab}	0.37 ± 0.05^{b}
Leu	0.64 ± 0.06^{a}	0.46 ± 0.05^{ab}	0.35 ± 0.05^{b}
Val	0.66 ± 0.06^{a}	0.47 ± 0.05^{ab}	0.41 ± 0.06^{b}
Total BCAA	0.64 ± 0.06^{a}	0.45 ± 0.05^{ab}	0.37 ± 0.05^{b}
His	0.77 ± 0.07^{a}	0.55 ± 0.06^{ab}	0.36 ± 0.05^{b}
Lys[2]	1.11 ± 0.12^{a}	0.78 ± 0.08^{ab}	0.47 ± 0.07^{b}
Met	0.85 ± 0.08^{a}	0.68 ± 0.08^{ab}	0.44 ± 0.06^{b}
Phe	0.69 ± 0.06^{a}	0.49 ± 0.05^{ab}	0.39 ± 0.05^{b}
Thr	0.64 ± 0.07^{a}	0.47 ± 0.05^{ab}	0.35 ± 0.05^{b}
Total EAA	0.72 ± 0.07^{a}	0.51 ± 0.06^{ab}	0.39 ± 0.05^{b}
Ala	0.60 ± 0.06^{a}	0.37 ± 0.04^{b}	0.30 ± 0.04^{b}
Arg	0.77 ± 0.07^{a}	0.53 ± 0.05^{b}	0.38 ± 0.05^{b}
Asp	0.71 ± 0.07^{a}	0.48 ± 0.05^{b}	0.34 ± 0.05^{b}
Glu	0.70 ± 0.07^{a}	0.52 ± 0.06^{ab}	0.39 ± 0.06^{b}
Ser	0.71 ± 0.07^{a}	0.50 ± 0.06^{ab}	0.37 ± 0.05^{b}
Tyr	0.77 ± 0.07	0.51 ± 0.06	0.59 ± 0.08
Total NEAA	0.71 ± 0.07^{a}	0.49 ± 0.05^{ab}	0.40 ± 0.05^{b}
Total LNAA	0.67 ± 0.06^{a}	0.47 ± 0.05^{ab}	0.42 ± 0.06^{b}

BCAA, branched-chain amino acids; EAA, essential amino acids; NEAA, non-essential amino acids; LNAA, large neutral amino acids.

[1] Values are means ± SEM, n = 4. Means in a row without a common superscript differ ($P \leq 0.05$) in a comparison between milk types.

[2] The lysine retention (greater than one) for piglets fed bovine milk may be due to residual protein retained in the stomach from the pre-fast milk meal.

On average across species, 67% of the total apparent AA absorption during the first 210 min post-feeding occurred in the first quarter of the small intestine (Table 3.4). Excluding lysine, the apparent AA absorption in the first quarter of the small intestine ranged between 15% and 38%. In contrast, only 10% to 15% of the AA absorption in the first three-quarters of the small intestine was accounted for by AA absorption in the second and third quarters of the small intestine. The apparent AA absorption at the terminal ileum was highly variable and did not differ ($P > 0.05$) from the apparent AA absorption determined over the proximal small intestinal regions (Table 3.5).

For all individual AA, both species (milk type) and location had a significant effect on the apparent AA absorption ($P \leq 0.05$; Table 3.4). Except for isoleucine, valine, phenylalanine, and tyrosine, the apparent absorption of other individual AA from piglets fed ovine milk at 210 min was greater ($P \leq 0.05$) than those fed bovine milk. For example, the apparent absorption of histidine was 3.1-fold higher in piglets fed ovine milk when compared to those fed bovine milk. A higher apparent absorption of alanine (+19%) was also observed for piglets fed caprine milk compared to those fed bovine milk ($P < 0.01$). Except for lysine, which had a higher ($P < 0.01$) apparent absorption in piglets fed ovine milk, there was no difference ($P > 0.05$) in the apparent absorption of any AA in piglets fed caprine or ovine milk. The apparent absorption of NEAA was higher ($P \leq 0.05$) in piglets fed ovine milk compared to those fed bovine milk.

Except for isoleucine and tyrosine, the apparent amount absorbed (mg AA disappearing per g protein consumed) of all AA was higher ($P \leq 0.05$) for piglets fed ovine milk than those fed bovine milk at 210 min (Table 3.6). A higher amount ($P < 0.01$) of valine was apparently absorbed by the piglets fed caprine milk compared to piglets fed bovine milk.

Table 3.4. Overall small intestinal apparent absorption of amino acids from raw bovine, caprine, and ovine milk, and absorption in the first quarter (25%) and the first three-quarters (75%) of the small intestine of piglets[1].

	Milk				Location[2] (%)			$P^{3,4}$	
	Bovine	Caprine	Ovine	SEM	25	75	SEM	Milk	Location
					%				
Ile	33.2	45.7	49.1	5.2	37.4	47.9	3.2	NS	***
Leu	30.2^{b}	40.9^{ab}	51.5^{a}	5.1	35.5	46.3	3.2	*	**
Val	28.4	40.5	44.4	5.4	32.3	43.2	3.3	NS	***
BCAA	29.5	41.1	46.2	5.3	35.1	42.8	3.3	NS	**
His	16.2^{b}	29.8^{ab}	49.1^{a}	5.8	25.3	38.0	3.7	*	**
Lys[5]	-20.1^{b}	1.4^{b}	33.4^{a}	8.3	-4.0	13.9	5.2	**	**
Met	9.3^{b}	14.7^{ab}	40.3^{a}	6.8	14.7	28.2	4.3	*	**
Phe	24.2	36.9	45.5	5.5	29.6	41.5	3.5	NS	**
Thr	24.5^{b}	36.7^{ab}	48.1^{a}	5.0	30.3	42.6	3.1	*	***
EAA	20.3	33.4	42.8	5.8	27.8	36.5	3.6	NS	**
Ala	28.2^{b}	47.3^{a}	55.6^{a}	3.9	38.3	49.1	2.5	**	***
Arg	9.9^{b}	25.6^{ab}	41.7^{a}	4.8	18.1	33.4	3.2	**	***
Asp	19.5^{b}	37.9^{ab}	51.6^{a}	5.5	30.6	42.1	3.6	**	***
Glu	24.7^{b}	36.5^{ab}	47.7^{a}	5.8	30.9	41.7	3.6	*	**
Ser	20.5^{b}	33.8^{ab}	46.2^{a}	5.5	27.2	39.8	3.5	*	***
Tyr	16.3	33.8	17.4	7.4	15.4	29.5	4.6	NS	**
NEAA	20.6^{b}	37.9^{ab}	48.0^{a}	5.7	28.9	37.3	3.5	*	**
LNAA	19.2	37.4	32.7	6.4	23.6	32.6	4.0	NS	**

SEM, pooled standard error of the mean; BCAA, branched-chain amino acids; EAA, essential amino acids; NEAA, non-essential amino acids; LNAA, large neutral amino acids; NS, not significant.

[1] Values are means ± SEM, n = 4. Means in a row without a common superscript differ ($P \leq 0.05$) in a comparison between milk types (a, b, c).

[2] Apparent absorption of the terminal ileum is not given as, except for three piglets, the amount of digesta recovered from the terminal ileum was insufficient for both titanium dioxide analysis and amino acid analysis. The terminal ileal absorption values were estimated using averages. The statistical analysis, including the terminal ileum, is shown in Supplementary Table S2.

[3] Significance levels are indicated as follows: * = $P \leq 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

[4] There was no significant ($P > 0.05$) milk x location interaction for any of the analysed amino acids. Thus, the interaction was removed from the final model.

[5] The negative lysine absorption result for piglets fed bovine milk may be due to gastric retention of the pre-fast milk meal protein (Table 3.3).

Table 3.5. Overall small intestinal apparent absorption (%) of dietary amino acids from raw bovine, caprine, and ovine milk, and absorption in the first quarter (25%), first three-quarters (75%), and for the whole small intestine (including terminal ileal material (100%))[1,2].

	Milk				Location (%)				P[4,5]	
	Bovine	Caprine	Ovine	SEM	25	75	100[3]	SEM	Milk	Location
					%					
Ile	33.8	46.9	49.7	5.2	37.4^{y}	47.9^{x}	45.2^{xy}	3.2	NS	***
Leu	29.9^{b}	43.2^{ab}	52.4^{a}	5.2	35.5^{y}	46.3^{x}	43.6^{xy}	3.2	*	***
Val	28.9	41.8	44.9	5.4	32.3^{y}	43.2^{x}	40.2^{xy}	3.4	NS	***
BCAA	29.4	43.6	48.0	5.3	35.1^{y}	42.8^{x}	43.0^{x}	3.2	NS	*
His	17.3^{b}	31.4^{ab}	50.1^{a}	5.9	25.3^{y}	38.0^{x}	35.3^{xy}	3.7	*	***
Lys[6]	-18.6^{c}	3.6^{ab}	35.0^{a}	8.4	-4.0^{y}	13.9^{x}	10.1^{xy}	4.5	**	***
Met	10.4^{b}	16.3^{ab}	41.3^{a}	6.9	14.7^{y}	28.2^{x}	25.2^{xy}	4.4	*	***
Phe	24.9	38.4	46.3	5.6	29.6^{y}	41.5^{x}	38.5^{xy}	3.5	NS	***
Thr	24.2^{b}	38.1^{ab}	48.6^{a}	5.0	30.3^{y}	42.6^{x}	38.0^{xy}	3.2	*	***
EAA	20.2	36.2	44.7	5.8	27.8^{y}	36.5^{x}	36.8^{x}	3.5	*	*
Ala	27.7^{b}	48.2^{a}	56.0^{a}	4.0	38.3^{y}	49.1^{x}	44.6^{xy}	2.5	**	***
Arg	10.0^{b}	27.3^{ab}	42.5^{a}	4.9	18.1^{y}	33.4^{x}	28.4^{x}	3.2	**	***
Asp	19.4^{b}	39.2^{ab}	52.3^{a}	5.5	30.6^{y}	42.1^{x}	38.3^{xy}	3.3	**	***
Glu	25.6^{b}	37.9^{ab}	48.6^{a}	5.8	30.9^{y}	42.0^{x}	39.5^{xy}	3.6	*	***
Ser	20.9^{b}	35.2^{ab}	46.9^{a}	5.5	27.2^{y}	39.8^{x}	35.9^{xy}	3.5	*	***
Tyr	17.4	35.5	18.0	7.4	15.4^{y}	29.5^{x}	25.9^{xy}	4.6	NS	***
NEAA	20.6	37.9	45.3	5.7	28.9^{y}	37.3^{x}	37.5^{x}	3.4	*	**
LNAA	19.2	37.4	32.7	6.4	23.6^{y}	32.6^{x}	33.1^{x}	3.9	NS	*

SEM, standard error of the mean; EAA, essential amino acids; BCAA, branched-chain amino acids; LNAA, long neutral amino acids; NS, not significant.

[1] Values are means ± SEM, n = 4. Means in a row without a common superscript differ in a comparison between milk types (a, b, c) or location (x, y, z).

[2] Except for three piglets, there were insufficient quantities of terminal ileum digesta for both marker and amino acid analysis. Therefore, the terminal ileal absorption values were estimated using averages.

[3] Based on the similarities in AA absorption at three-quarters of the small intestine and the small intestine overall, no further absorption was predicted for any amino acid after the first ¾ of the small intestine.

[4] Significance levels are indicated as follows: * = $P \leq 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

[5] There were no significant ($P > 0.05$) milk x location interactions. Thus, the interaction was removed from the final model.

[6] The negative lysine absorption result for piglets fed cow milk may be due to residual protein retained in the stomach from the pre-fast milk meal (Table 2).

For piglets fed ovine milk, a higher amount ($P < 0.01$) of the EAA histidine, lysine, and methionine, and the NEAA alanine, arginine, and asparagine were apparently absorbed than for piglets fed caprine milk.

As a large portion of the dietary AA content remained in the stomach of all piglets at 210 min, the absorption of AA entering the small intestine (i.e., available for uptake) was considered by correcting the dietary AA intake for AA retained in the stomach and then determining the apparent absorption of AA entering the small intestine. On average, across all raw milk types, the apparent absorption of all AA entering the small intestine was 81% within the first three-quarters of the small intestine (Table 3.7). The first quarter of the small intestine was responsible for around 58% of this apparent absorption. Except for valine and lysine, there was no difference ($P > 0.05$) in the apparent absorption of AA entering the small intestine in piglets fed raw bovine, caprine, and ovine milk. Valine entering the small intestine had a higher ($P \leq 0.05$) apparent absorption in piglets fed caprine milk than those fed ovine milk. The apparent absorption of lysine entering the small intestine varied ($P \leq 0.001$) across milk types (ovine milk > caprine milk > bovine milk).

Table 3.6. Overall amount of amino acids from raw bovine, caprine, and ovine milk apparently absorbed, which disappeared in the first quarter (25%) and the first three-quarters (75%) of the small intestine of piglets[1].

	Milk				Location (%)			$P^{2,3}$	
	Bovine	Caprine	Ovine	SEM	25	75	SEM	Milk	Location
	mg AA/g protein consumed								
Ile	13.8	20.5	22.6	2.3	16.6	21.3	1.4	NS	***
Leu	21.9^{b}	33.2^{ab}	47.5^{a}	4.4	29.7	38.7	2.8	**	**
Val	13.6^{b}	24.2^{a}	23.7^{a}	2.9	17.5	23.5	1.8	*	**
BCAA	47.8^{b}	76.3^{ab}	88.6^{a}	9.8	63.8	78.0	6.1	*	**
His	2.7^{b}	5.6^{b}	11.5^{a}	1.2	5.3	7.9	0.8	**	**
Lys[4]	-6.6^{b}	0.5^{b}	17.5^{a}	3.5	0.2	7.4	2.2	**	***
Met	1.6^{b}	2.5^{b}	9.1^{a}	1.4	3.1	5.6	0.8	**	**
Phe	8.8^{b}	15.2^{ab}	19.6^{a}	2.3	12.1	17.0	1.4	*	**
Thr	7.5^{b}	14.6^{ab}	19.0^{a}	1.9	11.4	16.0	1.2	**	***
EAA	59.9^{b}	113.0^{ab}	159.4^{a}	20.3	95.8	125.7	12.6	*	**
Ala	6.6^{b}	11.4^{b}	19.2^{a}	1.2	10.9	13.9	0.8	***	***
Arg	2.4^{b}	6.1^{b}	13.3^{a}	1.4	5.2	9.3	0.9	***	**
Asp	9.9^{b}	20.1^{b}	37.4^{a}	3.4	19.1	25.9	2.1	***	***
Glu	43.6^{b}	69.4^{ab}	102.3^{a}	11.6	61.1	82.4	7.1	*	**
Ser	7.9^{b}	13.5^{ab}	22.6^{a}	2.5	12.0	17.4	1.5	**	***
Tyr	5.8	11.1	5.2	2.3	5.1	9.6	1.4	NS	**
NEAA	72.2^{b}	128.2^{ab}	187. 7^{a}	23.0	113.4	145.2	14.0	*	**
LNAA	61.6	102.0	110.7	14.6	81.0	101.9	9.0	NS	**

SEM, pooled standard error of the mean; BCAA, branched-chain amino acids; EAA, essential amino acids; NEAA, non-essential amino acids; LNAA, large neutral amino acids; NS, not significant.

[1] Values are means ± SEM, n = 4. Means in a row without a common superscript differ ($P \leq 0.05$) in a comparison between milk types (a, b, c).

[2] Significance levels are indicated as follows: * = $P \leq 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

[3] There was no significant ($P > 0.05$) milk x location interaction for any of the analysed amino acids. Thus, the interaction was removed from the final model.

[4] The negative lysine absorption result for piglets fed bovine milk may be due to residual protein retained in the stomach from the pre-fast milk meal (Table 2).

Table 3.7. Overall small intestinal apparent absorption of available amino acids[1], and absorption in the first quarter (25%) and first three-quarters (75%) of the small intestine of piglets fed raw bovine, caprine, and ovine milk[2].

	Milk			Location (%)		$P^{3,4}$	
	Bovine	Caprine	Ovine	25	75	Milk	Location
			%				
Ile	81.6 ± 2.4	83.0 ± 2.4	77.4 ± 2.4	71.1 ± 3.9	90.2 ± 0.8	NS	***
Leu	80.1 ± 2.7	81.4 ± 2.7	77.5 ± 2.7	69.6 ± 4.6	89.8 ± 0.8	NS	***
Val	77.9 ± 2.6^{ab}	81.3 ± 2.6^{a}	74.0 ± 2.6^{b}	67.0 ± 3.6	88.4 ± 0.7	*	***
BCAA	81.5 ± 4.2	75.9 ± 4.2	72.2 ± 4.2	69.3 ± 3.2	83.7 ± 3.2	NS	**
His	65.2 ± 4.0	73.4 ± 4.0	72.1 ± 4.0	55.6 ± 6.3	84.9 ± 1.1	NS	***
Lys	7.0 ± 6.2^{c}	34.5 ± 6.2^{b}	60.9 ± 6.2^{a}	15.9 ± 5.0	52.4 ± 5.0	***	***
Met	47.1 ± 10.6	54.6 ± 10.6	70.1 ± 10.6	40.7 ± 7.0	73.9 ± 7.0	NS	***
Phe	74.1 ± 3.0	78.2 ± 3.0	72.8 ± 3.0	62.9 ± 5.1	87.2 ± 0.9	NS	***
Thr	66.8 ± 2.7	73.3 ± 2.7	70.7 ± 2.7	58.2 ± 4.3	82.3 ± 1.1	NS	***
EAA	65.9 ± 4.1	76.4 ± 4.1	65.2 ± 4.1	65.3 ± 5.0	81.1 ± 1.4	NS	**
Ala	72.1 ± 3.2	75.7 ± 3.2	78.7 ± 3.2	66.0 ± 2.5	85.0 ± 2.5	NS	***
Arg	41.2 ± 6.7	56.1 ± 6.7	66.9 ± 6.7	37.7 ± 4.9	71.7 ± 4.9	NS	***
Asp	66.2 ± 3.0	74.6 ± 3.4	73.5 ± 3.4	58.4 ± 5.4	84.8 ± 1.2	NS	***
Glu	79.1 ± 2.9	81.4 ± 2.9	76.8 ± 2.9	67.7 ± 5.0	90.4 ± 0.9	NS	***
Ser	66.7 ± 3.3	73.0 ± 3.3	69.5 ± 3.3	56.3 ± 5.4	83.2 ± 1.1	NS	***
Tyr	68.2 ± 8.5	69.7 ± 8.5	43.0 ± 8.5	43.5 ± 5.2	77.1 ± 5.2	NS	***
NEAA	70.5 ± 5.0	70.5 ± 5.0	69.7 ± 5.0	61.5 ± 3.8	79.1 ± 3.8	NS	**
LNAA	79.0 ± 4.7	74.2 ± 4.7	68.2 ± 4.7	65.9 ± 3.5	81.6 ± 3.5	NS	**

BCAA, branched-chain amino acids; EAA, essential amino acids; NEAA non-essential amino acids; LNAA, large neutral amino acids; NS, not significant.

[1] The dietary AA intake was corrected for AA retained in the stomach as only the AA entering the small intestine from the stomach are considered available for uptake from the small intestine.

[2] Values are means ± SEM, n = 4. Means in a row without a common superscript differ ($P \leq 0.05$) in a comparison between milk types (a, b, c).

[3] Significance levels are indicated as follows: * = $P \leq 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

[4] There were no significant ($P > 0.05$) milk x location interactions. Thus, the interaction was removed from the final model.

3.5. Discussion

This study is the first to report the apparent absorption of AA from raw bovine, caprine, and ovine milk throughout the small intestine. As hypothesised, differences in apparent AA absorption at 210 min postprandially were observed between piglets fed the different milk types, with generally greater apparent absorption observed in the first quarter of the small intestine compared to the subsequent 50% of the small intestine. At 210 min post-feeding, the entire milk meal had not yet transited the whole small intestine, so the current values do not represent the apparent extent of AA absorption in the small intestine overall but rather a single time point in the kinetics of apparent AA absorption.

On average, 13% of the TiO_2 was recovered in the large intestine across all animals, which indicates that part of the meal had transited the small intestine. The estimated apparent AA absorption in the small intestine was similar to that in the first three-quarters of the small intestine. However, the apparent AA absorption for the small intestine should be cautiously considered as the amount of digesta collected at the terminal ileum was often too small to accurately measure the AA and TiO_2 concentrations. Calculations based on mean TiO_2 values (across species) rather than the actual individual TiO_2 values could have exaggerated or masked any individual differences in the TiO_2 content in the terminal ileum, which in turn could have affected the apparent AA absorption estimates calculated over the small intestine. The small amount of terminal ileal digesta collected could be explained by the highly digestible nature of milk nutrients (Rutherfurd & Moughan, 2005).

In this study, on average, 73%, 52%, and 39% of the AA consumed by piglets fed raw bovine, caprine, or ovine milk, respectively, remained in the stomach at 210 min post-feeding, while the gastric TiO_2 retention was 49%, 59% and 47%, respectively. A study

using the same animal model and diets as the present study found residual material from the pre-washout milk meal in the stomach of 16 h fasted piglets (Roy et al., 2022). The piglets fed bovine milk retained 25% and 12% more of the protein consumed than those fed caprine and ovine milk, respectively (Roy et al., unpublished). Thus, the high level of inconsistency between the gastric retentions of the TiO_2 and the dietary AA in piglets fed bovine milk could be explained by a larger proportion of the protein from the pre-washout milk meal remaining in the stomach at 210 min.

In the same piglet study discussed previously, Roy et al. (2022) showed that the gastric emptying rate of dietary protein was faster for piglets fed raw caprine and ovine milk than bovine milk (7.1 and 8.2 vs 3.6% dietary protein/min × 10^{-3}, respectively). The gastric emptying of protein was associated with the structure (protein and lipid) and the strength of the gastric curd formed by each milk type. A similar association was observed between curd structure and curd strength with the gastric protein emptying rate in a study of growing pigs fed processed bovine milk (Chapter 4; Ahlborn et al., 2023). Thus, the greater proportion of AA remaining in the stomach of piglets fed bovine milk likely remained entrapped in the denser and firmer gastric curd.

As expected, the AA gastric retention at 210 min (mean across milk types) was inversely correlated ($r = -0.97$) to apparent AA absorption in the first three-quarters of the small intestine. Thus, the greater gastric emptying of caprine and ovine milk proteins compared to bovine milk protein partially explains their higher apparent AA absorption at 210 min. It has been demonstrated in pigs fed beef muscle protein that the amount of digested nitrogen entering the small intestine correlates positively to the apparent AA digestion and absorption in the first half of the small intestine (Montoya et al., 2018).

To better understand the absorption of milk proteins, the AA retained in the stomach were subtracted from those consumed to calculate the apparent absorption of AA that entered the small intestine (AA available for uptake). Based on correlations reported elsewhere (Montoya et al., 2018), the apparent absorption of AA entering the small intestine was used as a proxy for the degree of gastric protein hydrolysis to estimate whether the degree of hydrolysis of the milk proteins entering the small intestine differed across ruminant milk types. Proteins with a greater degree of gastric hydrolysis are expected to have greater apparent AA absorption. The small differences across milk types (species) and the lack of significant interactions between raw milk type and small intestinal location for the apparent absorption of AA available for uptake suggest that the protein of the different milk types entered the small intestine with a similar degree of hydrolysis after transiting the stomach.

Taken together, the differences in gastric AA retention and similarities in apparent absorption of available AA suggest that when the same amount of protein was consumed (2 g/kg BW), the differences observed in apparent AA absorption across piglets fed the different milk types are mainly ascribed to the amounts of AA retained in the stomach. It is important to note that the current results apply to infants and cannot be extrapolated to adult humans as the infant gastrointestinal tract is still comparatively immature (Grand et al., 1976; Henning & Kretchmer, 1973). Further, the current results cannot be extrapolated to consuming the same volume of each ruminant milk type, as the protein content across milk types differs. Further research is warranted to determine the rate of apparent AA absorption across milk types and in adult humans.

The results from the present study, together with those reported by Roy et al. (2022), show that the structural changes of milk in the stomach result in differences in the rate of release

of protein into the small intestine, which in turn affects the apparent absorption of AA. These findings corroborate the suggestions raised in preclinical and clinical human studies, where it is proposed that gastric emptying and small intestinal uptake are related to differences in the appearance of blood plasma AA over time across milk types and dairy products (Barbé et al., 2013; Boirie et al., 1997; Dangin et al., 2001; Milan et al., 2020). It is important to note that in terms of the rate of plasma AA appearance, other mechanisms (e.g., splanchnic metabolism) can also influence the relationship between AA uptake and appearance in the peripheral circulation (Remesy et al., 1978; Rérat et al., 1992), so more research is required to understand this relationship in the context of milk from different ruminant species.

The amounts of AA absorbed apparently absorbed at 210 min were, in general, higher for piglets fed raw ovine milk than for those fed bovine milk. Based on other studies, it could be expected that the differences in the amounts of AA apparently absorbed at 210 min, have implications for various aspects of protein metabolism in infants. The present results point to differences across the ruminant milk types in the kinetics of AA absorption from milk, and it remains to be established whether such differences translate to differences in overall small intestinal AA absorption.

For example, other aspects of protein metabolism observed in adult humans, such as postprandial protein deposition (Dangin et al., 2001), are also expected to be modulated by the amount of AA absorbed in infants. In addition, the hippocampal gene expression of some receptors of the neurotransmitter gamma-aminobutyric acid was higher in piglets fed raw ovine milk compared to those fed raw bovine milk (Jena et al., 2022), which has

implications for infant brain development and early cognitive function (Fleming et al., 2020; Lin et al., 2019).

It should be pointed out that in reporting apparent AA absorption, the endogenous losses for each protein were assumed to be similar and attempts to calculate the true AA absorption of the milk types by using reported endogenous losses in the stomach and small intestine were limited by a lack of appropriate literature. It is recommended that the true absorption of AA from bovine, caprine and ovine milk is investigated, as differences in small intestinal endogenous AA flows may influence the apparent absorption results presented here.

The presently reported results were collected using raw milk; however, as milk is usually processed to improve safety and preservation, raw milk is not commonly consumed. Thus, further research is warranted to determine the effect of processing on various parameters such as gastric emptying and AA absorption. Information on small intestinal AA absorption patterns across milk types and processing methods is expected to provide evidence to develop dairy products with benefits for specific aspects of human metabolism.

3.6. Conclusions

The present study found that at 210 min post-feeding, on average, 22%, 38%, and 46% of the AA consumed apparently absorbed within the first three-quarters of the small intestine of piglets fed bovine, caprine, and ovine milk, respectively. For most AA, at least two-thirds of the apparent AA absorption occurred in the first quarter of the small intestine. In general, the apparent small intestinal absorption of AA at 210 min was higher for piglets fed ovine milk than bovine milk, but similar between pigs fed ovine or caprine milk. The

difference in the apparent AA absorption was related to the amount of AA remaining in the stomach. When comparing the apparent absorption of the AA entering the small intestine, there were no differences in apparent small intestinal AA absorption across species for most AA.

This study provides a new understanding of the AA absorption following consumption of raw bovine, caprine, and ovine milk in the small intestine of infants. However, considering that milk is commonly processed before consumption or in preparation for dairy product production, further research is needed to understand the effect of processing on AA absorption throughout the small intestine.

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CHAPTER 4.

HEAT TREATMENT AND HOMOGENISATION OF BOVINE MILK LOOSENED GASTRIC CURD STRUCTURE AND INCREASED GASTRIC EMPTYING IN GROWING PIGS AS AN ADULT HUMAN MODEL

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HIGHLIGHTS

- This study investigated the impact of milk processing on *in vivo* gastric emptying and curd formation.
- Gastric emptying rates (dry matter and protein) followed the pattern ultra-high temperature treated milk > pasteurised milk > pasteurised non-homogenised milk = raw milk.
- Pasteurised non-homogenised milk only differed from raw milk in lipid emptying and late-digestion curd strength.
- The gastric emptying rate of lipid from ultra-high temperature treated milk was 18-fold higher than raw milk.
- Ultra-high temperature treated milk formed a 1.3-fold weaker gastric curd than raw milk.

Approximately 70% of the work contained in this chapter was contributed by the student.

4.1. Abstract

During gastric digestion, bovine milk forms a curd, which consists largely of protein and lipid. However, it is unknown how processing-induced changes to curd structure affects the gastric emptying of milk protein and lipid. This study aimed to determine the impact of heat treatment and homogenisation on gastric curd formation and gastric emptying of dry matter (DM), protein, and lipid from bovine milk fed to pigs as a human model. Growing pigs (n = 180, mean ± standard error of the mean (SEM) bodyweight 22.4 ± 0.13 kg) consumed raw, or pasteurised non-homogenised (PNH), or pasteurised homogenised (PH), or ultra-high temperature treated homogenised (UHT) milk diets. A protein-lipid-free lactose (PLFL) solution was also fed as a test diet. At 0, 20, 60, 120, 180 and 300 min postprandially the entire gastrointestinal tract was dissected out. The gastric chyme (curd and liquid) fractions were collected after separation using a mesh screen. The DM, protein, and lipid contents of these fractions were quantified. Confocal, transmission electron microscopy, cryo-scanning electron microscopy and rheological analyses were conducted to determine the micro- and macrostructure of the curd. Overall, both heat treatment and homogenisation influenced the *in vivo* gastric curd structure formed of bovine milk, although to different extents. The gastric emptying of DM, protein, and lipid increased with the extent of processing. Gastric emptying rates of DM and protein followed the pattern UHT > PH > PNH = raw, while emptying rates of lipid also differed between PNH and raw milk. Curd structure was the main gastric parameter affected in PNH milk.

Keywords: bovine milk, processing, gastric curd formation, gastric emptying, curd microstructure

4.2. Introduction

Milk has been a nutritional staple in the human diet for thousands of years and is consumed worldwide from infancy to adulthood. While milk from caprine and ovine species are popular in some Asian, African and Mediterranean areas, bovine milk is the most widely consumed milk in Western countries (*Food and Agriculture Organisation of the United Nations: FAOSTAT Statistical Database*, 2022). Bovine milk is an excellent source of nutrition, typically providing around 30 g/L of protein, 35 g/L of fat, calcium, and other minerals (Eigel et al., 1984; Jensen et al., 1991). Before consumption, commercial milk is often processed to improve safety, typically through heat treatment (pasteurisation and ultra-high temperature (UHT) treatment) and homogenisation. Processing induces various changes to the native milk protein and lipid structures present in milk (Corredig & Dalgleish, 1999; Dalgleish, 1990; Elfagm & Wheelock, 1978; Meisel & Hagemeister, 1984; Tunick et al., 2016; Wada & Lönnerdal, 2014; Ye et al., 2016b; Ye et al., 2004b). For example, during heat treatment, structural changes to the whey proteins result in denaturation and association with caseins (Ye et al., 2016a). Homogenisation disrupts the milk fat globule membrane and restructures the milk fat globule, resulting in smaller fat droplets and the adsorption of casein and whey proteins onto the surface of the droplets (Lee & Sherbon, 2002).

During gastric digestion, milk forms a curd, which consists mainly of protein and lipid (Roy et al., 2021). *In vitro* digestion studies with milk have shown that the formation and structure of the gastric curd were influenced by processing, due to changes in native protein and lipid structures as described above (Li et al., 2022; Mulet-Cabero et al., 2019; Ye et al., 2017; Ye et al., 2019). For example, heat-induced aggregation can disrupt gastric curd formation by restricting the ability of proteins to form a network (Ye et al.,

2016b, 2017). Such aggregation and subsequent effects on curd formation are greater in UHT-processed bovine milk than in pasteurised bovine milk (Ye et al., 2019). In addition, the adsorption of caseins and whey proteins onto the surface of homogenised fat droplets allows for incorporation of the fat droplets into the gastric curd matrix (Gallier et al., 2013; Ye et al., 2017). It is unknown if the structural changes in the gastric curd caused by milk processing can influence the gastric emptying rate of dry matter (DM), protein, and lipid into the small intestine. Such a relationship has been suggested in a recent review by Huppertz and Chia (2021). However, such a relationship still needs to be proven in a valid *in vivo* model.

These observations led to the hypothesis that heat-treated and homogenised bovine milk exhibit faster gastric emptying rates of DM, protein, and lipid due to forming a softer curd with a more open protein network. This study aimed to quantify the gastric emptying of DM, protein, and lipid of heat-treated and homogenised bovine milk along with the associated curd formation in a growing pig model of human metabolism. Bovine milk was selected as a milk model as the effects of heat treatment and homogenisation on its native protein and lipid structures have been well characterised (Anema & Klostermeyer, 1997; Elfagm & Wheelock, 1978; Guyomarc'h et al., 2003; Lee & Sherbon, 2002; Morr, 1989; Oldfield et al., 1998; Smits & Van Brouwershaven, 1980; Wada & Lönnerdal, 2014; Ye et al., 2004a). The growing pig was selected as a model for humans based on the digestive and physiological similarities of the gastrointestinal tract between both species (Rowan et al., 1994).

4.3. Methods

4.3.1. Animals, housing, and dietary treatments

This study was approved by the Massey University Animal Ethics Committee (application no. 19/83). Locally sourced Large White × Landrace entire male pigs (n = 180; 36 pigs per experimental diet; six pigs per experimental diet x time point combination), bodyweight 22.4 ± 0.13 kg, mean ± standard error of the mean (SEM)) were housed in individual metabolic crates at the Animal Production Unit of Massey University, Palmerston North.

The experimental treatments were raw, pasteurised non-homogenised (PNH), pasteurised homogenised (PH), or UHT bovine milk. The PH, PNH, and UHT milk types were commercially available processed products (Table 4.1), while the raw milk was locally sourced (Gorge Fresh Organics, Palmerston North, New Zealand).

Table 4.1: Processing conditions and source of the milk types and protein-lipid-free lactose solution used in the pig study.

Diet	Processing conditions	Source and diet preparation
Raw	N/A	Local supplier; consumed as sourced
Pasteurised non-homogenised (PNH)	75°C, 15 s	Commercially available trim milk mixed with commercially available fresh cream[1]
Pasteurised homogenised (PH)	75°C, 15 s, 160 bar	Commercially available; consumed as sourced
Ultra-high temperature homogenised (UHT)	140°C, 4 s, 160 bar	Commercially available; consumed as sourced[2]
Protein-lipid-free lactose (PLFL)	12% lactose in distilled water	Commercially available powdered lactose; solution prepared on-site

[1] Identical process was used to pasteurise the skim milk and cream.
[2] A preheating step (95°C, 90 s) was used to stabilise protein before UHT processing.

The raw, PH and UHT milk diets were fed as sourced, whereas PNH was prepared using simple manual agitation to combine pasteurised trim milk with pasteurised cream to the same lipid content as the PH milk. Another treatment included in the study was a protein-lipid-free lactose (PLFL) solution, which consisted of 12% lactose in distilled water. The PLFL treatment was included to allow correction for endogenous gastric luminal materials (Moughan & Rutherfurd, 2012), and for chyme remaining from the previous meals based on other studies showing some residual meal after an overnight fast (Roy et al., 2022).

4.3.2. Experimental design

A human-type meal (Table 4.2) was formulated based on the USDA's chemical composition data to meet the NRC nutrient requirements for growing pigs (NRC, 1998). The human-type diet and meal times were also designed to reflect typical Western meal composition and consumption patterns. On arrival, pigs were randomly allocated to each of the five dietary treatments. Over the first three days, the experimental treatments were gradually introduced by replacing the commercial diet provided on the farm (day 1; 33% experimental diet, 66% farm diet; day 2; 66% experimental diet, 33% farm diet; day 3 100% experimental diet) (Figure 4.1). On arrival, two meals (12:00 and 16:00 hours) were equally fed at a daily DM level of 4% based on individual body weight.

After the dietary transition, the pigs consumed their treatments at 09:00 hours (500 mL of raw, PH, PNH, UHT or PLFL) and human-type meals at 12:00 hours and 16:00 hours for seven days (Figure 3.1; Table 3.2). The pigs in the PLFL group received UHT milk for the first three days after the dietary transition, followed by the PLFL treatment for the final four days, as the PLFL diet for seven days would not provide sufficient nutrition.

During the first two days, the meals were kept in the feeder for up to 15 min, before refusals, if any, were collected and weighed. The feeders were then washed and kept emptied until the next feeding. From day three onwards, all pigs consumed their meals quickly, and within 5 to 10 min the feeders could be cleaned. This feeding method can be used in pigs as they have the digestive capacity for 'meal-eating' (Miller & Ullrey, 1987; Rowan et al., 1994). Thus, by the sampling day, pigs could consume their milk meals in approximately two minutes.

On day 10, pigs were fed lunch and dinner meals from 12:00 and 16:00 hours, respectively, at staggered times to accommodate the same fasting period at the sampling on day 11 (Figure 3.1). On day 11, fasted pigs (16 h) were either euthanised at time 0 min or fed 500 mL of milk type or PLFL solution for breakfast before being euthanised at either 20, 60, 120, 180, or 300 min.

Indigestible marker titanium dioxide (TiO_2, 0.38 ± 0.3 g) (Sigma Aldrich, St. Louis, MO, USA) was added to the breakfast meal to estimate meal absorption. The TiO_2 results were used for other analyses that are not reported here.

Table 4.2: Ingredient and calculated chemical composition of the human-type diet provided to the pigs at lunch and dinner[1]

	g fresh/kg DM	g DM/kg
Ingredient		
Milk	617	92
Whole grain bread (diced)	636	540
Peeled hard-boiled egg (minced)	740	186
Raw carrots (minced)	370	36
Canned, chopped fruits	555	108
Sucrose	12	12
Premix of vitamins and minerals[2]	6.2	6.2
Sodium chloride	3.6	3.6
Calcium carbonate	10	10
Dicalcium phosphate	6.2	6.2
Calculated composition		
Protein		171
Calcium		1.5
Phosphorus		4.2
Energy (kcal/kg)		3,369

[1] The chemical composition of the ingredients to formulate the diet was obtained from the USDA National Nutrient Database (https://ndb.nal.usda.gov/).

[2] Vitamin and mineral premixes were obtained from Vitec Nutrition Ltd (Auckland, New Zealand) and supplied (per kg of diet as-fed): Mn, 45 mg; Zn, 80 mg; Cu, 25 mg; Co, 0.5 mg; Se, 0.3 mg; Fe, 100 mg; I, 1.0 mg; Choline, 100 mg; all-trans retinylacetate, 3.0 mg; cholecalciferol, 0.05 mg; α-tocopherol, 50 mg; menadione, 2.0 mg; thiamin, 1.0 mg; riboflavin, 3.0 mg; nicotinic acid, 15 mg; pantothenic acid, 20 mg; pyridoxine, 2.0 mg; cyanocobalamin, 0.01 mg; folic acid, 0.5 mg; biotin, 0.1 mg.

Each pig was anaesthetised 15 min before its euthanasia time with a mix of Zoletil 100 (zolazepam and tiletamine, both 50 mg/mL; Virbac, Hamilton, NZ) reconstituted with 2.5 mL Ketamine and 2.5 mL Xylazine, both 100 mg/mL (Phoenix Pharm NZ, Auckland, New Zealand). The final solution contained 50 mg/mL of each drug and was administered at a dosage of 30-40 μL of the mixed solution/kg BW by intramuscular injection in the neck area. Following sedation, each pig was intravenously administered a second dose of the cocktail (30 μL/kg BW) to induce deep anaesthesia.

Once anaesthetised, the pigs were euthanised by an intracardiac injection of sodium pentobarbitone (0.3 mL/kg BW of Pentobarb 300, Provet NZ Pty Ltd, Christchurch, New Zealand). The abdomen was opened, and the stomach was clamped at the oesophageal and pyloric ends prior to being removed. The stomach was carefully removed with minimal movement, washed, dried, and weighed full before being opened by an incision along the lesser curvature. The pH of the gastric contents (chyme) at the proximal and distal regions was recorded, after which the solid (curd) and liquid fractions were separated using a 1 mm sieve. The pH of the liquid fraction was measured, and a photograph of the curd in the sieve was taken. The liquid fraction was weighed. Samples were collected from each fraction for confocal microscopy and from the solid fraction for rheology. Selected curd samples were collected for transmission electron microscopy (TEM) and cryo-scanning electron microscopy (cryo-SEM). The remaining material of each fraction was thoroughly mixed, frozen, freeze-dried, weighed, and ground. Samples were stored at -20 °C until analysis.

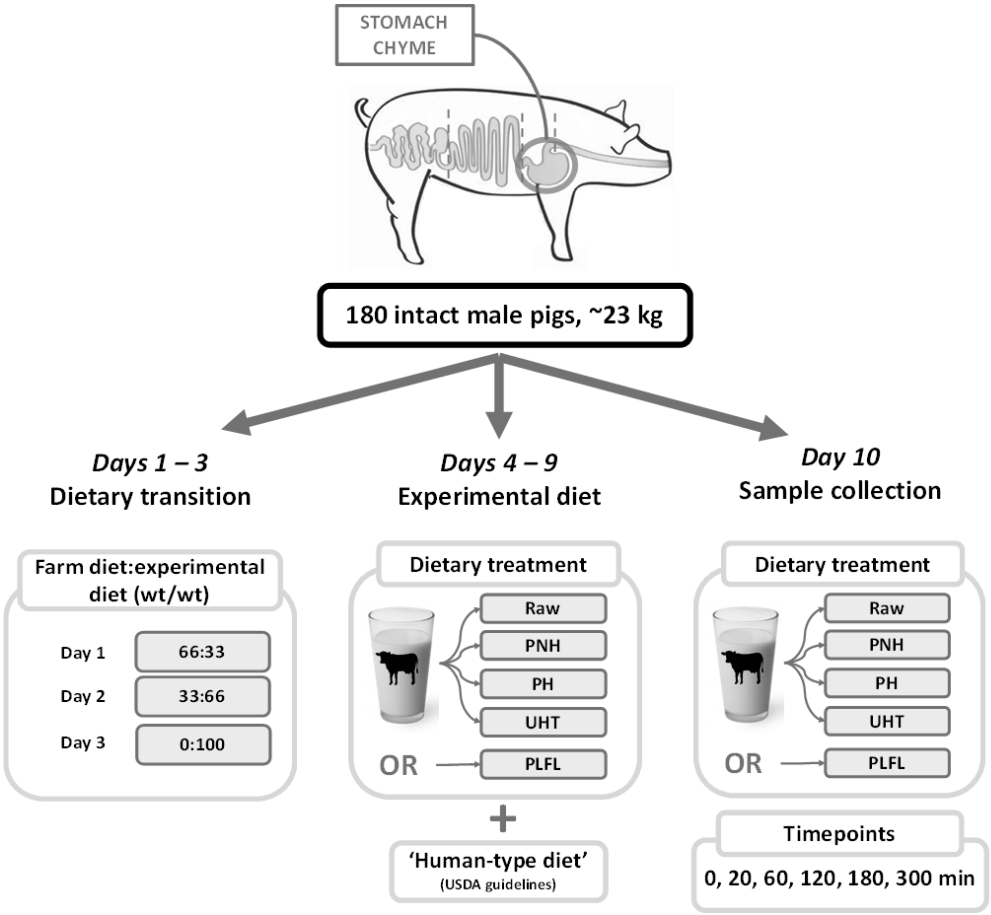


Figure 4.1: Experimental diagram of the pig study.
Pigs were gradually transitioned from the farm diet to the experimental diet in one-third increments over three days. The experimental diet was then consumed for seven days. On day 10, pigs consumed their milk diets and were euthanised at various time points. The entire gastric chyme was collected. Wt, weight; PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised; PLFL, protein-lipid free lactose solution.

4.3.3. Rheological analysis

Rheological measurements were conducted on milk and gastric curd samples within an hour of obtaining the samples from the pigs, following an existing protocol with minor modifications (Mulet-Cabero et al., 2019). An AR-G2 magnetic bearing rheometer (TA Instruments, Crawley, West Sussex, UK) fitted with a 40 mm diameter parallel steel plate geometry was used. Approximately 5 g of gastric curd was placed in the rheometer geometry, and the samples were pressed to the system's default gap of 2,000 μm. After pressing, approximately 2 g of the sample that moved out of the geometry was removed. The analysis was conducted on the remaining 3 g of the sample. Time sweep tests were performed at a frequency of 1 Hz, strain of 0.5 mm/mm, for 20 min at 37 °C.

4.3.4. Microscopy

Confocal microscopy and TEM analyses were conducted at the Manawatu Microscopy and Imaging Centre (Massey University, Palmerston North, New Zealand). A confocal laser scanning microscope (Leica SP5 DM6000B, Leica Microsystems, Heidelberg, Germany) was used to determine the microstructures of fresh curd and liquid samples. The fresh samples were immediately stained and imaged. Approximately 200 μg of the sample was transferred into an Eppendorf tube, and 5 μL of 1.0% (w/v) Fast Green and 10 μL of 0.1% (w/v) Nile Red were added to stain protein (He–Ne laser with an excitation at 633 nm) and oil (argon laser with an excitation line of 488 nm) phases, respectively, for at least 10 min. The stained samples were placed on concave confocal microscope slides (Sailing Medical-Lab Industries Co. Ltd., Suzhou, China), covered with coverslips, and observed using magnifications of ×40 and ×100 oil immersion lenses.

TEM availability limited the number of samples which could be imaged. Thus, milk and fresh curd samples from pigs that represented a milk type and post-feeding times of 20, 120, and 180 min were randomly selected throughout the six periods of the study. Selected samples were prepared and stored until analysis. However, PH milk and curd samples were accidentally discarded before being analysed. Sample preparation was carried out as described by Li et al. (2021) and imaged with a Tecnai G2 Spirit BioTWIN (FEI Company, Czech Republic) paired with a Veleta TEM camera (Olympus SIS Germany).

Cryo-SEM was conducted at the MacDiarmid Institute for Advanced Materials and Nanotechnology (Victoria University, Wellington, New Zealand). Randomly selected raw, PH, and UHT curd samples at 20 min and 180 min were snap-frozen in nitrogen slush directly after collection. Limited microscope availability did not allow for the analysis of all curd samples. Based on other imaging carried out in this study, the PNH curd samples were not imaged using cryo-SEM. The frozen samples were loaded into a cryo unit Gantan Alto 2500 (Gatan Inc., Pleasanton, CA, USA) and fitted onto the electron microscope at -120 °C. The fractured surfaces of the curds were sputter-coated with platinum under a current of 10 mA for 240 s. They were then inserted into a JEOL 6500F scanning electron microscope (JEOL Ltd, Tokyo, Japan) for imaging with accelerating voltages of 6 and 8 kV and a probe current of 22 nA. Multiple images were collected for each sample, and a representative image is presented in the results section.

4.3.5. Chemical compositional analysis

The milk types, curd, and liquid fraction samples were analysed for DM (AOAC 990.19 (AOAC, 2006)) and protein (nitrogen × 6.38) content (Koletzko & Shamir, 2006) using the Dumas method (AOAC 968.06 (AOAC, 2006)). The lipid content of a representative sample set (milk, liquid fraction, and curd fraction) was quantified using the Mojonnier method (AOAC 989.05 (AOAC, 2006)) and a modified petroleum ether/diethyl ether extraction. A significant correlation between both methods to determine lipid content was observed ($r = 0.998$, Appendix 2: Figure A2.1). Based on this high correlation, the remaining samples (350) were quantified using the modified method.

4.3.6. Modified petroleum ether/diethyl ether lipid defatting

Freeze-dried and ground curd and liquid fractions were weighed (1 g) in a glass tube before mixing with 5 mL petroleum ether and 5 mL diethyl ether. Solvents were added, vortexed for 30 s, stood for 5 min and centrifuged at 3,000 rpm for 7 min at room temperature. The solvent fraction was then discarded after removal using a water aspirator system (approximately 10 torrs). The complete procedure was repeated twice to remove residual lipid. The fraction remaining in the tube was left for 16 h at room temperature. The samples were then vortexed to help loosen the remaining material and incubated at 105 °C for one hour to remove solvent residues. The samples were then cooled for one hour in a desiccator and weighed to calculate the lipid content.

4.3.7. Statistical analyses

The present animal experiment was designed to investigate the gastric digestion of milk, as well as the small intestinal absorption and the appearance of amino acids (AA) in the circulatory system. Thus, it was important to cater to the greatest required number of animals. Based on previous studies with similar outcomes (Butteiger et al., 2013; Chen et al., 1962; Gaudichon et al., 1994; Montoya et al., 2018), six pigs were required to detect a difference in mean values powered over 80% at $P < 0.05$.

Statistical analyses were conducted using SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). First, a two-factor linear model including milk, time (as either a categorical or a numerical variable), and their interaction as fixed effects, was used to assess curd strength. For the pH of the proximal and distal chyme, a linear mixed model, including the fixed factors described above, and the pig (as repeated measures in space) included as a random effect, was used. Next, the log-likelihood ratio test was used to select the best polynomial model, and then compare models with time as numerical (selected polynomial) and categorical variables. The best model had time as a numerical variable for all response parameters.

In some pigs, part of the meal at 16:00 hours (human-type diet) on day 9 remained in the stomach on day 10, despite the 16 h fasting period. Where the gastric content did show more than 10% of the human-type diet remained, these samples were excluded from DM and protein analyses of the chyme, as the fibrous remnant material could influence the curd (e.g., formation, structure, and degradation) and subsequent gastric emptying (Bornhorst et al., 2014). Gastric contents with less than 10% of the human-type diet were corrected using average DM and protein contents found in the PLFL group for each post-

feeding time. Based on this, 20 pigs were removed from the analyses of DM and protein gastric emptying rates. The remaining parameters (rheometry, confocal microscopy, TEM, cryo-SEM) were quantified in the samples from these pigs using portions of curd samples free from the remnants of the 16:00 hour meal. A correction for lipid was omitted, as the lipid content of the remaining chyme for the pigs fed the PLFL solution was negligible. Excluded pigs were five for raw, one for PNH, six for PH, and eight for UHT.

The exponential power model was used to analyse the retention of DM, protein, and lipid (on a 100 mL milk basis) in the stomach as detailed by Montoya et al. (2014) (protein as an example):

$$\text{Remaining Protein (g)} = \alpha_0 \exp - (\kappa \times \text{time})^{\beta}$$

where the parameter α_0 is the amount of DM (g), protein, or lipid in the milk before being consumed, κ is the logarithmic slope of the curve (g/min), β is a dimensionless index for the shape of the curve, and time is in minutes. The parameters κ and β were then used to determine the half gastric emptying time ($T_{1/2}$):

$$\text{Gastric } T_{1/2\ \text{Protein}}\,(\text{min}) = (1/\kappa) \times (\log[1/0.5])^{(1/\beta)}$$

For analysis of the gastric emptying of the curd, it was assumed that the initial values (i.e., α_0) were the same as the milk consumed.

The normal distribution and the homogeneity of variance were evaluated for each statistical analysis, and the difference was declared significant if $P \leq 0.05$. Apart from the curd strength data (G*), which was natural log-transformed, all other data were normally distributed and showed homogeneity of variance. For the factorial models, means were

compared using an adjusted Tukey test, while for the non-linear model parameters, means were compared using a t-test.

4.4. Results

The pigs adapted well to the experimental diets, and after the third day they consumed all meals offered. One pig was excluded during the study due to coprophagia. Throughout the study, the remaining pigs were healthy.

The chemical composition of the milk treatments is shown in Table 4.3. The protein content of the milk samples ranged from 33 to 41 g/L, and the lipid content ranged from 33 to 44 g/L. The carbohydrate (average 46 g/L), sodium, and calcium contents were similar among milk treatments.

The gastric digestion of all milk types resulted in the formation of separable solid (curd) and liquid fractions, but the consistency and structure of the curd visually varied between treatments (Figure 4.2). After 300 min of digestion, there was insufficient gastric content in five of the six pigs fed the UHT milk to conduct any analyses.

Table 4.3: Chemical composition of raw and commercially processed bovine milk, and the protein-lipid-free lactose solution fed to the pigs.

	Raw[1]	PNH[2]	PH[2]	UHT[2]	PLFL[3]
Protein, g/L	41	36	33	34	0
Fat – total, g/L	44	34	34	33	0
Carbohydrates, g/L	46	46	47	44	117
Sodium, mg/L	481	320	328	319	0
Calcium, mg/L	1340	1203	1149	1154	5.2

[1] Locally sourced, single farm, bulk milk

[2] Commercially sourced

[3] Prepared on-site

PNH, pasteurised non-homogenised milk; PH, pasteurised homogenised milk; UHT, ultra-high temperature treated homogenised milk; PLFL, protein-lipid free lactose solution.

4.4.1. Gastric emptying of processed bovine milk

The total gastric DM (Figure 4.3a), protein (Figure 4.3c), and lipid (Figure 4.3e) emptying rates differed ($P \leq 0.05$) across milk types, but in general, they followed similar patterns. For instance, the pigs fed the UHT milk had faster ($P \leq 0.05$) gastric emptying rates of DM, protein, and lipid, compared to the other milk types. Pigs that received the raw and PNH milk had a slower ($P \leq 0.05$) gastric emptying of DM, protein, and lipid, compared to the UHT and PH milk types. Similar patterns were observed when only the curd was considered (Figure 4.3b, d, and f). In addition, the gastric emptying of DM, protein and lipid in the liquid fraction was similar ($P > 0.05$) between the pigs fed the different milk types (data not shown).

The ratio of protein to lipid retained in the gastric curd fraction varied with processing treatment (Figure 4.4). Further, pigs fed UHT milk had an inverse protein to lipid retention pattern, compared to pigs fed the raw, PNH, and PH milk types.

The differences in gastric emptying of the total (or curd) DM, protein, and lipid of the processed bovine milk types led to different kinetic parameters (κ and β) and $T_{1/2}$ and followed the pattern UHT > PH > PNH = raw (Table 4.4). The κ of DM, protein, and lipid in both the total content and curd of UHT milk was at least twice that ($P \leq 0.05$) of the raw milk. The differences in κ and β for total gastric content and curd were reflected in the $T_{1/2}$. For instance, the pigs fed the raw milk needed 228 min to empty half of the lipid consumed, while the pigs fed the UHT milk needed 11 min. In addition, high variability was observed in the amount of total gastric lipid of the raw milk, which can be explained by the large amount of lipid in the liquid fraction (Appendix 2: Figure A2.2).

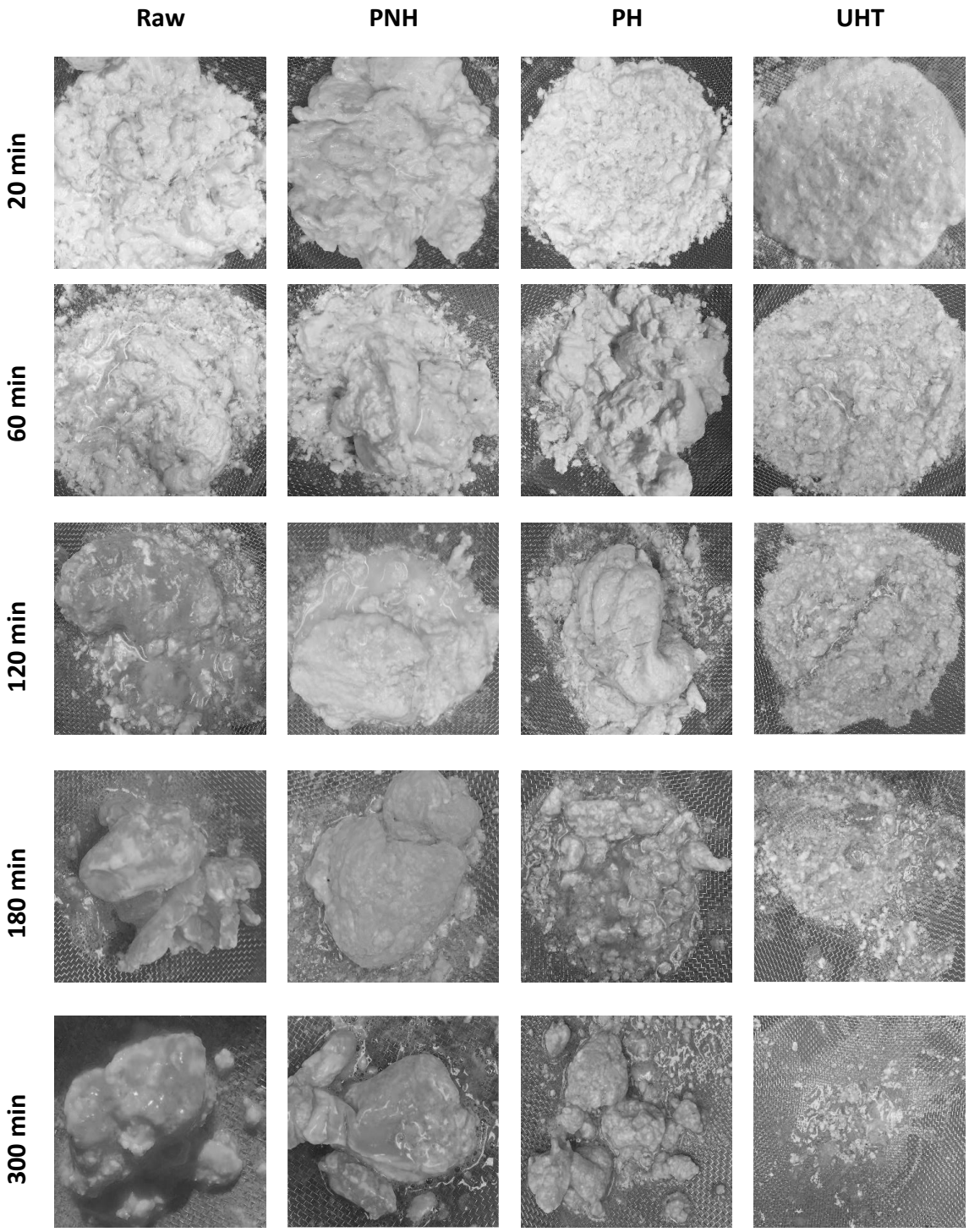


Figure 4.2*:* Representative images of fresh gastric curd (solid) collected at different post-feeding times from growing pigs fed different processed bovine milk types. PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT ultra-high temperature treated homogenised.

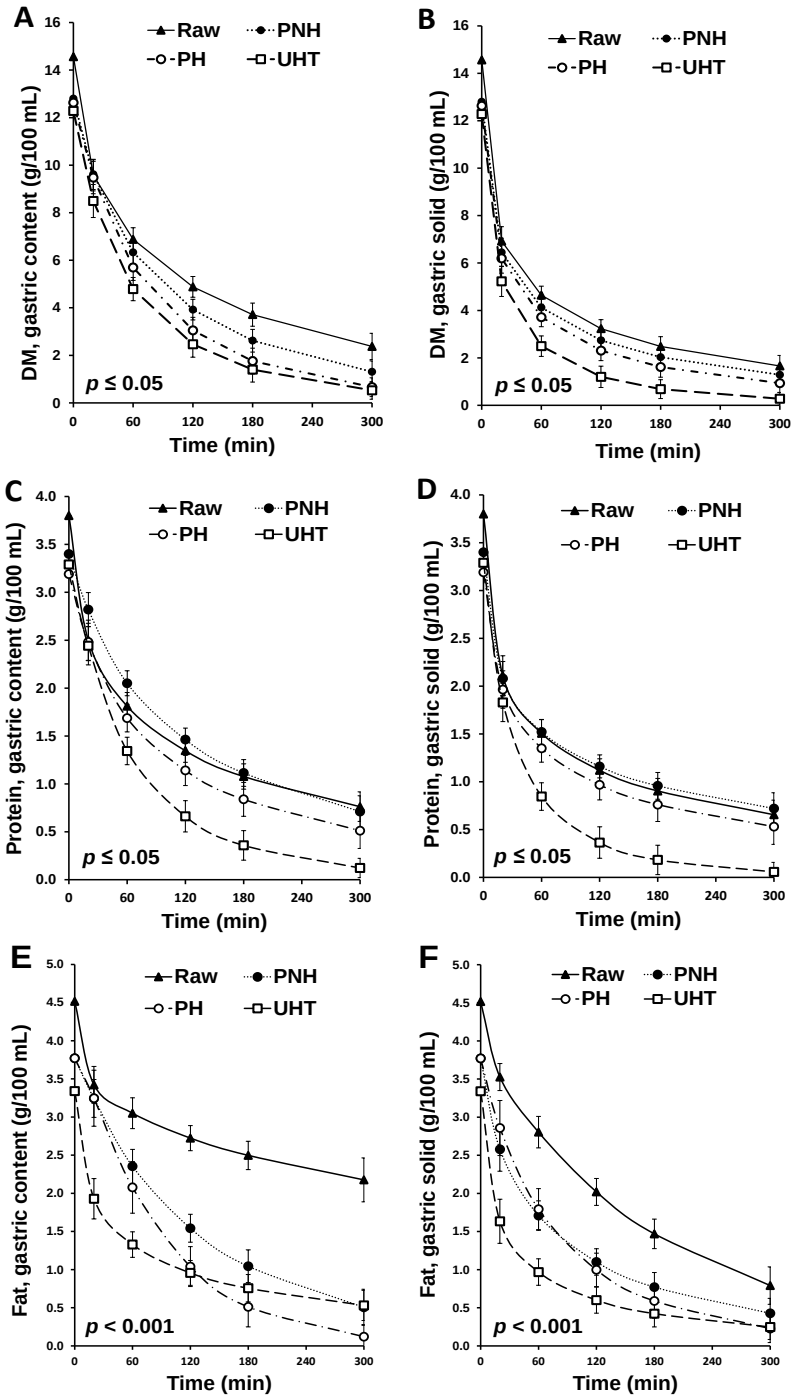


Figure 4.3: Gastric emptying of total gastric contents and gastric curd (solid) collected at different post-feeding times from growing pigs fed different processed bovine milk types.

A and B: total and curd dry matter (DM); **C and D:** total and curd protein; **E and F:** total and curd lipid. PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 4 – 6. The starting values for the curd were assumed to be the content of the milk consumed. Probability values in each panel reflect a comparison between the fitted curve of each treatment with the other treatments.

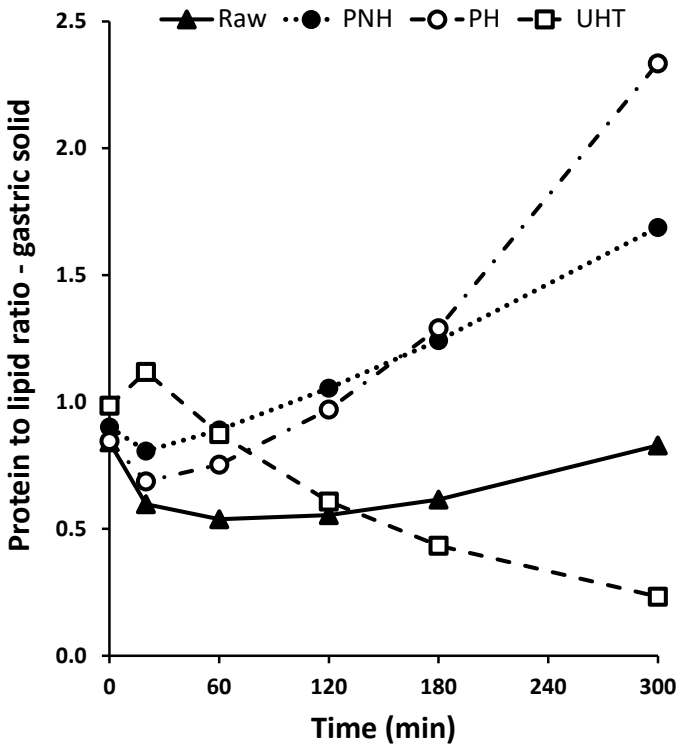


Figure 4.4: Changes to the protein to lipid ratio of the gastric solid (curd) collected at different post-feeding times from growing pigs fed different processed bovine milk types.
For each ratio the starting values (t = 0) are the ratio of protein to lipid in the milk. PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT ultra-high temperature treated homogenised. n = 5 or 6 for each treatment and time point combination.

Table 4.4: Gastric emptying parameters of the power exponential model (κ, β) and half-time (T1/2) of gastric emptying of total and gastric curd (solid) dry matter, protein, and lipid collected at different post-feeding times from growing pigs fed different processed bovine milk types.

	Total gastric content			Curd		
	$\kappa \times 10^{-3}$ (g/min)	β	$T_{1/2}$ (min)	$\kappa \times 10^{-3}$ (g/min)	β	$T_{1/2}$ (min)
DM						
Raw	9.1±1.4^{c}	0.57±0. 10^{d}	57±15.7^{ab}	21±4.2^{c}	0.41±0.075^{c}	19±8.0^{a}
PNH	12±1.4^{c}	0.68±0.10^{c}	49±4.6^{a}	27±5.6^{c}	0.42±0.078^{c}	15±4.1^{a}
PH	15±2.0^{b}	0.76±0.12^{a}	42±5.4^{ab}	32±6.9^{b}	0.44±0.084^{b}	14±3.6^{a}
UHT	18±2.6^{a}	0.70±0.12^{b}	32±4.9^{b}	49±12^{a}	0.51±0.12^{a}	10±3.5^{a}
Protein						
Raw	10±1.8^{c}	0.46±0.09^{c}	45±14^{ab}	16±3.8^{b}	0.38±0.09^{b}	25±11^{a}
PNH	8.4±1.2^{c}	0.59±0.10^{b}	64±9.6^{a}	15±3.5^{b}	0.36±0.09^{b}	24±10^{a}
PH	13±2.3^{b}	0.54±0.10^{b}	40±6.2^{b}	21±5.4^{b}	0.39±0.10^{b}	19±5.5^{a}
UHT	19±2.6^{a}	0.72±0.13^{a}	32±5.3^{b}	34±6.5^{a}	0.62±0.14^{a}	16±4.6^{a}
Lipid						
Raw	1.2±0.1^{d}	0.50±0.21^{b}	228±79^{a}	6.6±0.1^{c}	0.94±0.19^{a}	95±17^{a}
PNH	7.9±0.1^{c}	0.85±0.19^{b}	93±11.04^{a}	13±0.3^{b}	0.60±0.15^{ac}	43±12^{b}
PH	11±0.2^{b}	1.04±0.31^{a}	51±9.69^{b}	13±0.3^{b}	0.79±0.21^{b}	50±12^{b}
UHT	21±0.7^{a}	0.38±0.11^{b}	11±5.1^{c}	38±1.6^{a}	0.42±0.13^{abc}	7.5±4.0^{c}

DM, dry matter; PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 4 – 6. Means with different superscript letters within a column for DM, protein, or lipid differ ($P \leq 0.05$). Values are reported on a g/100 mL milk basis. κ, the slope of the curve, and β, the index for the shape of the curve, were used to determine the half gastric emptying time ($T_{1/2}$). The parameter alpha values (α_0) were the amount of DM, protein, or lipid in the milk before being consumed: DM, 13.0 g/100 mL; protein, 3.5 g/100 mL; lipid, 4.0 g/100 mL.

4.4.2. Structural changes in gastric contents of processed bovine milk

After 20 min of gastric digestion, a protein network was formed for all milk types with differences in density, structure, and lipid distribution as measured using confocal microscopy (Figure 4.5) or TEM (Figure 4.6). In the liquid fraction, the average size and amount of lipid droplets appeared visually lower in the PH and UHT milk types, compared to the raw and PNH milk types (Figure 4.7).

After 20 min, the raw and PNH curds formed a tight, more continuous protein network with larger, less distributed pores, whereas the PH and UHT curds were looser and crumblier, with a less dense network (Figure 4.5). For the raw, PNH and UHT curds, TEM in Figure 4.5 supported this observation. Although the PNH curd was looser than the raw curd, after 120 min, larger empty areas were observed within the network formed in the raw curd (Figure 4.6).

At 300 min of digestion, the raw curd continued to be tight, whereas the protein network of the PNH curd began loosening (Figure 4.5). Throughout digestion, the UHT curd network remained fragmented and open (Figures 4.5 and 4.6). In contrast to pigs fed PH and UHT milk, separated fat globules appeared to be suspended around, rather than inside, the protein network in the curd of pigs fed raw and PNH milk (Figure 4.5). As a result, the lipid in the raw and PNH curds formed comparatively large conglomerations, while the lipid in the PH and UHT curds showed minimal and smaller lipid conglomerations (Figure 4.5). This pattern continued up to 120 min of digestion. After 180 min of digestion, the lipid conglomerations in the curd of pigs fed the raw milk remained large and intact, but the lipid conglomerations in the curd of the pigs fed PNH were reduced in size and amount (Figure 4.5).

The cryo-SEM images further confirmed differences between the curd protein network formed by the digestion of raw, PH and UHT milk (Figures 4.8 and 4.9). For instance, at 20 and 180 min, the raw curds had fat globules embedded within pockets in the protein network, whereas the PH and UHT curds had smaller fat droplets bound to the protein network (Figure 4.9). At 180 min of digestion, the protein network around the fat globules in the raw milk curd was tighter compared to 20 min, with less space and smaller cavities visible (Figure 4.8). Over the digestion, the thickness of the protein network formed by the PH and UHT milk curds appeared to increase. However, the apparent density of the network appeared to increase for the PH curd and decrease for UHT milk curds (Figure 4.8). Up to 120 min of digestion, the liquid fraction of the raw and PNH milk chyme contained a high amount of lipid and larger lipid conglomerations, which decreased thereafter, especially for PNH (Figure 4.7). In contrast, the liquid fraction of the PH and UHT chyme showed small lipid conglomerations widely distributed throughout the sample, which remained over time (Figure 4.7).

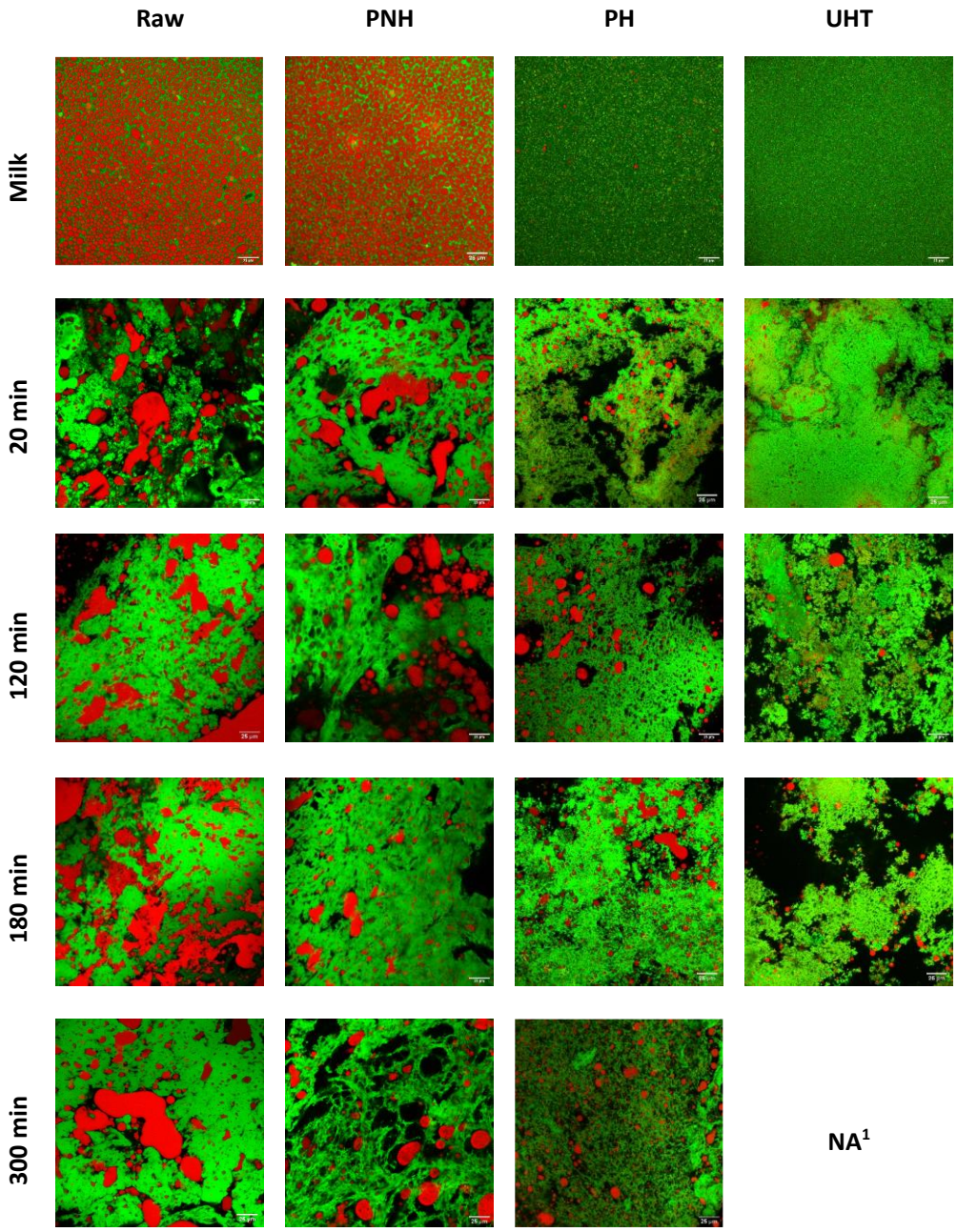


Figure 4.5: Representative confocal scanning microscopy images of milk and gastric curd (solid) collected at different post-feeding times from growing pigs fed different processed bovine milk types.
Protein appears green, while lipid appears red. PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. [1]NA, no available curd sample remained at 300 min. The scale bar is 25 µm.

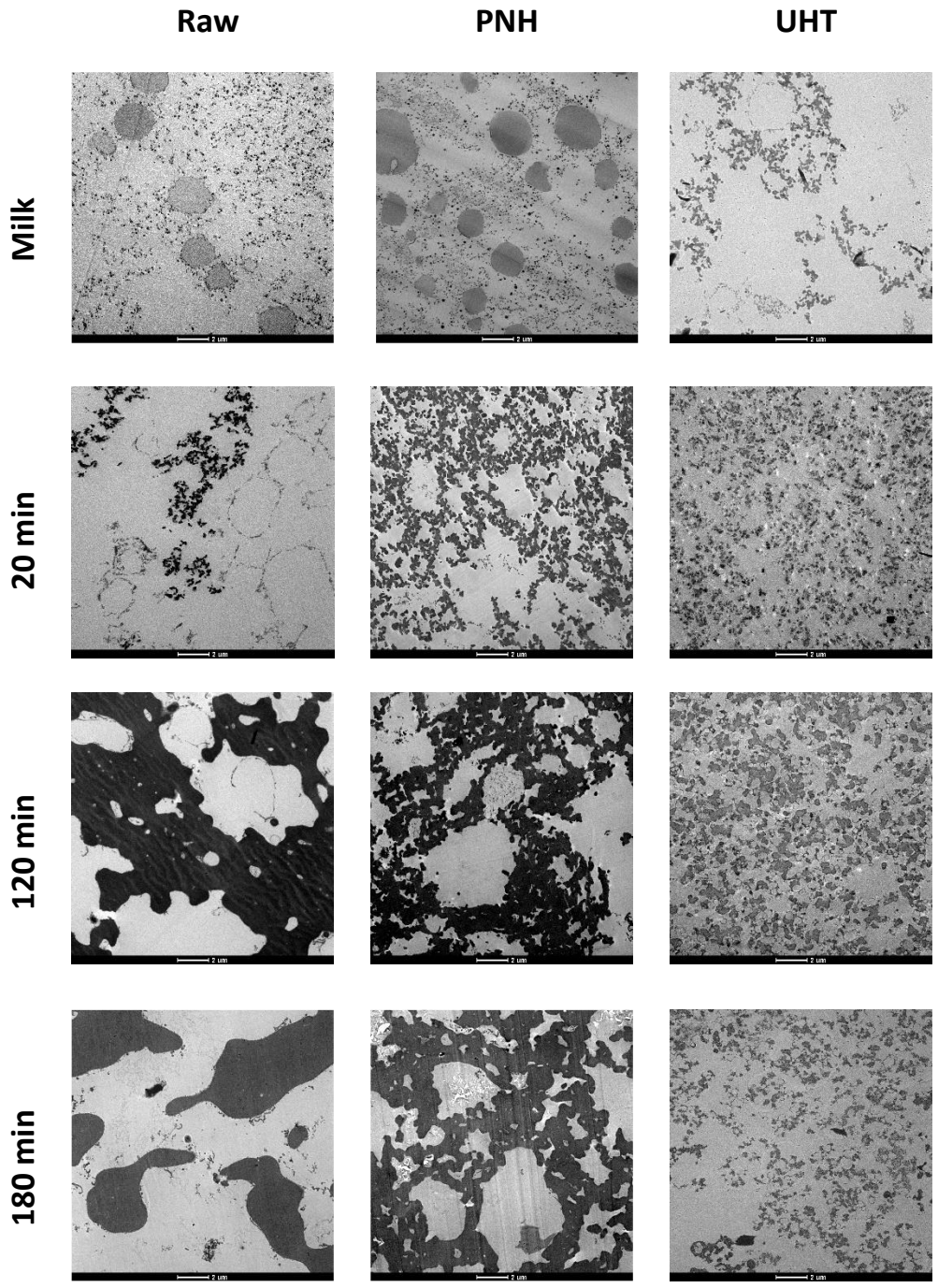


Figure 4.6: Transmission electron microscopy images of milk and gastric curd (solid) collected at different post-feeding times from growing pigs fed different processed bovine milk types.
Protein appears in the dark area, while lipid appears in the light grey area. PNH, pasteurised non-homogenised; UHT, ultra-high temperature treated homogenised.

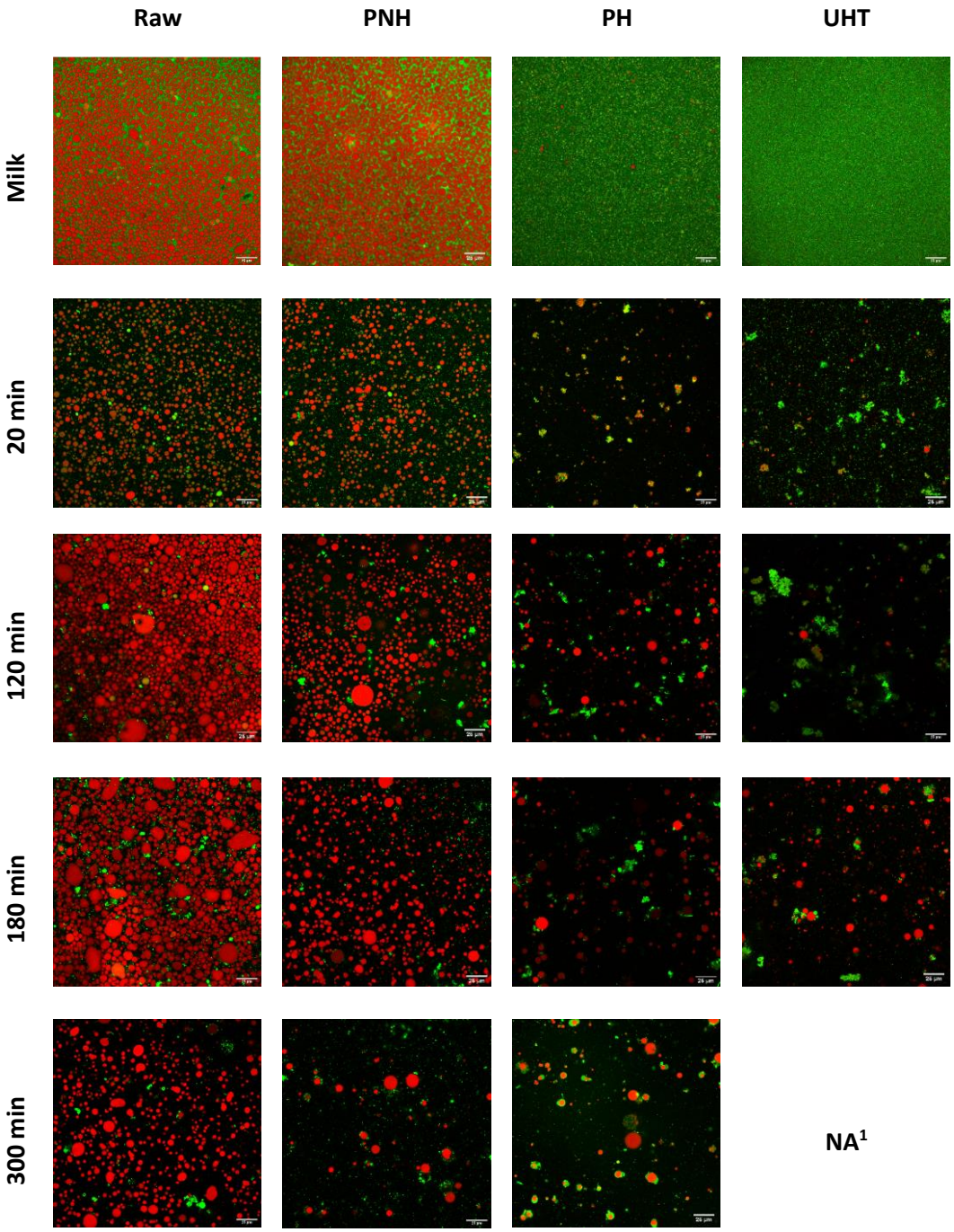


Figure 4.7*:* Representative confocal scanning microscopy images of milk and gastric liquid fraction collected at different post-feeding times from growing pigs fed different processed bovine milk types.
Protein appears green, while lipid appears red. PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. [1]NA, no available curd sample remained at 300 min.

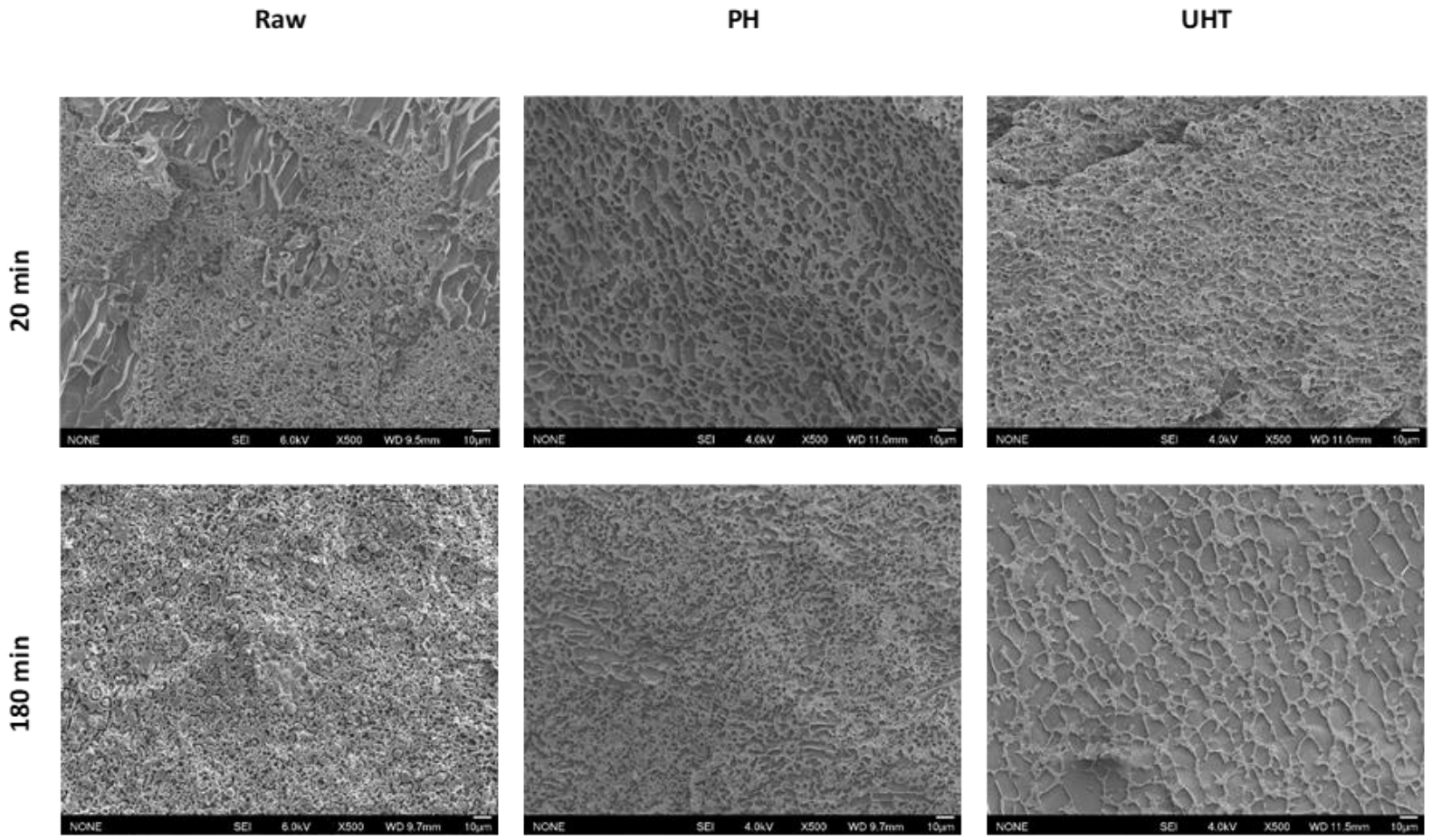


Figure 4.8. Cryo-scanning electron microscopy images of the protein network of gastric curd (solid) collected at selected post-feeding times from growing pigs fed different processed bovine milk types.
PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised.

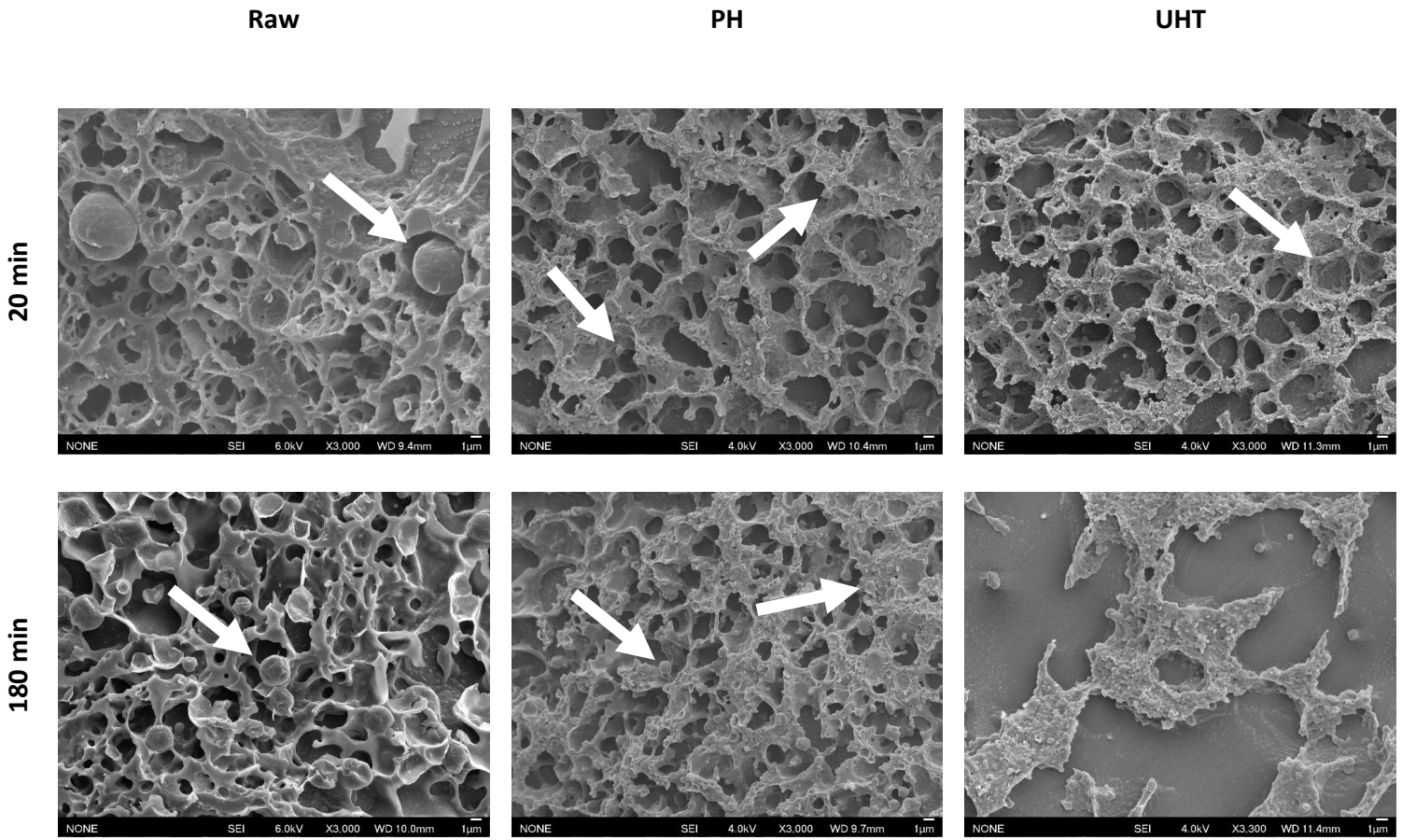


Figure 4.9: Cryo-scanning electron microscopy images of milk fat globules (raw) and restructured milk fat droplets (PH, UHT) in gastric curd (solid) collected at selected post-feeding times from growing pigs fed different processed bovine milk types.
White arrows indicate a fat globule (raw) or fat droplet (PH and UHT). Fat droplets could not be identified in the UHT 180 min curd. PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised.

4.4.3. Changes to curd strength over time

The G* value (oscillation stress (Pa) divided by strain) was applied to indicate the strength of the curd formed during gastric digestion. During the first 120 min of digestion, the strength increased similarly between milk types (2.1 to 3.0 Pa/h, for UHT and PNH, respectively) (Figure 4.10). However, after 120 min of digestion, homogenisation (PH vs PNH) and heating (raw vs PH or UHT vs PNH) led to less firm curd ($P \leq 0.05$) (Figure 4.10). As a result, the curd strength for the pigs fed UHT milk decreased from 4.2 Pa to 2.7 Pa after 120 min post-feeding ($P < 0.05$), whereas it remained constant for the pigs fed the other milk types (Figure 4.10).

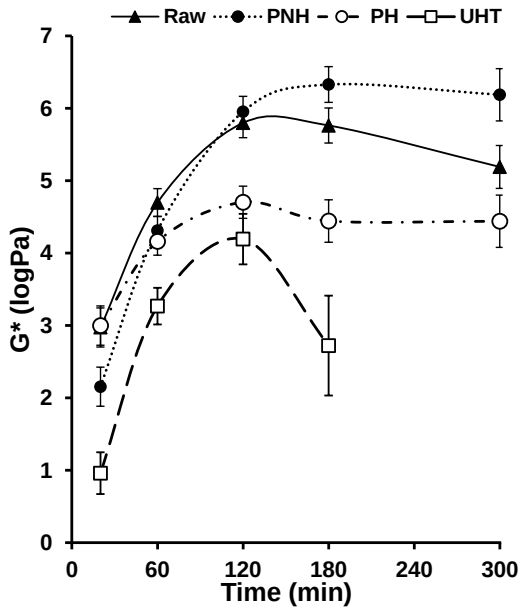
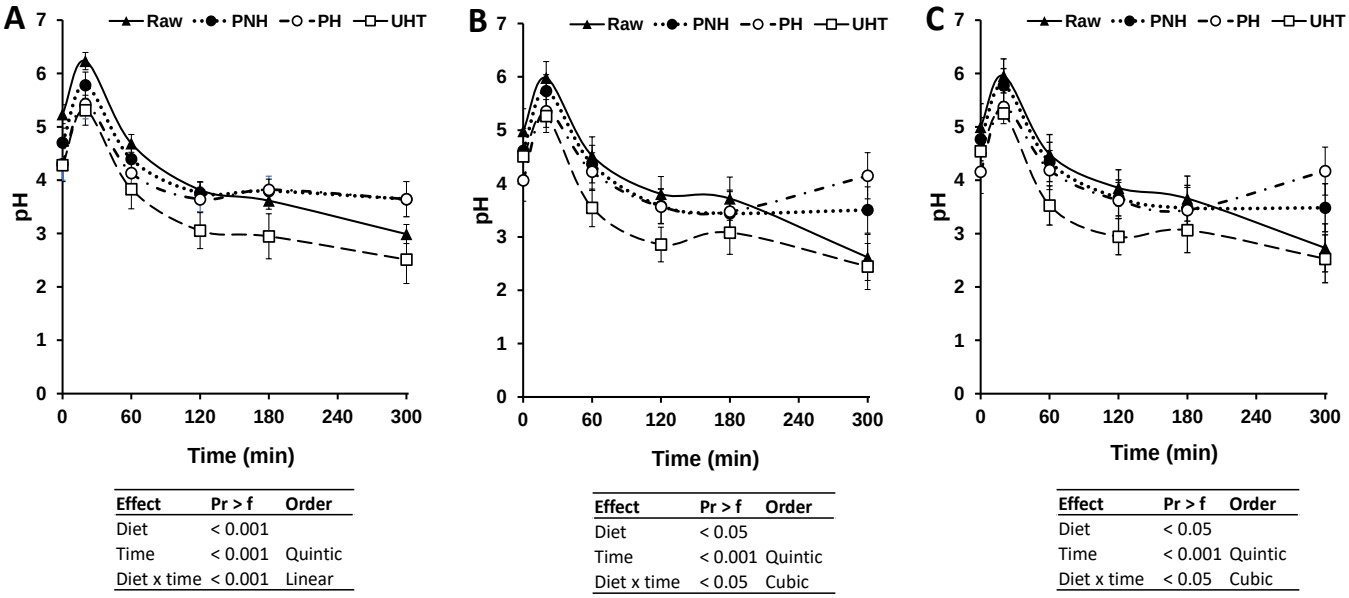


Figure 4.10*:* Strength (G*) of curds collected at different post-feeding times from growing pigs fed different processed bovine milk types.
Values are means ± standard errors, n = 5 - 6. PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. No available UHT curd sample remained at 300 min.

4.4.4. Gastric pH during digestion

The gastric digestion of all milk types resulted in a similar sharp increase of pH of proximal and distal chyme, and of the liquid fraction (Figure 4.11a, b, and c) during the first 20 min. Significant changes in chyme pH were also observed across milk types after 20 min and 120 min post-feeding ($P \leq 0.05$). After 120 min until 300 min, the pH of the proximal and distal stomach chyme and liquid fraction of the pigs fed the raw milk decreased by 0.8, 1.2 and 1.1 units, respectively ($P \leq 0.05$), while it plateaued ($P > 0.05$) for the pigs fed the PNH, PH and UHT milk types.



Effect	Pr > f	Order
Diet	< 0.001	
Time	< 0.001	Quintic
Diet x time	< 0.001	Linear

Effect	Pr > f	Order
Diet	< 0.05	
Time	< 0.001	Quintic
Diet x time	< 0.05	Cubic

Effect	Pr > f	Order
Diet	< 0.05	
Time	< 0.001	Quintic
Diet x time	< 0.05	Cubic

Figure 4.11*:* Changes in gastric pH values observed at different post-feeding times from growing pigs fed different processed bovine milk types.
Values are means ± standard errors, n = 5 - 6. **A**: proximal chyme; **B**: distal chyme; **C:** liquid fraction. PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised.

4.5. Discussion

This is the first *in vivo* study using the pig as an adult human model to report how heat processing and/or homogenisation of whole bovine milk affects the gastric emptying rates of DM, protein, and lipid, and how these results are linked to compositional and structural changes of the solid (curd) and liquid fraction of the gastric chyme. The results support the stated hypothesis that heat-treated and homogenised bovine milk exhibit faster gastric emptying rates of DM, protein, and lipid due to forming a softer curd with a more open protein network.

It is important to highlight that the PNH, PH, and UHT milk originated from a different source to the raw milk, which could have affected the comparison to the raw milk as a result of differences in protein, lipid and mineral composition. For example, differences in the amount of κ-casein present may impact pepsin-induced casein micelle aggregation during curd formation (Horne, 2020). In addition, 11 treatment x time combinations had a smaller sample size (n=4 or 5 vs 6) for some analyses due to remnant portions of the human-type evening meal received on day 10 in some animals. However, these limitations have been controlled where possible. For example, all processed milk used in this study was bulk milk from herds with similar compositions to minimise compositional variation. Some animals were given a PLFL meal to be able to consider remnants of the last human-type meal. Further, a review of other studies reporting the kinetic parameters of curd formation and digestion found that between two and four replicates were required to reach a power of at least 0.80 at a $P < 0.05$ significance level (Montoya et al., 2018; Roy et al., 2022; Tari et al., 2018). Thus, the limitations were expected to have had minimal effects on the findings.

4.5.1. Impact of heat treatment and homogenisation on gastric emptying

Differences in gastric emptying rates of DM were apparent across milk types. Both mild (pasteurisation) and strong (UHT) heat treatments combined with homogenisation (raw vs PH, raw

vs UHT) increased the emptying rate of total DM content and curd DM content, whereas mild heat treatment alone did not (raw vs PNH). The curd DM content represented 70% of the total gastric DM content for all treatments and post-feeding times. Similar patterns in the gastric emptying of total and curd DM content and total gastric DM content were found in piglets receiving either raw bovine, ovine, or caprine milk (Roy et al., 2022). These findings agree with an observational study with one mini-pig, where similar gastric emptying patterns of DM from raw, pasteurised, and UHT bovine milk were reported (Meisel & Hagemeister, 1984). However, they did not report gastric curd emptying.

The curd is comprised mainly of casein (Pfeil, 1984) and lipid (Ye et al., 2016a), as observed here and reported elsewhere. Thus, the gastric emptying of protein and lipid in the curd can be explained by total and curd DM contents. When compared to raw milk, the gastric emptying rate of protein ($\kappa_{protein}$) in the total gastric content was affected by both mild and strong heat treatments with homogenisation (PH and UHT). However, in the curd, only UHT increased $\kappa_{protein}$. These findings imply that the combination of heat treatment and homogenisation increased the total gastric protein emptying, while only strong heat treatment influenced gastric curd emptying.

Similar patterns were observed for κ_{lipid}, suggesting that the combination of heat treatment and homogenisation impacted total gastric κ_{lipid}, whereas the heat treatment alone affected curd κ_{lipid}. For example, the κ_{lipid} of UHT was at least two-fold that of PNH and PH in both the total gastric content and the curd, but differences between κ_{lipid} for PH and PNH were only significant for total gastric content.

The faster gastric emptying of protein and lipid into the small intestine due to processing might result in faster absorption of milk nutrients. It has been shown that the amount of digested protein (nitrogen) entering the small intestine modulates the rate of absorption in the first half of the small

intestine (Montoya et al., 2018). In addition, studies have shown a faster appearance of AA in peripheral blood for healthy human adults who consumed UHT milk compared to pasteurised milk (Lacroix et al., 2008), which could have implications for protein synthesis, insulin regulation and satiety (Dangin et al., 2001; Hall et al., 2003; Tang et al., 2009).

4.5.2. Impact of processing on curd structure and link to gastric emptying

The differences observed in gastric emptying rates of DM, protein, and lipid could be ascribed to changes in the macro- and micro-structure of the formed curds. The formation of curd from all milk types was mainly induced by the pepsin hydrolysis of κ-casein, which led to the coagulation of casein micelles at around pH 6 after 20 min of gastric digestion. With the further pH decrease to pH 2.5 - 4 at 300 min of gastric digestion, the structure of the clot became denser, likely due to gastric acid diffusing into the clot and the impact of gastric motility on the curd. In addition, as pH decreases, pepsin activity increases which could have continued altering the structure of curd. These changes in the curd structure during gastric digestion were dependent on the processing condition of milk, as the initial structure of the curd was different between the differently processed milk types.

The curd of pigs fed raw, PNH, and UHT milk types formed different protein networks. When compared to raw milk, heat treatment alone (PNH) led to a more fractionated protein network, and the addition of homogenisation (PH) followed by high-intensity heat treatment (UHT) led to progressively more brittle protein networks. Similar results have been shown in an *in vitro* dynamic model and in rats fed pasteurised and UHT milk and euthanised at various time points (Ye et al., 2019).

The differences in protein networks were likely partially due to changes in the initial structure of casein micelles, the bonding of whey proteins with the caseins due to heat treatment and the enzymatic hydrolysis of the resulting protein structures during gastric digestion (Corredig & Dalgleish, 1999; Dalgleish, 1990; Ye et al., 2016b). Other studies also show that heat treatment denatures whey proteins and leads to the formation of κ-casein-whey protein aggregates, both free and associated with the surface of the casein micelles (Anema & Klostermeyer, 1997; Anema & Li, 2003). These aggregates reduce the ability of casein micelles to associate with one another through steric hindering, which in turn results in the formation of a softer, more porous curd (Ye et al., 2016a, 2016b; Ye et al., 2019).

Mild and strong heat treatments combined with homogenisation (PH and UHT) led to the formation of a soft curd. However, the extent of heat treatment had a stronger influence on curd disintegration: UHT curd weakened rapidly after 120 min, PH curd did not. Visually, the protein network of the PH curd began disintegrating after 120 min, although the curd strength was maintained. There were no differences between the raw and PNH curd strength during the formation of the curd (up to 120 min) or $\kappa_{protein}$. However, at 120 min, when the strength of the curds was the highest, it was correlated to $\kappa_{protein}$, suggesting that it is one of the factors influencing $\kappa_{protein}$.

The faster curd disintegration and gastric emptying rate of protein could be explained by the weaker, more open curd structure resulting from the protein denaturation during heat treatment. Based on other studies, the more open structure could allow a greater surface area for enzymatic activity (Van Hooydonk, 1987) and a greater ingress of gastric fluid containing pepsin and hydrochloric acid (Kalantzi et al., 2006) into the curd. In another study, pigs fed raw, PH, and UHT bovine milk (one pig per diet and time point) had slower hydrolysis of casein in a stronger curd, resulting in slower gastric emptying of casein, compared to a weaker curd (Pfeil, 1984).

The pigs fed the PH or UHT milk treatment had smaller fat globules as homogenisation disrupted the native fat globules, forming small fat droplets (Robson & Dalgleish, 1984), resulting in a looser curd structure and faster total gastric κ_{lipid}, compared to the raw and PNH milk treatments. The cryo-SEM images of the gastric curd showed fat globules suspended amongst networks formed by the protein matrix in the raw milk curd. For the homogenised milk (PH and UHT) treatments, the small fat droplets were incorporated into the protein network, which occurred during curd formation via casein micelle aggregation by adsorption onto and around the fat droplets (Ye et al., 2017). The wide distribution of small fat droplets incorporated throughout the PH and UHT curd protein networks may have influenced curd integrity and strength by restricting the linking of the casein matrix (Gallier et al., 2012), as shown in curd used for cheese production (Kelly et al., 2008) and under gastric conditions (Wang et al., 2018). The smaller surface area of the homogenised fat droplet may have also impacted curd disintegration and subsequent emptying by indirectly increasing proteolysis, as shown in an *in vitro* comparison of non-homogenised and homogenised infant formula (Bourlieu et al., 2015).

The curd from the pigs fed the unhomogenised (raw and PNH) milk treatments had greater gastric lipid coalescence due to the tightening of the casein matrix (Gallier et al., 2013). In addition, the coalesced lipids of these milk types were entrapped within the rigid and intact protein matrix of the curd, resulting in a higher concentration of lipid remaining in the curd and a subsequent smaller κ_{lipid} when compared to UHT. The $T_{1/2}$ lipid of raw curd was two-fold larger than that of PNH curd, indicating that pasteurisation increases the release of lipid into the small intestine.

4.6. Conclusions

This study demonstrated the impacts of commercial processing treatments of bovine milk on the gastric emptying of DM, protein, and lipid and curd formation in a pig model of adult human

metabolism. The UHT milk treatment had the greatest impact on gastric emptying of DM and protein: it was faster with UHT treatment, followed by PH and PNH treatments, compared to raw milk. In addition, the gastric emptying of lipid was also faster in the pigs fed the PNH treatment than the raw milk treatment.

The differences in gastric emptying of DM, protein and lipid between processing treatments were ascribed to differences in the structure of the curds formed and their disintegration. In particular, heat treatments resulted in a looser protein network, and homogenisation led to the incorporation of restructured lipid droplets into the curd matrix.

This study provided a new understanding of the gastric emptying of processed bovine milk DM, protein, and lipid. Whether these effects can improve nutrient absorption in the small intestine and subsequent release into the systemic circulation for metabolism remains to be confirmed.

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CHAPTER 5.

HEAT TREATMENT AND HOMOGENISATION INFLUENCE THE GASTRIC DIGESTION OF BOVINE MILK PROTEIN IN GROWING PIGS AS AN ADULT HUMAN MODEL

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HIGHLIGHTS

- Heat treatment increased the rate of apparent gastric protein hydrolysis and the gastric emptying rate of digested protein.
- Ultra-high temperature treatment increased the total amount of digested protein emptied from the stomach at 300 min postprandially.
- The gastric emptying of amino acids was mainly affected by homogenisation.

Approximately 70% of the work contained in this chapter was contributed by the student.

5.1. Abstract

Globally, milk is processed to extend shelf life and presentation. Processing influences the structure of the curd formed during gastric digestion, which may alter gastric milk protein hydrolysis and impact amino acid (AA) release into the small intestine. The present study hypothesised that a weaker, looser curd formation could increase gastric protein hydrolysis, and lead to a faster gastric emptying of digested protein and AA into the small intestine. Nine-week-old growing pigs (n=180) consumed either raw, pasteurised non-homogenised (PNH), pasteurised-homogenised (PH), or ultra-high-temperature homogenised (UHT) bovine milk for ten days. Gastric solid and liquid fractions were collected at 0, 20, 60, 120, 180, and 300 min postprandially. The apparent degree of gastric protein hydrolysis, apparent gastric disappearance of individual proteins, and the gastric emptying of digested protein and AA were determined. During the first 60 min, the rate of gastric protein hydrolysis was fastest for pigs fed UHT milk (0.29%/min vs on average 0.07%/min for pigs fed raw, PNH and PH milk). Differences in the degree of gastric protein hydrolysis were reflected in the rate of digested protein entering the small intestine. The AA gastric half-time was generally shorter for pigs fed PH and UHT milk than pigs fed raw and PNH milk. For example, the gastric release of total essential AA was more than two-fold faster (P<0.01) for pigs fed PH and UHT milk compared to pigs fed raw or PNH milk. In conclusion, although heat and homogenisation influenced the gastric protein digestion parameters, homogenisation was the main process affecting the rate of AA entering the small intestine.

Keywords: bovine milk, processing, gastric hydrolysis, gastric protein digestion, amino acids

5.2. Introduction

Globally, milk is an essential source of macro- and micro-nutrients; of these components, the protein content is an important driver of its nutritional value. Bovine milk protein is comprised of caseins and whey proteins, which represent around 80% and 20% of the total milk protein, respectively (Lucey et al., 2017). When raw milk is consumed, the milk caseins (α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein) coagulate under the action of pepsin in the stomach, forming a solid (curd) phase which is susceptible to enzymatic degradation (Ye et al., 2019). In contrast, the whey proteins (β-lactoglobulin and α-lactalbumin) do not coagulate and are largely resistant to pepsin hydrolysis (Kitabatake & Kinekawa, 1998). However, in most Western countries, milk is mostly consumed after undergoing commercial processing, which is usually a combination of heat treatment and homogenisation.

Both heat treatment and homogenisation have been shown to influence bovine milk gastric curd formation (Chapter 4; Ahlborn et al., 2023a; Mulet-Cabero et al., 2019), as a result of processing-induced changes to the native structure of milk proteins and their assemblies (Gallier et al., 2013; Ye et al., 2016a, 2016b). The specific conditions under which bovine milk is processed (e.g., temperature, time, pressure) play a role in determining the extent of the changes. For example, high-heat treated (140 °C) homogenised bovine milk forms a loose, soft curd, whereas the curd formed by mild-heat treated (75 °C) homogenised milk was comparatively stronger, with a tighter protein network (Chapter 4; Ahlborn et al., 2023a; Ye et al., 2019).

Processing-related changes to the curd structure are thought to influence the total gastric protein hydrolysis and emptying, and the gastric digestion of individual proteins of differently processed milk types. For example, high heat treatment combined with homogenisation increased the gastric disappearance of casein *in vitro* (Mulet-Cabero et al., 2019; Ye et al., 2019), and also increased the

rate of total protein gastric emptying *in vivo* (Chapter 4; Ahlborn et al., 2023). These studies suggested that a looser curd structure combined with processing-induced changes in the native protein structures (e.g., denaturation) could facilitate enzymatic hydrolysis by proteases of both host (pepsin) and/or milk (e.g., plasmin) origin.

Differences in both gastric protein hydrolysis and protein emptying of processed bovine milk could result in different releases of digested protein into the small intestine. Both the rate and amount of protein leaving the stomach have been shown to influence total nitrogen and amino acid (AA) absorption in the small intestine (Gaudichon et al., 1994; Montoya et al., 2018), which can alter the appearance of AA in the circulation (Boirie et al., 1997; Dangin et al., 2001). However, the impact of processing on whole bovine milk gastric protein hydrolysis and the release of digested protein and AA into the small intestine is unknown.

Based on the softer curd structure, more open protein network, greater protein hydrolysis, and faster gastric emptying of protein reported for heated, homogenised milk during digestion, it was hypothesised that mild or strong heat-treatment combined with homogenisation increase the flow of digested protein and AA into the small intestine. To test this hypothesis the apparent gastric degree of total protein hydrolysis, the gastric emptying of digested protein, the apparent gastric disappearance of individual proteins and the gastric emptying of AA following consumption of raw, pasteurised non-homogenised (PNH), pasteurised homogenised (PH), or ultra-high temperature treated homogenised (UHT) bovine milk was determined in pigs at 0, 20, 60, 120, 180, and 300 min postprandially. The growing pig was selected as a model for the adult human, due to similarities in the gastrointestinal tract of both species (Guilloteau et al., 2010; Moughan et al., 1992; Sciascia et al., 2016). In addition, a ‘meal’ feeding approach can be used in pigs as they have

the digestive capacity and adaptability required for ‘meal-eating’ similar to that of an adult human (Miller & Ullrey, 1987; Rowan et al., 1994).

5.3. Materials and Methods

5.3.1. Animals, dietary treatments, and experimental design

This study was approved by the Massey University Animal Ethics Committee (application no. 19/83). The animals, housing, dietary treatments, and experimental design of the pig study have been reported previously (Chapter 4; Ahlborn et al., 2023a). In brief, 144 locally sourced Large White × Landrace entire male pigs (bodyweight 22.4 ± 0.13 kg, mean ± standard error of the mean (SEM)), were housed in individual metabolic crates at the Animal Production Unit of Massey University, Palmerston North.

The experimental treatments were raw, PNH, PH, or UHT bovine milk. The PH, PNH, and UHT milk types were commercially available processed products, while the raw milk was locally sourced (Gorge Fresh Organics, Palmerston North, New Zealand). The raw, PH, and UHT milk were fed as sourced, whereas PNH was prepared using simple gentle manual agitation to combine pasteurised trim milk with pasteurised cream to the same lipid content as the PH milk. A human-type meal was also formulated to emulate a typical Western diet, using the USDA's chemical composition data of each ingredient to meet the NRC nutrient requirements for growing pigs (NRC, 1998). The preparation and composition of the dietary treatments and human-type meal have been reported previously (Chapter 4; Ahlborn et al., 2023a).

The pigs were randomly distributed between the four dietary treatments (36 pigs per experimental diet; 6 pigs per experimental diet x time point combination).

After arrival to the facility, the pigs underwent a three-day gradual dietary transition to the experimental diets, after which they consumed their milk treatments (breakfast; 500 mL of raw, PH, PNH, or UHT) and human-type meals (lunch and dinner) for seven days. On sampling day, fasted pigs (16 h) were either euthanised at time 0 min or fed 500 mL of milk type for breakfast before being euthanised at either 20, 60, 120, 180, or 300 min. The indigestible marker titanium dioxide (TiO_2, 0.38 ± 0.3 g) (Sigma Aldrich, St. Louis, MO, USA) was included in the breakfast meal to estimate meal flow throughout the small intestine (results not reported here).

Each pig was anesthetised 15 min before its euthanasia time with a mix of Zoletil 100 (zolazepam and tiletamine, both 50 mg/mL; Virbac, Hamilton, NZ) reconstituted with 2.5 mL Ketamine and 2.5 mL Xylazine, both 100 mg/mL (Phoenix Pharm NZ, Auckland, New Zealand). The final solution contained 50 mg/mL of each drug and was administered at a dosage of 30 - 40 µL of the mixed solution/kg BW by intramuscular injection in the neck area. Following sedation, each pig was intravenously administered a second dose of the cocktail (30 µL/kg BW) to induce deep anaesthesia. Once anaesthetised, the pigs were euthanised by an intracardiac injection of sodium pentobarbitone (0.3 mL/kg BW of Pentobarb 300, Provet NZ Pty Ltd, Christchurch, New Zealand).

Sample collection has been previously described (Chapter 4; Ahlborn et al., 2023a). In brief, the abdomen was opened, and the stomach was clamped and removed with minimal movement, washed, dried, and weighed full before being opened by an incision along the lesser curvature. The solid (curd) and liquid fractions were separated using a 1 mm sieve. Gastric solid and liquid sub-samples were collected for various other analyses, and the remaining material of each fraction was thoroughly mixed, immediately frozen, freeze-dried, weighed, and ground. Freeze-dried and ground chyme (solid and liquid fractions) and milk samples were defatted using a diethyl-

ether/petroleum ether extraction method described elsewhere (Chapter 4; Ahlborn et al., 2023a). Samples were stored at -20 °C until analysis.

5.3.2. Apparent degree of total gastric protein hydrolysis

The apparent degree of total gastric protein hydrolysis was determined by measuring the free amino groups in the total gastric chyme and gastric solid and liquid fractions. The free and total amino groups present in each milk type were also measured. All samples were defatted. No correction for the endogenous production of free amino groups was made; thus, only the apparent degree of total gastric protein hydrolysis is reported.

The free amino groups in the chyme fractions were then quantified using the *o*-phthaldialdehyde method (Church et al., 1983; Montoya et al., 2014). To quantify the total amino groups in each milk type, samples were hydrolysed for 24 h in HCl 6 M at 110°C. The resulting hydrolysed samples were dried using a vacuum evaporator, followed by two water washes to remove traces of HCl. The final dried samples were then analysed for amino groups as described above.

5.3.3. Amino acid analysis

Defatted chyme and milk samples were analysed for AA content by 24 h hydrochloric acid hydrolysis with *o*-phthaldialdehyde pre-column derivatisation, followed by reverse-phase chromatography (Rutherfurd et al., 2012). Because of their role in human health, the physiologically relevant AA groups (branched-chain AA (BCAA); long neutral AA (LNAA); essential AA (EAA); non-essential AA (NEAA); total AA (TAA)) were also calculated for each chyme and milk sample.

5.3.4. Electrophoresis analysis (SDS-PAGE)

The apparent gastric disappearance of milk proteins in the curd and liquid samples were analysed using reducing sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). As no correction was done for endogenous proteins, only the apparent disappearance of each protein was reported.

Defatted chyme and milk samples were mixed with tricine sample buffer (0.2 M Tris-HCl buffer pH 6.8, 4.3 M glycerol, 69.4 mM SDS) containing β-mercaptoethanol (1:100 w:v) for 60 min. The samples were then held at 100 °C for 10 min before being centrifuged at 4,000 rpm for 5 min at room temperature. Extracted samples were loaded onto 16.5% tris-tricine precast gels (Bio-Rad Laboratories Pty Ltd., Auckland, New Zealand) and run using a Criterion cell (Bio-Rad Laboratories Pty Ltd., Auckland, New Zealand). The well loadings (10 – 30 µL) were adjusted according to the dietary treatment and time point of each sample to avoid band oversaturation. A molecular weight standard ladder (Mark 12, 2.5 – 200 kDa, Invitrogen NZ Ltd, Auckland, New Zealand) and a control milk sample were loaded in each gel. Gels were run at a constant voltage of 125 V for 105 min and stained in Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories Pty Ltd., Auckland, New Zealand). De-stained gels were imaged using a GelDoc XR (Bio-Rad Laboratories Pty Ltd., Auckland, New Zealand). ImageLab (software version 6.1, Bio-Rad Laboratories Pty Ltd., Auckland, New Zealand) was used to identify intact proteins against the molecular weights of the ladder, and the band intensities were then measured horizontally. Band intensities were reported in arbitrary density units. As known total protein quantities were loaded into each lane, the intensity of each band in milk and chyme (solid plus liquid fractions) was calculated using the grams of total protein in each sample to semi-quantitively estimate the total apparent band intensity for each identified protein in each sample.

5.3.5. Calculations

In some pigs, part of the pre-fast meal (human-type diet) remained in the stomach despite the 16 h fasting period. Pigs with gastric chyme which contained more than 10% of the pre-fast meal at sample collection were excluded from all analyses, as the remnant material could alter the curd (e.g., formation, structure, and digestion) and subsequent gastric emptying (Bornhorst et al., 2014). Based on this, 19 pigs were removed (raw, five pigs; PNH, one pig; PH, six pigs; UHT, seven pigs).

The formation, strength, and structure of the gastric curd have been shown to impact the gastric emptying of total protein from processed bovine milk, as a large portion of the protein from processed milk is retained in the gastric curd during digestion (Chapter 4; Ahlborn et al., 2023a; Ye et al., 2019). Thus, it is important to understand the disappearance of individual proteins and AA from the gastric curd as well as the total chyme. Therefore, the degree of gastric hydrolysis, apparent gastric individual protein disappearance, and gastric AA emptying of the curd and total chyme are reported. The AA content and gastric protein disappearance in the liquid fraction were also measured and used in the results of the total chyme only, as the rapid gastric emptying of the liquid phase did not allow reliable modelling of the gastric AA emptying and the disappearance of individual proteins from the stomach. The degree of gastric hydrolysis of the liquid fraction is also reported.

The apparent degree of gastric protein hydrolysis was calculated as the number of free amino groups (free NH_2) in the chyme relative to the total amino groups (total NH_2) in the milk. However, over time, the chyme is released into the small intestine and its chemical composition changes. Thus, in the calculations, the total and free NH_2 were expressed per g protein of the chyme sample.

Based on this, the apparent degree of total protein hydrolysis in the stomach at each time point was calculated as follows (apparent degree of hydrolysis in chyme at 20 min as an example):

$$\text{Apparent degree of protein hydrolysis}_{20\ \text{min}}\ (\%) =$$

$$(\text{free } NH_{2\ 20\ \text{min}} / \text{ total retained } NH_{2\ \text{Milk, 20 min}}) \times 100$$

where free NH_2 is the apparent amount of free amino groups in each gastric fraction (liquid or solid) alone or combined (total chyme) per g of protein retained in that fraction, and retained total $NH_{2\ \text{Milk}}$ is the apparent amount of total amino groups from the milk per g of protein retained in the stomach.

The amount of free amino groups in the milk before digestion were subtracted from both the free NH_2 after each post-feeding time in the chyme and the total NH_2 in the milk to calculate a corrected degree of hydrolysis as follows (corrected apparent degree of hydrolysis in chyme at 20 min as an example):

$$\text{Corrected apparent degree of protein hydrolysis}_{20\ \text{min}}\ (\%) =$$

$$(\text{Free } NH_{2\ \text{Chyme, 20 min}} - \text{free } NH_{2\ \text{Milk, 20 min}} / \text{ Total } NH_{2\ \text{Milk, 20 min}} - \text{free } NH_{2\ \text{Milk, 20 min}}) \times 100$$

The apparent amount of free amino groups from the milk remaining in the stomach at each time point was adjusted using the amount of protein retained as described above for the total amino groups. The corrected degree of hydrolysis is only reported for the total gastric chyme, as it was not possible to determine the amount of free amino groups of the milk remaining in the solid and liquid fractions before digestion.

The amount of digested protein released into the small intestine was calculated as reported by Montoya et al. (2018):

$$\text{Digested protein released into the small intestine}_{20\ \text{min}}\ (\text{g}) = \text{protein released}_{20\ \text{min}}\ (\text{g}) \times \text{coefficient of total apparent hydrolysis}_{20\ \text{min}}$$

The apparent degree of protein hydrolysis in the stomach and the release of digested protein into the small intestine represent the overall gastric protein digestion. However, milk protein is composed of different casein and whey proteins (Roy et al., 2022). As the curd rheological and structural properties and amounts formed during gastric digestion differed across processed milk types (Chapter 4; Ahlborn et al., 2023a), it is expected that within each milk, individual proteins are digested and emptied at different rates. The SDS-PAGE method was used to determine the digestion of individual bovine milk proteins (αs_2-casein, αs_1-casein, β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin), This semiquantitative method allowed investigation of the disappearance of the selected milk proteins from the stomach of pigs as described elsewhere (Montoya et al., 2014). Concerning the apparent disappearance of individual proteins, it is important to consider the limitations of the methodology as described elsewhere (Montoya et al., 2014). For example, the amount of the individual proteins in each chyme and milk sample was estimated using the band intensity. Thus, the outcomes of apparent disappearance should be interpreted with caution.

The apparent disappearance of intact individual proteins from the stomach was calculated as follows (αs_2-casein in chyme at 20 min as an example):

$$\text{Apparent disappearance}_{\alpha s2\text{-casein, 20 min}}\ (\%) =$$

$$(\text{chyme band intensity}_{\alpha s2\text{-casein, 20 min}}\ (\text{arbitrary density units})/\ \text{milk band intensity}_{\alpha s2\text{-casein}}\ (\text{arbitrary density units}))\ x100$$

For each milk type, distinct curds were formed after 20 min of digestion, and it has been shown that caseins preferentially remain in the curd, whereas whey proteins are found in the liquid fraction (Roy et al., 2022). Thus, for the curd, the gastric disappearance of each protein band was calculated by considering the estimated total band intensity of each individual protein at 60, 120, 180, and 300 min relative to the total band intensity of that individual protein in the curd at 20 min.

The different curd properties and amounts remaining in the stomach over time across processed bovine milk types as described above could have resulted in different amounts of AA released into the small intestine. Thus, the gastric emptying rate of individual and total AA was calculated as follows (EAA in chyme at 20 min as an example):

$$\text{EAA retention}_{20\text{ min}}\ (\%) = (\text{Chyme EAA}_{20\text{ min}}\ (\text{mg}) / \text{Milk EAA}_{20\text{ min}}\ (\text{mg})) \times 100$$

5.3.6. Statistical analysis

The study was designed to investigate the gastric digestion of milk, as well as the small intestinal absorption and the appearance of AA in the circulatory system. Thus, the sample size selected considered the greatest required number of animals for several of the response variables. Based on previous studies with similar outcomes (Butteiger et al., 2013; Chen et al., 1962; Gaudichon et al., 1994; Montoya et al., 2018), six pigs were required to detect a difference in mean values of each parameter, powered over 80% at $P < 0.05$.

A linear model including milk type, time and their interaction as fixed effects was used to analyse the apparent gastric degree of total protein hydrolysis and the apparent release of digested protein into the small intestine. Models using time as a categorical or numerical variable were tested. In all cases the best fitting model used time as a numerical variable. The log-likelihood ratio test was used to select the best polynomial model. Factors which were not significant were removed from the model, except for each main factor.

For the individual proteins, the gastric apparent disappearance half-time (i.e., time taken for the intensity of each individual protein band in the milk to halve) was calculated using the Michaelis-Menten non-linear model as described by Montoya et al. (2014):

$$\text{Apparent gastric disappearance (\%)} = \alpha \times \text{time}/(\beta + \text{time})$$

where α is the maximum possible disappearance (i.e., 100%), and β is the half-time of the fitted curve. The gastric disappearance of individual proteins from the curd was modelled as described above, except that α (100%) was the intensity of each individual protein band in the curd at 20 min.

The power exponential model was used to analyse the retention of individual and total AA (mg AA/g protein consumed) in the stomach as detailed by Montoya et al. (2014) (EAA as an example):

$$\text{Gastric retention}_{\text{EAA}}\ (\text{mg}) = \alpha_0 \exp - (\kappa \times \text{time})^{\beta}$$

where the parameter α_0 is the amount of consumed AA (mg), κ is the slope of the curve (mg/min), β is a dimensionless index for the shape of the curve, and time is in minutes. The parameters κ and β were then used to determine the gastric emptying half-time ($T_{1/2}$):

$$\text{Gastric } T_{1/2\,\text{EAA}}\ (\text{min}) = (1/\kappa) \times (\log[1/0.5])^{(1/\beta)}$$

For the gastric AA emptying of the curd, it was assumed that the initial values (i.e., α_0) were the same as the milk consumed.

For all non-linear models, the full (i.e., individual model for each milk type) and the reduced model (i.e., a single model for all milk types) were fitted, and an *F* test was used to identify the best fitting model. For each statistical analysis, the normal distribution and the homogeneity of variance were evaluated. The parameters (κ, β, $T_{1/2}$ for gastric AA retention; β for gastric protein disappearance) were compared using a t-test, and the difference was declared significant if $P \leq 0.05$.

5.4. Results

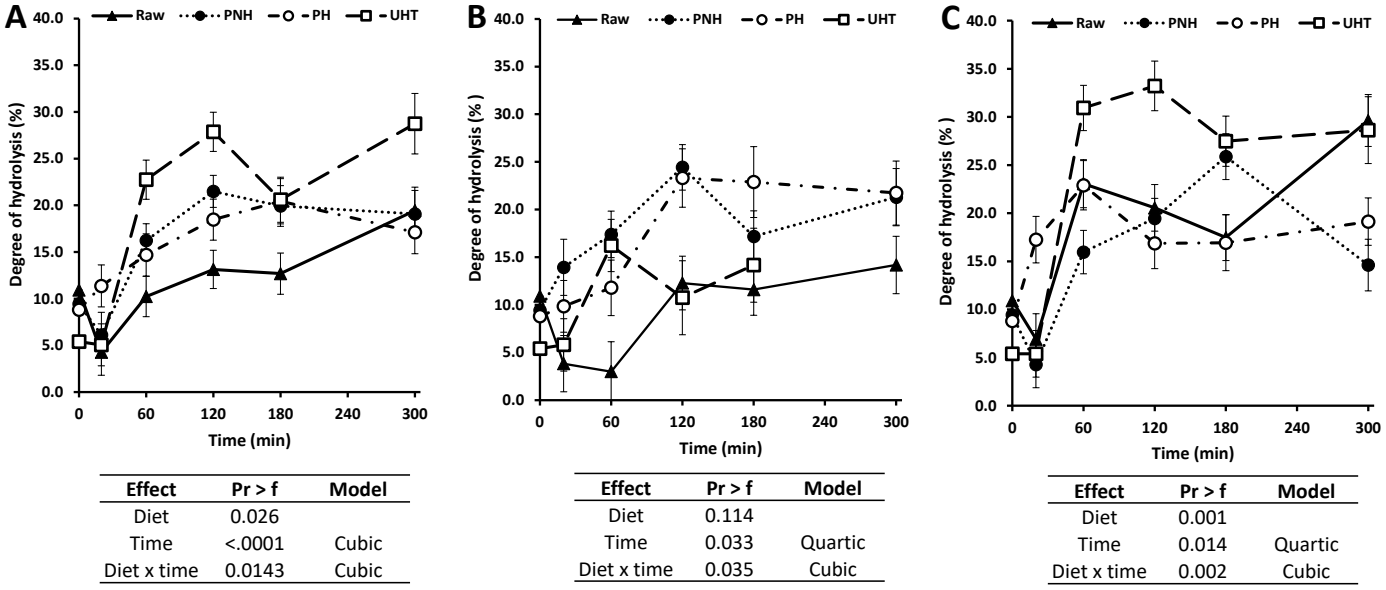
The pigs adapted well to the experimental diets, and after the third day they consumed all meals offered. One pig was excluded during the study due to coprophagia. Throughout the study, the remaining pigs were healthy. For all milk types, the milk in the stomach separated into two distinguishable gastric fractions (solid and liquid) within 20 min of digestion. The macro- and microstructure of the curd (solid fraction) and the gastric emptying of dry matter, total protein, and total fat from each fraction varied between milk types, as discussed elsewhere (Chapter 4; Ahlborn et al., 2023a). After 300 min of digestion, there was insufficient gastric content in five of the six pigs fed the UHT milk to conduct any analyses. For these pigs, in all analyses, the amount of free amino groups, AA, and individual proteins was set to zero.

5.4.1. Apparent degree of protein hydrolysis in the stomach

For the total chyme, and the curd and liquid fractions in the stomach, the interaction between diet and time had a significant effect ($P \leq 0.05$) on the apparent degree of protein hydrolysis (Figure 5.1A - C). Within the total chyme and the liquid phase, the apparent degree of protein hydrolysis

in the stomach of pigs fed UHT milk increased at a rate of 0.3%/min and 0.4%/min within the first 60 min of digestion respectively, whereas the apparent degree of protein hydrolysis in the total chyme and liquid phase of the other processed milk types (PH and PNH) increased on average 0.1%/min and 0.2%/min respectively.

Throughout the digestion time, the degree of gastric protein hydrolysis in the pigs fed UHT milk remained in general higher than other milk types until 300 min of digestion. In the curd, the apparent degree of gastric protein hydrolysis of pigs fed PNH and PH increased at an average of 0.25%/min up to 120 min, whereas for pigs fed raw milk the apparent degree of hydrolysis increased at 0.02%/min.



Effect	Pr > f	Model
Diet	0.026	
Time	<.0001	Cubic
Diet x time	0.0143	Cubic

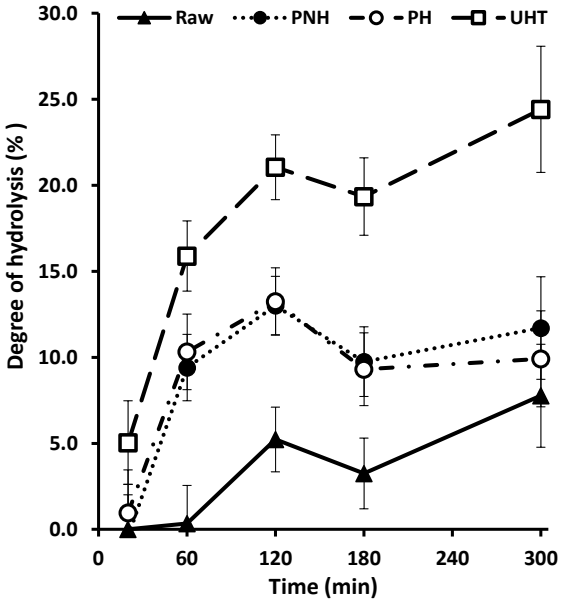
Effect	Pr > f	Model
Diet	0.114	
Time	0.033	Quartic
Diet x time	0.035	Cubic

Effect	Pr > f	Model
Diet	0.001	
Time	0.014	Quartic
Diet x time	0.002	Cubic

Figure 5.1: Apparent degree of hydrolysis of total gastric chyme (**A**), curd fraction (**B**), and liquid fraction (**C**) retained at different post-feeding times of pigs fed different processed bovine milk types.
PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 4 – 6. The starting values are the proportion of total amino groups that were free in each milk before they were given to the pigs. At 300 min, insufficient UHT curd remained for analysis.

When the apparent degree of gastric protein hydrolysis in the total chyme was corrected for the free amino groups present in the milk before being consumed, the interaction effect between diet and post-feeding time disappeared ($P > 0.05$). At each time point, the degree of gastric protein hydrolysis was higher for pigs fed UHT milk than those fed any other milk ($P < 0.05$; Figure 5.2).

The degree of protein hydrolysis between 20 and 60 min for the pigs fed raw milk increased at 0.5%/h, whereas it increased on average 15%/h for pigs fed the other milk types. These differences remained up to 180 min, but by 300 min the apparent degree of gastric protein hydrolysis of pigs fed raw milk was similar to that of pigs fed PNH and PH milk, which were below that of pigs fed UHT milk.

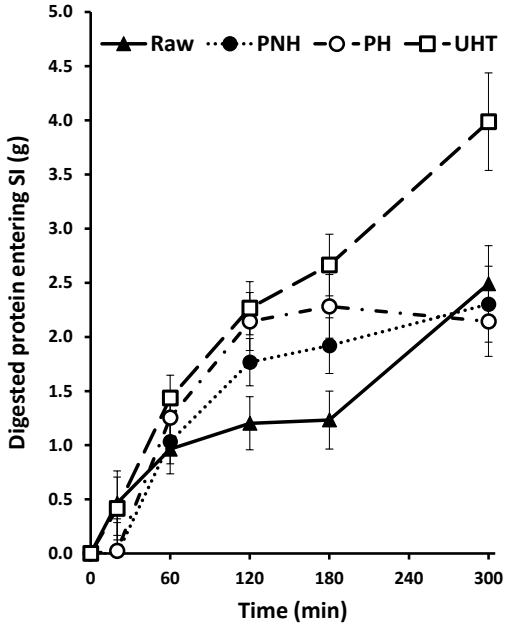


Effect	Pr > f	Model
Diet	0.022	
Time	0.026	Quartic
Diet x time	0.1002	

Figure 5.2: Apparent degree of hydrolysis in total gastric chyme retained at different post-feeding times for pigs fed different processed bovine milk types, corrected for free amino groups present in each milk before being consumed.
PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 4 – 6.

5.4.2. Apparent release of digested protein into the small intestine

Throughout the digestion time, differences in the apparent amount of digested protein that entered the small intestine were observed between pigs fed the different milk types ($P \leq 0.05$; Figure 5.3). Between 20 and 120 min, the apparent rate of digested protein entering the small intestine increased rapidly (on average 1.0 g/h) for pigs fed PNH, PH and UHT milk, compared to pigs fed raw milk (0.4 g/h). After 120 min, the apparent rate of digested protein entering the small intestine of pigs fed UHT milk continued to increase, whereas the apparent rate of digested protein entering the small intestine of pigs fed PNH and PH plateaued. At 300 min, the amount of digested protein entering the small intestine was similar between pigs fed raw, PNH, and PH milk.



Effect	Pr > f	Model
Diet	0.31	
Time	0.04	Cubic
Diet x time	0.04	Quadratic

Figure 5.3: Apparent amount of digested protein that entered the small intestine at different post-feeding times for pigs fed different processed bovine milk types.
PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 4 – 6.

5.4.3. Gastric disappearance of individual milk proteins

For all milk types, bands at around 25 – 35, 21 – 24, 19 – 21, 16 – 18, and 13 – 15 kDa were found (Appendix 3, Figure A3.1a - d). The bands were identified as αs_2-casein, an overlap of αs_1-casein and β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin respectively, based on previously reported molecular weights for bovine milk proteins in SDS-PAGE gels (Bär et al., 2019; Hinz et al., 2012; Ye et al., 2016b). As expected, casein proteins (αs_2-casein, αs_1-casein/β-casein, and κ-casein) were mainly present in the gastric curd, whereas the whey proteins (β-lactoglobulin, and α-lactalbumin) were mainly present in the liquid phase.

The apparent disappearance of αs_2-casein, αs_1-casein/β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin from the total gastric chyme at 300 min of gastric residence time was on average 89%, 93%, 85%, 98%, and 100% respectively (Figure 5.4a - e**)**. The apparent gastric disappearance half-times of the combined αs_1-casein and β-casein from the total chyme was similar between pigs fed raw, PNH, and PH milk, but longer than for those fed UHT milk (Figure 5.5a - d).Thus, for raw, PNH and PH milk more time was needed for half of the consumed αs_1-casein/β-casein to leave the stomach, compared to UHT milk. The apparent gastric disappearance half-time of κ-casein was more than three-fold shorter ($P < 0.01$) for pigs fed UHT milk than for those fed raw or PNH milk, and at least four-fold longer for pigs fed raw milk than pigs fed the other milk types. Pigs fed PH milk had a minimum two-fold longer ($P \leq 0.01$) β-lactoglobulin apparent gastric disappearance half-time than pigs fed the other milk types, for which there was no difference ($P > 0.05$). There was no difference ($P > 0.05$) in the apparent gastric disappearance half-time of αs_2-casein or α-lactalbumin from the total chyme of pigs fed the different milk types.

For the curd, at 300 min of gastric residence time, between 84% and 97% of the curd casein content observed at 20 min had apparently disappeared (Figure 5.6). The apparent gastric disappearance

half-time of αs_1-casein and β-casein from the curd was three-fold shorter ($P \leq 0.01$) for pigs fed UHT than the other milk types, whereas for raw, PNH and PH the half-time was similar ($P > 0.05$) (Figure 5.7). The curd apparent gastric disappearance half-time of κ-casein was at least two-fold longer ($P \leq 0.01$) for pigs fed PH milk, than those fed the other milk types.

Within each milk type, differences in the total chyme apparent gastric disappearance half-times were observed across individual proteins (Figure 5.5). In general, the observed patterns were consistent for all milk types. For example, the apparent gastric disappearance half-time of β-lactoglobulin and α-lactalbumin was shorter than any other individual protein, for each milk type.

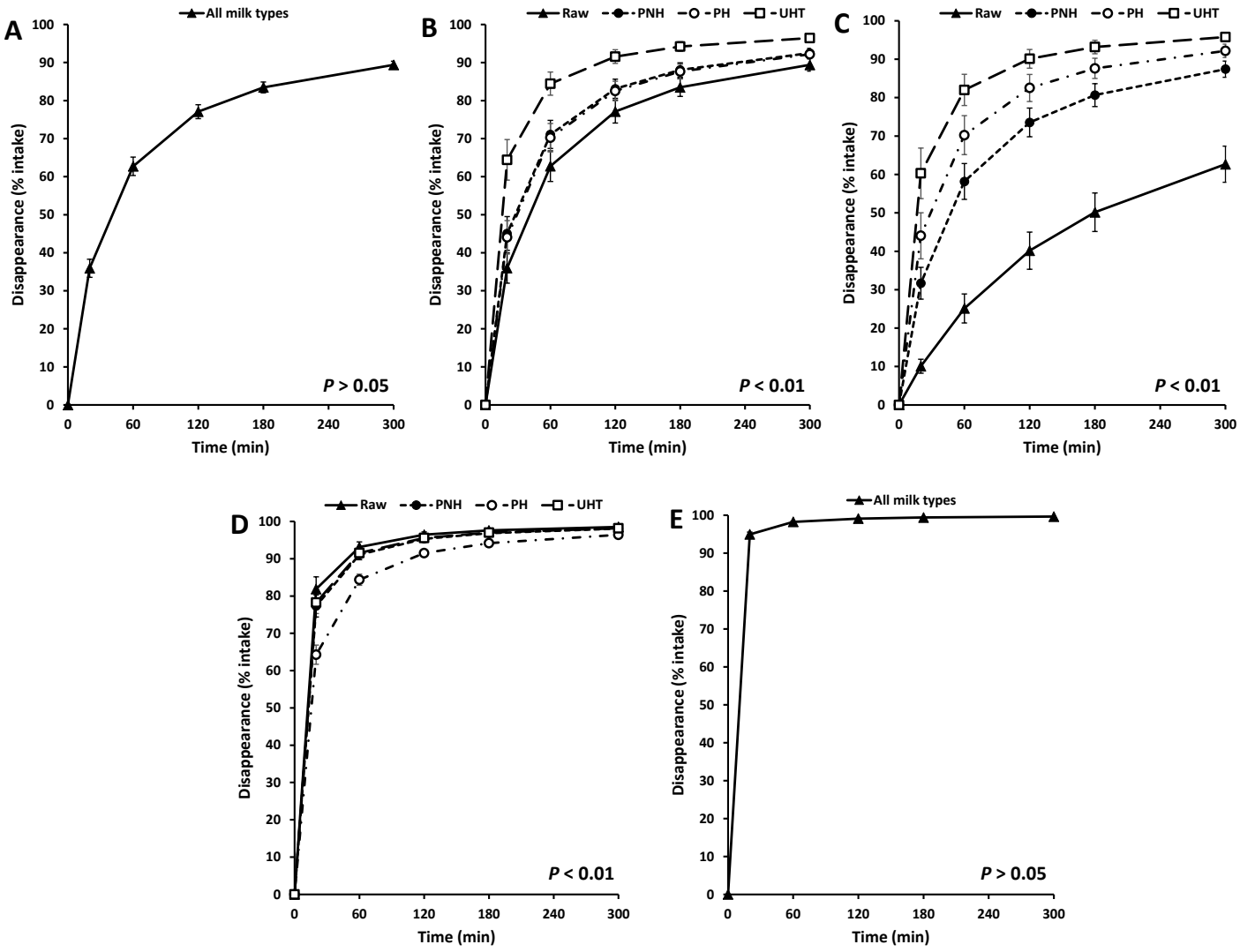


Figure 5.4: Apparent disappearance of individual proteins in total gastric chyme collected at different post-feeding times from growing pigs fed different processed bovine milk types. **A:** αs_2-casein; **B:** combined αs_1-casein and β-casein; **C:** κ-casein; **D:** β-lactoglobulin; **E:** α-lactalbumin. PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 4 – 6. Probability values in each panel reflect a comparison between fitted curves of apparent gastric disappearance across milk types. There was no difference ($P > 0.05$) in the disappearance of αs_2-casein and α-lactalbumin from pigs fed the different milk types.

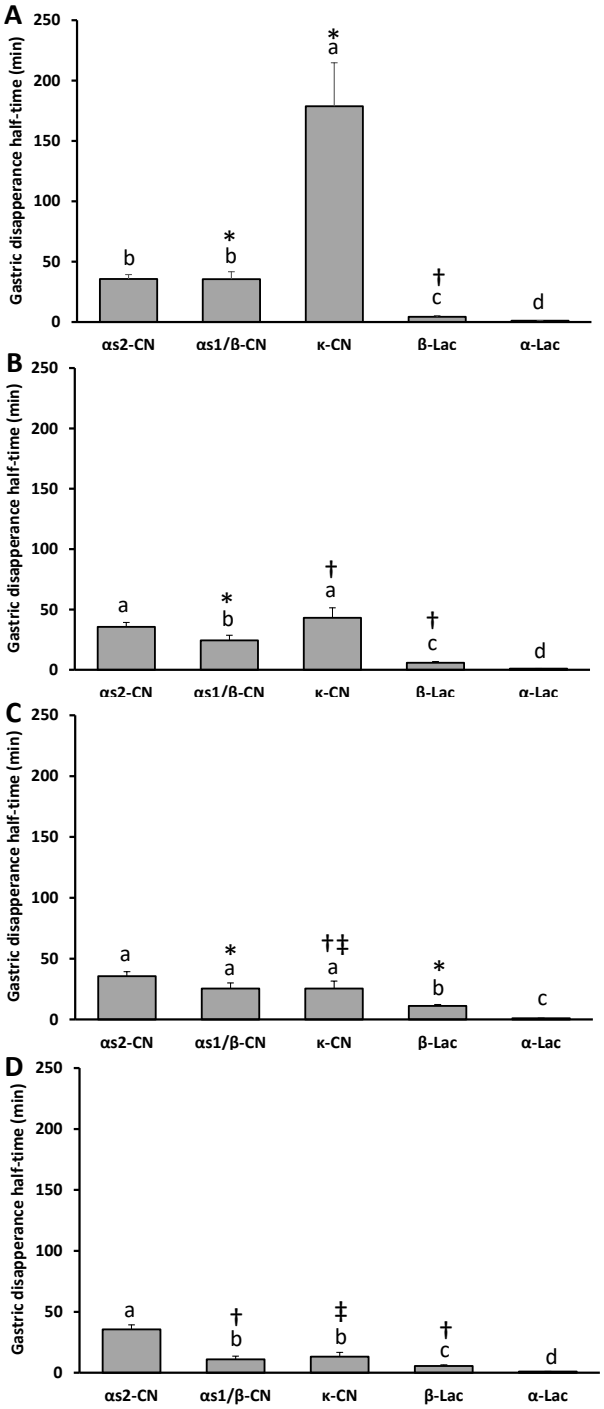


Figure 5.5: Half-time ($T_{1/2}$) of individual proteins which apparently disappeared from total gastric chyme collected at different post-feeding times of growing pigs fed different processed bovine milk types.
A: raw; **B:** pasteurised non-homogenised; **C:** pasteurised homogenised; **D:** ultra-high temperature treated homogenised. Proteins with different symbols (*, †, ‡) have a different half-time across milk types ($P \leq 0.05$). Proteins with different letters (a, b, c, d) have different half-time within each milk type ($P \leq 0.05$). αs2-CN, αs_2-casein; αs_1/β-CN, combined αs_1-casein and β-casein; κ-CN, κ-casein; β-lac, β-lactoglobulin; α-lac, α-lactalbumin. Values are means ± SEM, n = 4 – 6.

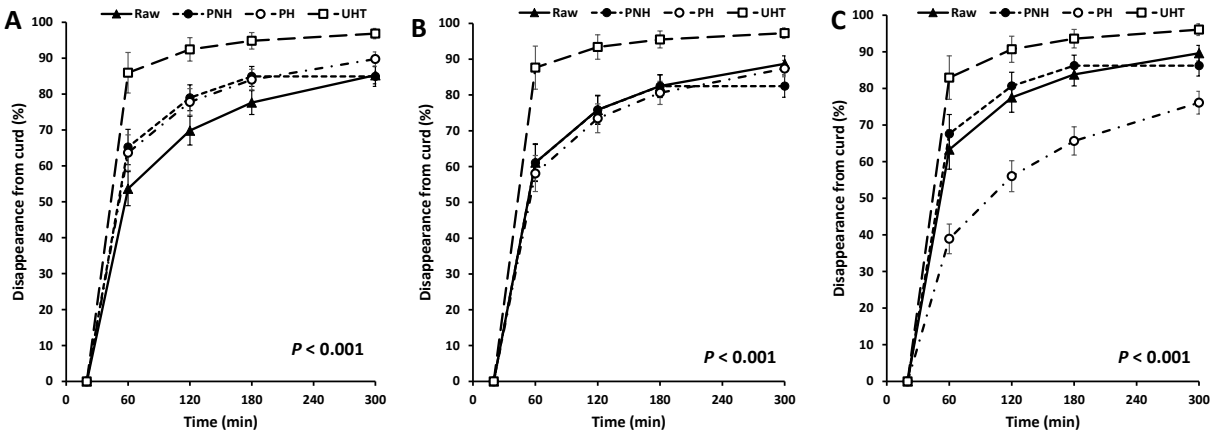


Figure 5.6: Apparent disappearance of individual proteins from gastric curd (solid) collected at different post-feeding times of growing pigs fed different processed bovine milk types.
At 20 min of digestion distinct curds were formed for all milk types. Thus, the concentration of each individual protein at 20 min was used as the initial (or 100%) value. No whey proteins were identified in the gastric curd. **A:** αs_2-casein; **B:** αs_1-casein and β-casein; **C:** κ-casein. PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 4 – 6. Probability values in each panel reflect a comparison between the fitted curve of each treatment with the other treatments.

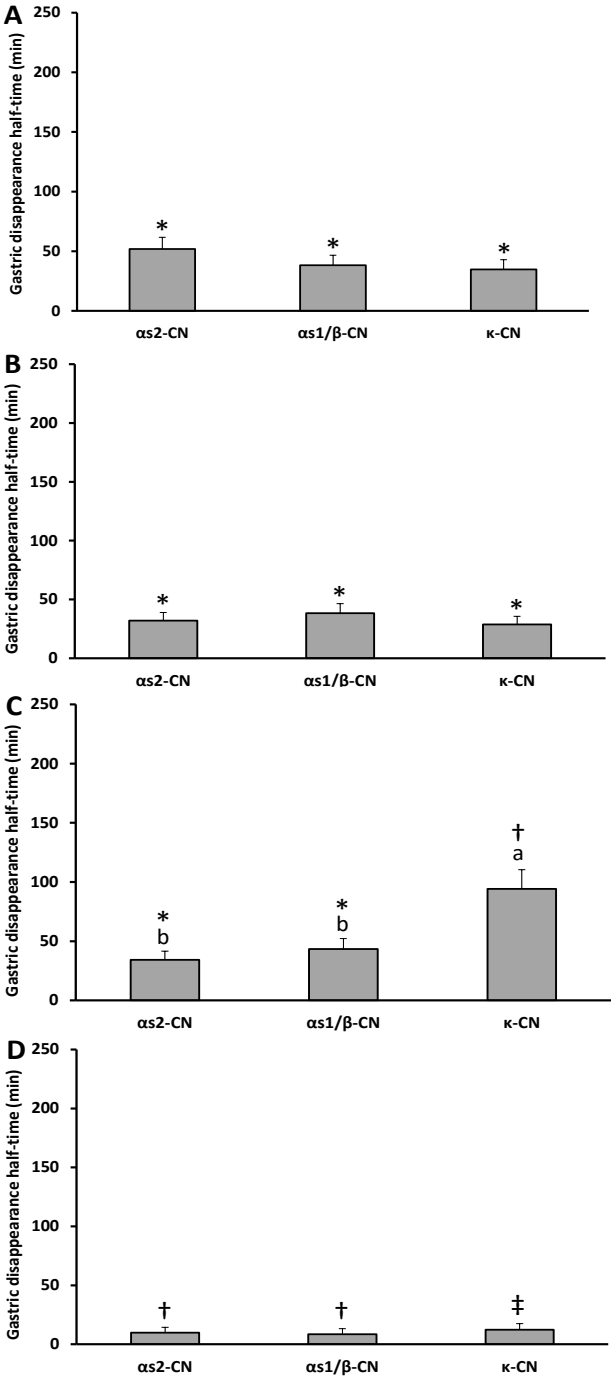


Figure 5.7: Half-time ($T_{1/2}$) of individual proteins which apparently disappeared from gastric curd collected at different post-feeding times of growing pigs fed different processed bovine milk types.
At 20 min of digestion, distinct curds were formed for all milk types; thus, for the apparent disappearance from the curd, the amount of each individual protein at 20 min was used as the 100% value. No whey proteins were identified in the gastric curd. **A:** raw; **B:** pasteurised non-homogenised; **C:** pasteurised homogenised; **D:** ultra-high temperature treated homogenised. Proteins with different symbols (*, †, ‡) have a different half-time across milk types ($P \leq 0.05$). Proteins with different letters (a, b, c, d) have different half-time within each milk type ($P \leq 0.05$). αs2-CN, αs_2-casein; αs_1/β-CN, combined αs_1-casein and β-casein; κ-CN, κ-casein; β-lac, β-lactoglobulin; α-lac, α-lactalbumin. Values are means ± SEM, n = 4 – 6.

5.4.4. Gastric emptying of amino acids

Except for glycine and alanine, differences in the gastric emptying of AA were observed across the differently processed milk types (Figure 5.8). For example, Figure 5.9a - d shows the gastric emptying of EAA and NEAA. In the total gastric chyme (curd plus liquid), the gastric emptying rate (κ) of each AA was at least two-fold faster ($P \leq 0.05$) for pigs fed UHT milk than those fed raw or PNH milk (Figure 5.8). Except for lysine, a two-fold faster ($P \leq 0.05$) gastric emptying rate for each AA was also observed in pigs fed PH milk, than for those fed raw milk (Figure 5.8). There were no differences ($P > 0.05$) in the gastric emptying rates of any AA from pigs fed the raw and PNH milk types, or from pigs fed PH and UHT milk types (Figure 5.8).

Differences in the gastric emptying rates of the total chyme were reflected in the time taken to empty half of the AA intake ($T_{1/2}$, half-time), where generally the half-time of AA in pigs fed PH and UHT milk did not differ from each other ($P > 0.05$) but were more than 1.7-fold shorter ($P \leq 0.05$) than the half-time of pigs fed raw milk (Figure 5.10). There was no difference in the total chyme half-time of any AA for pigs fed raw or PNH milk. The κ and half-time of the AA in the curd fraction followed the same pattern as the total chyme (Tables 5.1 and 5.2).

Within each milk type, differences in the emptying rates and half-time across AA were observed (Figures 5.8 and 5.10). Although some patterns were consistent for all milk types (e.g., glycine had the longest half-time for all milk types) in general, the differences across AA were specific to each milk type. For example, within the pigs fed UHT milk, the half-time of glycine was at least 2.5 times longer ($P \leq 0.05$; Figure 5.10d) than the half-time of any other AA.

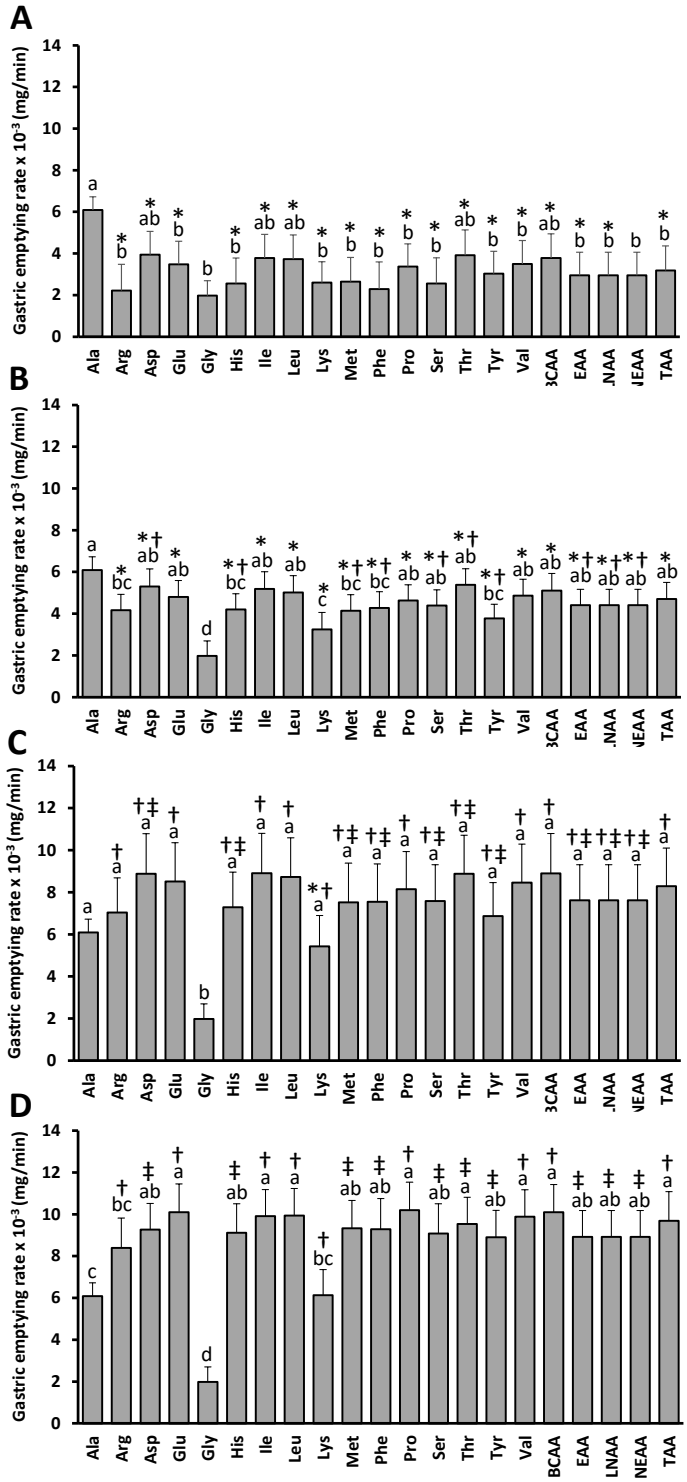


Figure 5.8: Gastric emptying rates of amino acids (AA) in total gastric chyme collected at different post-feeding times from growing pigs fed different processed bovine milk types. **A:** Raw milk; **B:** pasteurised non-homogenised milk; **C:** pasteurised homogenised milk; **D:** ultra-high temperature treated homogenised milk. AA with different symbols (*, †, ‡) have a different emptying rate across milk types ($P \leq 0.05$). AA with different letters (a, b, c, d) have different emptying rates within each milk type ($P \leq 0.05$). Values are means ± SEM, n = 4 – 6.

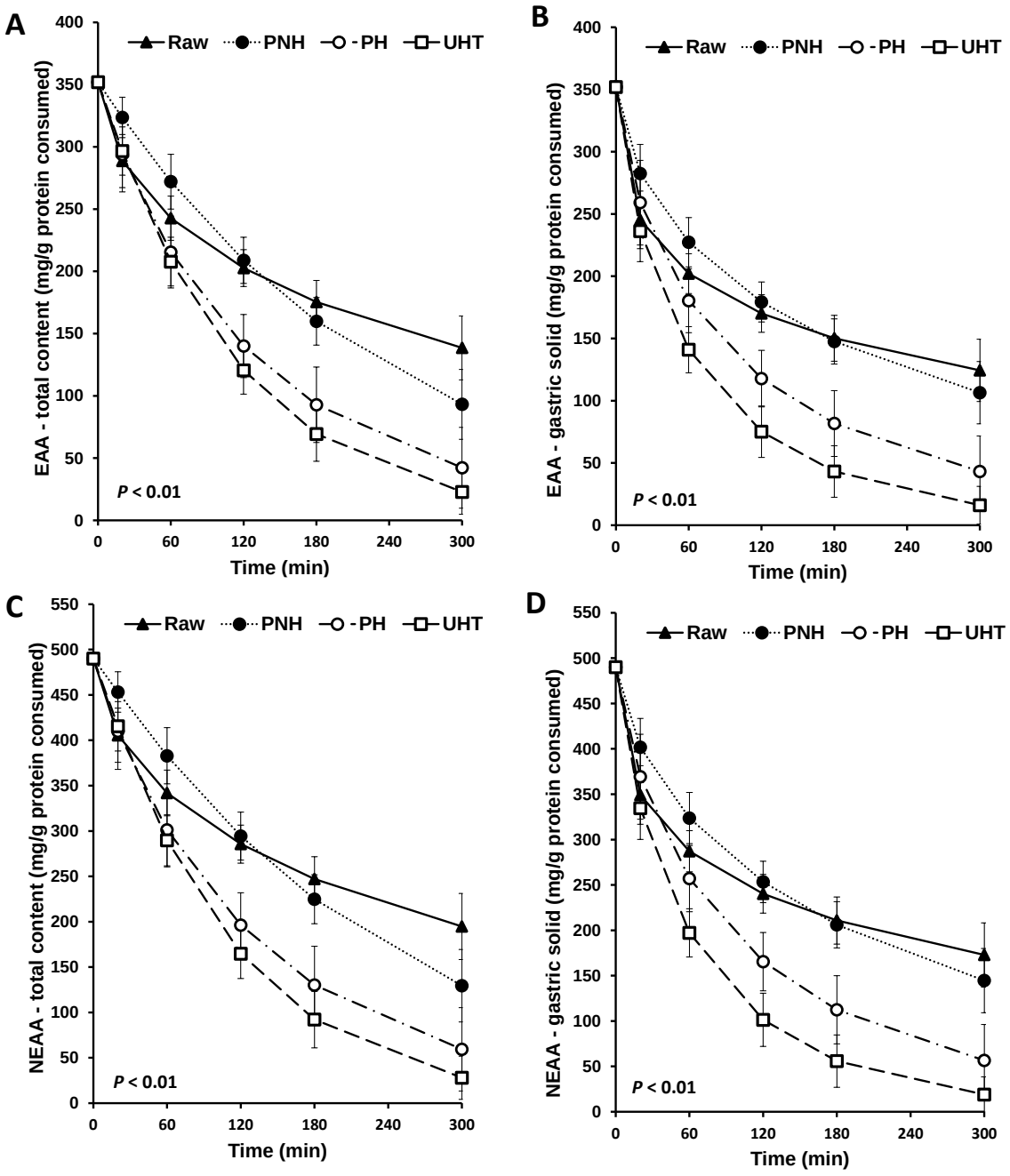


Figure 5.9: Gastric emptying of essential and non essential amino acids (AA) in total gastric chyme and gastric curd (solid fraction) collected at different post-feeding times from growing pigs fed different processed bovine milk types.
A and B: total and curd essential AA; **C and D**: total and curd non-essential AA. PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 4 – 6. Probability values in each panel reflect a comparison between the fitted curve of each treatment with the other treatments.

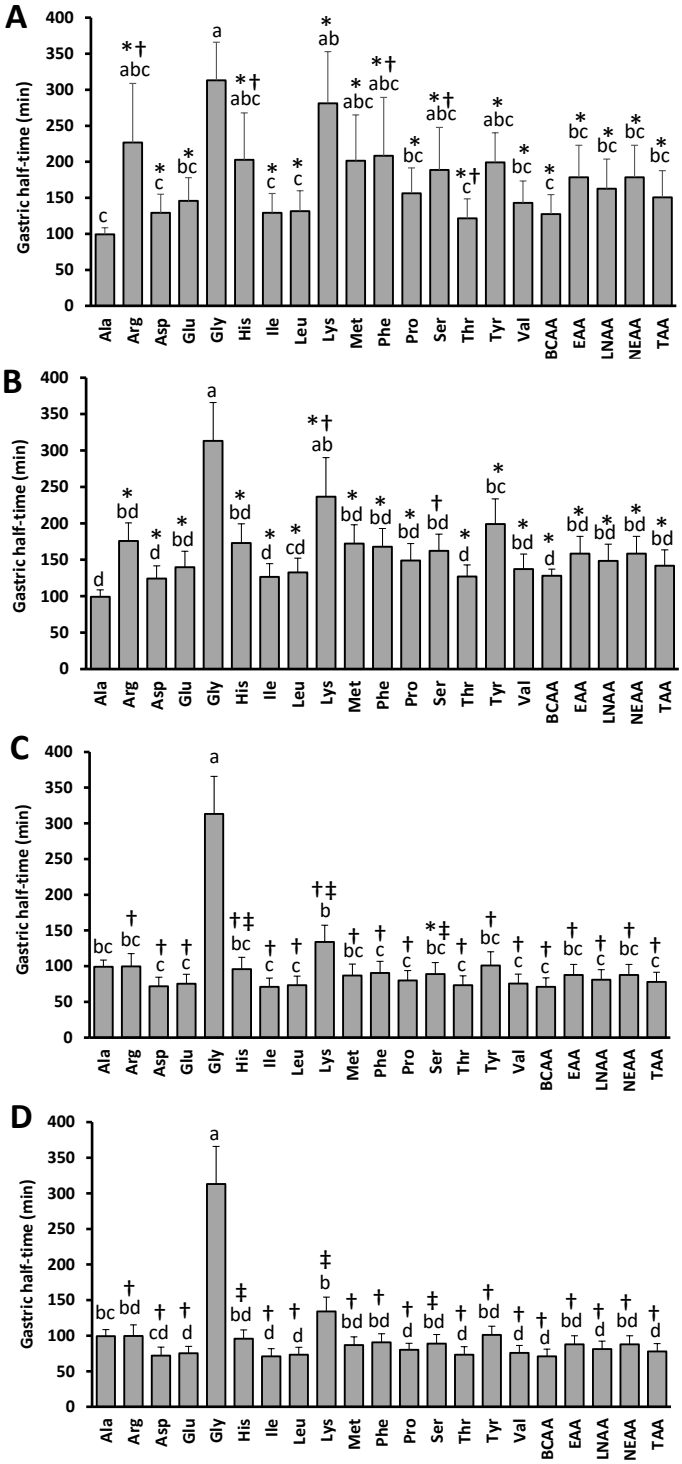


Figure 5.10: Gastric emptying half-time ($T_{1/2}$) of amino acids (AA) in total gastric chyme collected at different post-feeding times from growing pigs fed different processed bovine milk types.

A: Raw milk; **B:** pasteurised non-homogenised milk; **C:** pasteurised homogenised milk; **D:** ultra-high temperature treated homogenised milk. AA with different symbols (*, †, ‡) have different half-times across milk types ($P \leq 0.05$). AA with different letters (a, b, c, d) have different half-times within each milk type ($P \leq 0.05$). Values are means ± SEM, n = 4 – 6.

Table 5.1: Gastric emptying rate (κ) of amino acids in gastric solid (curd) collected at different post-feeding times from growing pigs fed different processed bovine milk types.

	Raw	PNH	PH	UHT
		$\kappa \times 10^{-3}$ *(mg/min)*		
Ala	8.90 ± 1.20	8.90 ± 1.20	8.90 ± 1.20	8.90 ± 1.20
Arg	2.75 ± 1.78^{b}	4.12 ± 1.07^{b}	8.45 ± 2.05^{a}	14.2 ± 3.1^{a}
Asp	5.5 ± 2.01^{b}	6.03 ± 1.43^{b}	11.4 ± 2.73^{ab}	15.4 ± 2.74^{a}
Glu	4.64 ± 1.68^{b}	5.05 ± 1.15^{b}	10.6 ± 2.46^{a}	16.5 ± 2.93^{a}
Gly	3.42 ± 1.04	3.42 ± 1.04	3.42 ± 1.04	3.42 ± 1.04
His	3.15 ± 1.72^{b}	4.13 ± 1.04^{b}	8.79 ± 2.11^{a}	15.1 ± 3.09^{a}
Ile	5.22 ± 1.94^{b}	5.69 ± 1.33^{b}	11.5 ± 2.76^{a}	16.5 ± 2.93^{a}
Leu	5.42 ± 1.96^{b}	5.59 ± 1.27^{b}	11.5 ± 2.73^{a}	16.7 ± 2.91^{a}
Lys	2.28 ± 1.46^{c}	2.77 ± 1.19^{bc}	6.6 ± 1.83^{ab}	10.8 ± 2.65^{a}
Met	3.16 ± 1.67^{b}	4.01 ± 1.13^{b}	9.28 ± 2.42^{a}	15.1 ± 2.91^{a}
Phe	2.87 ± 1.83^{b}	4.22 ± 1.07^{b}	9.06 ± 2.21^{a}	15.1 ± 3.11^{a}
Pro	4.38 ± 1.57^{b}	4.75 ± 1.05^{b}	10.1 ± 2.31^{a}	16.9 ± 3.01^{a}
Ser	3.37 ± 1.84^{b}	4.44 ± 1.09^{b}	9.26 ± 2.22^{a}	15.1 ± 3.05^{a}
Thr	5.71 ± 2.35^{c}	6.02 ± 1.33^{bc}	11.6 ± 2.84^{ab}	16.7 ± 3.17^{a}
Tyr	2.22 ± 1.57^{c}	3.58 ± 1^{bc}	8.08 ± 2.07^{ab}	13.8 ± 2.92^{a}
Val	4.67 ± 1.76^{b}	5.14 ± 1.2^{b}	10.6 ± 2.5^{a}	16.1 ± 2.84^{a}
BCAA	5.21 ± 1.91^{b}	5.53 ± 1.28^{b}	11.3 ± 2.7^{a}	16.6 ± 2.92^{a}
EAA	3.69 ± 1.72^{b}	4.44 ± 1.14^{b}	9.47 ± 2.29^{a}	14.8 ± 2.8^{a}
LNAA	3.97 ± 1.81^{b}	4.66 ± 1.2^{b}	10.1 ± 2.49^{a}	15.9 ± 3.04^{a}
NEAA	3.69 ± 1.64^{b}	4.48 ± 1.09^{b}	9.3 ± 2.17^{a}	14.8 ± 2.74^{a}
TAA	4.33 ± 1.85^{b}	4.96 ± 1.21^{b}	10.5 ± 2.53^{a}	16.5 ± 3.11^{a}

PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised, BCAA, branch-chain amino acids; EAA, essential amino acids; LNAA, long neutral amino acids; NEAA, non-essential amino acids; TAA, total amino acids. Values are means ± SEM, n = 4 – 6. Means with different superscript letters within a row differ ($P \leq 0.05$). The parameter alpha values (α_0) were the amount of each amino acid (mg/g protein) in the milk before being consumed. For alanine and glycine, a reduced model best described the gastric emptying of the different milk types; thus, the figures reported for each milk are the same.

Table 5.2: Gastric emptying half-time ($T_{1/2}$) of amino acids in gastric solid collected at different post-feeding times from growing pigs fed different processed bovine milk types.

	Raw	PNH	PH	UHT
		T ½ (min)		
Ala	51.9 ± 6.9	51.9 ± 6.9	51.9 ± 6.9	51.9 ± 6.9
Arg	137.8 ± 50.6^{ab}	148.3 ± 25.5^{a}	75.6 ± 20.0^{bc}	42.0 ± 12.9^{c}
Asp	64.8 ± 18.0^{ab}	81.5 ± 16. 3^{a}	49.7 ± 14.8^{ab}	39.0 ± 10.6^{b}
Glu	87.5 ± 22.6^{ab}	107.8 ± 20.2^{a}	55.2 ± 15.3^{bc}	37.3 ± 9.1^{c}
Gly	31.0 ± 3.8	31.0 ± 3.8	31.0 ± 3.8	31.0 ± 3.8
His	127.9 ± 43.6^{ab}	148.1 ± 26.5^{a}	71.8 ± 18.7^{bc}	41.0 ± 11.0^{c}
Ile	68.0 ± 19.0^{ab}	87.8 ± 17.3^{a}	48.7 ± 14.6^{ab}	36.4 ± 9.6^{b}
Leu	66.3 ± 18.9^{ab}	91.7 ± 17.7^{a}	49.1 ± 14.6^{b}	36.5 ± 9.1^{b}
Lys	231.7 ± 83.6^{a}	221.7 ± 68.2^{a}	100.1 ± 27.5^{ab}	56.5 ± 16.5^{b}
Met	129.0 ± 44.1^{ab}	142.8 ± 26.9^{a}	63.7 ± 18.7^{bc}	42.2 ± 10.4^{c}
Phe	127.0 ± 49.8^{ab}	143.0 ± 25.3^{a}	69.1 ± 18.3^{b}	40.8 ± 10.9^{b}
Pro	99.0 ± 25.3^{ab}	121.2 ± 21.8^{a}	59.3 ± 15.8^{bc}	37.2 ± 8.5^{c}
Ser	108.1 ± 35.9^{ab}	131.8 ± 22.6^{a}	66.1 ± 18.2^{bc}	39.8 ± 11.1^{c}
Thr	53.0 ± 18.9^{ab}	85.6 ± 15.5^{a}	47.9 ± 15.3^{bc}	34.3 ± 10.0^{c}
Tyr	205.1 ± 99.6^{ab}	184.2 ± 35.3^{a}	81.5 ± 20.1^{bc}	47.6 ± 11.5^{c}
Val	83.0 ± 21.7^{ab}	103.3 ± 19.6^{a}	54.5 ± 15.4^{bc}	38.3 ± 9.5^{c}
BCAA	69.8 ± 19.3^{ab}	92.5 ± 18.0^{a}	49.8 ± 14.7^{b}	36.6 ± 9.3^{b}
EAA	106.0 ± 30.6^{ab}	125.3 ± 23.4^{a}	63.1 ± 17.7^{bc}	41.5 ± 10.8^{c}
LNAA	92.8 ± 27.4^{ab}	114.1 ± 22.1^{a}	56.9 ± 16.9^{bc}	37.7 ± 10.1^{c}
NEAA	112.2 ± 31.8^{ab}	129.1 ± 22.9^{a}	66.0 ± 17.5^{bc}	42.5 ± 10.6^{c}
TAA	83.9 ± 24.6^{ab}	106.7 ± 20.2^{a}	54.9 ± 16.2^{bc}	36.1 ± 9.7^{c}

PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised, BCAA, branch-chain amino acids; EAA, essential amino acids; LNAA, long neutral amino acids; NEAA, non-essential amino acids; TAA, total amino acids. Values are means ± SEM, n = 4 – 6. Means with different superscript letters within a row differ ($P \leq 0.05$). The parameter alpha values (α_0) were the amount of each amino acid (mg/g protein) in the milk before being consumed. For alanine and glycine, a reduced model best described the gastric emptying of the different milk types; thus, the figures reported for each milk are the same.

5.5. Discussion

This study is the first to compare *in vivo* gastric protein hydrolysis, milk protein disappearance from the stomach, gastric AA emptying, and release of digested protein into the small intestine of heat treated and/or homogenised whole bovine milk, using the growing pig as a model for the adult human. The results support the hypothesis that heat-treatment combined with homogenisation increased the amount of both digested protein in the stomach and AA emptying from the stomach.

Homogenisation (PNH vs PH) did not impact the apparent degree of gastric protein hydrolysis in the total chyme over time. In contrast, heat treatment (raw vs PNH, PH vs UHT) appeared to impact the apparent degree of total gastric protein hydrolysis over time, although the extent varied with the intensity of treatment. However, at 300 min, the overall apparent degree of protein hydrolysis in the total chyme was similar for the raw and the mild heat-treated milk types. As the effect of gastric protein emptying was removed by adjusting the apparent degree of hydrolysis for protein retention, this result indicates that, for raw, PNH, and PH milk, a similar proportion of total protein had been apparently hydrolysed by 300 min. Similar degree of hydrolysis results have been reported for heat-treated and homogenised bovine milk *in vitro* (Mulet-Cabero et al., 2019).

When the degree of hydrolysis was corrected for the free amino groups remaining from the milk at each time point, the differences between milk types generally did not change, indicating that, overall, the milk free amino groups did not influence the apparent degree of protein hydrolysis result observed previously.

Previous *in vivo* studies showed that the gastric curd formed by processed bovine milk became softer and more fractionated as the extent of processing increased (raw > PNH > PH > UHT milk) (Chapter 4; Ahlborn et al., 2023a; Ye et al., 2019). It is expected that a more open curd structure

would provide an increased surface area for enzymatic activity (Van Hooydonk, 1987), as well as allowing further ingress of gastric fluid containing pepsin and hydrochloric acid (Kalantzi et al., 2006), which may increase gastric protein hydrolysis. Thus, for pigs fed UHT milk, the relatively high apparent degree of protein hydrolysis could be partially explained by the generally softer, more open curd structure formed by UHT milk. In comparison, the tighter curd structure reported for raw, PNH, and PH milk (Chapter 4; Ahlborn et al., 2023a) may have trapped the free amino groups in the curd, resulting in a slower release into the liquid phase. A similar curd effect has been reported in previous research by Mulet-Cabero et al. (2019) during the *in vitro* digestion of similarly processed milk. In addition, between 20 min and 60 min, an approximately 30-fold slower corrected apparent rate of gastric protein hydrolysis was observed for pigs fed raw milk compared to those fed the processed milk types. The slow apparent initial rate of gastric protein hydrolysis for pigs fed raw milk suggests some curd-related hindrance towards the early protein digestion of raw milk; possibly partially a result of the tighter curd structure formed by raw milk (Ye et al., 2016b).

Besides curd structure, the apparent degree of gastric protein hydrolysis results may also be influenced by differing protease activities in pigs fed different milk types. An *in vitro* study has shown that native milk proteases played a greater role on the digestion of milk proteins than pepsin (Leite et al, unpublished). Processing could change native milk protease activity (France et al., 2021; Leite et al., 2021), and this could be one of the factors influencing the degree of gastric protein hydrolysis observed here.

It has been previously hypothesised that the more open curd structure formed by UHT milk and the increased release of hydrolysed protein from the curd could result in a faster emptying of digested protein from the stomach, compared to raw, PNH and PH milk (Ye et al., 2019). The

gastric emptying of total protein has been shown to be influenced by differences in curd formation as a result of heating and homogenisation (Chapter 4; Ahlborn et al., 2023a). For example, the total protein gastric emptying rate followed the pattern UHT > PH > PNH = raw. In the present study, up to 120 min, although the degree of gastric protein hydrolysis was higher for pigs fed UHT milk, the apparent rate of release of digested protein into the small intestine was similar for pigs fed the PNH, PH, and UHT milk. This finding is explained by a higher proportion of free NH_2 observed in the gastric liquid fraction of pigs fed UHT milk, compared to the other milk types. In addition, the release of digested protein into the small intestine is thought to be regulated by a feedback mechanism between the stomach, duodenum and brain, with the digestive and absorptive capacities in the first part of the small intestine acting as the limiting factor (McHugh & Moran, 1979). It is expected that this mechanism could play a role in modulating the release of digested protein into the small intestine observed here.

A continuous flow of digested protein into the small intestine after 120 min was observed only for pigs fed UHT milk, and for pigs fed raw milk after 180 min. Ahlborn et al. (2023a) (Chapter 4) showed that in pigs fed UHT and raw milk, the gastric curd began softening and loosening after 120 and 180 min of digestion respectively, whereas the curd formed by PNH and PH milk did not. Thus, the continued increase in the rate of digested protein released into the small intestine of pigs fed raw and UHT milk could be explained by the impact of curd disintegration, and the associated increased access to proteins as a result of a looser curd structure, as discussed previously.

The protein content of bovine milk is comprised of ~80% caseins, of which αs_1-casein, β-casein, and κ-casein make up 38%, 35% and 12% respectively (Huppertz, 2013; Lucey et al., 2017). Once in the stomach, caseins are susceptible to hydrolysis by both gastric and native milk proteases, whereas whey proteins show some resistance to gastric hydrolysis (Dupont & Tome, 2014; Peram

et al., 2013; Prado et al., 2006). In the present study, the digestion of individual proteins was determined by measuring their disappearance in SDS-PAGE, with different disappearance across milk caseins ascribed to processing. For example, pigs fed UHT milk had a shorter apparent disappearance half-time of combined αs_1-casein and β-casein, and κ-casein than pigs fed raw milk, which aligns with the higher apparent degree of gastric protein hydrolysis observed for pigs fed UHT milk. Within the curd, αs_1-casein, β-casein, and κ-casein are involved in the formation of curd via casein micelles; a process triggered by the enzymatic protein hydrolysis by pepsin. The protein structure of the coagulated caseins has been shown to be altered by strong heat treatment, as a result of denatured β-lactoglobulin association with casein micelles via κ-casein. The β-lactoglobulin-κ-casein association results in a reduced susceptibility to pepsin-induced coagulation and a subsequently weaker curd, which leads to an increased casein hydrolysis (Ye et al., 2016a; Ye et al., 2019). Thus, the observed difference in αs_1-casein and β-casein, and κ-casein disappearance between raw and heated milk was expected, and is in line with results reported using *in vitro* semi-dynamic digestion models, and in rats (Wada & Lönnerdal, 2014; Ye et al., 2016a, 2016b, 2017; Ye et al., 2019). In addition, the longer half-time of disappearance for κ-casein from pigs fed raw milk was likely a consequence of the tighter curd structure and reduced curd permeability, as a result of stronger pepsin-induced casein coagulation, compared with the other milk types.

No difference was observed in the apparent disappearance of αs_2-casein from pigs fed the different milk types. However, αs_2-casein is located at the centre of the casein micelle and does not react with denatured whey proteins (Horne, 2020; Huppertz et al., 2017). Thus, the relatively sheltered location and low reactivity may have contributed to the result observed in the present study.

For all milk types, the whey proteins emptied faster than the casein proteins, as whey proteins are soluble and therefore present in the liquid phase, which empties rapidly (Kitabatake & Kinekawa,

1998). In the present study, no difference in the apparent disappearance of α-lactalbumin from pigs fed the different milk types was observed. It is possible that any potential differences in pepsin-induced α-lactalbumin hydrolysis were obscured by gastric emptying. Despite heat treatment increasing the susceptibility of β-lactoglobulin to pepsin, it was expected that the apparent gastric disappearance of β-lactoglobulin may be decreased as a result of heat-induced binding with casein micelles (Ye et al., 2016b). However, in the present study, the disappearance half-time of β-lactoglobulin was longer for pigs fed PH milk, than those fed the other milk types.

As expected, differences in both the amount of digested protein entering the small intestine and disappearance patterns of each milk type resulted in different rates of gastric emptying of AA. The gastric emptying of AA from pigs fed PH and UHT milk was faster than for pigs fed raw milk, which aligns with the differences in apparent degree of gastric protein hydrolysis from pigs fed these milk types. However, the gastric emptying half-time of most AA from pigs fed PH was faster than for pigs fed PNH milk, whereas the apparent degree of hydrolysis and the amount of digested protein entering the small intestine of pigs fed PNH and PH milk were similar. This result implies that a greater portion of the protein from PH milk left the stomach in a less hydrolysed state than that from PNH milk.

A shorter gastric emptying half-time was observed for most AA of pigs fed PH and UHT milk, compared to pigs fed raw or PNH milk. The similarity in emptying rate and gastric half-time of AA for pigs fed the two unhomogenised milk types, and the two homogenised milk types indicated that the rate and profile of AA entering the small intestine was mainly affected by homogenisation, whereas the effect of heat treatment was less pronounced. While previous research showed both heat treatment and homogenisation affected the gastric emptying of total protein, the consumption of non-homogenised bovine milk resulted in a higher amount of lipid in the rapidly emptied gastric

liquid phase, compared to homogenised milk (Chapter 4; Ahlborn et al., 2023a). Therefore, the slower emptying of AA from pigs fed non-homogenised milk could be partially influenced by the stomach-gut-brain feedback loop acting to regulate nutrient flows into the small intestine (McHugh & Moran, 1979), as discussed previously.

Overall, the disappearance of individual proteins was mainly affected by high heat treatment combined with homogenisation, whereas the apparent gastric hydrolysis of bovine milk protein was influenced by both mild (raw vs PNH) and strong heat treatment (PH vs UHT). The difference in the effect of processing on the degree of gastric protein hydrolysis and individual protein disappearance from the stomach suggests that gastric emptying contributes strongly to the results reported for individual protein disappearance, both here and reported elsewhere in the literature. Despite the influence of heat treatment on the apparent disappearance of individual protein, the emptying of AA from the stomach was mainly influenced by homogenisation (PNH vs PH, PNH vs UHT), rather than heat treatment (raw vs PNH; PH vs UHT). From a physiological perspective, the release (rate and amount) of AA into the small intestine can be considered as the final stage of gastric protein digestion. Thus, the findings from the present study, when taken together, indicate that homogenisation plays an important role in controlling the outcomes of gastric protein digestion of processed bovine milk. The differing responses of the various protein digestion parameters to the different processing treatments reported here highlights the need to fully understand each aspect of gastric protein digestion and emptying in the context of milk processing.

It could be expected that the differences in gastric protein hydrolysis and AA emptying observed throughout the gastric digestion time in the present study will impact small intestinal AA absorption kinetics. Recently, an *in vivo* study reporting the apparent small intestinal AA absorption at 210 min post-feeding of raw bovine, caprine and ovine milk in the infant model showed that the gastric

retention of AA was the main driver of their digestibility (Chapter 3; Ahlborn et al., 2023b). A study in mini-pigs feeding ^{15}N labelled bovine milk and yoghurt suggested that the kinetics of milk protein absorption are largely controlled by gastric retention of total nitrogen (Gaudichon et al., 1994). In addition, a study feeding beef muscle protein showed that both the rate of release of protein from the stomach and the amount of digested protein released has an impact on small intestinal absorption (Montoya et al., 2018). The modulation of small intestinal AA absorption may have further implications for the appearance of AA in the circulation, with impacts on human body function, such as muscle protein synthesis (Dangin et al., 2003; Hamarsland et al., 2019). Thus, further research determining the influence of heat treatment and homogenisation on both the small intestinal rate and location of absorption of milk protein would be valuable and could contribute to the development of processing treatments which optimise gastric digestion and subsequent nutritional outcomes.

When taken together with the previously reported differences in curd characteristics, the results reported here suggest that differences in the gastric digestion and AA emptying of differently processed bovine milk could be attributed to differences in the curd structures described in Chapter 4. While curd structure was impacted by both heat treatment and homogenisation, the present study highlights the role of homogenisation in the emptying of AA as the final outcome of gastric digestion. It is important to note that these results relate to processed bovine milk, and it cannot be assumed that the digestion of processed milk from other sources (e.g., non-bovine ruminants) would result in the same observations. In addition, other processing techniques (e.g., spray-drying for formula production) may alter the results reported here.

5.6. Conclusions

The present study demonstrated the impact of common bovine milk processing techniques (heat treatment and homogenisation) on the total gastric protein hydrolysis, the individual protein disappearance, and the release of digested protein and AA into the small intestine in the growing pig as a model for the adult human. Differences observed between pigs fed raw, PNH, PH, and UHT milk were related to the different processing treatments of the consumed milk.

Overall, while high heat treatment combined with homogenisation (UHT) had the greatest impact on individual protein disappearance, both mild (PNH and PH) and strong heat treatment (UHT) impacted the apparent degree of total protein hydrolysis in the stomach, and the rate of digested protein entering the small intestine. The release of AA into the small intestine was mainly impacted by homogenisation. This study provides a new understanding of how processing treatments affect protein digestion (total protein hydrolysis and individual protein disappearance) in the stomach, altering the release of AA into the small intestine. Further research is needed to understand the effect of these findings on the small intestinal AA absorption and blood circulation AA appearance.

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CHAPTER 6.

HEAT TREATMENT AND HOMOGENISATION OF BOVINE MILK INFLUENCED TRUE SMALL INTESTINAL ABSORPTION KINETICS AND BLOOD PLASMA CONCENTRATIONS OF AMINO ACIDS IN THE GROWING PIG AS A MODEL OF THE ADULT HUMAN

This chapter is in preparation for submission to The Journal of Nutrition (impact factor 4.20): Ahlborn, N. G., Montoya, C. A., Roy, N. C., Ye, A., Samuelsson, L. M., & McNabb, W. C. Heat treatment and homogenisation of bovine milk influenced true small intestinal absorption kinetics and plasma concentrations of amino acids in the growing pig as a model of the adult human.

HIGHLIGHTS

- Homogenisation increased the true absorption kinetics for most amino acids in the small intestine.
- The extent of true small intestinal absorption of amino acids ranged between 51% to 93%.
- Differences in true small intestinal absorption kinetics were modulated by the gastric retention of amino acids.
- Postprandial amino acid concentrations in the blood circulation were inconsistent with small intestinal amino acid absorption patterns.

The student contributed approximately 70% of the research in this chapter.

6.1. Abstract

Bovine milk is commonly consumed after undergoing heat treatment and homogenisation. Such processing treatment has been shown to alter the gastric digestion of milk proteins, which influence the release of total amino acid (AA) and digested protein into the small intestine. However, the impact of processing on true small intestinal AA absorption and blood AA appearance is unknown. This study aimed to determine the impact of heat treatment and homogenisation of bovine milk on the true small intestinal AA absorption kinetics, and the blood plasma AA concentrations in the growing pig as an adult human model. Raw, pasteurised non-homogenised (PNH), pasteurised homogenised (PH), or ultra-high temperature treated homogenised (UHT) bovine milk was fed to 180 nine-week-old pigs for ten days. A group of pigs (36) were fed a protein-fat free solution on sampling day to allow for correction of AA recovered between the stomach and terminal ileum which did not originate from the final milk meal. The final milk meal contained the indigestible marker titanium oxide (TiO_2) as a meal tracer. Entire stomach, small intestine, caecum, and colon contents, as well as portal and peripheral blood samples were collected at 0, 20, 60, 120, 180, and 300 min postprandially to determine true absorption of AA in the small intestine, and concentration of AA in the portal and peripheral blood plasma. The true small intestinal absorption kinetics of most AA were faster ($P \leq 0.05$) in pigs fed homogenised milk (PH and UHT), compared to pigs fed non-homogenised milk (raw and PNH). For instance, for essential AA (EAA), the time taken to reach a true overall absorption of 50% (T_{50}) was at least 35% shorter in pigs fed homogenised milk ($P \leq 0.05$) than for those fed non-homogenised milk. Heat treatment also altered small intestinal absorption kinetics, although fewer AA were affected by heat treatment than homogenisation. When corrected for gastric AA retention, there were minimal differences in the true AA absorption in the small intestine between milk treatments, indicating that gastric retention

plays a key role in the determination of absorption. The postprandial plasma concentration of AA in the portal and peripheral blood over time did not reflect the true small intestinal AA absorption. In conclusion, homogenisation was the main process affecting the true absorption kinetics of AA in the small intestine in pigs fed differently processed bovine milk.

Keywords: bovine milk, processing, true amino acid absorption, plasma amino acid concentration, pigs, small intestine

6.2. Introduction

Most milk available for human consumption is processed using either heat treatment or homogenisation, or in combination. It is well documented that the application of such processes leads to structural changes in proteins and lipids naturally present in milk (Corredig & Dalgleish, 1999; Kitabatake & Kinekawa, 1998; Lee & Sherbon, 2002).

The consumption of heat treated and homogenised bovine milk has been shown to lead to the formation of gastric curd with differing characteristics *in vitro* (Mulet-Cabero et al., 2019; Ye et al., 2016, 2017) and *in vivo* (Chapter 4) (Ahlborn et al., 2023a; Ye et al., 2019). For example, the curd of ultra-high temperature treated homogenised (UHT) milk has a porous, open protein network, forming a weak curd, whereas raw milk forms a less porous, comparatively stronger structure (Chapter 4) (Ahlborn et al., 2023a). As a result of differing curd structures, gastric protein hydrolysis and gastric protein emptying were altered, resulting in different releases of amino acids (AA) and digested protein into the small intestine (Chapter 5). Studies in piglets have shown that the gastric release kinetics of total nitrogen modulates small intestinal AA absorption (Chapter 3) (Ahlborn et al., 2023b; Gaudichon et al., 1994; Montoya et al., 2018). Thus, it is expected that the small intestinal AA absorption could vary for each milk treatment.

The nutritional value of milk is generally described as excellent, particularly in terms of ileal protein absorption (Dupont & Tome, 2014), which is typically assessed by considering AA disappearance at the ileum of cannulated pigs (Hodgkinson et al., 2022). However, it has been suggested that changes to native milk nutrient structures caused by common processing treatments could alter small intestinal AA absorption. For example, the authors of a study reporting a faster postprandial appearance of leucine in the peripheral blood of pigs fed heated milk (90 °C, 10 min), compared to pigs fed unheated milk postulated that their results could be explained by an increased

AA absorption, resulting from the increased gastric hydrolysis of whey proteins after UHT treatment (Barbé et al., 2013). However, the influence of heat treatment and homogenisation of bovine milk on the kinetics of AA absorption in the small intestine has not yet been investigated. Differences in small intestinal absorption also have implications for a number of metabolic processes throughout the body, such as skeletal muscle protein synthesis (Dangin et al., 2002; Koopman et al., 2009b; Tang et al., 2009)

Based on the processing-induced increase in gastric milk protein hydrolysis, and the resulting faster release of AA and digested protein into the small intestine, it was hypothesised that the heat treatment and homogenisation of bovine milk would result in a higher rate and extent of AA absorption (disappearance) in the small intestine. The aim of this study was to investigate the impact of heat treatment and homogenisation of bovine milk on the true small intestinal AA absorption kinetics and the concentration of AA in the portal and peripheral blood plasma using the growing pig as a model for the adult human.

To determine true small intestinal AA absorption, a dietary AA recovery approach was used, with AA flow corrected for any AA recovered between the stomach and terminal ileum which did not originate from the final milk meal (i.e., residual AA from the previous meal, and endogenous AA not reabsorbed in the small intestine) (Fuller & Tome, 2005; Moughan, 2023). As small intestinal AA absorption is considered complete at the end of the terminal ileum (van der Wielen et al., 2017), the true absorption determined in the present study was considered a measure of overall small intestinal AA absorption.

Due to the extensive similarities between the pig and human gastrointestinal tract, as well as their feeding habits (Guilloteau et al., 2010; Moughan et al., 1992; Sciascia et al., 2016), the pig was

selected as a model for the adult human. Further, the pig has been shown to be an excellent predictor of the extent of ileal AA absorption of different foods with varying digestibility in the human (Hodgkinson et al., 2022). Although rates of absorption have not been compared between pigs and human, the pig can be considered the optimal *in vivo* model for the determination of absorption in the human.

6.3. Materials and Methods

6.3.1. Animals, dietary treatments, and experimental design

This study was approved by the Massey University Animal Ethics Committee (approval no. 19/83). The animals, housing, dietary treatments, and experimental design of the pig study have been reported previously (Chapter 4) (Ahlborn et al., 2023a). In brief, 180 9-week-old locally sourced Large White × Landrace entire male pigs (bodyweight 22.4 ± 0.13 kg, mean ± standard error of the mean (SEM)), were housed in individual metabolic crates at the Animal Production Unit of Massey University, Palmerston North, New Zealand.

The experimental treatments were raw, pasteurised non-homogenised (PNH), pasteurised homogenised (PH), or UHT bovine milk. A protein- and fat-free (PFF) solution was included as a fifth treatment to allow for the correction of endogenously released AA, as well as residual AA remaining from the previous (evening) meal. The PH, PNH, and UHT milk were commercially available processed products, while the raw milk was purchased locally (Gorge Fresh Organics, Palmerston North, New Zealand). The raw, PH, and UHT milk were fed as sourced, whereas PNH was prepared using simple gentle manual agitation to combine pasteurised trim milk with pasteurised cream to the same lipid content as the PH milk. A human-type meal was also formulated to emulate a typical Western diet, using the USDA's chemical composition data of each

ingredient to meet the NRC nutrient requirements for growing pigs (NRC, 1998). The preparation and composition of the dietary treatments and human-type meal have been reported previously (Chapter 4) (Ahlborn et al., 2023a).

On arrival to the facility, the pigs were randomly distributed between the five dietary treatments (36 pigs per experimental diet; 6 pigs per experimental diet x time point combination). The pigs underwent a three-day gradual dietary transition to the experimental diets, after which they consumed their milk treatments (breakfast; 500 mL of raw, PH, PNH, or UHT) and human-type meals (lunch and dinner) for seven days. On sampling day, fasted pigs (16 h) were either euthanised at time 0 min or fed 500 mL of one of the milk treatments, or the PFF solution for breakfast before being euthanised at either 20, 60, 120, 180, or 300 min as described previously (Chapter 4) (Ahlborn et al., 2023a). The breakfast meal contained the indigestible marker titanium dioxide (TiO_2, 0.38 ± 0.3 g) (Sigma Aldrich, St. Louis, MO, USA) to measure small intestinal meal transit.

Each pig was anaesthetised 15 min before its euthanasia time with a mix of Zoletil 100 (zolazepam and tiletamine, both 50 mg/mL; Virbac, Hamilton, NZ) reconstituted with 2.5 mL Ketamine and 2.5 mL Xylazine, both 100 mg/mL (Phoenix Pharm NZ, Auckland, New Zealand). Once anaesthetised, the abdomen was opened and blood samples were drawn from the portal vein and the inferior vena cava using 6 mL anticoagulant ACD vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). After centrifugation at 1,500 *g* for 15 min at room temperature the resulting plasma was transferred into cryotubes and immediately frozen. Plasma samples were stored at -80 °C until analysis of AA concentration.

The gastrointestinal tract was clamped at the oesophagus, pylorus, ileal caecal junction, and rectum. The complete gastrointestinal tract was then dissected into the stomach, small intestine, and large

intestine. The small intestine was uncoiled and the terminal ileal section (approximately 20 cm before the ileal-caecal junction) was isolated using clamps. The proximal and distal small intestinal sections were identified and separated by evenly clamping the remaining section in half. The complete digesta from the stomach, each small intestinal section, and the entire large intestine were collected as described previously (Chapter 3) (Ahlborn et al., 2023b; Montoya et al., 2022), and immediately frozen, freeze-dried, weighed, and ground. Freeze-dried and ground contents were stored at -20 °C until analysis.

Throughout the study, samples from each milk type, as well as the PFF solution, were collected and freeze-dried for AA analysis (Chapter 4) (Ahlborn et al., 2023a).

6.3.2. Chemical analyses of gastrointestinal samples

The freeze-dried stomach and small intestinal contents were analysed for AA concentration by 24 h hydrochloric acid hydrolysis with *o*-phthaldialdehyde pre-column derivatisation, followed by reverse-phase chromatography (Rutherfurd et al., 2012). Due to a high fat content, the stomach chyme was defatted prior to hydrolysis (Chapter 4) (Ahlborn et al., 2023a). The stomach, small intestinal, caecal, and colonic contents were also analysed for TiO_2 concentration (Short et al., 1996).

6.3.3. Amino acid analysis of plasma samples

The plasma samples were prepared and analysed for AA concentration by the Liggins Institute, Auckland, New Zealand. The automated AA sample preparation for ultra performance liquid chromatography (UPLC) assays was performed using the EpMotion 5075 platform (Eppendorf,

Hamburg, Germany). This process involves two primary procedures: protein precipitation (procedure 1) and fluorescent tagging (procedure 2).

In each well of a 1.2 mL 96-well deep plate, 160 µL of 0.04 M sulphuric acid (Scharlau, Barcelona, Spain) containing 15 µM L-Nor-Valine (Sigma-Aldrich, Saint Louis, MO, USA) as internal standard was added using a TM 1000-8 eight-channel pipetting tool, followed by 20 µL of plasma sample, quality control one (human plasma provided by Auckland Biobank, NZ) or quality control two (300 µM Metabolomics AA Mix Standard (Cambridge Isotope Laboratories, Tewksbury, MA, USA)) by a TS 50 single-channel pipettor. After 2 min of shaking, 20 µL of 10% sodium tungstate (Acros Organics (Thermo Fisher Scientific), Waltham, MA, USA) was added to precipitate the protein, utilising the TM 1000-8 tool. The mixture was shaken for 3 min, held at 4 °C for 3 min, and shaken for an additional 3 min. The plate subsequently centrifuged at 1000 *g* for 10 min at 4 °C.

A tagging plate was prepared by adding 140 µL of 0.2 M borate buffer (pH 8.8 (Sigma-Aldrich, Saint Louis, MO, USA) adjusted with fresh 5 M NaOH) to each well of a new 1.2 mL 96-well deep plate, using the TM 1000-8 pipetting tool. Next, 20 µL of each of 5 standards, QC or the supernatant from the previous procedure were dispensed into the tagging plate using a TM 50-8 eight-channel pipetting tool. After 3 min of shaking, 10 µL of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate reagent (2.8 mg/mL in acetonitrile) (Cohen & Michaud, 1993) were added to the plate for fluorescent tagging purposes by the TM 50-8 pipettor, followed by an immediate 5 min of shaking. The plate was then sealed and heated at 55 °C for 10 min before being subjected to UPLC assay, as described by Prodhan et al. (2018).

Across all assays, the overall coefficient of variation of the AA quality control ranged from 1.0 – 5.9% (mean 2.4%), and for the plasma quality control, with the exception of histidine (CV 19.3%), this ranged from 4.7 – 9.7% (mean 7.4%).

6.3.4. Calculations

It is important to note that the term ‘absorption’ utilised here differs from the term ‘ileal digestibility’ reported elsewhere (Cervantes-Pahm et al., 2014; Deglaire et al., 2009; Fuller & Tomé, 2005; Hodgkinson et al., 2022). In contrast to the cannulated pig methodology commonly used for the determination of ileal digestibility, the approach used here is based on small intestinal contents collected from the entire small intestine (i.e., from the duodenal bulb to the ileocecal junction in the terminal ileum) at various postprandial time points, which allows for determination of the kinetics of absorption in the small intestine. This difference is important to note, as small intestinal absorption kinetics cannot be determined in cannulated pigs, due to the restricted location of sample collection (i.e., the terminal ileum), and because several hours are required for the experimental meal to reach the terminal ileum, which limits the determination of absorption values prior to this time point. It is important to note that the following methodology describes the determination of AA disappearance from the small intestine. However, in the disappearance of AA from the small intestine is assumed to equate to AA absorption (Holmes et al., 1974), and is thus reported accordingly.

The amount of AA released into the large intestine at each postprandial time point (t_i) relative to meal flow was calculated for pigs fed each milk type ($milk_j$) as described by Ahlborn et al. (2023b) (Chapter 3):

$$\text{AA content}_{\text{released into colon, milk}_{j,\,t_i}}\ (\text{mg}) =$$

$$(\text{AA content}_{\text{terminal ileum, milk}_{j,\,t_i}}\ (\text{mg}) \times \text{TiO}_2\ \text{content}_{\text{colon, milk}_{j,\,t_i}}\ (\text{mg}))/\ \text{TiO}_2\ \text{content}_{\text{terminal ileum, milk}_{j,\,t_i}}\ (\text{mg})$$

The total amount of AA in the stomach and small intestine was used with the amount of AA released into the colon to determine the AA recovered in the gastrointestinal tract (GIT) at each time point. In order to report true AA absorption, the AA recovered at each time point in pigs fed each milk type was corrected for the amount of AA recovered in the stomach and small intestine of pigs fed the PFF solution, as follows:

$$\text{True AA content}_{\text{GIT, milk}_{j,\,t_i}}\ (\text{mg}) =$$

$$(\text{AA content}_{\text{stomach, milk}_{j,\,t_i}}\ (\text{mg}) - \text{AA content}_{\text{stomach, PFF, }t_i}\ (\text{mg})) + (\text{AA content}_{\text{small intestine, milk}_{j,\,t_i}}\ (\text{mg})$$

$$- \text{AA content}_{\text{small intestine, PFF, }t_i}\ (\text{mg})) + (\text{AA content}_{\text{milk}_j\text{, released into colon, }t_i}\ (\text{mg})$$

$$- \text{AA content}_{\text{PFF, released into colon, }t_i}\ (\text{mg}))$$

The true AA absorption of each milk type at each postprandial time point (t_i) was then calculated as follows:

$$\text{True AA absorption}_{\text{milk}_{j,\,t_i}}\ (\%) =$$

$$(\text{AA content}_{\text{milk}_j}\ (\text{mg}) - \text{true AA content}_{\text{GIT, milk}_{j,\,t_i}}\ (\text{mg}))\ /\text{AA content}_{\text{milk}_j}\ (\text{mg}) \times 100$$

A study in piglets fed bovine, caprine and ovine milk at a single time point demonstrated the modulatory role of gastric AA retention on small intestinal absorption (Chapter 3) (Ahlborn et al., 2023b). Thus, the true absorption of AA available for uptake was calculated by considering only the AA released into the small intestine at each time point:

$$\text{AA content}_{\text{entering small intestine, milk}_{j,\,t_i}}\ (\text{mg}) = \text{AA content}_{\text{milk}_j}(\text{mg}) - \text{AA content}_{\text{stomach, milk}_{j,\,t_i}}\ (\text{mg})$$

$$\text{True AA absorption}_{\text{available, milk}_{j,\,ti}}(\%) =$$

$$(\text{AA content}_{\text{entering small intestine, milk}_{j,\,ti}}(\text{mg}) - \text{true AA content}_{\text{small intestine, milk}_{j,\,ti}}(\text{mg})$$

$$- \text{true AA content}_{\text{released into colon, milk}_{j,\,ti}}(\text{mg}))/ \text{AA content}_{\text{entering small intestine, milk}_{j,\,ti}}(\text{mg}) \times 100$$

Due to their importance for human metabolism, the true absorption of AA, absorption of available AA, and plasma concentration of the physiologically relevant AA groups branched-chain AA (BCAA), long neutral AA (LNAA), essential AA (EAA), non-essential AA (NEAA), and total AA (TAA) were calculated.

6.3.5. Statistical analysis

Using previous studies reporting small intestinal AA absorption and peripheral blood AA appearance as primary outcomes (Butteiger et al., 2013; Chen et al., 1962; Montoya et al., 2018), it was determined that a sample size of six pigs per diet and time point group was needed to power the study over 80% at $P < 0.05$.

All statistical analyses were carried out in SAS (version 9.4; SAS Institute Inc., Cary, NC, USA; RRID:SCR_008567). For each statistical analysis, the normal distribution and homogeneity of variance were assessed.

To test the reliability of TiO_2 as a meal flow marker, a simple linear correlation analysis of the release of TiO_2 and DM from the stomach was carried out.

For the analysis of AA absorption kinetics, a logistic function was used to fit kinetic curves for the absorption of AA of pigs fed each milk type, as described elsewhere (Montoya et al., 2023):

$$\text{Relative absorption}_{\text{Time}} = \alpha / [1 + \exp^{[\beta - (\gamma \text{Time})]}])$$

In the logistic model, α is the asymptote describing the extent of absorption, which is considered equivalent to the ileal digestibility as measured in cannulated pig studies. The parameter β responsible for horizontal curve movement, and γ describes the slope. For each AA, the full and reduced models were compared and the best fitting model was selected using an F-test.

In order to compare the absorption kinetics, the T_{50} of each AA was calculated from the fitted models for the different milk treatments. The T_{50} is the time taken for absorption of 50% of the AA consumed. Where only the AA available for uptake are considered, the T_{50} is the time taken for absorption of 50% of the AA entering the small intestine. For the determination of T_{50}, the relationships $\gamma = \alpha/2$ and $t_{50} = \beta/\gamma$ were used with α set to 100% for each curve. For each AA and AA groups, the T_{50} was compared between milk treatments via a T-test, and the difference was determined to be significant if $P \leq 0.05$. Trends were reported if $P < 0.1$.

For the analysis of plasma AA concentrations over time, a linear model with milk type, postprandial time, and the milk type x postprandial time interaction as fixed effects was used. The use of time as either a categorical and numerical value was tested, and for all AA the best fitting model used time as a categorical value.

6.4. Results

Throughout the study, all pigs remained healthy and consumed their experimental meals within five minutes. During sample collection, one pig was found to be coprophagic and was subsequently excluded from all analyses.

Throughout the postprandial period, the TiO_2 transited along the GIT (Appendix 4, Table A4.1). For all milk treatments, the release of TiO_2 and DM from the stomach were strongly correlated (r

= 0.81, Figure 6.1), which indicated that the TiO_2 can be reliably used to estimate milk flow into the small intestine, and subsequent AA absorption.

Within the PFF group, the amount of individual AA present in the stomach and small intestine was highest within the first 20 min post-feeding. The amount decreased until 60 min, after which the amounts of AA generally remained stable until 300 min (total AA as an example; Figure 6.2). This result was reflected in the portal and peripheral plasma concentration of individual and grouped AA which appeared to be elevated at 20 min postprandially and decreased at 60 min postprandially (TAA as a summary; Figure 6.3).

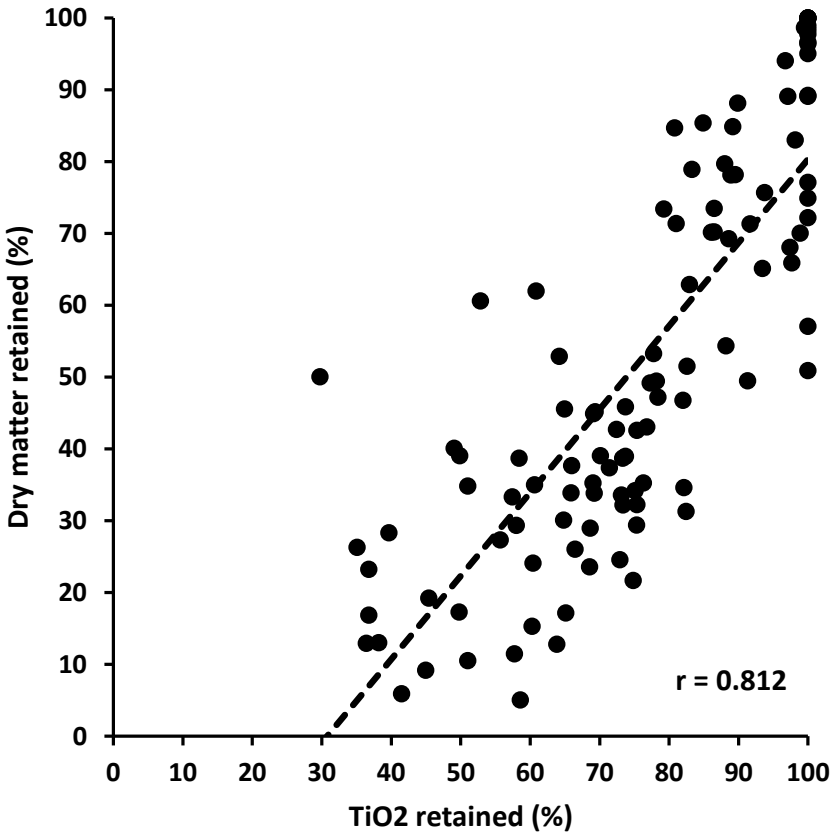


Figure 6.1: Correlation between the retention of dry matter and indigestible marker titanium dioxide (TiO_2) in the stomach of pigs fed different processed bovine milk treatments at post-feeding times (20 min – 300 min).

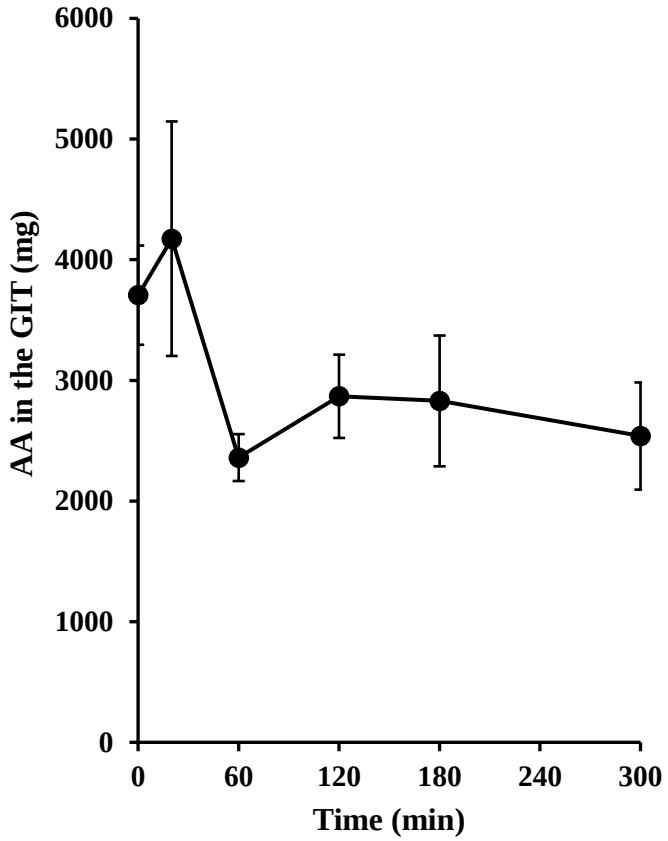


Figure 6.2: Total amino acids (AA) in the gastrointestinal tract (GIT; stomach plus small intestine) at different postprandial time points of pigs fed a protein-fat free solution.
The amount of individual AA followed a similar pattern. Values are means ± SEM, n = 6 for each postprandial time.

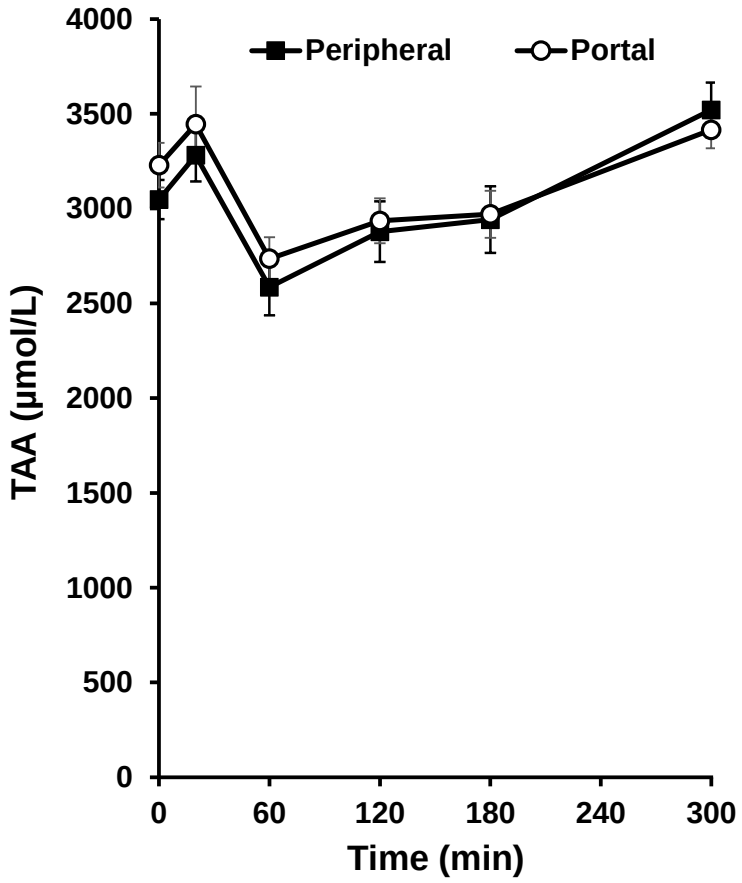


Figure 6.3: Postprandial concentrations of total amino acids (TAA) in portal and peripheral blood plasma of pigs fed a protein and fat free solution.
Values are means ± SEM, n = 6 for each postprandial time.

6.4.1. True small intestinal amino acid absorption kinetics

The true AA absorption kinetics differed ($P \leq 0.05$) between pigs fed the different milk treatments, as summarised for EAA and NEAA (Figures 6.4a - b). Across individual AA, the extent of absorption (α) ranged from 51% (raw milk, arginine) to 93% (PH milk, isoleucine) (Table 6.1). Except for leucine, methionine, threonine, asparagine, glutamine, proline, tyrosine, and NEAA, pigs fed PH milk had a 30% greater ($P \leq 0.05$) extent of absorption than pigs fed raw or PNH milk (Table 6.1). However, pigs fed PH milk tended ($P < 0.1$) toward a higher extent of absorption of lysine, leucine, methionine, serine, tyrosine and NEAA than pigs fed raw milk (Appendix 4, Table A4.2). While there was no difference ($P > 0.05$) in the extent of absorption of any AA between pigs fed PH and pigs fed UHT milk, or between pigs fed raw and pigs fed PNH milk, the differences in the extent of absorption of lysine, alanine, and arginine between pigs fed PH and pigs fed UHT milk tended towards significance ($P < 0.1$) (Appendix 4, Table A4.2). Except for arginine, which was higher ($P \leq 0.05$), and histidine and alanine, which tended ($P < 0.1$) to be higher in pigs fed UHT milk than raw milk, the extent of absorption of all other AA was similar ($P > 0.05$) between pigs fed raw and pigs fed UHT milk.

Differences in AA T_{50} were observed across pigs fed the different milk treatments (Figure 6.5). In general, for pigs fed raw or PNH milk, the T_{50} of individual AA was at least 20% greater ($P \leq 0.05$) (Table 6.2) than for pigs fed PH or UHT milk. The T_{50} of the EAA isoleucine, valine, histidine, methionine, phenylalanine, as well as the NEAA tyrosine was also at least 19% shorter ($P \leq 0.05$) for pigs fed UHT milk than for those fed PH milk. For example, in pigs fed UHT milk, the T_{50} of phenylalanine was 123 min, compared to 150 min for pigs fed PH milk. The differences in T_{50} of arginine, BCAA, and TAA between pigs fed PH or UHT milk also tended ($P < 0.1$) toward significance (Appendix 4, Table A4.3). Except for methionine, the T_{50} of all other individual AA

did not differ ($P > 0.05$) between pigs fed raw or PNH milk. The T_{50} of methionine for pigs fed raw milk was 63 min shorter ($P \leq 0.05$) than for those fed PNH milk.

When considering the true absorption of AA available for uptake, i.e., only AA released into the small intestine, the extent of true absorption ranged from 87% to 100% across AA (data not shown). The extent of absorption of AA was similar ($P > 0.05$) between pigs fed the different milk treatments. Of the individual and grouped AA, only the absorption kinetics of available glutamine and methionine were different ($P \leq 0.05$) between pigs fed the different milk treatments (Figure 6.6a – b). The T_{50} of available glutamine for pigs fed PNH milk was at least half of that of pigs fed raw, PH or UHT milk (Figure 6.6a). The T_{50} of available methionine followed the pattern UHT > raw = PH > PNH (Figure 6.6b). The difference in the T_{50} of available methionine of pigs fed raw or PH milk also tended toward significance ($P < 0.1$).

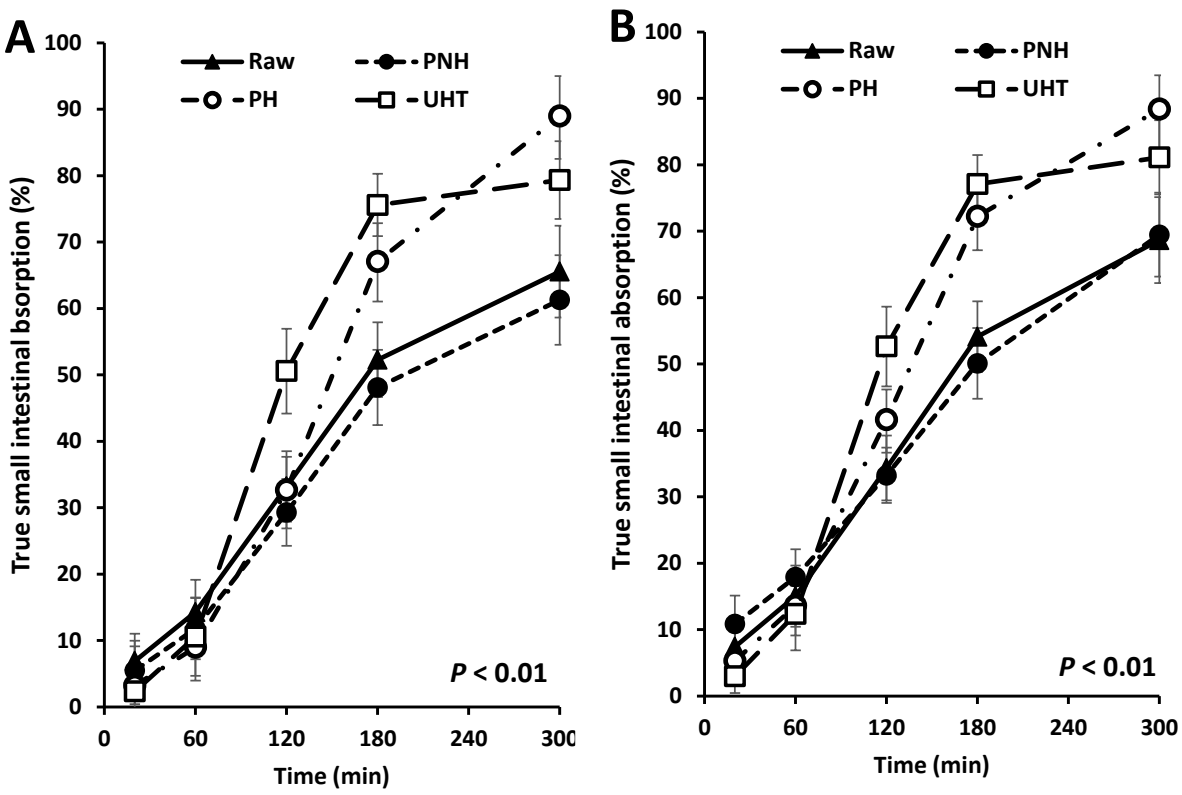


Figure 6.4: True small intestinal absorption of essential (**A**) and non-essential (**B**) amino acids over time in pigs fed processed bovine milk treatments.
PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 5 – 6 per milk type and postprandial time combination. Probability values in each panel reflect a comparison between the fitted curve of each treatment with the other treatments.

Table 6.1: Extent of true small intestinal absorption (α) of amino acids in growing pigs fed differently processed bovine milk.

	Raw	PNH	PH	UHT
		%		
Ile	72.8 ± 7.9^{bc}	66.5 ± 6.8^{c}	92.8 ± 6.7^{a}	83.3 ± 5.5^{ab}
Leu	71.8 ± 8.0	78.6 ± 13.7	90.3 ± 6.7	83 ± 5.5
Val	69.7 ± 8.1^{bc}	66.9 ± 8.1^{c}	90.4 ± 7.0^{a}	80.6 ± 5.6^{ab}
BCAA	71.5 ± 7.9^{b}	71 ± 8.9^{b}	91.2 ± 6.7^{a}	82.3 ± 5.4^{ab}
His	62.7 ± 9.1^{bc}	53.6 ± 9.0^{c}	89.1 ± 7.6^{a}	78.7 ± 6.5^{ab}
Lys	59.8 ± 9.9	64.5 ± 17.6	86.2 ± 7.3	72 ± 6.9
Met	69.6 ± 10.9^{ab}	49.4 ± 6.4^{b}	88 ± 7.3^{a}	83.2 ± 6.3^{a}
Phe	66.4 ± 9.4^{b}	61.5 ± 9.3^{b}	90.4 ± 7.3^{a}	80.6 ± 6^{ab}
Thr[1]	69.1 ± 4.2	69.1 ± 4.2	69.1 ± 4.2	69.1 ± 4.2
EAA	67 ± 8.6^{b}	62.6 ± 8.3^{b}	90.1 ± 7.2^{a}	79.4 ± 5.9^{ab}
Ala	56.8 ± 6.0^{bc}	52.4 ± 7.8^{c}	89.1 ± 8.5^{a}	71.9 ± 7.1^{ab}
Arg	50.7 ± 7.9^{c}	59.5 ± 12.2^{bc}	88.1 ± 8.0^{a}	69.9 ± 6.3^{ab}
Asp[1]	75.3 ± 3.8	75.3 ± 3.8	75.3 ± 3.8	75.3 ± 3.8
Glu	80.2 ± 9.9	87.4 ± 16.4	86.7 ± 5.6	86.1 ± 5.5
Pro	74.3 ± 9.6	85.9 ± 22.8	87.6 ± 6.4	83.1 ± 5.7
Ser	65.2 ± 8.9^{b}	61.4 ± 8.2^{b}	86 ± 7.5^{a}	74.4 ± 6.7^{ab}
Tyr	67.7 ± 10.7	63.2 ± 13.1	87.5 ± 6.9	80.2 ± 5.8
NEAA	70.3 ± 8.3	74.8 ± 11.5	89.3 ± 6.7	81.2 ± 5.6
LNAA	67.1 ± 8.9^{b}	62.5 ± 8.4^{b}	89.8 ± 7.2^{a}	79.3 ± 5.9^{ab}
TAA	69.5 ± 8.5^{b}	70.5 ± 10.4^{ab}	90.2 ± 6.9^{a}	80.9 ± 5.7^{ab}

PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised, BCAA, branch-chain amino acids; EAA, essential amino acids; LNAA, long neutral amino acids; NEAA, non-essential amino acids; TAA, total amino acids. Values are means ± SEM, n = 5 – 6 for each milk type by postprandial time combination. Means with different superscript letters within a row differ ($P \leq 0.05$).

[1] For asparagine and threonine, a reduced model best described the absorption of the different milk treatments; thus, the figures reported for each milk are the same.

Table 6.2: Time to reach a true small intestinal amino acid absorption of 50% (T_{50}) in growing pigs fed differently processed bovine milk.

	Raw	PNH	PH	UHT
		min		
Ile	174.0 ± 15.7[a]	192.9 ± 16.4[a]	142.8 ± 8.7[b]	116.3 ± 7.1[c]
Leu	178.4 ± 16.6[a]	180.6 ± 15.7[a]	129.6 ± 8.7[b]	116.6 ± 7.0[b]
Val	190.7 ± 17.3[a]	207.7 ± 16.4[a]	145.8 ± 9.0[b]	122.6 ± 8.0[c]
BCAA	181.0 ± 16.6[a]	192.5 ± 15.2[a]	138.1 ± 8.7[b]	118.3 ± 7.3[b]
His	211.1 ± 22.4[a]	253.1 ± 29.1[a]	155.6 ± 6.8[b]	125.4 ± 8.9[c]
Lys	261.3 ± 19.6[a]	270.3 ± 17.8[a]	160.5 ± 8.5[b]	156 ± 9.2[b]
Met	191.6 ± 20.1[b]	254.4 ± 29.2[a]	159.1 ± 6.8[b]	115.9 ± 6.5[c]
Phe	204.8 ± 20.7[a]	230.8 ± 20.9[a]	150.4 ± 11.8[b]	122.6 ± 8.1[c]
Thr[1]	208.3 ± 28.8	208.3 ± 28.8	208.3 ± 28.8	208.3 ± 28.8
EAA	204.6 ± 19.2[a]	224.4 ± 18.7[a]	151.5 ± 8.7[b]	128.2 ± 8.2[c]
Ala	171.7 ± 12.4	257.7 ± 28.2	165.1 ± 7.3	145.3 ± 12.3
Arg	257.1 ± 26.7[a]	261.8 ± 21.4[a]	162.4 ± 6.7[b]	136.7 ± 17.4[b]
Asp[1]	184.8 ± 20.8	184.8 ± 20.8	184.8 ± 20.8	184.8 ± 20.8
Glu	173.7 ± 14.2[a]	164.9 ± 14.1[a]	118.1 ± 8.1[b]	113.5 ± 5.9[b]
Pro	189.8 ± 16.6[a]	184.2 ± 17.1[a]	128.1 ± 8.0[b]	118.5 ± 7.3[b]
Ser	215.7 ± 19.3[a]	236.5 ± 19.2[a]	160.8 ± 8.6[b]	143.7 ± 11.0[b]
Tyr	217.5 ± 20.5[a]	250.9 ± 22.3[a]	153.8 ± 8.0[b]	124.8 ± 7.3[c]
NEAA	194.3 ± 17.9[a]	198.7 ± 15.9[a]	138.5 ± 8.2[b]	124.8 ± 7.9[b]
LNAA	206.8 ± 19.3[a]	226.8 ± 18.7[a]	152.0 ± 8.7[b]	128.4 ± 8.1[b]
TAA	197.3 ± 18.3[a]	207.8 ± 16.7[a]	143.3 ± 8.4[b]	125.5 ± 7.8[b]

PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised, BCAA, branch-chain amino acids; EAA, essential amino acids; LNAA, long neutral amino acids; NEAA, non-essential amino acids; TAA, total amino acids. Values are means ± SEM, n = 5 – 6. Means with different superscript letters within a row differ ($P \leq 0.05$).

[1] For asparagine and threonine, a reduced model best described the absorption of the different milk treatments; thus, the figures reported for each milk are the same.

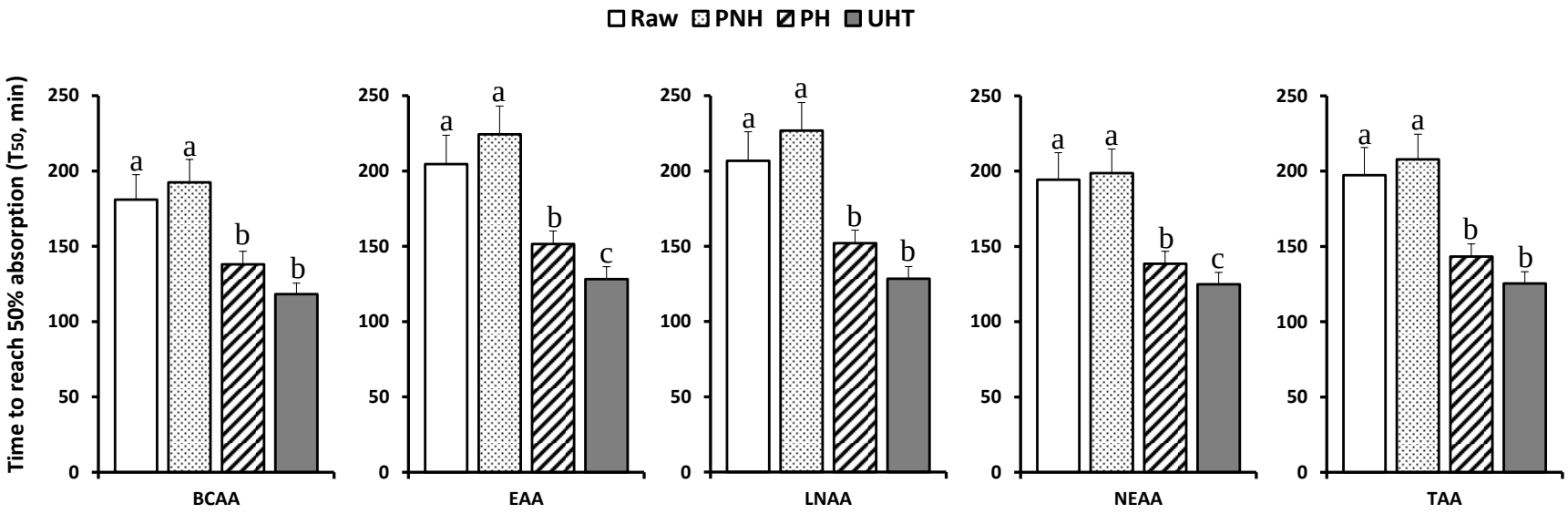
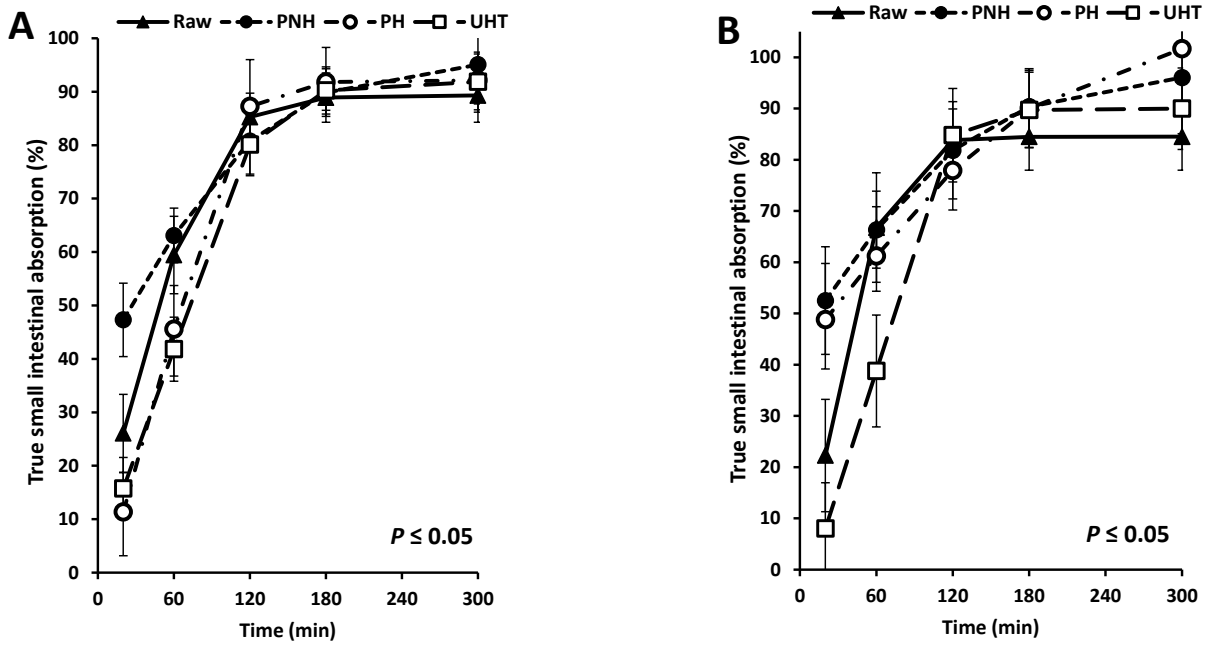


Figure 6.5: Time taken to reach a true small intestinal absorption of 50% (T_{50}) for grouped amino acids (AA) in pigs fed different bovine milk treatments.
BCAA, branched chain AA; EAA, essential AA; LNAA, long neutral AA; NEAA, non-essential AA; TAA, total AA; PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 5 – 6 per milk type and postprandial time combination. Bars with different letters (a, b, c) have different T_{50} across milk treatments ($P \leq 0.05$).



	Raw	PNH	PH	UHT
α	89.3 ± 5.0	95.9 ± 9.0	92.1 ± 5.9	91.9 ± 5.3
T_{50}	55.0 ± 5.0^{b}	25.5 ± 5.0^{c}	69.2 ± 5.0^{ab}	74.1 ± 5.0^{a}

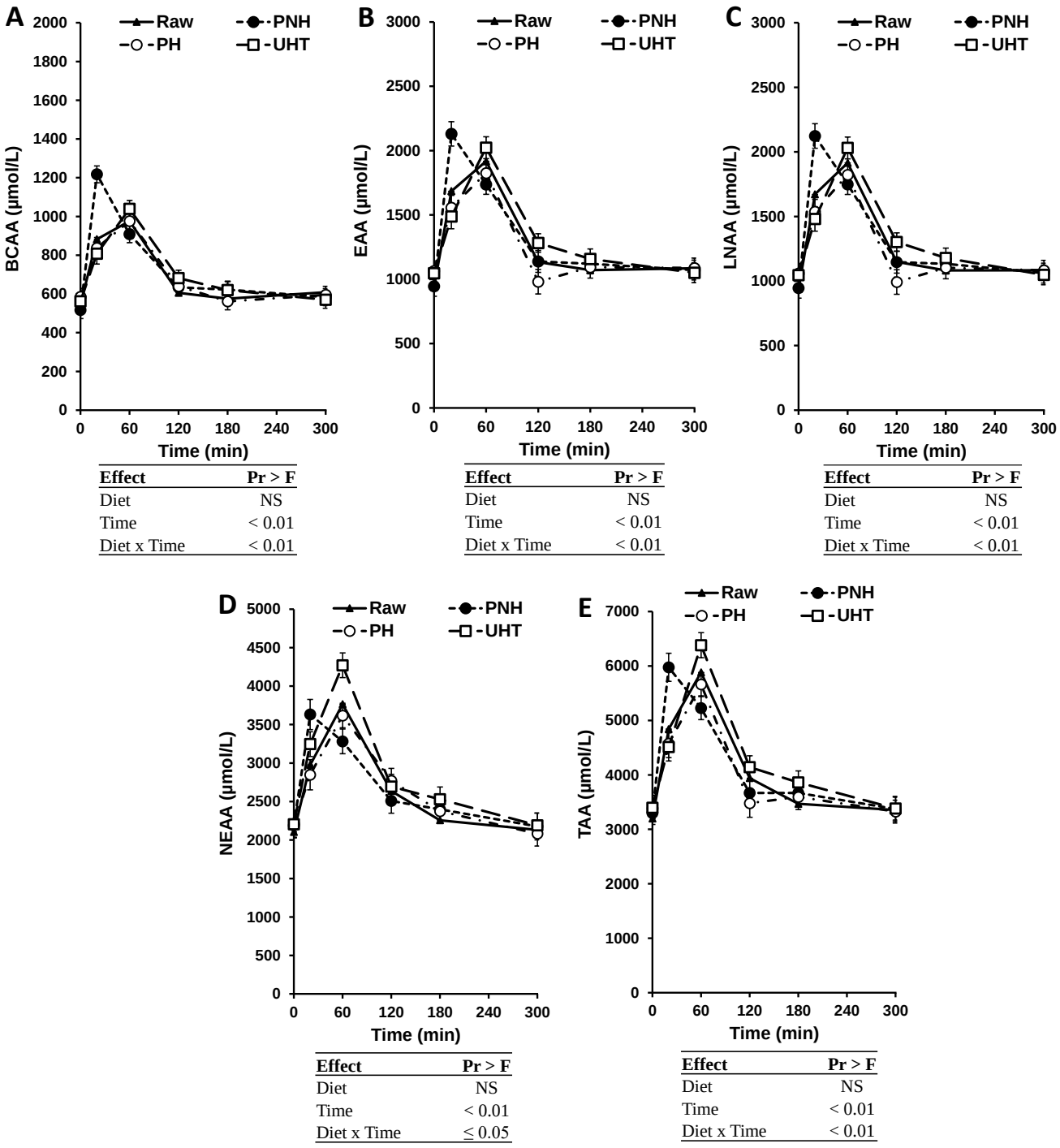
	Raw	PNH	PH	UHT
α	84.5 ± 6.5	97.2 ± 14.8	100.0 ± 17.9	90.0 ± 8.0
T_{50}	48.0 ± 9.1^{b}	10.7 ± 3.3^{c}	27.7 ± 8.4^{b}	75.7 ± 8.5^{a}

Figure 6.6: True absorption of glutamine (**A**) and methionine (**B**) released into the small intestine of pigs fed different processed bovine milk treatments.
The extent of true absorption (α) and time to reach a true absorption of 50% (T_{50}) are included as measures of small intestinal absorption kinetics. PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 5 – 6 per milk type and postprandial time combination. Probability values in each panel reflect a comparison between the fitted curve of each treatment with the other treatments.

6.4.2. Concentration of amino acids in the blood circulation

The interaction between milk type and postprandial time had a significant effect ($P < 0.01$) on the portal concentrations of BCAA, EAA, LNAA, NEAA and TAA (Figure 6.7a - e). For pigs fed raw, PH, or UHT milk, the postprandial portal AA concentration increased until 60 min, after which point the concentrations rapidly decreased (Appendix 4, Table A4.4). For pigs fed PNH milk, portal AA concentrations were highest at 20 min and decreased thereafter. In all pigs, portal AA concentrations had returned to the fasted level from 180 min postprandially.

In the peripheral circulation, there was a significant ($P \leq 0.05$) interaction between milk type and postprandial time for the plasma concentration of EAA and LNAA (Figure 6.8a and c), whereas the plasma concentrations of EAA, NEAA, and TAA was influenced ($P < 0.01$) by postprandial time only (Figure 6.8b, d, and e). The peripheral plasma concentration of individual AA in pigs fed all milk treatments generally increased up to 60 min (Appendix 4, Table A4.5). After 120 min, peripheral blood plasma AA concentrations were close to the fasted level.



Effect	Pr > F
Diet	NS
Time	< 0.01
Diet x Time	< 0.01

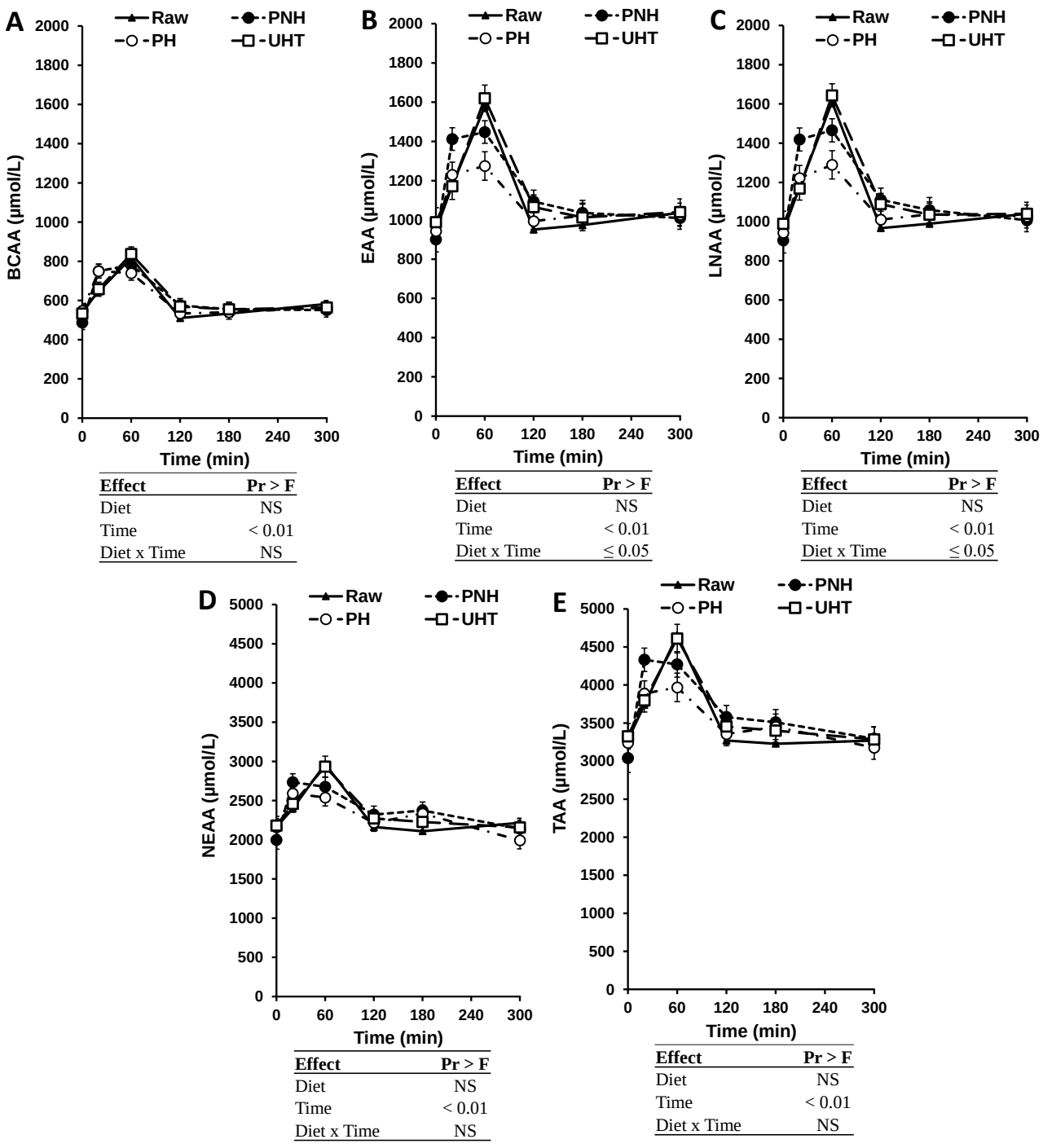
Effect	Pr > F
Diet	NS
Time	< 0.01
Diet x Time	< 0.01

Effect	Pr > F
Diet	NS
Time	< 0.01
Diet x Time	< 0.01

Effect	Pr > F
Diet	NS
Time	< 0.01
Diet x Time	≤ 0.05

Effect	Pr > F
Diet	NS
Time	< 0.01
Diet x Time	< 0.01

Figure 6.7: Postprandial plasma concentrations of branched chain (BCAA, **A**), essential (EAA, **B**), non-essential (NEAA, **C**), long neutral (LNAA, **D**), and total (TAA, **E**) amino acids in portal blood of pigs fed different processed bovine milk treatments.
PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 5 – 6 per milk type and postprandial time combination.



Effect	Pr > F
Diet	NS
Time	< 0.01
Diet x Time	NS

Effect	Pr > F
Diet	NS
Time	< 0.01
Diet x Time	≤ 0.05

Effect	Pr > F
Diet	NS
Time	< 0.01
Diet x Time	≤ 0.05

Effect	Pr > F
Diet	NS
Time	< 0.01
Diet x Time	NS

Effect	Pr > F
Diet	NS
Time	< 0.01
Diet x Time	NS

Figure 6.8: Postprandial plasma concentrations of branched chain (BCAA, **A**), essential (EAA, **B**), non-essential (NEAA, **C**), long neutral (LNAA, **D**), and total (TAA, **E**) amino acids in peripheral blood of pigs fed different processed bovine milk treatments.
PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 5 – 6 per milk type and postprandial time combination.

6.5. Discussion

This study is the first reporting the influence of heat treatment and homogenisation of bovine milk on true small intestinal absorption kinetics and plasma concentrations of AA. As hypothesised, PH and UHT treatment influenced the true small intestinal AA absorption. Further, this study demonstrated that portal and peripheral plasma AA concentration as a proxy for true absorption does not provide reliable conclusions.

For most AA, the separation between the non-homogenised (i.e., raw and PNH) and homogenised (PH and UHT) milk treatments was clearly visible in the rates of absorption throughout the linear phase of the curve, which typically occurred between 60 min and 180 min for most AA. For most AA, the rates of absorption in the linear phase were faster for pigs fed PH or UHT milk, than for those fed raw or PNH milk. For example, between 60 and 180 min, the rate of absorption of TAA was 0.31%/min and 0.27%/min in pigs fed raw and PNH milk respectively, compared to 0.48%/min and 0.53%/min in pigs fed PH and UHT milk, respectively.

Generally, homogenisation (PH and UHT) increased the extent of true AA absorption in the small intestine. For instance, the extent of true absorption of BCAA, EAA, LNAA and TAA was 38%, 40%, 40%, and 28% higher for pigs fed PH milk than those fed PNH milk, respectively. However, the extent of absorption of BCAA, EAA, LNAA, NEAA and TAA did not differ between pigs fed PH and pigs fed UHT milk, or between pigs fed raw milk and pigs fed PNH milk, which suggests that heat treatment alone has minimal effect on the extent of absorption in the small intestine. Despite this result, differences in the extent of absorption of some AA between pigs fed PH milk and pigs fed UHT milk approached significance, suggesting that stronger heat treatment of milk may have some effect on the extent of absorption.

The clear separation of the true small intestinal AA absorption kinetics between the non-homogenised (raw and PNH) and homogenised (PH and UHT) milk treatments indicated that homogenisation resulted in a greater amount of AA absorbed comparatively earlier in the postprandial period for pigs fed PH or UHT milk, compared to those fed raw or PNH milk. This was reflected in the time taken to reach a true absorption of 50% (T_{50}). For example, the T_{50} of most AA in pigs fed raw or PNH milk was similar and greater than the T_{50} observed in pigs fed PH or UHT milk. Previous research in pigs fed the same milk treatments reported a faster gastric release of BCAA, EAA, NEAA and TAA into the small intestine of pigs fed PH or UHT milk than for pigs fed raw or PNH milk (Chapter 5).

In addition, the same study described in Chapter 5 demonstrated that in pigs fed PH or UHT milk, a greater amount of digested protein was released into the small intestine at a faster rate than in pigs fed raw milk. Protein which is already partially hydrolysed on entry into the small intestine requires less time for absorption, as less breakdown is required in the small intestine (Koopman et al., 2009b). Further, the rate and amount of digested protein entering the small intestine have been shown to influence the rate of absorption in the proximal and medial small intestine (Montoya et al., 2018). Therefore, it is expected that the increased small intestinal AA absorption observed for pigs fed PH or UHT milk could be explained by increased rates and amounts of digested protein and AA released into the small intestine.

Differences in the release of digested protein and AA into the small intestine were accredited to processing-induced differences in the structure of the curd formed in the stomach (Chapter 5). For example, the curd of pigs fed UHT milk was softer with a loose structure, which appeared to promote rapid curd disintegration, greater protein hydrolysis, and a faster rate of AA release into the small intestine, compared with the curd of pigs fed raw or PNH milk. As such, it is proposed

that the differences in AA absorption kinetics observed for the processed milk treatments were linked to initial differences in gastric curd structure, and the effect of this on gastric digestion and emptying. Such a relationship between gastric transit and postprandial AA absorption kinetics has also been previously postulated in a review by Horstman and Huppertz (2022).

To add to the importance of the effect of gastric emptying on AA absorption, when only the AA available for uptake were considered, the differences in small intestinal AA absorption kinetics between milk treatments mostly disappeared, indicating that the gastric retention of AA is one of the main determinants of both the extent and the kinetics of AA absorption. This effect is consistent with a previous single time point study by Ahlborn et al. (2023b) (Chapter 3), which reported a similar influence of gastric retention on the apparent absorption of AA in piglets fed from raw bovine, caprine, or ovine milk. Thus, the results from the present study, taken together with that of the aforementioned piglet study, confirm the modulatory role of gastric retention on both the kinetics and the extent of absorption of milk AA.

The influence of the processing and time interaction on portal AA concentration was characterised by a maximal concentration of AA at 20 min in pigs fed PNH milk, which was not observed in pigs fed raw, PNH, or UHT milk until 60 min. For example, at 20 min, the portal BCAA concentration in pigs fed PNH milk was 38% higher than that in pigs fed raw, PH, or UHT milk. Considering the kinetics of true absorption in pigs fed the different milk treatments, this early peak in portal AA concentration for pigs fed PNH was unexpected, and the reasons behind this observation are unclear. However, previous research in piglets fed raw bovine, caprine, and ovine milk has shown that at 210 min, 67% of AA absorption occurring in the small intestine occurred in the first quarter of the small intestine (Chapter 3) (Ahlborn et al., 2023b). Despite a large proportion of absorption occurring in the proximal region of the small intestine, differences in absorption detected in the

proximal small intestine may not necessarily be detectable in later regions of the small intestine, as demonstrated in a study reporting AA absorption in beef-fed pigs (Montoya et al., 2018). Thus, it could be possible that differences in absorption occurring early in the small intestine are not detected through determination of the whole true absorption.

The peripheral blood plasma concentrations of histidine, methionine, phenylalanine, threonine, asparagine, glycine, tryptophan, tyrosine, EAA, and LNAA were influenced by the interaction between processing and time. In the peripheral blood plasma, the concentration curves for these AA were typically characterised by a higher maximum concentration for pigs fed raw or UHT milk than pigs fed PNH or PH milk. The result for pigs fed raw and UHT milk aligns with a study in mini-pigs receiving either unheated or heated skim milk, which found similar EAA concentrations over time for each milk type (Barbé et al., 2013). It is important to note that in that study, all milk treatments used were reconstituted from powdered form. Thus, the drying treatment could have altered the protein structures in the milk (Singh, 2007), which could in turn have affected the peripheral blood plasma AA concentration over time (Le Feunteun et al., 2014). In contrast to the present results, a study by Horstman et al. (2021) showed that consumption of full-fat PH or full-fat UHT milk standardised for volume and protein content resulted in similar maximum or time of maximum postprandial peripheral serum concentrations of EAA in humans. However, as with the present study, no intrinsic AA labelling was used for either of the aforementioned studies, so the results are influenced by whole-body metabolism, as well as the circulatory presence of endogenous and residual dietary AA and should be interpreted with caution.

In both the portal and peripheral blood plasma, the AA concentrations of pigs fed the different milk treatments generally stabilised at similar levels after 120 min. This result may be partially explained by the similar, slower rate of absorption observed for most available AA for all milk treatments

after 120 min. It is expected that this observation could be the direct result of a reduced flow of digested protein and AA into the small intestine after 120 min, as reported in Chapter 5.

In the pigs fed the PFF solution, an increase in AA concentration in portal and peripheral blood plasma was seen at 20 min, followed by a decrease at 60 min. It is expected that this observation is a result of the physiological response to an anticipated feed, which includes a release of enzymes such as lipase and pancreatic polypeptides, which are subsequently reabsorbed (Power & Schulkin, 2008). Thus, it could be assumed that a portion of the plasma AA concentration at 20 min postprandially could be ascribed to endogenous AA production specific to that time point for pigs fed differently processed bovine milk. Further, the peripheral blood plasma also contains AA of microbial origin (Metges et al., 2006), which can be up to 20% of total body input, as is the case for leucine (Raj et al., 2008). Therefore, the effect of intestinal luminal metabolism and the contribution of non-dietary AA to both portal and peripheral blood plasma AA concentrations should not be neglected. These limitations restrict the conclusions that can be drawn from portal and peripheral blood AA concentrations.

Some studies attempt to use the concentration of AA in the blood circulation as a proxy for the estimation of small intestinal absorption. However, while the results from the present study show processing does affect postprandial AA concentration in the blood plasma over time, they also demonstrate that, in the context of milk, AA concentration patterns in plasma were not consistently reflective of small intestinal AA absorption. In addition, the present study draws attention to the importance of AA metabolism in the small intestinal tissue, and highlights the needs to use tracer methodology, such as isotopically labelled AA described by de Lange et al. (1990); Engelen et al. (2014); Hess et al. (1998); Moughan (2023) to relate absorption with circulatory AA flows.

6.6. Conclusions

This study is the first to report the impact of pasteurisation, UHT treatment and homogenisation of bovine milk on the true absorption of AA in the small intestine in the growing pig as an adult human model. Homogenisation increased the rate and extent of true absorption of most AA, resulting in a greater amount of AA being absorbed comparatively earlier in the postprandial period for pigs fed PH or UHT milk, compared to those fed raw or PNH milk. Heat treatment also had an effect on true AA absorption, although the influence of heat treatment was less pronounced than that of homogenisation.

The results from the present study aligned with patterns previously reported for the gastric release of digested protein and AA from processed bovine milk into the small intestine. Further, the results demonstrate that, in the context of milk digestion, both the rate and amount of AA released from the stomach have a key role in determining small intestinal AA absorption. However, the differences in small intestinal AA absorption across the various processed milk treatments did not consistently reflect the concentrations of AA in the portal or peripheral blood plasma, indicating that plasma AA concentration alone is not representative of absorption.

This study provided new knowledge on the true absorption of AA from heat treated and homogenised bovine milk. Whether these differences translate to indicative differences in protein metabolism (e.g., protein synthesis) remains to be confirmed.

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CHAPTER 7.

GENERAL DISCUSSION

HIGHLIGHTS

- Processing-induced differences in gastric curd structure influenced the gastric emptying kinetics of protein, lipid, digested protein, and amino acids.
- The kinetics of true small intestinal amino acid absorption of processed bovine milk were mainly increased by homogenisation; heat treatment had a less pronounced effect.
- Small intestinal amino acid absorption kinetics were modulated by gastric amino acid retention, as a result of differences in curd structures.
- Small intestinal amino acid absorption kinetics were not reflected in circulatory amino acid concentrations.

The research in this PhD dissertation aimed to determine the impact of heat treatment (pasteurisation and ultra-high temperature (UHT) treatment) and homogenisation of bovine milk on the digestion of protein and absorption of amino acids (AA) in the pig as a model for the human.

Until now, studies investigating the digestion of protein and absorption of AA after the intake of heated and heated-homogenised milk have focused on gastric coagulation, curd structure, and curd disintegration. These studies have mostly used *in vitro* models of gastric digestion. Of the three studies carried out *in vivo*, the sample size was small (n = 1 – 3), or the rat was used, which limits the relevance of the results to humans. Further, no studies have determined the small intestinal absorption of AA from the consumption of processed bovine milk.

Therefore, this PhD dissertation is the first research reporting the impact of heat treatment and homogenisation of bovine milk on the *in vivo* gastric release of AA into the small intestine and the true *in vivo* small intestinal AA absorption. Furthermore, this is the first research report describing the modulatory effect of gastric curd formation, protein digestion, and gastric emptying on the small intestinal AA absorption of different milk types in the pig as a model for the human.

As hypothesised, the different curd structures formed during the gastric digestion of heat treated and homogenised bovine milk resulted in different gastric digestion characteristics, which in turn affected the protein and AA release into the small intestine, subsequently impacting small intestinal AA absorption. However, the influence of heat and homogenisation on small intestinal AA absorption was not reflected in the concentration of AA in the blood plasma. The thesis findings are summarised in Figure 7.1.

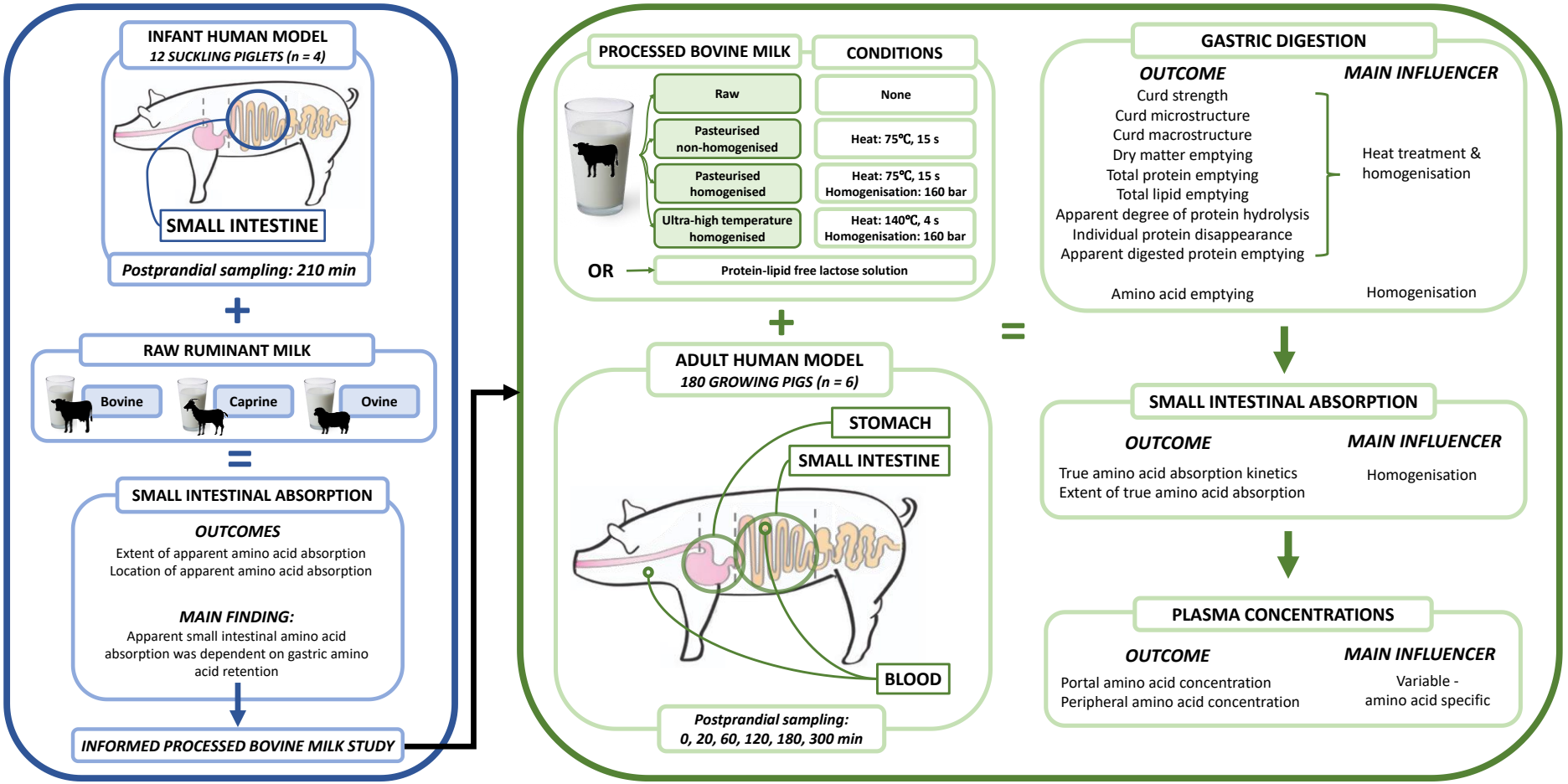

Figure 7.1: An overview of the dissertation research outcomes.

7.1. Key findings

The present thesis research began with an exploratory study to establish whether the consumption of raw bovine, caprine and ovine milk with known differences in gastric curd formation and protein emptying would result in detectable differences in small intestinal AA absorption at 210 min postprandially. For this purpose, the apparent small intestinal absorption of AA was determined in piglets as an *in vivo* model of the human infant (Chapter 3). This research showed that raw bovine, caprine, and ovine milk had different apparent small intestinal absorption at 210 min postprandially. However, the differences in apparent small intestinal AA absorption between milk types were dependent on gastric AA retention, likely resulting from differences in gastric curd structure. Chapter 3 also highlighted the modulatory effect of gastric protein behaviour on the *in vivo* small intestinal AA digestibility of raw milk. In addition, the research in Chapter 3 demonstrated that, as expected, for most AA at least two-thirds of the apparent AA absorption at 210 min occurred within the first quarter of the small intestine.

Based on the importance of the relationship between gastric protein behaviour and the small intestinal digestibility of milk protein shown in Chapter 3, the processed bovine milk protein digestibility section of the thesis research began by investigating the effect of pasteurisation, UHT treatment, and homogenisation on the *in vivo* gastric curd formation and dry matter (DM), protein, and lipid macronutrient emptying (Chapter 4). Both heating and homogenisation altered the structure of the gastric curd which became progressively more fractionated and porous as the extent of processing increased. Further, homogenisation (pasteurised homogenised (PH) and UHT treated milk) resulted in smaller fat globules incorporated into the protein network, whereas the rigid protein matrix of the unhomogenised milk (raw and pasteurised non-homogenised (PNH) milk) contained large groups of coalesced milk fat globules. Based on the existing literature, the

differences in curd structure could be explained by processing-induced changes to native casein micelles, whey protein and milk fat globules structures (Anema & Li, 2003; Corredig & Dalgleish, 1999; De Kruif et al., 2012; Lee & Sherbon, 2002; Ye et al, 2004a; Ye et al, 2004b).

The more fractionated curd structure formed by PH and UHT milk resulted in a softer curd, compared to raw and PNH milk. The softer, more open protein network formed by the PH and UHT milk was hydrolysed more rapidly than that of raw and PNH milk, which in turn resulted in a faster gastric emptying of DM, protein and lipid. In contrast, the mild heat treatment (pasteurisation) of milk without the addition of a homogenisation step did not influence the curd strength, or gastric emptying of DM or total protein from the curd or the total chyme, compared to raw milk. However, mild heat treatment alone did increase the gastric release of lipid from the total gastric contents, and from the curd.

Other studies have suggested that the release of lipid from gastric curd is coupled to curd protein release (Roy et al., 2022; Ye et al., 2016). In the present study, a strong positive linear correlation ($r = 0.94$) was observed between the release of protein and lipid from the gastric curd of pigs fed each milk treatment (Chapter 4) (Figure 7.2). This correlation suggested that the relationship between curd protein network disintegration, and lipid release into the gastric liquid phase also persisted for processed bovine milk treatments.

While both heat treatment and homogenisation altered the protein to lipid retention ratio of the gastric curd, strong heat treatment combined with homogenisation (UHT) resulted in an inverse protein to lipid retention ratio, compared to mild heat treatment (PNH) or mild heat treatment combined with homogenisation (PH). The difference in protein to fat ratio of the curd formed from UHT milk compared to the other milk treatments is likely explained by the loose initial curd

structure, and more rapid lipid emptying kinetics observed for pigs fed UHT milk, as described in Chapter 4.

Differences in gastric curd structure, and the release of DM, protein, and lipid from the curd were reflected in the emptying of total gastric contents, whereas the gastric liquid phase had minimal impact on these overall outcomes of gastric digestion. Thus, this thesis research showed that the emptying of the total gastric content was determined by the curd disintegration and emptying behaviours.

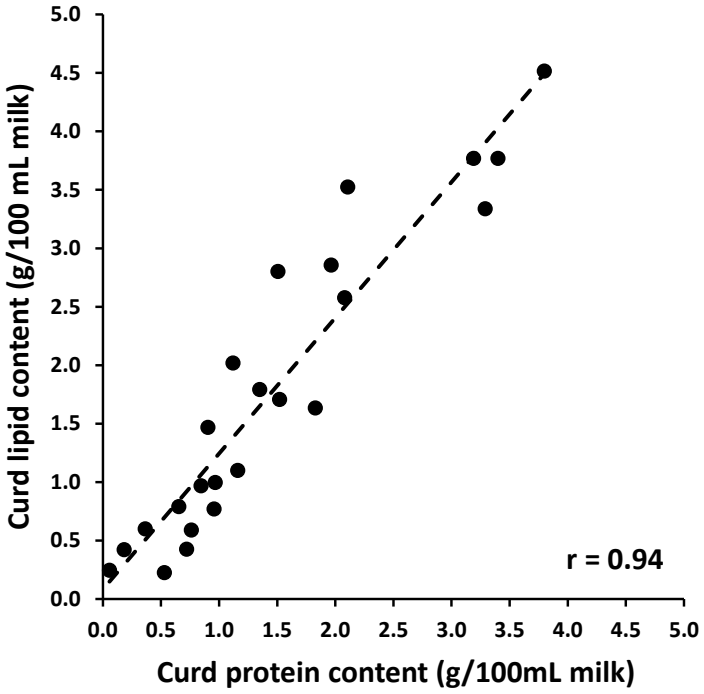

Figure 7.2: Correlation between the amount of total protein and total lipid retained in the stomach solid (curd) of pigs fed raw, pasteurised non-homogenised, pasteurised homogenised, or ultra-high temperature treated bovine milk, as sampled at 20, 60, 120, 180, and 300 min postprandially.
Fitted means for each treatment and time point combination are shown, n = 5 or 6 for each treatment and time point combination.

The altered curd structure and gastric emptying characteristics caused by heat treatment and homogenisation had subsequent effects on the gastric protein digestion, and the flow of digested protein and AA into the small intestine (Chapter 5). Both heat treatment and homogenisation increased the apparent degree of gastric protein hydrolysis, the gastric disappearance of individual proteins, and the release of digested protein into the small intestine. However, the rate and amount of most AA entering the small intestine, which could be considered as the final outcomes of gastric digestion, were mainly increased by homogenisation. In contrast, heat treatment had a less pronounced effect on gastric AA emptying.

The influence of homogenisation on the flow of AA into the small intestine was directly reflected in the true small intestinal AA absorption kinetics (Chapter 6). In the small intestine, faster absorption kinetics were observed for most AA from the homogenised milk treatments, compared to those from the non-homogenised milk treatments. Homogenisation also increased the extent of true small intestinal AA absorption. Similar to the gastric release of AA, the effect of heat treatment on the rate and extent of true small intestinal AA absorption was less obvious.

Across the differently processed bovine milk types, a strong negative linear correlation was observed between the rate of AA entering the small intestine and the rate of true small intestinal AA absorption ($r = -0.94$) (Chapter 6) (Figure 7.3). This finding supported the results of Chapter 3, which showed that the absorption of milk AA in the small intestine was influenced by the release of AA from the stomach.

When considering the relationship between curd structure and gastric emptying discussed in Chapters 4 and 5, in conjunction with the correlation of small intestinal AA absorption with gastric AA release, it is clear that differences in AA absorption are the result of differences in gastric milk

coagulation. For example, the slower true small intestinal AA absorption kinetics observed for pigs fed raw and PNH milk could be explained by the slower release of AA into the small intestine, compared to pigs fed PH and UHT milk. The slower AA gastric emptying kinetics for these pigs was explained by the dense and strong curd formed during gastric digestion.

Similarly, the plateaued rate of absorption observed after 180 min for most AA in pigs fed UHT can be explained by the reduced rate of AA entering the small intestine, as a result of the small amount of the consumed protein remaining in the stomach at 180 and 300 min; a scenario which arose due to the rapid protein emptying over the postprandial digestion period.

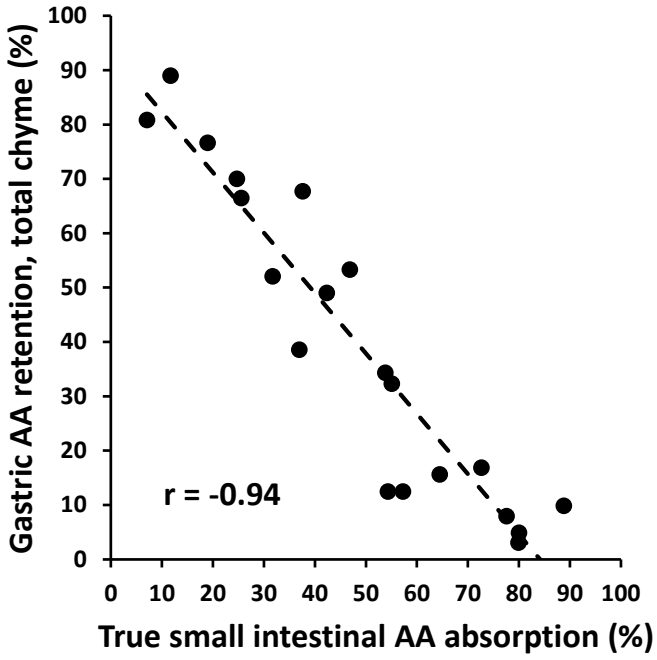

Figure 7.3: Correlation between the gastric retention of total amino acids (TAA) in the total gastric contents, and the true overall absorption of TAA in the small intestine of pigs fed raw, pasteurised non-homogenised, pasteurised homogenised, or ultra-high temperature treated bovine milk, as sampled at 20, 60, 120, 180, and 300 min postprandially.
Fitted means for each treatment and time point combination are shown, n = 5 or 6 for each data point.

Boirie et al. (1997), Horstman and Huppertz (2022), and Boulier et al. (2023) have proposed that the gastric emptying kinetics determined by curd structure could be the main modulator of small intestinal absorption kinetics. However, this thesis research is the first to demonstrate this relationship, by linking gastric curd characteristics with gastric protein digestion, gastric protein and AA emptying, and true small intestinal AA absorption.

Research by Roy et al. (2022) reported different curd structure formation and protein emptying kinetics for piglets fed raw bovine, caprine, and ovine milk. The single time point study described in Chapter 3 showed a correlation between the apparent small intestinal absorption of raw ruminant milk and gastric AA retention. Thus, when this correlation and the results of Roy et al. (2022) are taken with the findings from Chapter 6, it could be proposed that a similar dependent relationship between gastric curd formation and small intestinal AA absorption kinetics might also be observed for raw bovine, caprine, and ovine milk.

As a final physiological outcome, the postprandial plasma concentration of AA in the portal vein and in peripheral blood of pigs fed each processed milk treatment was compared. As expected, the effect of processing on plasma AA concentration was more visible in the portal vein than in peripheral blood. However, the clear effect of homogenisation on true small intestinal AA absorption kinetics was not reflected in plasma AA concentrations. Thus, the findings from Chapter 6 demonstrate that plasma AA concentration is an inaccurate proxy for gastric AA emptying or small intestinal AA absorption, and as such this area remains to be investigated. Together with the strong relationship between gastric AA release and small intestinal AA absorption, these results suggest that, in the absence of tracer methodologies, the gastric AA emptying is a better indicator of AA absorption than plasma concentrations. However, the limitation of this approach is that

human gastric AA emptying cannot be easily assessed, which increases research reliance on *in vitro* and *in vivo* models.

It is accepted that the ability of dietary protein to influence aspects of human metabolism, such as protein synthesis, is determined largely by both a food's AA profile and its digestion and AA absorption kinetics (Pennings et al., 2011; Trommelen et al., 2020). Based on the results from this thesis research, it is suggested that the differently processed bovine milk treatments could differentially impact postprandial protein synthesis, as a result of altered curd structure, protein digestion characteristics, and small intestinal AA absorption kinetics. Of particular relevance is the potential to modulate the postprandial anabolic muscle response by the application of heat treatment and homogenisation, which could be exploited to provide superior nutrition options for populations requiring individual protein demands, such as the elderly.

7.2. Strengths

A strength of this thesis research was the use of the pig as a human model. Besides the close similarity of the pig and human gastrointestinal tract, the pig model also allowed for the collection of various samples from locations that are difficult to access in humans, and in greater volume than is possible in the rat. This provided the opportunity to gain a detailed understanding of the gastrointestinal digestion of processed milk. For example, the overall true small intestinal AA absorption kinetics would be exceptionally difficult to determine in the human, as the collection of the entire duodenal and jejunal contents is invasive. In contrast to *in vitro* digestion models, digestion and absorption in the pig model means that physiological functions such as gastric peristaltic movements, enzymatic release, nutrients flows, and gastrointestinal feedback mechanisms are accounted for.

Another strength was the scale of the study carried out. The use of a sufficiently large sample size (180 pigs; n = 6 per treatment and time point combination) for the outcome with the lowest detectable difference (small intestinal AA absorption and AA plasma concentration) powered at an appropriate level (80%), means that the conclusions drawn from the results can be expected to be reliable.

The inclusion of a protein-fat-free solution as an experimental meal allowed for the samples collected from the processed-milk fed groups to be corrected for endogenous AA, as well as residual AA from the pre-fast meal. Furthermore, some pigs had residual pre-fast meal remaining in the stomach, which could be corrected for using the amount and composition of pre-fast meal retained in some of the pigs fed the protein-fat free solution.

A further strength of the growing pig study was that the gastrointestinal content and blood samples were collected from the same animals in the same study. This allowed for an in-depth understanding of the links between each of the digestion and absorption stages. In addition, this approach ensured consistency of factors such as milk composition, milk processing conditions, milk storage, animal demographics, feeding routines, and animal handling throughout the study, thus avoiding the effects of uncontrolled extraneous variables on the outcomes from each gastrointestinal section.

In terms of application of the thesis research, the pigs in the processed bovine milk study consumed commercially available processed bovine milk, which can be easily accessed through supermarkets in most Western countries. With the exception of the PNH milk, which was prepared by combining skim milk with cream, the milk was consumed as purchased, which makes the present findings relevant to the standard Western household consumer.

7.3. Limitations

During the thesis research some limitations were identified. While the effects of the limitations on the results were minimised as much as possible, some aspects remain to be considered.

While determining the apparent small intestinal AA absorption of raw bovine, caprine, and ovine milk in the piglet model, the smaller physical size of the animal limited the amount of terminal ileal digesta that could be collected, which meant that some estimations were required, based on two animals. As a result, the overall small intestinal AA absorption could not be determined. In addition, the small group size (n = 4) means that the reported results could be influenced by intra-individual variation.

The study used for the determination of small intestinal AA absorption of raw bovine, caprine, and ovine milk did not include a protein fat free treatment to correct for endogenous and pre-fast dietary AA. Thus, the results were not solely representative of dietary AA, and only apparent AA absorption could be determined.

The milk consumed by the piglets was standardised for protein content, which resulted in the consumption of differing volumes, due to differences in nutrient composition of bovine, ovine, and caprine milk. As volume can affect gastric emptying, it is possible that this could in turn have affected the release of AA into the small intestine, which could subsequently have impacted the apparent AA absorption.

Blood collected from the portal vein over the study time course contains the AA transported from the whole gastrointestinal tract, although these AA concentrations have been influenced by gastrointestinal epithelial cell metabolism (Stoll et al., 1998). In contrast, blood collected from the inferior vena cava vein can be considered peripheral blood, as the circulating AA concentrations

have already been additionally influenced by liver metabolism, which altogether can metabolise close to 60% of dietary nitrogen (Fouillet et al., 2003). Besides dietary AA, the presence of AA from endogenous origin contributes to the circulating AA. Therefore, the use of portal and peripheral blood for the estimation of dietary AA appearance is not an accurate approach. In addition, blood flow was not measured, which meant that blood AA fluxes could not be determined.

During the collection of portal and peripheral blood of pigs fed differently processed bovine milk, the pigs were deeply anaesthetised by the intravenous administration of a drug cocktail containing ketamine, xylazine, zolazepam, and tiletamine. There is some *in vivo* evidence that the use of intravenous anaesthetics, including ketamine and xylazine, could have vasodilatory properties (Irwin et al., 2023; Yamazaki et al., 1995), which may result in an increased splanchnic blood flow (Harper & Chandler, 2016). The use of these drugs could have affected the concentrations of AA in the portal and peripheral blood at each time point, which is especially important for the relevance of these measures to the postprandial peripheral blood collection from non-anaesthetised humans. However, this relationship has not been consistently reported *in vivo* or in humans.

Some anaesthetic drugs are reported to impact aspects of gastrointestinal digestion, such as gastric emptying. However, there is minimal research on the effects of anaesthesia by ketamine, xylazine, zolazepam, and tiletamine on the gastrointestinal function relative to digestion and absorption. In any case, for both pig studies all gastrointestinal sample collection was carried out within 10 min of full anaesthetisation. Thus, in pigs sampled at least 60 min postprandially the effect of anaesthesia on the gastrointestinal transit of the milk meal is expected to be minimal, due to the short sampling timeframe relative to the postprandial time point. In pigs sampled at 20 min postprandially, the gastric results showed that the milk meal had not yet been released from the

stomach. As such any potential effect of anaesthesia on gastrointestinal motility should not have affected the results.

Finally, it is important to note that the thesis research was carried out in the pig as a model for the human. While the morphological and functional aspects of gastrointestinal digestion are similar between the two species, it cannot be guaranteed that the effects of heat treatment and homogenisation on the digestion and absorption of bovine milk protein observed here will be replicated in the human.

7.4. Future directions

The research presented in this dissertation has highlighted various aspects of processed milk digestion and absorption which warrant further investigation. These aspects are described in the following suggestions, which might guide future research on the digestion and absorption of processed milk.

1. The differences in the apparent amount of AA absorbed from raw bovine, caprine, and ovine milk at 210 min suggest that the raw ruminant milk types each have differing small intestinal AA absorption kinetics. However, this aspect has not yet been investigated. Therefore, a study determining the rates of small intestinal AA absorption of raw bovine, caprine, and ovine milk is warranted. This study should include a protein-fat-free treatment, to allow for the correction of endogenous and residual dietary AA.

2. The research in Chapter 3 demonstrated that the majority of raw milk AA absorption occurred in the first quarter of the small intestine. However, for heat treated and homogenised bovine milk, only the overall small intestinal AA absorption of processed

bovine milk was determined. Thus, it could be expected that AA absorption in different locations of the small intestine could also be influenced by processing, as a result of differences in gastric emptying kinetics. Therefore, it is suggested that the small intestinal AA absorption kinetics of differently processed bovine milk in different locations throughout the small intestine is investigated.

3. Other factors which could influence gastric protein emptying were not investigated in this study. For example, the semiquantitative approach used to estimate individual protein emptying in this thesis suggests that the processing influenced the disappearance of casein and whey protein fractions, which might indicate the individual proteins from differently processed bovine milk could have differing rates of release into the small intestine. However, this observation is a combination of the gastric digestion and gastric emptying, and the influence of processing-induced structural changes of individual proteins on gastric digestion and gastric emptying remains to be quantified. To this end, the analysis of peptide kinetics in the stomach and small intestine after the consumption of processed bovine milk is warranted.

4. The use of portal and peripheral blood for the estimation of dietary AA appearance is an inaccurate approach. Thus, to accurately determine the postprandial blood plasma concentrations and flows of AA from heat treated and homogenised milk it is suggested that a study determining the blood flow and AA appearance kinetics of isotope-labelled proteins of processed milk is carried out.

5. Extracted dairy proteins with differing absorption rates resulting in altered plasma AA concentrations have been shown to differentially affect various metabolic processes

throughout the body, such as skeletal muscle protein synthesis (Koopman et al., 2009; Tang et al., 2009). Therefore, a study to determine the metabolic effect of the processed bovine milk treatments is warranted. When used with the present results, a metabolic study (i.e., investigating postprandial protein metabolism markers in the circulatory system) could be valuable for the design of processing treatments and products which enhance the nutritional outcomes of dairy consumption, especially for specific populations with modified protein needs, such as the elderly (Moore et al., 2015).

6. Extensive research efforts should be made to understand the relevance of the current knowledge of milk protein digestion and AA absorption in the pig model to the human. Alongside confirming the persistence of the observed results in humans, such validation could provide a valuable basis for the future use of digestion outcomes determined in humans (i.e., blood AA appearance) as indicators for changes occurring during the gastrointestinal digestion (i.e., gastric AA emptying). However, it is expected that this research could be challenging, due to the limitations associated with invasive human sample collection.

7. While this thesis research focussed on protein digestion and AA absorption, Chapter 4 showed that heat treatment and homogenisation also altered the behaviour of milk lipid in the stomach and increased the rate of release of lipid into the small intestine. Based on the altered plasma concentrations of fatty acids after the consumption of pasteurised and UHT treated bovine milk (Nuora et al., 2018), it could be expected that different gastric lipid emptying could affect lipid absorption in the small intestine. Samples were collected for these analyses, however the impact of Covid-19 on the thesis timeline meant that the analyses could not be included. Therefore, the investigation of the impact of processing

on milk lipid small intestinal digestion and absorption kinetics, and appearance in the lymph and blood circulatory systems remains to be determined.

8. In the context of nutritional research, the pig is generally accepted as the best model for the human at various life stages (Roura et al., 2016). The animal model plays a particularly essential role in scenarios where conducting the same research in the human would result in a loss of detail (i.e., due to sample collection limitations), or where dynamic physiological processes cannot be sufficiently simulated *in vitro*. In this way, the pig makes valuable contributions to the advancement of scientific knowledge. However, an increasing ethical pressure to avoid animal models in research scenarios has become apparent, and reliable alternative approaches must be identified. Currently, *in vitro* gastric models have been shown to accurately simulate the *in vivo* gastric digestion of differently processed bovine milk, but dynamic small intestinal models which incorporate a physiologically relevant simulated absorption aspect still need to be developed. Therefore, a concerted research effort should be made to develop accurate, repeatable *in vitro* small intestinal absorption models. These gastrointestinal *in vitro* models should be validated against both the pig and the human for various foods with different compositions and structural matrices. This development and validation would provide the detail necessary to create a thorough understanding of gastrointestinal digestive behaviour when other test foods are applied, without a reliance on animal models. A further alternative to the use of animals in nutritional research is the application of mathematical models, such as those developed by Strathe et al. (2008) and Le Feunteun et al. (2014). However, these rely on the input of various fixed parameters (i.e., gastric retention time) specific to the food of

interest. To this end, it is suggested that the current thesis research could be used to inform and refine such mathematical models for the application to other processed dairy products.

9. This research used the growing pig as a model for the healthy adult. However, the findings from the present research are especially relevant for the development of milk-based products which can meet specific protein-related nutritional needs. Thus, efforts should be made to understand the translation of the current results to demographic groups with altered protein requirements, such as the elderly. While the use of an older pig as a model for the elderly human would provide fundamental knowledge, such research efforts should also be validated in the elderly human.

7.5. Conclusions

Overall, this thesis showed that both heat treatment and homogenisation influenced the digestion of protein and absorption of AA in the growing pig as a model for the adult human fed whole milk. Further, this thesis is the first research to demonstrate that the true small intestinal AA absorption in these pigs is modulated by gastric retention, as a result of differences in curd structures.

The heat treatment and homogenisation of bovine milk altered the curd structures formed during *in vivo* gastric digestion, which increased the gastric emptying of DM, protein, and lipid. However, the release of digested protein and AA into the small intestine was mainly increased by homogenisation, with minimal effect observed for heat treatment. This pattern was reflected in the small intestinal AA absorption kinetics, where homogenisation increased the rate and extent of milk AA absorption. Heat treatment also had a less pronounced effect of small intestinal AA absorption. Differences in small intestinal AA absorption kinetics of pigs fed the different bovine milk processing treatments were dependent on gastric AA retention. The influence of gastric AA

retention on small intestinal AA absorption was also consistent for raw bovine, caprine and ovine milk in piglets as a human infant model.

The differences in small intestinal AA absorption between differently processed bovine milk types were not consistently reflected in the concentration of AA in the peripheral and portal blood plasma. This finding indicated that plasma AA concentration alone is not representative of the true small intestinal AA absorption in pigs fed processed bovine milk. Based on the outcomes of the thesis research, it is suggested that, in the absence of tracer methodology, the gastric AA emptying is a better predictor of small intestinal AA absorption than plasma AA concentrations.

Overall, homogenisation of bovine milk had a strong effect on the overall protein digestion and AA absorption outcomes in the pig as an adult human model. Heat treatment had a less pronounced effect. The manipulation of curd structure through processing is an approach for the modification of the small intestinal AA absorption kinetics of bovine milk but not of circulatory AA concentrations. The new knowledge gained through this research could be used to design milk products which optimise the delivery of milk protein and AA to the small intestine. It is not yet clear whether these differences translate to indicative differences in blood AA appearance kinetics, and subsequently whole-body or tissue-specific protein metabolism (e.g., protein synthesis).

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APPENDIX 1.

CHAPTER 3: SUPPLEMENTARY METHODS

Method A1.1. Gastric chyme defatting

Freeze-dried and ground gastric curd (solid fraction) and liquid fraction were weighed (1 g) in a glass tube before addition of petroleum ether and diethyl ether at a 1:5:5 ratio. Solvents were added, vortexed for 30 s, stood for 5 min, and centrifuged at 3,000 rpm for 7 min at room temperature. The solvent fraction was removed using a water-vacuum system. The same procedure was repeated twice to remove residual solvent. The samples were then incubated at 105 °C for one hour, cooled, and the remaining lipid-free sample material was weighed.

APPENDIX 2.

CHAPTER 4: SUPPLEMENTARY FIGURES

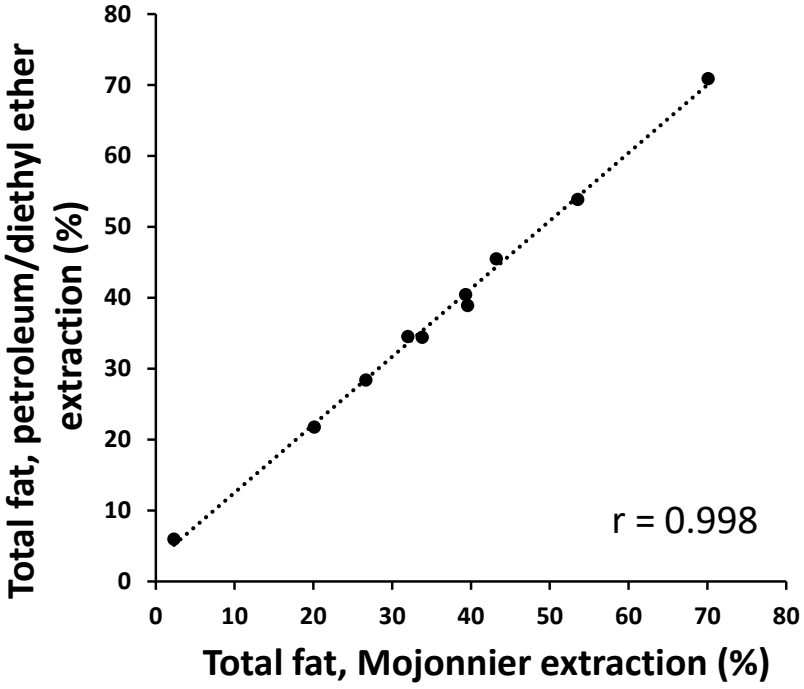


Figure A2.1: Correlation between Mojonnier and petroleum/diethyl ether lipid extraction methods for a representative sample set of milk (4) and curd (6) collected at different post-feeding times of growing pigs fed different processed bovine milk types.

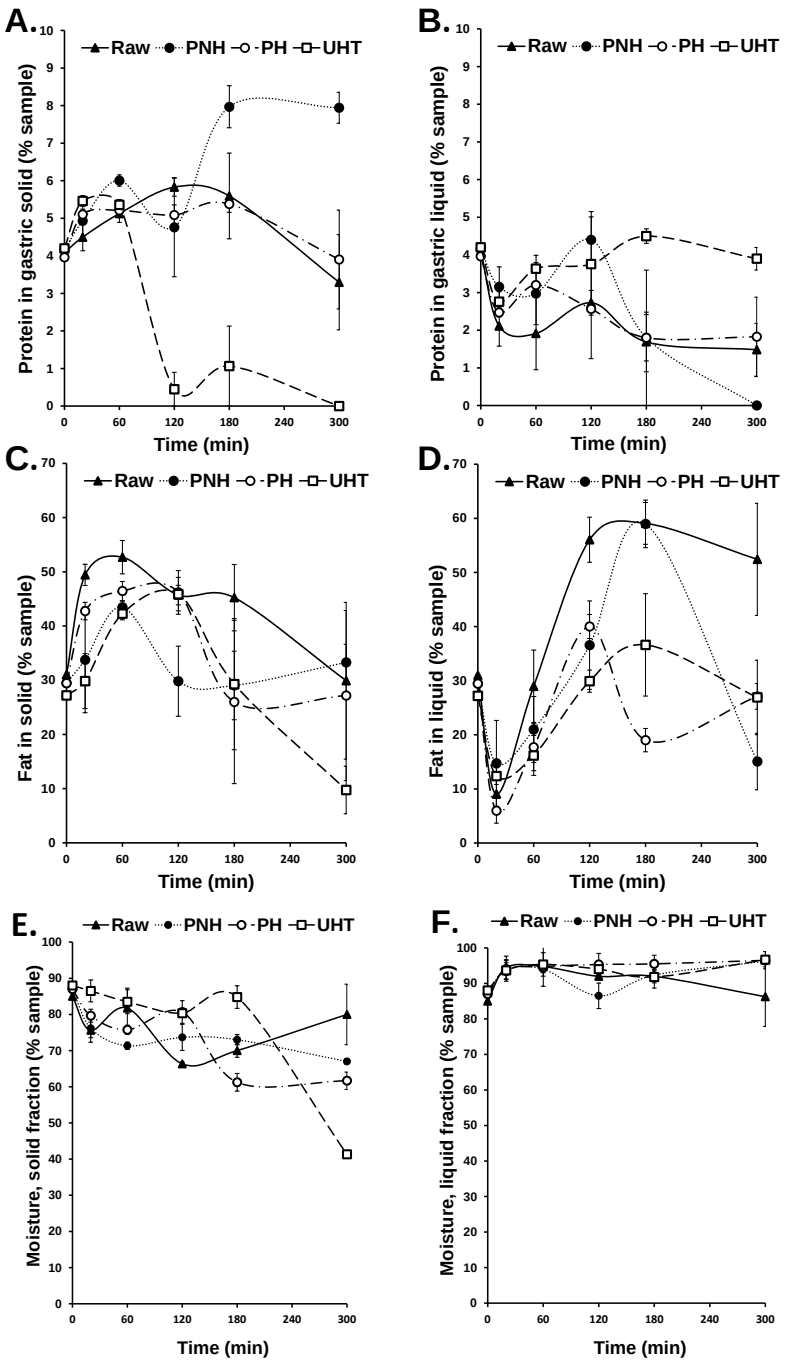
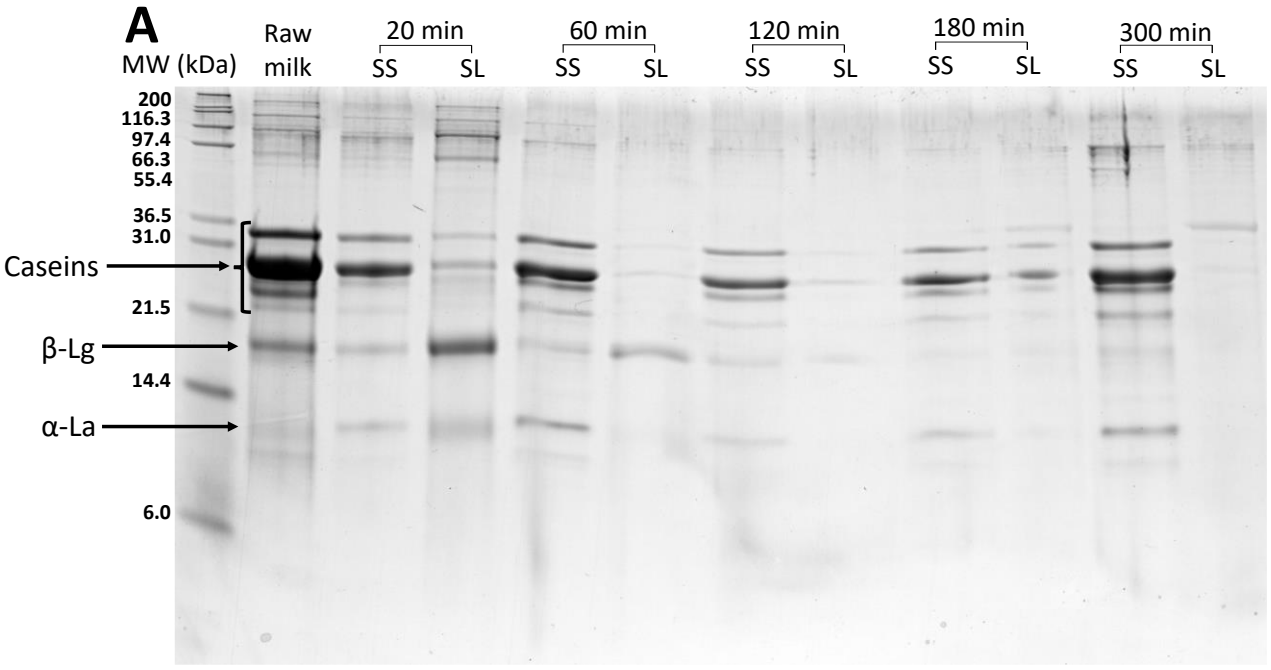
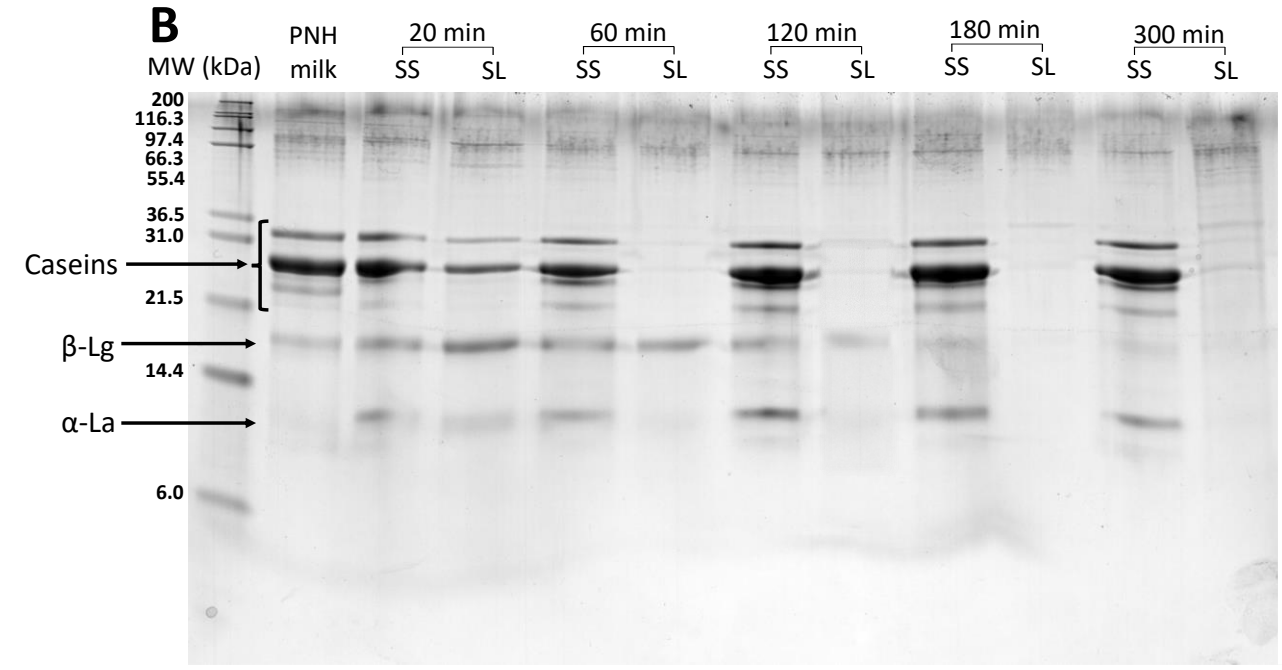


Figure A2.2: Observed concentrations of protein and lipid (on a dry matter basis) and moisture in gastric curd (solid) and liquid fractions collected at different post-feeding times of growing pigs fed different processed bovine milk types.
A and B: curd and liquid protein; **C and D:** curd and liquid lipid; **E and F:** curd and liquid moisture. PNH, pasteurized non-homogenized; PH, pasteurized homogenized; UHT, ultra-high temperature treated homogenized. Values are means ± SEM, n = 4 – 6. The starting values for the curd and liquid fractions were assumed to be the concentrations of the milk consumed.

APPENDIX 3.

CHAPTER 5: SUPPLEMENTARY FIGURES


A
MW (kDa)
Raw milk
20 min
60 min
120 min
180 min
300 min
SS
SL
200
116.3
97.4
66.3
55.4
36.5
31.0
21.5
14.4
6.0
Caseins
β-Lg
α-La


B
MW (kDa)
PNH milk
20 min
60 min
120 min
180 min
300 min
SS
SL
200
116.3
97.4
66.3
55.4
36.5
31.0
21.5
14.4
6.0
Caseins
β-Lg
α-La

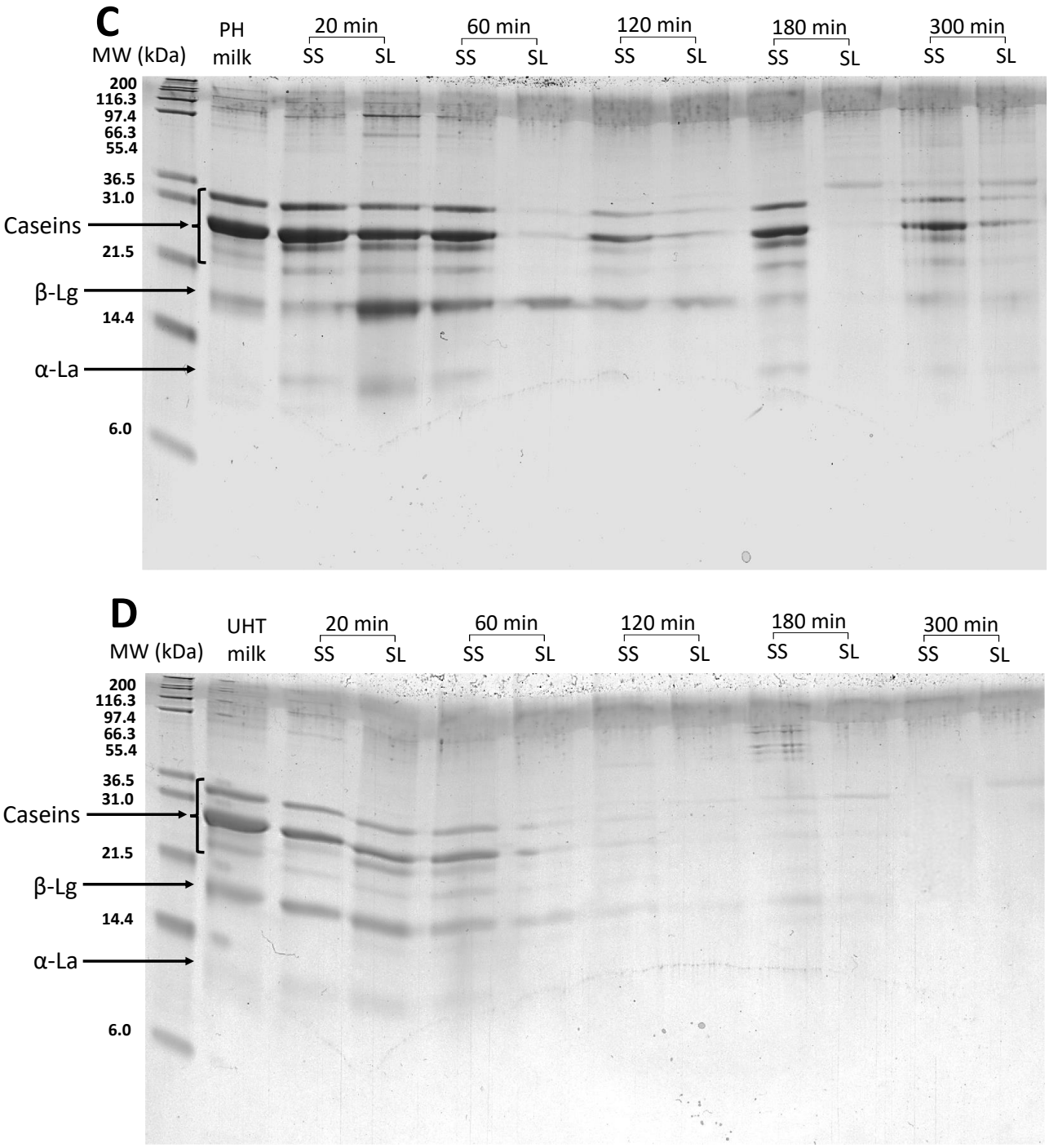


Figure A3.1: Milk proteins in the gastric solid (SS) and liquid (SL) fractions collected at different postprandial time points from pigs fed differently processed bovine milk types, as identified by sodium dodecyl-sulphate polyacrylamide gel electrophoresis.
A, raw milk; B, pasteurised non-homogenised milk (PNH); C, pasteurised homogenised milk (PH); D, ultra-high temperature treated milk (UHT). Samples from the same milk type and time point combination were pooled; for each lane n = 5 – 6. In each gel the milk type consumed was included in lane 2. β-Lg, β-lactoglobulin; α-la, α-lactalbumin.

APPENDIX 4.

CHAPTER 6: SUPPLEMENTARY TABLES

Table A4.1: Recovery of titanium dioxide (TiO_2) content in the gastrointestinal tract of pigs fed different processed bovine milk treatments at different post-feeding times.

	Time	Stomach	PSI	DSI	TI	Colon	Recovered
				% consumed			
Raw	20	76.1 ± 3.1	6.3 ± 1.6	2.7 ± 1.1	0.2 ± 0	24.4 ± 2.8	109.6 ± 6.2
	60	64.6 ± 0.7	3.6 ± 1	9.5 ± 2	0.5 ± 0.2	29 ± 3.1	107.1 ± 4.1
	120	68 ± 4.1	1.6 ± 0.4	14 ± 2.7	0.4 ± 0.1	22 ± 3.5	106 ± 3.7
	180	50.4 ± 7.2	4.5 ± 2.2	16.6 ± 2.7	3.6 ± 2.3	26.7 ± 3.1	101.8 ± 5.2
	300	35.4 ± 11.3	5.1 ± 1.8	19.9 ± 6.6	1.6 ± 1.3	36.8 ± 7	98.9 ± 3.6
PNH	20	74.7 ± 3.1	6 ± 1.7	2.2 ± 1.1	0.3 ± 0.1	19.4 ± 2.5	102.6 ± 4.7
	60	68.4 ± 3	2.6 ± 0.4	8.1 ± 1	0.4 ± 0.2	31 ± 4.4	110.4 ± 2.4
	120	59.4 ± 5.7	6.3 ± 2.8	16.1 ± 2.9	0.9 ± 0.5	24.9 ± 5.6	107.6 ± 3.8
	180	41.5 ± 9.7	4 ± 1.4	30.3 ± 2.9	1.6 ± 0.6	25.9 ± 5.7	103.4 ± 3.3
	300	18.2 ± 3.3	3.6 ± 2.2	27 ± 2.3	8.7 ± 2.3	37.7 ± 2.5	95.1 ± 2.8
PH	20	68.6 ± 4.7	4.8 ± 1.3	0.8 ± 0.4	0.2 ± 0.1	22.3 ± 3.3	96.8 ± 3.9
	60	67.5 ± 2	3.2 ± 0.7	9.1 ± 1.8	1.1 ± 0.3	25.9 ± 3.6	106.8 ± 2.7
	120	67.1 ± 7.8	4.2 ± 2.2	13.1 ± 4.1	0.7 ± 0.5	27.5 ± 6.4	112.6 ± 4.1
	180	26 ± 6.7	4.5 ± 1.8	44.3 ± 9.2	3.4 ± 1.3	24.3 ± 3.5	102.5 ± 4.1
	300	19.7 ± 7	1.9 ± 0.6	26.8 ± 4.2	8.1 ± 2	45.7 ± 6.3	102.3 ± 3
UHT	20	69.5 ± 4.8	6.1 ± 1.5	1 ± 0.6	0.2 ± 0.1	22.7 ± 2	99.4 ± 4.3
	60	60.4 ± 4.5	5.1 ± 1.1	12.8 ± 2.5	0.4 ± 0.1	19 ± 1.8	97.7 ± 2.6
	120	35.1 ± 6.4	4.4 ± 2	18.2 ± 4.4	0.4 ± 0.1	23.3 ± 1.8	81.3 ± 10.5
	180	50.7 ± 3.9	3 ± 1.3	24.7 ± 3.9	1.9 ± 0.9	25.8 ± 3.2	106.2 ± 4.7
	300	23.4 ± 8.4	2.4 ± 1	25.2 ± 6.7	4.8 ± 2.6	44.2 ± 6.9	100.1 ± 4.4

PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised; PSI, proximal small intestine; DSI, distal small intestine; TI, terminal ileum. Values are means ± SEM, n = 5 – 6 per milk type and postprandial time combination.

Table A4.2: Extent of true small intestinal absorption (α) of amino acids in growing pigs, including amino acids where differences in means between pigs fed different processed bovine milk treatments are approaching significance.

	Raw	PNH	PH	UHT
		%		
Leu	71.8 ± 8.0*	78.6 ± 13.7	90.3 ± 6.7*	83 ± 5.5
His	62.7 ± 9.1^{b*c}	53.6 ± 9.0^{c}	89.1 ± 7.6^{a}	78.7 ± 6.5^{ab*}
Lys	59.8 ± 9.9	64.5 ± 17.6	86.2 ± 7.3*	72 ± 6.9*
Met	69.6 ± 10.9$^{a*b\wedge}$	49.4 ± 6.4$^{b\wedge}$	88 ± 7.3^{a*}	83.2 ± 6.3^{a}
Phe	66.4 ± 9.4^{b}	61.5 ± 9.3^{b*}	90.4 ± 7.3^{a}	80.6 ± 6^{ab*}
EAA	67 ± 8.6^{b}	62.6 ± 8.3^{b*}	90.1 ± 7.2^{a}	79.4 ± 5.9^{ab*}
Ala	56.8 ± 6.0$^{b\wedge c}$	52.4 ± 7.8^{c}	89.1 ± 8.5^{a*}	71.9 ± 7.1$^{a*b\wedge}$
Arg	50.7 ± 7.9^{c}	59.5 ± 12.2^{bc}	88.1 ± 8.0^{a*}	69.9 ± 6.3^{a*b}
Ser	65.2 ± 8.9^{b*}	61.4 ± 8.2^{b}	86 ± 7.5^{a*}	74.4 ± 6.7^{ab}
Tyr	67.7 ± 10.7*	63.2 ± 13.1$^{\wedge}$	87.5 ± 6.9$^{*\wedge}$	80.2 ± 5.8
NEAA	70.3 ± 8.3*	74.8 ± 11.5	89.3 ± 6.7*	81.2 ± 5.6
LNAA	67.1 ± 8.9^{b}	62.5 ± 8.4^{b*}	89.8 ± 7.2^{a}	79.3 ± 5.9^{ab*}
TAA	69.5 ± 8.5^{b}	70.5 ± 10.4^{a*b}	90.2 ± 6.9^{a*}	80.9 ± 5.7^{ab}

PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised, EAA, essential amino acids; LNAA, long neutral amino acids; NEAA, non-essential amino acids; TAA, total amino acids. Values are means ± SEM, n = 5 – 6 for each milk type by postprandial time combination. Means with different superscript letters within a row differ ($P \leq 0.05$). Means with similar symbols (*, ^) within a row indicate differences between milk treatments that are approaching significance ($P < 0.1$).

Table A4.3: Time to reach a true small intestinal amino acid absorption of 50% (T_{50}) in growing pigs, including amino acids where differences in means between pigs fed different processed bovine milk treatments are approaching significance.

	Raw	**PNH**	**PH**	**UHT**
			min	
BCAA	181.0 ± 16.6^{a}	192.5 ± 15.2^{a}	$138.1 \pm 8.7^{b*}$	$118.3 \pm 7.3^{b*}$
Met	$191.6 \pm 20.1^{b*}$	254.4 ± 29.2^{a}	$159.1 \pm 6.8^{b*}$	115.9 ± 6.5^{c}
Arg	257.1 ± 26.7^{a}	261.8 ± 21.4^{a}	$162.4 \pm 6.7^{b*}$	$136.7 \pm 17.4^{b*}$
TAA	197.3 ± 18.3^{a}	207.8 ± 16.7^{a}	$143.3 \pm 8.4^{b*}$	$125.5 \pm 7.8^{b*}$

PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised, BCAA, branch-chain amino acids; TAA, total amino acids. Values are means ± SEM, n = 5 – 6. Means with different superscript letters within a row differ ($P \leq 0.05$). Means with similar symbols (*, ^) within a row indicate differences between milk treatments that are approaching significance ($P < 0.1$).

Table A4.4. Postprandial concentration of individual and grouped amino acids in portal blood plasma of pigs fed raw, pasteurised non-homogenised (PNH), pasteurised homogenised (PH), and ultra-high temperature treated (UHT) bovine milk.

		Raw	PNH	PH	UHT	*P*		
	min	*µmol/L*				Diet	Time	Diet x time
Ile	0	122.8 ± 12.1	115.5 ± 12.1	123.7 ± 12.1	118.0 ± 13.2	-	<0.01	-
	20	214.9 ± 12.1	273.6 ± 12.1	220.1 ± 13.2	201.6 ± 13.2			
	60	234.2 ± 12.1	215.4 ± 12.1	233.7 ± 12.1	250.2 ± 12.1			
	120	130.8 ± 12.1	140.7 ± 12.1	141.1 ± 12.1	150.3 ± 11.2			
	180	128.2 ± 12.1	137.7 ± 12.1	120.9 ± 12.1	134.1 ± 12.1			
	300	131.4 ± 12.1	131.2 ± 12.1	129.5 ± 12.1	124.5 ± 12.1			
Leu	0	145.0 ± 17.2	134.0 ± 17.2	164.6 ± 17.2	159.1 ± 18.9	≤0.05	<0.01	<0.01
	20	303.5 ± 18.9	449.6 ± 17.2	273.9 ± 21.1	260.3 ± 21.1			
	60	320.6 ± 17.2	295.5 ± 17.2	320.3 ± 17.2	332.6 ± 17.2			
	120	176.0 ± 17.2	193.7 ± 17.2	188.4 ± 17.2	205.5 ± 16.0			
	180	168.1 ± 17.2	184.8 ± 17.2	166.2 ± 17.2	183.3 ± 17.2			
	300	187.7 ± 17.2	182.1 ± 17.2	184.0 ± 17.2	170.1 ± 17.2			
Val	0	278.8 ± 18.9	267.2 ± 18.9	297.2 ± 18.9	284.6 ± 20.8	-	<0.01	≤0.05
	20	407.3 ± 18.9	494.2 ± 18.9	406.1 ± 20.8	379.0 ± 20.8			
	60	421.4 ± 18.9	397.6 ± 18.9	422.2 ± 18.9	456.4 ± 18.9			
	120	298.8 ± 18.9	299.8 ± 18.9	310.0 ± 18.9	326.0 ± 17.5			
	180	278.9 ± 18.9	300.2 ± 18.9	274.5 ± 18.9	302.1 ± 18.9			
	300	289.6 ± 18.9	270.2 ± 18.9	281.9 ± 18.9	275.3 ± 18.9			
BCAA	0	546.5 ± 43.7	516.6 ± 43.7	585.6 ± 43.7	561.7 ± 47.9	-	<0.01	<0.01
	20	882.6 ± 47.9	1217.4 ± 43.7	837.6 ± 53.6	807.5 ± 53.6			
	60	976.3 ± 43.7	908.4 ± 43.7	976.2 ± 43.7	1039.2 ± 43.7			
	120	605.6 ± 43.7	634.2 ± 43.7	639.4 ± 43.7	681.8 ± 40.5			
	180	575.1 ± 43.7	622.6 ± 43.7	561.6 ± 43.7	619.6 ± 43.7			
	300	608.8 ± 43.7	583.5 ± 43.7	595.4 ± 43.7	569.8 ± 43.7			

His	0	33.6 ± 4.2	39.7 ± 6.2	52.2 ± 6.4	45.5 ± 10.5	≤0.05	<0.01	-
	20	60.2 ± 4.2	73.6 ± 7.6	59.8 ± 7.0	72.7 ± 9.6			
	60	72.2 ± 4.5	66.3 ± 6.2	65.7 ± 6.4	102.0 ± 9.6			
	120	45.2 ± 4.2	45.0 ± 6.2	42.4 ± 7.0	50.5 ± 8.9			
	180	36.8 ± 4.2	39.1 ± 6.2	41.5 ± 6.4	46.1 ± 9.6			
	300	39.9 ± 4.2	45.8 ± 6.2	38.1 ± 6.4	41.8 ± 9.6			
Lys	0	86.0 ± 19.3	70.5 ± 19.3	80.1 ± 19.3	92.6 ± 21.2	-	<0.01	-
	20	250.8 ± 21.2	303.0 ± 19.3	230.4 ± 21.2	221.8 ± 19.3			
	60	282.6 ± 19.3	233.1 ± 19.3	245.7 ± 19.3	269.0 ± 19.3			
	120	131.9 ± 19.3	143.9 ± 21.2	113.9 ± 21.2	168.3 ± 17.9			
	180	132.9 ± 19.3	129.3 ± 19.3	126.6 ± 19.3	130.1 ± 19.3			
	300	140.3 ± 19.3	129.9 ± 19.3	131.2 ± 19.3	126.3 ± 19.3			
Met	0	27.9 ± 7.0	51 ± 7.0	39.8 ± 7.0	33.4 ± 7.7	-	<0.01	-
	20	74.8 ± 7.0	94 ± 7.7	74.8 ± 7.7	83.5 ± 7.0			
	60	85.6 ± 7.0	64.6 ± 7.0	66.4 ± 7.0	86.1 ± 7.0			
	120	38.9 ± 7.0	40.0 ± 7.0	40.3 ± 7.0	50.6 ± 6.5			
	180	36.5 ± 7.0	37.8 ± 7.0	42.3 ± 7.0	45.5 ± 7.0			
	300	34.8 ± 7.0	33.5 ± 7.0	32.4 ± 7.0	29.9 ± 7.0			
Phe	0	64.9 ± 10.3	75.3 ± 10.3	83.3 ± 10.3	83.9 ± 11.3	-	<0.01	-
	20	132.1 ± 11.3	175.6 ± 12.7	131.3 ± 12.7	146.2 ± 11.3			
	60	143.6 ± 10.3	126.9 ± 10.3	131.6 ± 10.3	157.4 ± 10.3			
	120	81.0 ± 10.3	85.4 ± 10.3	85.6 ± 10.3	94.9 ± 9.6			
	180	68.9 ± 10.3	71.2 ± 10.3	77.1 ± 10.3	86.7 ± 10.3			
	300	83.2 ± 10.3	85.7 ± 10.3	79.4 ± 10.3	76.8 ± 10.3			
Thr	0	141.7 ± 16.8	148.9 ± 16.8	165.2 ± 16.8	173.4 ± 18.4	-	<0.01	-
	20	242.3 ± 16.8	295.3 ± 18.4	226.5 ± 18.4	241.2 ± 16.8			
	60	315.0 ± 16.8	268.3 ± 16.8	270.6 ± 16.8	344.7 ± 16.8			
	120	183.7 ± 16.8	184.5 ± 16.8	209.4 ± 16.8	188.3 ± 15.5			
	180	169.6 ± 16.8	174.8 ± 16.8	176.3 ± 18.4	179.5 ± 16.8			
	300	152.9 ± 18.4	151.6 ± 16.8	161.8 ± 16.8	162.5 ± 16.8			

EAA	0	940.5 ± 77.5	945.3 ± 77.5	1064.7 ± 77.5	1047 ± 84.9	-	<0.01	<0.01
	20	1685.2 ± 84.9	2130.3 ± 94.9	1556.7 ± 94.9	1487.8 ± 94.9			
	60	1917.4 ± 84.9	1737.3 ± 77.5	1825.8 ± 77.5	2022.6 ± 84.9			
	120	1133.9 ± 77.5	1138.4 ± 84.9	981.2 ± 94.9	1281.8 ± 71.7			
	180	1071.1 ± 77.5	1119.9 ± 77.5	1093.7 ± 84.9	1158.0 ± 77.5			
	300	1085.1 ± 84.9	1071.3 ± 77.5	1087.3 ± 77.5	1050.8 ± 77.5			
Ala	0	286.0 ± 50.3	273.6 ± 51.9	272.9 ± 43.4	254.1 ± 58.8	-	<0.01	-
	20	575.2 ± 50.3	661.0 ± 51.9	528.8 ± 47.6	533.7 ± 53.7			
	60	774.3 ± 50.3	626.6 ± 51.9	796.3 ± 43.4	849.9 ± 53.7			
	120	479.1 ± 50.3	452.6 ± 51.9	521.4 ± 43.4	514.2 ± 49.7			
	180	380.6 ± 50.3	369.4 ± 51.9	364.1 ± 43.4	415.8 ± 53.7			
	300	379.7 ± 50.3	353.3 ± 51.9	301.0 ± 43.4	385.6 ± 53.7			
Arg	0	105.4 ± 13.8	119.7 ± 19.8	121.3 ± 16.5	128.1 ± 21.1	-	<0.01	-
	20	172.8 ± 13.8	218.3 ± 21.7	178.4 ± 18.0	190.9 ± 19.3			
	60	169.6 ± 13.8	174.7 ± 19.8	155.1 ± 16.5	206.6 ± 19.3			
	120	100.0 ± 13.8	112.9 ± 19.8	115.9 ± 16.5	103.1 ± 17.9			
	180	96.9 ± 13.8	108.1 ± 19.8	103.0 ± 16.5	109.0 ± 19.3			
	300	101.8 ± 13.8	111.7 ± 19.8	99.2 ± 16.5	93.6 ± 19.3			
Asp	0	43.6 ± 9.6	43.2 ± 9.6	49.2 ± 9.6	51.2 ± 10.6	-	<0.01	<0.01
	20	100.4 ± 9.6	152.9 ± 9.6	94.6 ± 10.6	114.7 ± 9.6			
	60	139.8 ± 9.6	116.7 ± 9.6	126.1 ± 9.6	156.6 ± 9.6			
	120	72.2 ± 9.6	69.4 ± 9.6	80.1 ± 9.6	76.4 ± 8.9			
	180	51.7 ± 9.6	58.1 ± 9.6	57.6 ± 9.6	64.3 ± 9.6			
	300	52.3 ± 9.6	50.4 ± 9.6	44.8 ± 9.6	48.9 ± 9.6			
Asx	0	19.4 ± 2.1	16.8 ± 2.1	17.2 ± 2.1	16.7 ± 2.3	-	<0.01	<0.01
	20	23.6 ± 2.1	29.4 ± 2.1	22.3 ± 2.3	20.4 ± 2.1			
	60	30.8 ± 2.1	23.7 ± 2.1	33.2 ± 2.1	32.9 ± 2.1			
	120	16.9 ± 2.1	20.2 ± 2.1	17.8 ± 2.1	20.7 ± 1.9			
	180	17.0 ± 2.1	16.8 ± 2.1	20.5 ± 2.1	16.7 ± 2.1			
	300	16.7 ± 2.1	16.3 ± 2.3	18.0 ± 2.1	18.2 ± 2.1			

Cit	0	69.2 ± 4.6	73.4 ± 4.6	76.6 ± 4.6	76.2 ± 4.7	-	<0.01	<0.01
	20	78.3 ± 3.8	82.5 ± 3.7	85.7 ± 3.8	85.3 ± 3.8			
	60	87.5 ± 4.3	91.7 ± 4.2	94.9 ± 4.3	94.4 ± 4.2			
	120	84.9 ± 4.0	89.1 ± 3.9	92.3 ± 4.0	91.8 ± 3.9			
	180	72.7 ± 4.7	76.9 ± 4.7	80.1 ± 4.7	79.6 ± 4.7			
	300	64.4 ± 5.0	68.6 ± 4.9	71.8 ± 5.0	71.3 ± 4.9			
Gln	0	349.5 ± 28.1	373.4 ± 28.1	361.8 ± 30.8	375.3 ± 30.8	-	<0.01	-
	20	471.5 ± 28.1	535.8 ± 30.8	458.6 ± 30.8	501.4 ± 28.1			
	60	544.5 ± 28.1	532.4 ± 28.1	523.6 ± 28.1	622.7 ± 28.1			
	120	407.6 ± 28.1	390.8 ± 28.1	440.8 ± 28.1	429.0 ± 26.1			
	180	377.6 ± 28.1	390.3 ± 28.1	384.1 ± 28.1	400.4 ± 28.1			
	300	341.0 ± 28.1	337.8 ± 28.1	325.1 ± 28.1	318.7 ± 28.1			
Glu	0	193.3 ± 17.4	146.2 ± 17.4	170.2 ± 17.4	153.6 ± 19.0	-	<0.01	-
	20	183.0 ± 17.4	207.5 ± 17.4	188.9 ± 19.0	190.8 ± 17.4			
	60	284.0 ± 17.4	247.5 ± 17.4	283.3 ± 17.4	291.8 ± 17.4			
	120	172.9 ± 17.4	194.2 ± 17.4	175.9 ± 17.4	178.1 ± 17.4			
	180	198.5 ± 17.4	172.4 ± 17.4	201.7 ± 17.4	168.6 ± 17.4			
	300	185.4 ± 17.4	208.6 ± 17.4	213.6 ± 17.4	185.8 ± 17.4			
Gly	0	701.3 ± 48.0	788.5 ± 48.0	790.0 ± 48.0	780.8 ± 52.5	-	<0.01	<0.01
	20	805.3 ± 48.0	971.4 ± 48.0	780.0± 52.5	812.8 ± 48.0			
	60	851.5 ± 48.0	720.2 ± 48.0	772.3 ± 48.0	959.9 ± 48.0			
	120	769.7 ± 48.0	654.2 ± 48.0	757.4 ± 48.0	703.2 ± 44.4			
	180	655.1 ± 48.0	754.1 ± 48.0	720 ± 48.0	761.2 ± 48.0			
	300	653.8 ± 52.5	695.0 ± 48.0	644.4 ± 48.0	690.6 ± 48.0			
Orn	0	42.3 ± 4.7	43.8 ± 4.7	41.8 ± 4.7	40.9 ± 5.2	-	<0.01	-
	20	59.0 ± 4.7	57.0 ± 4.7	53.5 ± 5.2	53.0 ± 4.7			
	60	69.9 ± 4.7	66.1 ± 4.7	66.3 ± 4.7	71.4 ± 4.7			
	120	52.2 ± 4.7	60.1 ± 4.7	56.7 ± 4.7	55.4 ± 4.4			
	180	46.3 ± 4.7	49 ± 4.7	55.5 ± 4.7	47.6 ± 4.7			
	300	45.1 ± 4.7	39.1 ± 4.7	49.9 ± 4.7	38.6 ± 4.7			

Pro	0	243.6 ± 29.7	252.1 ± 29.7	254.8 ± 29.7	248.6 ± 32.5	≤0.05	<0.01	<0.01
	20	443.1 ± 32.5	619.3 ± 29.7	441.8 ± 32.5	505.8 ± 29.7			
	60	580.3 ± 29.7	506.7 ± 29.7	564.2 ± 29.7	682.4 ± 29.7			
	120	380.8 ± 29.7	378.2 ± 29.7	403.7 ± 29.7	435.2 ± 27.5			
	180	292.5 ± 29.7	323.9 ± 29.7	319.1 ± 29.7	344.8 ± 29.7			
	300	274.5 ± 29.7	262 ± 29.7	254.1 ± 29.7	269.2 ± 29.7			
Ser	0	99.7 ± 16.0	100.7 ± 16.0	114.2 ± 16.0	114.6 ± 17.5	-	<0.01	<0.01
	20	203.7 ± 16.0	267.8 ± 16.0	197.4 ± 17.5	216.0 ± 16.0			
	60	245.3 ± 16.0	195.5 ± 16.0	234.7 ± 16.0	288.6 ± 16.0			
	120	140.3 ± 16.0	142.6 ± 16.0	162.3 ± 16.0	144.5 ± 14.8			
	180	107.6 ± 16.0	122.4 ± 16.0	116.5 ± 16.0	141.6 ± 16.0			
	300	113.0 ± 16.0	104.1 ± 16.0	106.8 ± 16.0	106.4 ± 16.0			
Tau	0	112.8 ± 10.5	120.6 ± 10.5	106.4 ± 10.5	103.3 ± 11.5	-	<0.01	-
	20	136.9 ± 10.5	165.9 ± 10.5	135.7 ± 11.5	143.6 ± 10.5			
	60	141.6 ± 10.5	142.0 ± 10.5	153.6 ± 10.5	144.6 ± 11.5			
	120	119.7 ± 10.5	100.4 ± 10.5	100.5 ± 10.5	126.9 ± 9.7			
	180	95.0 ± 10.5	100.7 ± 10.5	104.4 ± 10.5	127.2 ± 10.5			
	300	98.1 ± 10.5	108.1 ± 10.5	106.4 ± 10.5	102.0 ± 10.5			
Trp	0	39.9 ± 6.0	43.3 ± 6.0	58.7 ± 6.0	56.5 ± 6.6	-	<0.01	-
	20	59.7 ± 6.0	84.1 ± 6.6	62.6 ± 6.6	66.9 ± 6.0			
	60	70.0 ± 6.0	69.7 ± 6.0	69.6 ± 6.0	68.4 ± 6.6			
	120	47.6 ± 6.0	47.6 ± 6.0	45.6 ± 6.0	47.4 ± 5.6			
	180	51.3 ± 6.0	45.1 ± 6.0	46.4 ± 6.0	50.6 ± 6.0			
	300	54.3 ± 6.0	41.3 ± 6.0	49.1 ± 6.0	43.7 ± 6.0			
Tyr	0	62.2 ± 8.2	75.3 ± 11.3	76.8 ± 9.7	81.6 ± 20.3	-	<0.01	-
	20	118.7 ± 9	168.6 ± 13.8	111.7 ± 11.8	159.9 ± 18.5			
	60	145.4 ± 8.2	137.5 ± 11.3	128.5 ± 9.7	179.1 ± 18.5			
	120	96.3 ± 8.2	92.9 ± 11.3	95.0 ± 9.7	114.8 ± 17.1			
	180	79.4 ± 8.2	84.1 ± 11.3	88.1 ± 9.7	106.5 ± 18.5			
	300	83.1 ± 8.2	76.2 ± 11.3	75.2 ± 9.7	73.5 ± 18.5			

LNAA	0	937.8 ± 77.3	945.2 ± 77.3	1058.3 ± 77.3	1044.7 ± 84.6	-	<0.01	<0.01
	20	1671.7 ± 84.6	2123.4 ± 94.6	1537.1 ± 94.6	1480.8 ± 94.6			
	60	1914.9 ± 84.6	1747.8 ± 77.3	1822.7 ± 77.3	2030.9 ± 84.6			
	120	1149.1 ± 77.3	1146.5 ± 84.6	992.3 ± 94.6	1301.8 ± 71.5			
	180	1081.6 ± 77.3	1132.8 ± 77.3	1102.3 ± 84.6	1177.8 ± 77.3			
	300	1083.5 ± 84.6	1061.8 ± 77.3	1083.2 ± 77.3	1047.5 ± 77.3			
NEAA	0	2104.1 ± 159.9	2189.4 ± 159.9	2211.4 ± 175.2	2204.8 ± 175.2	-	<0.01	≤0.05
	20	2976.4 ± 175.2	3630.9 ± 195.8	2847.8 ± 195.8	3246.4 ± 159.9			
	60	3765.3 ± 159.9	3281.4 ± 159.9	3617.1 ± 159.9	4270.5 ± 159.9			
	120	2635.8 ± 159.9	2507.9 ± 159.9	2770.3 ± 159.9	2694.4 ± 159.9			
	180	2256.9 ± 159.9	2399.6 ± 159.9	2374.6 ± 159.9	2528.9 ± 159.9			
	300	2130.9 ± 175.2	2176.1 ± 175.2	2082.1 ± 159.9	2190.6 ± 159.9			
TAA	0	3199.6 ± 210.5	3299 ± 210.5	3385.3 ± 230.6	3395.9 ± 230.6	-	<0.01	<0.01
	20	4842.8 ± 230.6	5976.4 ± 257.9	4574.8 ± 257.9	4512.5 ± 257.9			
	60	5883.9 ± 230.6	5226.9 ± 210.5	5662.8 ± 210.5	6383.1 ± 230.6			
	120	3941.5 ± 210.5	3669.4 ± 230.6	3480.4 ± 257.9	4141.5 ± 210.5			
	180	3469.2 ± 210.5	3669.1 ± 210.5	3595.3 ± 230.6	3861.6 ± 210.5			
	300	3355.8 ± 230.6	3374.8 ± 230.6	3325.8 ± 210.5	3382.0 ± 210.5			

AA, amino acids; BCAA, branched chain AA; EAA, essential AA; LNAA, long neutral AA; NEAA, non-essential AA; TAA, total AA; PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 5 – 6 per milk type and postprandial time combination.

Table A4.5. Postprandial concentration of individual and grouped amino acids in peripheral blood plasma of pigs fed raw, pasteurised non-homogenised (PNH), pasteurised homogenised (PH), and ultra-high temperature treated (UHT) bovine milk.

		Raw	PNH	PH	UHT	*P*		
		µmol/L				Diet	Time	Diet x time
Ile	0	117.2 ± 9.3	106.4 ± 8.5	113.9 ± 8.5	109.5 ± 9.3	-	<0.01	-
	20	137.2 ± 8.5	155.5 ± 8.5	166.0 ± 8.5	144.0 ± 8.5			
	60	184.1 ± 8.5	175.1 ± 8.5	162.4 ± 8.5	188.9 ± 8.5			
	120	105.1 ± 8.5	121.9 ± 8.5	109.1 ± 8.5	118.0 ± 7.9			
	180	115.5 ± 8.5	116.6 ± 8.5	114.1 ± 8.5	114.2 ± 8.5			
	300	121.7 ± 8.5	121.5 ± 8.5	120.0 ± 8.5	120.6 ± 8.5			
Leu	0	140.6 ± 14.2	119.1 ± 13.0	147.7 ± 13.0	145.3 ± 14.2	-	<0.01	-
	20	191.7 ± 13.0	239.8 ± 13.0	224.8 ± 13.0	194.3 ± 13.0			
	60	254.1 ± 13.0	243.3 ± 13.0	228.6 ± 13.0	260.6 ± 13.0			
	120	141.4 ± 13.0	167.0 ± 13.0	148.3 ± 13.0	162.2 ± 12.0			
	180	148.5 ± 13.0	155.2 ± 13.0	154.9 ± 13.0	157.4 ± 13.0			
	300	171.8 ± 13.0	162.6 ± 13.0	169.4 ± 13.0	162.1 ± 13.0			
Val	0	287.9 ± 17.3	261.5 ± 15.8	286.3 ± 15.8	278.1 ± 17.3	-	<0.01	-
	20	317.0 ± 15.8	354.8 ± 15.8	359.0 ± 15.8	319.3 ± 15.8			
	60	379.3 ± 15.8	362.5 ± 15.8	348.5 ± 15.8	388.0 ± 15.8			
	120	264.0 ± 15.8	285.3 ± 15.8	276.3 ± 15.8	288.8 ± 14.6			
	180	269.1 ± 15.8	284.0 ± 15.8	271.1 ± 15.8	283.3 ± 15.8			
	300	289.0 ± 15.8	267.1 ± 15.8	273.9 ± 15.8	281.5 ± 15.8			
BCAA	0	545.7 ± 39.1	487.0 ± 35.7	547.9 ± 35.7	532.9 ± 39.1	-	<0.01	-
	20	645.9 ± 35.7	750.1 ± 35.7	749.7 ± 35.7	657.6 ± 35.7			
	60	817.5 ± 35.7	780.9 ± 35.7	739.5 ± 35.7	837.4 ± 35.7			
	120	510.4 ± 35.7	574.2 ± 35.7	533.6 ± 35.7	569.0 ± 33.1			
	180	533.1 ± 35.7	555.8 ± 35.7	540.0 ± 35.7	554.8 ± 35.7			
	300	582.5 ± 35.7	551.1 ± 35.7	563.3 ± 35.7	564.1 ± 35.7			

His	0	36.1 ± 4.7	38.1 ± 4.4	33.3 ± 2.0	35.7 ± 6.5	<0.01	<0.01	<0.01
	20	34.0 ± 4.3	49.7 ± 4.4	36.1 ± 2.0	35.2 ± 5.9			
	60	63.3 ± 4.3	41.9 ± 4.4	35.9 ± 2.0	58.6 ± 5.9			
	120	37.5 ± 4.3	35.9 ± 4.4	34 ± 1.8	42.5 ± 5.5			
	180	32.5 ± 4.3	39.8 ± 4.4	36.1 ± 2.0	38.6 ± 5.9			
	300	40.2 ± 4.3	36.5 ± 4.4	33.6 ± 1.8	45.3 ± 5.9			
Lys	0	71.8 ± 17.0	59.0 ± 15.5	80.5 ± 15.5	96.9 ± 17.0	-	<0.01	-
	20	125.6 ± 15.5	163.4 ± 15.5	160.5 ± 15.5	114.2 ± 15.5			
	60	188.1 ± 15.5	181.9 ± 15.5	157.1 ± 15.5	200.1 ± 15.5			
	120	106.6 ± 15.5	128.6 ± 15.5	107.9 ± 15.5	131.1 ± 14.3			
	180	112.0 ± 15.5	97.8 ± 15.5	106.0 ± 15.5	110.7 ± 15.5			
	300	123.3 ± 15.5	117.3 ± 15.5	114.0 ± 15.5	105.7 ± 15.5			
Met	0	27.1 ± 4.3	38.9 ± 4.3	33.2 ± 4.0	29.5 ± 4.3	-	<0.01	<0.01
	20	40.4 ± 4.0	54.2 ± 4.0	44.1 ± 4.0	44.4 ± 4.0			
	60	64.8 ± 4.3	51.6 ± 4.0	38.1 ± 4.3	65.1 ± 4.0			
	120	29.2 ± 4.0	37.7 ± 4.0	31.4 ± 4.0	41.5 ± 3.7			
	180	33.0 ± 4.0	32.8 ± 4.0	43.0 ± 4.0	37.7 ± 4.0			
	300	33.0 ± 4.0	30.2 ± 4.0	29.2 ± 4.0	30.6 ± 4.0			
Phe	0	64.0 ± 5.9	66.7 ± 5.4	63.8 ± 5.4	68.1 ± 5.9	≤0.05	<0.01	<0.01
	20	84.5 ± 5.4	107.0 ± 5.4	92.6 ± 5.4	88.1 ± 5.4			
	60	112.0 ± 5.9	98.7 ± 5.4	86.9 ± 5.4	113.5 ± 5.4			
	120	57.7 ± 5.4	84.2 ± 5.4	62.8 ± 5.4	71.0 ± 5.0			
	180	56.9 ± 5.4	64.0 ± 5.4	71.9 ± 5.4	66.1 ± 5.4			
	300	74.6 ± 5.4	73.5 ± 5.4	73.4 ± 5.4	79.0 ± 5.4			
Thr	0	143.8 ± 14.4	147.8 ± 13.1	154.8 ± 13.1	163.5 ± 14.4	-	<0.01	≤0.05
	20	188.6 ± 13.1	216.8 ± 13.1	193 ± 13.1	180.4 ± 13.1			
	60	274.4 ± 13.1	232.3 ± 13.1	204.5 ± 13.1	272.1 ± 13.1			
	120	158.2 ± 13.1	174.8 ± 13.1	174.0 ± 13.1	159.4 ± 12.2			
	180	164.3 ± 13.1	174.7 ± 14.4	177.9 ± 13.1	158.7 ± 13.1			
	300	154.3 ± 14.4	153.7 ± 13.1	159.2 ± 13.1	165.7 ± 13.1			

EAA	0	947.6 ± 63.5	900.2 ± 63.5	942.9 ± 63.5	988.1 ± 63.5	-	<0.01	≤0.05
	20	1175.3 ± 58.0	1411.8 ± 58.0	1229.6 ± 63.5	1171.7 ± 58.0			
	60	1577.4 ± 71.0	1447.7 ± 58.0	1274.9 ± 71.0	1619.9 ± 58.0			
	120	950.7 ± 58.0	1093.8 ± 58.0	993.5 ± 58.0	1065.2 ± 53.7			
	180	973.5 ± 58.0	1035.8 ± 63.5	1020.8 ± 63.5	1012.6 ± 58.0			
	300	1036.5 ± 63.5	1009.8 ± 58.0	1026.3 ± 58.0	1039.6 ± 58.0			
Ala	0	259.4 ± 34.1	237.7 ± 31.1	236.7 ± 31.1	216.4 ± 34.1	-	<0.01	-
	20	344.5 ± 31.1	348.6 ± 31.1	372.8 ± 31.1	311.8 ± 31.1			
	60	427.0 ± 31.1	390.5 ± 31.1	345.8 ± 31.1	485.4 ± 31.1			
	120	313.5 ± 31.1	282.7 ± 31.1	303.2 ± 31.1	343.5 ± 28.8			
	180	297.5 ± 31.1	289.8 ± 31.1	290.7 ± 31.1	301.2 ± 31.1			
	300	342.7 ± 31.1	328.3 ± 31.1	263.6 ± 31.1	362.5 ± 31.1			
Arg	0	96.6 ± 10.6	90.6 ± 10.6	87.2 ± 9.7	89.3 ± 10.6	-	<0.01	-
	20	102.5 ± 9.7	128.1 ± 9.7	120.5 ± 9.7	108.6 ± 9.7			
	60	145.2 ± 9.7	129.6 ± 9.7	100.1 ± 9.7	143.8 ± 9.7			
	120	72.8 ± 9.7	95.4 ± 9.7	72.6 ± 9.7	81.3 ± 9.0			
	180	77.8 ± 9.7	96.5 ± 9.7	99.0 ± 9.7	79.7 ± 9.7			
	300	90.3 ± 9.7	86.8 ± 9.7	86.3 ± 9.7	89.9 ± 9.7			
Asp	0	41.6 ± 4.6	36.9 ± 4.2	39.9 ± 4.2	41.6 ± 4.6	≤0.05	<0.01	<0.01
	20	54.0 ± 4.2	67.9 ± 4.2	56.5 ± 4.2	58.8 ± 4.2			
	60	90.8 ± 4.2	75.6 ± 4.2	61.7 ± 4.2	89.6 ± 4.2			
	120	46.4 ± 4.2	53.9 ± 4.2	48.2 ± 4.2	47.9 ± 3.9			
	180	40.9 ± 4.2	52.3 ± 4.2	47.7 ± 4.2	46.0 ± 4.2			
	300	47.9 ± 4.2	46.9 ± 4.2	40.5 ± 4.2	47.3 ± 4.2			
Asx	0	8.8 ± 1.5	6.3 ± 1.4	9.1 ± 1.4	7.6 ± 1.5	-	-	-
	20	6.7 ± 1.4	9.2 ± 1.4	10.7 ± 1.4	7.6 ± 1.4			
	60	9.9 ± 1.5	9.0 ± 1.5	9.2 ± 1.4	9.0 ± 1.5			
	120	5.8 ± 1.4	9.4 ± 1.4	6.7 ± 1.4	7.1 ± 1.3			
	180	8.8 ± 1.4	8.4 ± 1.4	7.2 ± 1.4	7.4 ± 1.4			
	300	6.7 ± 1.4	8.5 ± 1.4	10.1 ± 1.4	9.9 ± 1.4			

Cit	0	48.8 ± 5.2	54.3 ± 4.7	50.0 ± 4.7	50.7 ± 5.2	-	<0.01	-
	20	48.0 ± 4.7	51.4 ± 4.7	47.2 ± 4.7	49.4 ± 4.7			
	60	59.8 ± 4.7	57.2 ± 4.7	64.9 ± 4.7	61.5 ± 4.7			
	120	52.1 ± 4.7	56.7 ± 4.7	60.3 ± 4.7	57.1 ± 4.4			
	180	51.6 ± 4.7	54.6 ± 4.7	50.9 ± 4.7	52.0 ± 4.7			
	300	48.8 ± 4.7	44.8 ± 4.7	58.5 ± 4.7	50.3 ± 4.7			
Gln	0	417.2 ± 30.6	450.9 ± 27.9	436.8 ± 27.9	458.5 ± 30.6	-	<0.01	-
	20	472.5 ± 27.9	539.1 ± 27.9	494.7 ± 27.9	490.3 ± 27.9			
	60	592.2 ± 27.9	557.7 ± 27.9	540.9 ± 27.9	583.6 ± 27.9			
	120	424.2 ± 27.9	472.7 ± 27.9	464.4 ± 27.9	459.6 ± 25.8			
	180	429.2 ± 27.9	469.8 ± 27.9	469.0 ± 27.9	434.6 ± 27.9			
	300	408.9 ± 27.9	417.8 ± 27.9	358.2 ± 27.9	408.0 ± 27.9			
Glu	0	153.0 ± 20.6	95.5 ± 18.8	156.0 ± 18.8	121.3 ± 20.6	-	≤0.05	-
	20	107.0 ± 18.8	131.1 ± 18.8	144.7 ± 18.8	116.1 ± 18.8			
	60	187.8 ± 18.8	165.7 ± 18.8	153.0 ± 18.8	150.0 ± 20.6			
	120	106.7 ± 18.8	154.3 ± 18.8	118.2 ± 18.8	116.7 ± 17.4			
	180	142.6 ± 18.8	137.6 ± 18.8	117.9 ± 18.8	112.5 ± 18.8			
	300	124.7 ± 18.8	138.5 ± 18.8	162.0 ± 18.8	129.9 ± 20.6			
Gly	0	755.8 ± 55.9	746.9 ± 51.0	765.6 ± 51.0	796.9 ± 55.9	-	-	<0.01
	20	766.2 ± 51.0	862.3 ± 51.0	814.1 ± 51.0	792.3 ± 51.0			
	60	911.3 ± 51.0	697.4 ± 51.0	694.5 ± 51.0	885.3 ± 51.0			
	120	683.4 ± 51.0	694.4 ± 51.0	682.6 ± 51.0	640.4 ± 47.2			
	180	662.8 ± 51.0	804.2 ± 51.0	775.7 ± 51.0	723.9 ± 51.0			
	300	722.9 ± 51.0	683.2 ± 51.0	637.6 ± 51.0	760.6 ± 51.0			
Orn	0	49.1 ± 6.0	47.8 ± 5.5	51.3 ± 5.5	58.8 ± 6.0	-	<0.01	-
	20	53.9 ± 5.5	56.7 ± 5.5	61.8 ± 5.5	56.0 ± 5.5			
	60	74.3 ± 5.5	71.4 ± 5.5	67.8 ± 5.5	75.9 ± 5.5			
	120	57.7 ± 5.5	54.1 ± 5.5	58.1 ± 5.5	58.8 ± 5.1			
	180	48.8 ± 5.5	50.1 ± 5.5	59.3 ± 5.5	50.9 ± 5.5			
	300	46.4 ± 5.5	43.2 ± 5.5	51.8 ± 5.5	39.4 ± 5.5			

Pro	0	236.5 ± 17.0	242.6 ± 15.5	243.3 ± 15.5	250.9 ± 17.0	≤0.05	<0.01	-
	20	326.2 ± 15.5	383.0 ± 15.5	340.9 ± 15.5	347.9 ± 15.5			
	60	450.7 ± 17.0	423.9 ± 15.5	399.3 ± 15.5	475.8 ± 19.0			
	120	321.5 ± 15.5	332.2 ± 15.5	324.9 ± 15.5	367.9 ± 14.4			
	180	268.8 ± 15.5	297.5 ± 15.5	312.9 ± 15.5	312.1 ± 15.5			
	300	272.9 ± 15.5	251.6 ± 15.5	245.1 ± 15.5	255.0 ± 15.5			
Ser	0	125.5 ± 11.1	106.5 ± 10.1	121.4 ± 10.1	129.1 ± 11.1	-	<0.01	-
	20	133.6 ± 10.1	150.5 ± 10.1	146.3 ± 10.1	139.4 ± 10.1			
	60	189.2 ± 10.1	141.2 ± 10.1	139.8 ± 10.1	164.6 ± 11.1			
	120	114.2 ± 10.1	123.8 ± 10.1	116.2 ± 10.1	111.8 ± 9.3			
	180	108.8 ± 10.1	131.1 ± 10.1	127.3 ± 10.1	121.8 ± 10.1			
	300	117.4 ± 10.1	109.6 ± 10.1	115.9 ± 10.1	127.5 ± 10.1			
Tau	0	121.6 ± 10.8	105.2 ± 9.8	91.6 ± 9.8	97.3 ± 10.8	-	<0.01	-
	20	113.2 ± 9.8	129.3 ± 9.8	115.1 ± 9.8	112.6 ± 9.8			
	60	124.3 ± 10.8	126.7 ± 9.8	126.4 ± 9.8	129.6 ± 10.8			
	120	99.1 ± 9.8	109.8 ± 9.8	87.0 ± 9.8	89.6 ± 9.8			
	180	96.0 ± 9.8	112.6 ± 9.8	99.7 ± 9.8	109.2 ± 9.8			
	300	86.6 ± 9.8	98.4 ± 9.8	106.5 ± 9.8	122.0 ± 9.8			
Trp	0	59.0 ± 5.5	47.7 ± 5.0	54.3 ± 5.0	61.5 ± 5.5	-	<0.01	≤0.05
	20	56.4 ± 5.0	70.7 ± 5.0	58.4 ± 5.0	51.8 ± 5.0			
	60	69.5 ± 5.0	60.4 ± 5.0	60.2 ± 5.0	73.1 ± 5.0			
	120	51.2 ± 5.0	58.4 ± 5.0	49.9 ± 5.0	50.6 ± 4.6			
	180	41.7 ± 5.0	58.3 ± 5.0	54.3 ± 5.0	46.0 ± 5.0			
	300	53.5 ± 5.0	47.6 ± 5.0	53.8 ± 5.0	49.2 ± 5.0			
Tyr	0	67.2 ± 7.4	68.6 ± 6.8	63.3 ± 6.8	68.5 ± 7.4	<0.01	<0.01	<0.01
	20	84.8 ± 6.8	114.1 ± 6.8	88.7 ± 6.8	85.2 ± 6.8			
	60	130.6 ± 6.8	116.3 ± 6.8	94.5 ± 6.8	137.3 ± 6.8			
	120	73.9 ± 6.8	101.8 ± 6.8	79.2 ± 6.8	95.0 ± 6.3			
	180	72.3 ± 6.8	87.0 ± 6.8	86.4 ± 6.8	88.9 ± 6.8			
	300	80.7 ± 6.8	71.1 ± 6.8	72.8 ± 6.8	79.3 ± 6.8			

NEAA	0	2161.6 ± 118.2	1998.1 ± 118.2	2159.2 ± 107.9	2180.2 ± 118.2	-	<0.01	-
	20	2397.9 ± 107.9	2733.9 ± 107.9	2589.9 ± 107.9	2458.0 ± 107.9			
	60	2966.2 ± 132.2	2676.2 ± 118.2	2538.8 ± 107.9	2933.4 ± 132.2			
	120	2162.2 ± 107.9	2320.4 ± 107.9	2216.1 ± 107.9	2271.2 ± 99.9			
	180	2109.4 ± 107.9	2374.1 ± 107.9	2333.7 ± 107.9	2228.1 ± 107.9			
	300	2215.0 ± 107.9	2142.3 ± 107.9	1992.1 ± 107.9	2157.1 ± 118.2			
LNAA	0	950.8 ± 64.6	904.3 ± 64.6	942.5 ± 64.6	988.5 ± 64.6	-	<0.01	≤0.05
	20	1175.6 ± 58.9	1418.8 ± 58.9	1221.9 ± 64.6	1168.7 ± 58.9			
	60	1609.5 ± 64.6	1465.3 ± 58.9	1289.6 ± 72.2	1643.6 ± 58.9			
	120	966.9 ± 58.9	1111.4 ± 58.9	1009.9 ± 58.9	1089.2 ± 54.6			
	180	988.8 ± 58.9	1058.6 ± 64.6	1036.8 ± 64.6	1035.4 ± 58.9			
	300	1039.6 ± 64.6	1007.4 ± 58.9	1025.7 ± 58.9	1039.9 ± 58.9			
TAA	0	3279.9 ± 167.6	3038.9 ± 187.3	3238.3 ± 167.6	3324.4 ± 167.6	-	<0.01	-
	20	3740.3 ± 153.0	4331.7 ± 153.0	3886.3 ± 167.6	3798.3 ± 153.0			
	60	4653.6 ± 216.3	4270.4 ± 167.6	3968.1 ± 187.3	4609.9 ± 187.3			
	120	3269.7 ± 153.0	3578.1 ± 153.0	3354.6 ± 153.0	3455.6 ± 153.0			
	180	3227.6 ± 153.0	3508.7 ± 167.6	3450.5 ± 167.6	3400.7 ± 153.0			
	300	3270.4 ± 167.6	3293.6 ± 153.0	3176.7 ± 153.0	3285.0 ± 167.6			

AA, amino acids; BCAA, branched chain AA; EAA, essential AA; LNAA, long neutral AA; NEAA, non-essential AA; TAA, total AA; PNH, pasteurised non-homogenised; PH, pasteurised homogenised; UHT, ultra-high temperature treated homogenised. Values are means ± SEM, n = 5 – 6 per milk type and postprandial time combination

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This appendix comprises the Massey University DRC 16 forms describing the candidate's contribution to the material in this dissertation which is published or intended for publication. A summary of each form is included at the beginning of each relevant chapter.

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Student name:	Natalie G. M. Ahlborn
Name and title of main supervisor:	Prof. Dr. Warren McNabb
In which chapter is the manuscript/published work?	Chapter 2
What percentage of the manuscript/published work was contributed by the student?	90%

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The student was responsible for:
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First draft of the manuscript
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Data visualization
First draft of the manuscript

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		Volume	3
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Volume / Edition	3	**Issue, if Republishing an Article From a Serial**	1
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Article Title	Dissociation and coagulation of caseins and whey proteins in concentrated skim milk heated by direct steam injection	End Page	826
		Issue	6
		Volume	96
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Title, Description or Numeric Reference of the Portion(s)	Figure 10: Schematic representation of the proposed model of heat-induced dissociation and coagulation of caseins and whey proteins in CSM derived from the distribution analysis of individual proteins	Title of the Article / Chapter the Portion Is From	Dissociation and coagulation of caseins and whey proteins in concentrated skim milk heated by direct steam injection
		Author of Portion(s)	Dumpler, Joseph; Wohlschläger, Heidi; Kulozik, Ulrich
Editor of Portion(s)	Dumpler, Joseph; Wohlschläger, Heidi; Kulozik, Ulrich	Publication Date of Portion	2017-02-01
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1) **Definitions.** For purposes of these General Terms, the following definitions apply:

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"Order Confirmation" is the confirmation CCC provides to the User at the conclusion of each Marketplace transaction. "Order Confirmation Terms" are additional terms set forth on specific Order Confirmations not set forth in the General Terms that can include terms applicable to a particular CCC transactional licensing service and/or any Rightsholder-specific terms.

"Rightsholder(s)" are the holders of copyright rights in the Works for which a User obtains licenses via the Marketplace platform, which are displayed on specific Order Confirmations.

"Terms" means the terms and conditions set forth in these General Terms and any additional Order Confirmation Terms collectively.

"User" or "you" is the person or entity making the use granted under the relevant License. Where the person accepting the Terms on behalf of a User is a freelancer or other third party who the User authorized to accept the General Terms on the User's behalf, such person shall be deemed jointly a User for purposes of such Terms.

"Work(s)" are the copyright protected works described in relevant Order Confirmations.

2) **Description of Service.** CCC's Marketplace enables Users to obtain Licenses to use one or more Works in accordance with all relevant Terms. CCC grants Licenses as an agent on behalf of the copyright rightsholder identified in the relevant Order Confirmation.

3) **Applicability of Terms.** The Terms govern User's use of Works in connection with the relevant License. In the event of any conflict between General Terms and Order Confirmation Terms, the latter shall govern. User acknowledges that Rightsholders have complete discretion whether to grant any permission, and whether to place any limitations on any grant, and that CCC has no right to supersede or to modify any such discretionary act by a Rightsholder.

4) **Representations; Acceptance.** By using the Service, User represents and warrants that User has been duly authorized by the User to accept, and hereby does accept, all Terms.

5) **Scope of License; Limitations and Obligations.** All Works and all rights therein, including copyright rights, remain the sole and exclusive property of the Rightsholder. The License provides only those rights expressly set forth in the terms and conveys no other rights in any Works

6) **General Payment Terms.** User may pay at time of checkout by credit card or choose to be invoiced. If the User chooses to be invoiced, the User shall: (i) remit payments in the manner identified on specific invoices, (ii) unless otherwise specifically stated in an Order Confirmation or separate written agreement, Users shall remit payments upon receipt of the relevant invoice from CCC, either by delivery or notification of availability of the invoice via the Marketplace platform, and (iii) if the User does not pay the invoice within 30 days of receipt, the User may incur a service charge of 1.5% per month or the maximum rate allowed by applicable law, whichever is less. While User may exercise the rights in the License immediately upon receiving the Order Confirmation, the License is automatically revoked and is null and void, as if it had never been issued, if CCC does not receive complete payment on a timely basis.

7) **General Limits on Use.** Unless otherwise provided in the Order Confirmation, any grant of rights to User (i) involves only the rights set forth in the Terms and does not include subsequent or additional uses, (ii) is non-exclusive and non-transferable, and (iii) is subject to any and all limitations and restrictions (such as, but not limited to, limitations on duration of use or circulation) included in the Terms. Upon completion of the licensed use as set forth in the Order Confirmation, User shall either secure a new permission for further use of the Work(s) or immediately cease any new use of the Work(s) and shall render inaccessible (such as by deleting or by removing or severing links or other locators) any further copies of the Work. User may only make alterations to the Work if and as expressly set forth in the Order Confirmation. No Work may be used in any way that is unlawful, including without limitation if such use would violate applicable sanctions laws or regulations, would be defamatory, violate the rights of third parties (including such third parties' rights of copyright, privacy, publicity, or other tangible or intangible property), or is otherwise illegal, sexually explicit, or obscene. In addition, User may not conjoin a Work with any other material that may result in damage to the reputation of the Rightsholder. Any unlawful use will render any licenses hereunder null and void. User agrees to inform CCC if it becomes aware of any infringement of any rights in a Work and to cooperate with any reasonable request of CCC or the Rightsholder in connection therewith.

8) **Third Party Materials.** In the event that the material for which a License is sought includes third party materials (such as photographs, illustrations, graphs, inserts and similar materials) that are identified in such material as having been used by permission (or a similar indicator), User is responsible for identifying, and seeking separate licenses (under this Service, if available, or otherwise) for any of such third party materials; without a separate license, User may not use such third party materials via the License.

9) **Copyright Notice.** Use of proper copyright notice for a Work is required as a condition of any License granted under the Service. Unless otherwise provided in the Order Confirmation, a proper copyright notice will read substantially as follows: "Used with permission of [Rightsholder's name], from [Work's title, author, volume, edition number and year of copyright]; permission conveyed through Copyright Clearance Center, Inc." Such notice must be provided in a reasonably legible font size and must be placed either on a cover page or in another location that any person, upon gaining access to the material which is the subject of a permission, shall see, or in the case of republication Licenses, immediately adjacent to the Work as used (for example, as part of a by-line or footnote) or in the place where substantially all other credits or notices for the new work containing the republished Work are located. Failure to include the required notice results in loss to the Rightsholder and CCC, and the User shall be liable to pay liquidated damages for each such failure equal to twice the use fee specified in the Order Confirmation, in addition to the use fee itself and any other fees and charges specified.

10) **Indemnity.** User hereby indemnifies and agrees to defend the Rightsholder and CCC, and their respective employees and directors, against all claims, liability, damages, costs, and expenses, including legal fees and expenses, arising out of any use of a Work beyond the scope of the rights granted herein and in the Order Confirmation, or any use of a Work which has been altered in any unauthorized way by User, including claims of defamation or infringement of rights of copyright, publicity, privacy, or other tangible or intangible property.

11) **Limitation of Liability.** UNDER NO CIRCUMSTANCES WILL CCC OR THE RIGHTSHOLDER BE LIABLE FOR ANY DIRECT, INDIRECT, CONSEQUENTIAL, OR INCIDENTAL DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF

BUSINESS PROFITS OR INFORMATION, OR FOR BUSINESS INTERRUPTION) ARISING OUT OF THE USE OR INABILITY TO USE A WORK, EVEN IF ONE OR BOTH OF THEM HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. In any event, the total liability of the Rightsholder and CCC (including their respective employees and directors) shall not exceed the total amount actually paid by User for the relevant License. User assumes full liability for the actions and omissions of its principals, employees, agents, affiliates, successors, and assigns.

12) **Limited Warranties.** THE WORK(S) AND RIGHT(S) ARE PROVIDED "AS IS." CCC HAS THE RIGHT TO GRANT TO USER THE RIGHTS GRANTED IN THE ORDER CONFIRMATION DOCUMENT. CCC AND THE RIGHTSHOLDER DISCLAIM ALL OTHER WARRANTIES RELATING TO THE WORK(S) AND RIGHT(S), EITHER EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. ADDITIONAL RIGHTS MAY BE REQUIRED TO USE ILLUSTRATIONS, GRAPHS, PHOTOGRAPHS, ABSTRACTS, INSERTS, OR OTHER PORTIONS OF THE WORK (AS OPPOSED TO THE ENTIRE WORK) IN A MANNER CONTEMPLATED BY USER; USER UNDERSTANDS AND AGREES THAT NEITHER CCC NOR THE RIGHTSHOLDER MAY HAVE SUCH ADDITIONAL RIGHTS TO GRANT.

13) **Effect of Breach.** Any failure by User to pay any amount when due, or any use by User of a Work beyond the scope of the License set forth in the Order Confirmation and/or the Terms, shall be a material breach of such License. Any breach not cured within 10 days of written notice thereof shall result in immediate termination of such License without further notice. Any unauthorized (but licensable) use of a Work that is terminated immediately upon notice thereof may be liquidated by payment of the Rightsholder's ordinary license price therefor; any unauthorized (and unlicensable) use that is not terminated immediately for any reason (including, for example, because materials containing the Work cannot reasonably be recalled) will be subject to all remedies available at law or in equity, but in no event to a payment of less than three times the Rightsholder's ordinary license price for the most closely analogous licensable use plus Rightsholder's and/or CCC's costs and expenses incurred in collecting such payment.

14) **Additional Terms for Specific Products and Services.** If a User is making one of the uses described in this Section 14, the additional terms and conditions apply:

a) ***Print Uses of Academic Course Content and Materials (photocopies for academic coursepacks or classroom handouts).*** For photocopies for academic coursepacks or classroom handouts the following additional terms apply:

i) The copies and anthologies created under this License may be made and assembled by faculty members individually or at their request by on-campus bookstores or copy centers, or by off-campus copy shops and other similar entities.

ii) No License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied) (ii) permit "publishing ventures" where any particular anthology would be systematically marketed at multiple institutions.

iii) Subject to any Publisher Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the academic pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to no more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular anthology, whether photocopied or electronic, at more than one institution of learning;

E) in the case of a photocopy permission, no materials may be entered into electronic memory by User except in order to produce an identical copy of a Work before or during the academic term (or analogous period) as to which any particular permission is granted. In the event that User shall choose to retain materials that are the subject of a photocopy permission in electronic memory for purposes of producing identical copies more than one day after such retention (but still within the scope of any permission granted), User must notify CCC of such fact in the applicable permission request and such retention shall constitute one copy actually sold for purposes of calculating permission fees due; and

F) any permission granted shall expire at the end of the class. No permission granted shall in any way include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied).

iv) Books and Records; Right to Audit. As to each permission granted under the academic pay-per-use Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any photocopies sold or by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this License for any reason.

b) ***Digital Pay-Per-Uses of Academic Course Content and Materials (e-coursepacks, electronic reserves, learning management systems, academic institution intranets).*** For uses in e-coursepacks, posts in electronic reserves, posts in learning management systems, or posts on academic institution intranets, the following additional terms apply:

i) The pay-per-uses subject to this Section 14(b) include:

A) **Posting e-reserves, course management systems, e-coursepacks for text-based content,** which grants authorizations to import requested material in electronic format, and allows electronic access to this material to members of a designated college or university class, under the direction of an instructor designated by the college or university, accessible only under appropriate electronic controls (e.g., password);

B) **Posting e-reserves, course management systems, e-coursepacks for material consisting of photographs or other still images not embedded in text,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorization: to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above, including any necessary resizing, reformatting or modification of the resolution of such requested material (provided that such modification does not alter the underlying editorial content or meaning of the requested material, and provided that the resulting modified content is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms), but not including any other form of manipulation, alteration or editing of the requested material;

C) **Posting e-reserves, course management systems, e-coursepacks or other academic distribution for audiovisual content,** which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorizations: (i) to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above; (ii) to display and perform the requested material to such members of such class in the physical classroom or remotely by means of streaming media or other video formats; and (iii) to "clip" or reformat the requested material for purposes of time or content management or ease of delivery, provided that such "clipping" or reformatting does not alter the underlying editorial content or meaning of the requested material and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms. Unless expressly set forth in the relevant Order Conformation, the License does not authorize any other form of manipulation, alteration or editing of the requested material.

ii) Unless expressly set forth in the relevant Order Confirmation, no License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied or, in the case of Works subject to Sections 14(b)(1)(B) or (C) above, as described in such Sections) (ii) permit "publishing ventures" where any particular course materials would be systematically marketed at multiple institutions.

iii) Subject to any further limitations determined in the Rightsholder Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the electronic course content pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to not more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular materials, whether photocopied or electronic, at more than one institution of learning;

E) electronic access to material which is the subject of an electronic-use permission must be limited by means of electronic password, student identification or other control permitting access solely to students and instructors in the class;

F) User must ensure (through use of an electronic cover page or other appropriate means) that any person, upon gaining electronic access to the material, which is the subject of a permission, shall see:

- a proper copyright notice, identifying the Rightsholder in whose name CCC has granted permission,
- a statement to the effect that such copy was made pursuant to permission,
- a statement identifying the class to which the material applies and notifying the reader that the material has been made available electronically solely for use in the class, and
- a statement to the effect that the material may not be further distributed to any person outside the class, whether by copying or by transmission and whether electronically or in paper form, and User must also ensure that such cover page or other means will print out in the event that the person accessing the material chooses to print out the material or any part thereof.

G) any permission granted shall expire at the end of the class and, absent some other form of authorization, User is thereupon required to delete the applicable material from any electronic storage or to block electronic access to the applicable material.

iv) Uses of separate portions of a Work, even if they are to be included in the same course material or the same university or college class, require separate permissions under the electronic course content pay-per-use Service. Unless otherwise provided in the Order Confirmation, any grant of rights to User is limited to use completed no later than the end of the academic term (or analogous period) as to which any particular permission is granted.

v) Books and Records; Right to Audit. As to each permission granted under the electronic course content Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any electronic copies used by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this license for any reason.

c) ***Pay-Per-Use Permissions for Certain Reproductions (Academic photocopies for library reserves and interlibrary loan reporting) (Non-academic internal/external business uses and commercial document delivery).*** The License expressly excludes the uses listed in Section (c)(i)-(v) below (which must be subject to separate license from the applicable Rightsholder) for: academic photocopies for library reserves and interlibrary loan reporting; and non-academic internal/external business uses and commercial document delivery.

i) electronic storage of any reproduction (whether in plain-text, PDF, or any other format) other than on a transitory basis;

ii) the input of Works or reproductions thereof into any computerized database;

iii) reproduction of an entire Work (cover-to-cover copying) except where the Work is a single article;

iv) reproduction for resale to anyone other than a specific customer of User;

v) republication in any different form. Please obtain authorizations for these uses through other CCC services or directly from the rightsholder.

Any license granted is further limited as set forth in any restrictions included in the Order Confirmation and/or in these Terms.

d) ***Electronic Reproductions in Online Environments (Non-Academic-email, intranet, internet and extranet).*** For "electronic reproductions", which generally includes e-mail use (including instant messaging or other electronic transmission to a defined group of recipients) or posting on an intranet, extranet or Intranet site (including any display or performance incidental thereto), the following additional terms apply:

i) Unless otherwise set forth in the Order Confirmation, the License is limited to use completed within 30 days for any use on the Internet, 60 days for any use on an intranet or extranet and one year for any other use, all as

measured from the "republication date" as identified in the Order Confirmation, if any, and otherwise from the date of the Order Confirmation.

ii) User may not make or permit any alterations to the Work, unless expressly set forth in the Order Confirmation (after request by User and approval by Rightsholder); provided, however, that a Work consisting of photographs or other still images not embedded in text may, if necessary, be resized, reformatted or have its resolution modified without additional express permission, and a Work consisting of audiovisual content may, if necessary, be "clipped" or reformatted for purposes of time or content management or ease of delivery (provided that any such resizing, reformatting, resolution modification or "clipping" does not alter the underlying editorial content or meaning of the Work used, and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular License described in the Order Confirmation and the Terms.

15) **Miscellaneous.**

a) User acknowledges that CCC may, from time to time, make changes or additions to the Service or to the Terms, and that Rightsholder may make changes or additions to the Rightsholder Terms. Such updated Terms will replace the prior terms and conditions in the order workflow and shall be effective as to any subsequent Licenses but shall not apply to Licenses already granted and paid for under a prior set of terms.

b) Use of User-related information collected through the Service is governed by CCC's privacy policy, available online at www.copyright.com/about/privacy-policy/.

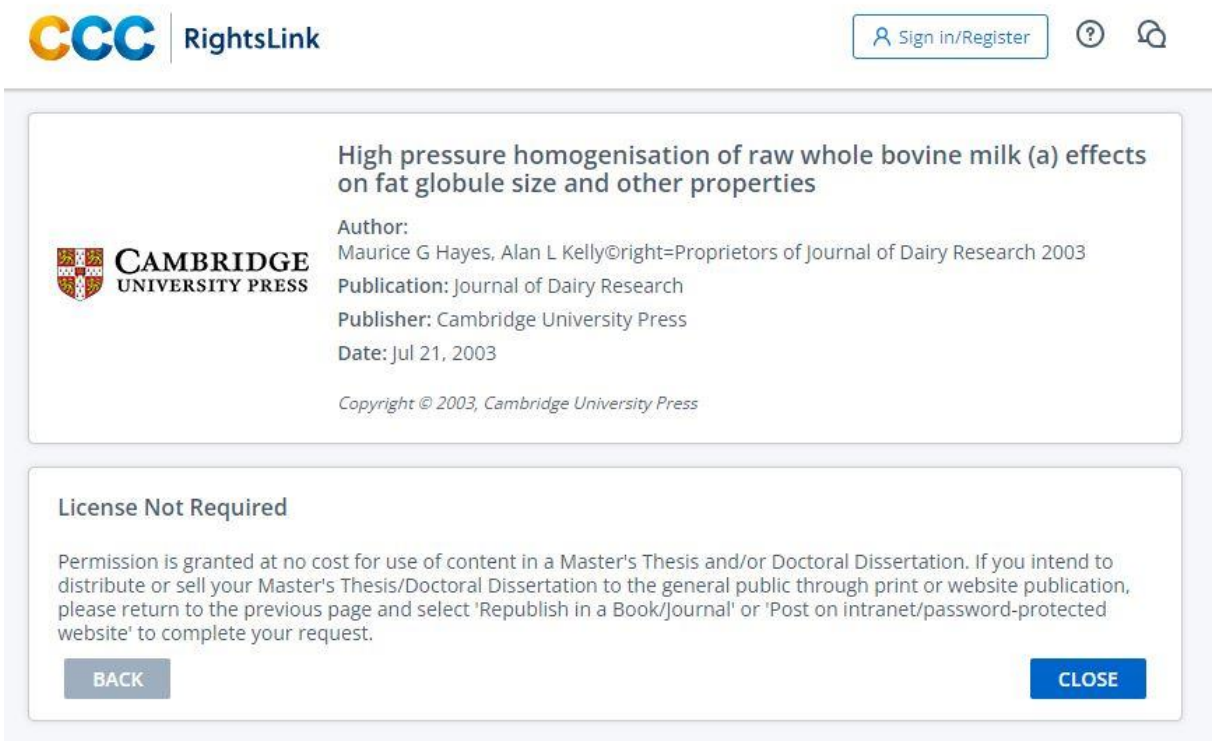
c) The License is personal to User. Therefore, User may not assign or transfer to any other person (whether a natural person or an organization of any kind) the License or any rights granted thereunder; provided, however, that, where applicable, User may assign such License in its entirety on written notice to CCC in the event of a transfer of all or substantially all of User's rights in any new material which includes the Work(s) licensed under this Service.

d) No amendment or waiver of any Terms is binding unless set forth in writing and signed by the appropriate parties, including, where applicable, the Rightsholder. The Rightsholder and CCC hereby object to any terms contained in any writing prepared by or on behalf of the User or its principals, employees, agents or affiliates and purporting to govern or otherwise relate to the License described in the Order Confirmation, which terms are in any way inconsistent with any Terms set forth in the Order Confirmation, and/or in CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Confirmation, and whether such writing appears on a copy of the Order Confirmation or in a separate instrument.

e) The License described in the Order Confirmation shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such License shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.

Last updated October 2022

Publication licence – Figure 2.3



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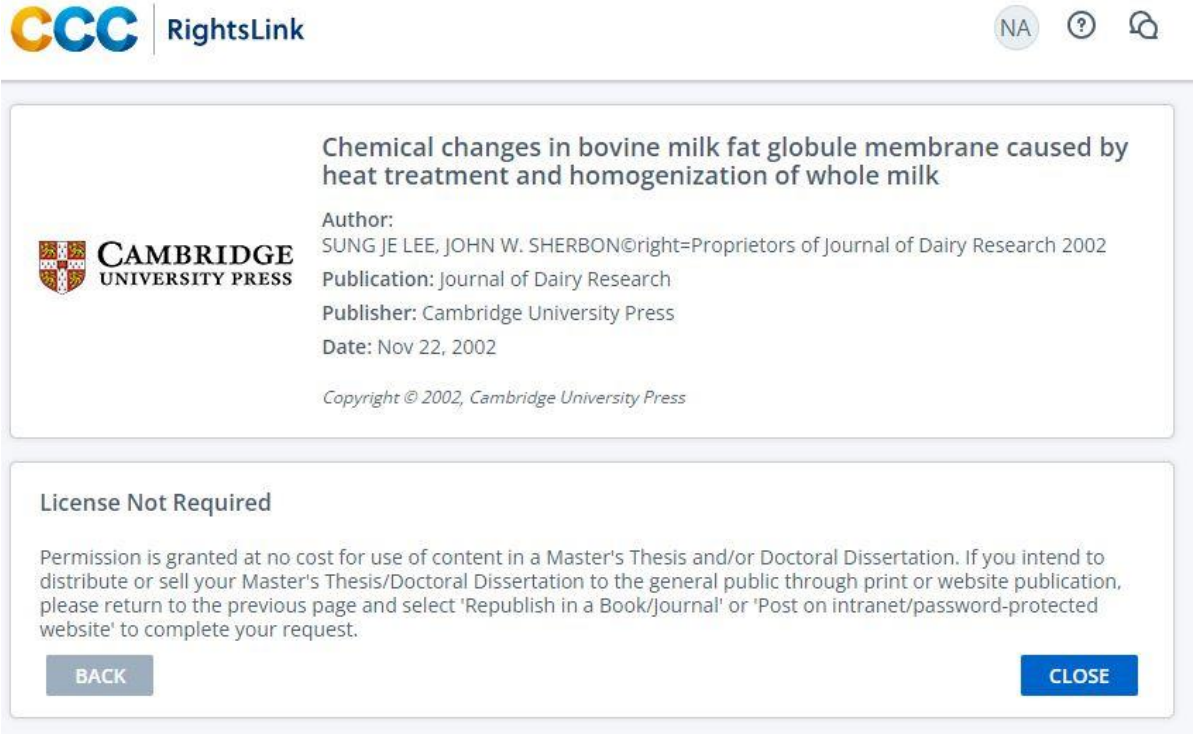

Publication licence – Figure 2.6

Marketplace

This is a License Agreement between Natalie G. M. Ahlborn ("User") and Copyright Clearance Center, Inc. ("CCC") on behalf of the Rightsholder identified in the order details below. The license consists of the order details, the Marketplace Permissions General Terms and Conditions below, and any Rightsholder Terms and Conditions which are included below.

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Order Date	21-Nov-2023	Type of Use	Republish in a thesis/dissertation
Order License ID	1418552-1		
ISSN	0268-005X	Publisher	ELSEVIER BV
		Portion	Image/photo/illustration

LICENSED CONTENT

Publication Title	Food hydrocolloids	Rightsholder	Elsevier Science & Technology Journals
Article Title	Formation of a structured clot during the gastric digestion of milk: Impact on the rate of protein hydrolysis	Publication Type	Journal
		Start Page	478
		End Page	486
Date	01/01/1986	Volume	52
Language	Dutch		
Country	Netherlands		

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Portion Type	Image/photo/illustration	Distribution	Worldwide
Number of Images / Photos / Illustrations	1	Translation	Original language of publication
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NEW WORK DETAILS

Title	THE EFFECT OF MILK PROCESSING ON PROTEIN DIGESTION AND ABSORPTION IN THE GASTROINTESTINAL TRACT OF PIGS AS A MODEL HUMAN	Institution Name	Massey University
		Expected Presentation Date	2024-03-01
Instructor Name	Natalie Ahlborn		

ADDITIONAL DETAILS

Order Reference Number	N/A	The Requesting Person / Organization to Appear on the License	Natalie G. M. Ahlborn

REQUESTED CONTENT DETAILS

Title, Description or Numeric Reference of the Portion(s)	Figure 3: Images of clots formed during the gastric digestion (SGF with pepsin) of 200 g of unheated (top row) and heated (bottom row) of skim milk at different digestion times.	Title of the Article / Chapter the Portion Is From	Formation of a structured clot during the gastric digestion of milk: Impact on the rate of protein hydrolysis
		Author of Portion(s)	Ye, Aiqian; Cui, Jian; Dalgleish, Douglas; Singh, Harjinder
Editor of Portion(s)	Ye, Aiqian; Cui, Jian; Dalgleish, Douglas; Singh, Harjinder	Issue, if Republishing an Article From a Serial	N/A
Volume / Edition	52	Publication Date of Portion	2016-01-01
Page or Page Range of Portion	478-486		

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1) **Definitions.** For purposes of these General Terms, the following definitions apply:

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