Studies on the gastric digestion of plant-based alternative milks

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Abstract

Plant-based alternative milks are colloidal dispersions consisting of extracted plant protein, oil bodies and other biopolymers that resemble cow milk in appearance. In recent years, plant-based alternative milks have become increasingly popular because of their sustainability and perceived health benefits. However, little is known about the structural changes and colloidal stability of these milks during gastric digestion and how these changes impact the delivery and absorption of nutrients.

The aim of this PhD project was to understand the digestion behaviour of plant-based alternative milk in the gastric environment, with a focus on the changes in microstructure, colloidal stability, physiochemical properties and protein hydrolysis and their direct consequences on the kinetics of nutrient release and delivery. The impact of cow milk protein on the digestion behaviour of oat-based milk was also explored. State-of-the-art dynamic *in vitro* and *in vivo* gastric digestion models were employed for this project.

The results demonstrated that plant-based alternative milks made with different plant materials (almonds, soybeans and oats) behaved differently under gastric conditions, in particular, in terms of changes in microstructures, colloidal stability and protein digestibility. Almond milk oil bodies flocculated, coalesced and then quickly layered into an upper lipid-rich layer and a lower aqueous phase upon gastric digestion. Soymilk coagulated and formed small-sized particles which sedimented rapidly. In contrast, no significant changes in the structure and colloidal stability were observed in oat milk. These variations in colloidal stability resulted in different gastric release profiles of protein and lipid. These results highlight the role of intragastric structural properties as a determining factor in controlling the kinetics of delivery of macronutrients. This study

also clearly showed the influence of cow milk protein on the structural and colloidal stability of the oat milk-cow milk blend during gastric digestion.

The findings from this thesis provide new knowledge and understanding of the gastric digestion of plant-based alternative milks and how their behaviours are different from cow milk. The knowledge gained from this PhD project may provide valuable information into the tailored design of novel plant-based alternative milk products or milk blended products for specific consumers' needs.

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Table of contents

List of figures

List of tables

List of abbreviations

BSA	Bovine serum albumin
CLSM	Confocal laser scanning microscopy
DSC	Differential scanning calorimetric
ED	Emptied digesta
EDTA	Ethylenediaminetetraacetic acid
FAMEs	Fatty acid methyl esters
GC	Gas chromatography
HGL	Human gastric lipase
HGS	Human Gastric Simulator
PAGE	Polyacrylamide gel electrophoresis
PDCAAS	Protein digestibility corrected amino acid scores
pI	Isoelectric point
SDS	Sodium dodecyl sulfate
SGF	Simulated gastric fluid
TAG	Triacylglycerols
UHT	Ultra-high temperature processing
α-la	α-lactalbumin
β-lg	β-lactoglobulin

List of peer-reviewed publications, posters, and presentations

Publications

1. **Wang, X.**, Ye, A., & Singh, H. (2020). Structural and physicochemical changes in almond milk during *in vitro* gastric digestion: impact on the delivery of protein and lipids. *Food & Function, 11*(5), 4314-4326. https://doi.org/10.1039/C9FO02465D

2. **Wang, X.**, Ye, A., Dave, A., & Singh, H. (2021). *In vitro* digestion of soymilk using a human gastric simulator: Impact of structural changes on kinetics of release of proteins and lipids. *Food Hydrocolloids, 111*, 106235. https://doi.org/10.1016/j.foodhyd. 2020.106235

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Oral presentations

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Poster

1. **Wang, X**., Ye, A., Dave, A., & Singh, H. (2022, 31st October – 3rd November) *Modelling gastric digestion behaviour of oat milk and an oat milk-bovine skim milk blend*. [Poster presentation]. The 21st IUFoST World Food Congress of Food Science and Technology, Singapore.

Chapter 1 Introduction

In recent years, there has been growing consumer interest in replacing cow milk with plant-based alternative milks in the diet (Sethi et al., 2016). Plant-based alternative milks have become a fast-growing area of functional and speciality foods across the globe (Sethi et al., 2016). The overall sales of the plant-based beverage in the US market have grown up to 30% from 2010 to 2015, with almond and coconut milk leading the 2014 increases (DMI.2014; IFT, 2015). People consume plant-based alternative milks for a variety of reasons, including lactose intolerance, cow milk allergy, ethical considerations, environmental issues and lifestyle choices (Sethi et al., 2016; Valencia‐Flores et al., 2013). Moreover, plant-based alternative milks have been shown to have several health benefits, such as cholesterol reduction and regulation of blood glucose levels, which may appeal to health-conscious consumers (Sethi et al., 2016).

Plant-based alternative milks are water extracts of nuts, cereals, legumes, pseudocereals and oil seeds (Mäkinen et al., 2016). Unlike cow milk, plant-based alternative milks vary widely in nutritional quality and most have a very low protein content (Jeske et al., 2017). The development of plant-based milks with nutritional values that are comparable with those of cow milk has been attempted (Vogelsang-O'Dwyer et al., 2021). The gastric digestion behaviour of cow milk has been studied extensively, both *in vivo* and *in vitro* (Boirie et al., 1997; Mulet-Cabero et al., 2019; Roy et al., 2022; Tunick et al., 2016; Ye et al., 2019). In cow milk, the caseins exist in the form of casein micelles, which are known to coagulate in the human stomach, whereas the whey proteins remain soluble and pass into the small intestine more rapidly (Boirie et al., 1997; He & Giuseppin, 2014; Mahe et al., 1992). During the gastric digestion of cow milk, coagulation and the

resulting curd structure have been reported to influence the rates of protein hydrolysis and gastric emptying of both proteins and lipids (Roy et al., 2022; Ye et al., 2016b).

However, little is known about the gastric digestion behaviour of plant-based alternative milks, and how their gastric digestion compares with that of cow milk. Most previous studies investigated the effect of processing treatment on the sensory and physicochemical properties (Bernat et al., 2015; Devnani et al., 2020; Manzoor, 2017), while others on the digestion of plant-based alternative milks were focused on the bioaccessibility of their bioactive compounds (Rodríguez-Roque et al., 2013; Wu et al., 2012). Recently, the static digestion behaviour of plant-based alternative milk was studied *in vitro* (Gallier & Singh, 2012a; Liu et al., 2019). The static digestion model has several advantages, such as being rapid and simple and having relatively good reproducibility. However, it is less representative of the real gastric environment than a dynamic gastric digestion model (Lucas-González et al., 2018), which provides an understanding of the changes in food structures and the rate of release of macronutrients into the small intestine for further digestion and absorption.

For the purpose either of using plant-based milk in the diet or the design of novel alternative milk products, it is very important to understand their digestion behaviours, in particular under dynamic digestion conditions and physiological-relevant conditions. The current knowledge of the preparation process of plant-based alternative milks, the structural and physicochemical properties of the plant protein and lipid, and the physiological condition of the human stomach are reviewed in **Chapter 2.** The factors that potentially affect the gastric digestion of plant-based alternative milks are also outlined. The general methods used in this thesis are described in **Chapter 3**; however, the specific method used for the preparation of different milk samples and analyses are listed in **Chapters 4-7** separately. The *in vitro* gastric digestion of three selected plant-

based alternative milks (almond milk, soymilk and oat milk) and a milk blend (oat milk-bovine skim milk blend) were investigated using a dynamic digestion model-the Human Gastric Simulator (HGS) in **Chapters 4-6**. The HGS has been widely recognized as a useful tool to study the physical and chemical properties of food materials under simulated gastric conditions and the simulated gastric contraction forces have been verified as physiological relevant to that developed *in vivo* (Kong & Singh, 2010). Using this model, the simulated gastric secretion (dynamic pH and enzyme concentration) and gastric emptying can be precisely controlled. Finally, the gastric digestion of isocaloric and iso-macronutrient cow milk, almond milk and oat milk were compared in rats euthanized at different post-feeding times (**Chapter 7**).

To understand gastric digestion of plant-based alternative milks from the viewpoints of food structure and physicochemical properties and colloid stability, and their direct consequence on the delivery of macronutrients, the key objectives of this thesis were:

Objective 1: To investigate the gastric digestion behaviour of almond milk and its consequence on the kinetics of delivery of macronutrients using a dynamic digestion model – the HGS (**Chapter 4**).

The findings from chapter 4 have been written as a paper and published as Wang, X., Ye, A., & Singh, H. (2020). Structural and physicochemical changes in almond milk during *in vitro* gastric digestion: impact on the delivery of protein and lipids. *Food & Function, 11*(5), 4314-4326. https://doi.org/10.1039/c9fo02465d

Objective 2: To investigate the *in vitro* gastric digestion behaviour of soymilk and the effects on macronutrient delivery using a dynamic digestion model-the HGS (**Chapter 5**).

Chapter 5 has been written as a paper and published as Wang, X., Ye, A., Dave, A., & Singh, H. (2021). *In vitro* digestion of soymilk using a human gastric simulator: Impact of structural changes on the kinetics of release of proteins and lipids. *Food Hydrocolloids*, *111*, 106235. https://doi.org/10.1016/j.foodhyd.2020.106235.

Objective 3: To investigate the *in vitro* gastric digestion behaviour of oat milk and the impact on macronutrient delivery using a dynamic digestion model- the HGS (**Chapter 6**).

Objective 4: To investigate the *in vitro* gastric digestion behaviour of a plant-dairy milk blend (an oat milk–bovine skim milk blend) and to understand the role of dairy proteins in the gastric colloidal stability of the plant-based alternative milk (**Chapter 6**).

The results of objectives 3 and 4 have been combined in chapter 6 and written into a paper and published as Wang, X., Ye, A., Dave, A., & Singh, H. (2022). Structural changes in oat milk and an oat milk–bovine skim milk blend during dynamic *in vitro* gastric digestion. *Food Hydrocolloids*, *124*, 107311. https://doi.org/10.1016/j.foodhyd.2021.107311

Objective 5: To confirm the *in vitro* gastric digestion of selected plant-based alternative milk in an animal model and to understand the difference and similarities of their digestion behaviour compared to cow milk (**Chapter 7**).

The findings from this chapter have been written into a paper and published as Wang, X., Wolber, F. M., Ye, A., Stroebinger, N., Hamlin, A., Zhu, X., Montoya, C., & Singh, H. (2022). Gastric digestion of cow milk, almond milk and oat milk in the rat. *Food & Function*, *13*(21), 10981-10993.

Chapter 2 Literature review

2.1 Introduction

Over the last decade, the consumption of cow milk decreased while the consumption of non-dairy alternative milk increased dramatically (McCarthy et al., 2017). From 2010 to 2015, overall sales of plant-based beverages grew by up to 30% in the United States, with a 13.7% increase in 2014 alone (DMI.2014; IFT, 2015). In New Zealand, sales of almond milk (NZ$ 14.4 million in 2017) more than doubled from 2015 to 2017 (CPG, FMCG, & RETAIL, 2017). Other plant-based alternative milks available on the New Zealand market include soy, rice, coconut and oat milks. Soymilk led sales in 2016, achieving a market of NZ$ 18.2 million nationally (CPG, FMCG, & RETAIL, 2017). The increase in sales of plant-based alternative milk appears to be driven by several reasons, for example, lactose intolerance, allergies, animal welfare, environmental concerns, increased health concerns as well as veganism (Sethi et al., 2016; Valencia-Flores et al., 2013).

Indeed, plant-based alternative milks have become a fast-growing functional and speciality food segment globally (Sethi et al., 2016). This chapter aims to provide background information and identify the research gap. The manufacturing processes of plant-based alternative milks are introduced. Considering that plant-based alternative milks are used by many consumers to replace cow milk, the composition and structural properties of macronutrients in plant-based alternative milk and cow milk are summarized. The digestion behaviour of plant-based alternative milk and cow milk under gastric conditions is reviewed. However, little information about the digestive behaviour of plant-based alternative milks is available. In addition, there is a significant gap in scientific knowledge that needs to be bridged regarding the effects of the structure and

gastric stability of plant-based alternative milks on their digestion behaviour under gastric conditions.

2.2 Plant-based alternative milks

2.2.1 General classification, nutritional composition, and benefits of plant-based alternative milks

Plant-based alternative milks are aqueous extracts of nuts, cereal, legumes, pseudocereals or oil seeds that resemble cow milk in appearance (Maekinen et al., 2016). A variety of traditional plant-based alternative milks are available around the world, for example, Sikhye (a traditional sweet rice drink from South Korea), Horchata (plant milk made from tiger nuts), and soymilk (a traditional beverage originating from China) (Chen, 1988; Cortés et al., 2005). The most widely consumed plant-based alternative milk in the world is soymilk, and the first commercially successful product was launched in Hong Kong in 1940 (Chen, 1988). Other plant-based alternative milks that dominate the dairy alternative market include coconut, oat, and almond milk.

Plant-based alternative milks can be classified into five categories according to different plant origins as follows (Sethi et al., 2016):

(1) Nut sourced: almond, coconut, hazelnut, pistachio, walnut milk;

(2) Legume sourced: soy, peanut, lupin and cowpea milk;

(3) Cereal sourced: oat, rice, corn and spelt milk;

(4) Seed sourced: sesame, flax, hemp and sunflower milk;

(5) Pseudo-cereal sourced: teff, amaranth and quinoa milk.

The nutritional composition of plant-based alternative milk varies widely (McCarthy et al., 2017). The comparison of the nutritional composition of cow milk and plant-based alternative milk has also drawn the attention of researchers (Chalupa-Krebzdak et al., 2018) (Table 2.1). However, beyond basic nutrition, plant-based alternative milks are free of cholesterol and lactose, making them a suitable alternative for consumers suffering from, for example, heart disease or lactose intolerance, etc. (Sethi et al., 2016). In addition, they contain bioactive components that are associated with many health benefits (Table 2.2) (Sethi et al., 2016).

Table 2.1 *Nutritional comparison of selected, commercially available plant-based milk alternatives.*

Type (per 100 ml)	Calories (kcal)	Protein (g)	Fat (g)	Carbohydrates (g)	Dietary fibres (g)	Calcium (% daily value)	Iron	Vitamin A
Soymilk (Silk)	33	3	1.7	1.7	0.4	13	/	4
Quinoa milk (Ecomil)	43	2	2.5	3.8	/	/	/	/
Rice milk (Pacific)	54	0.4	0.8	11.3	0	13	2.5	4
Ota milk (Oatly)	33	1	1.7	6.7	0.8	6	0	4
Sesame milk (Ecomil)	58	1	2.5	6.9	0.2	/	/	/
Almond milk (Silk)	17	0.4	1.3	0.8	0.4	8	0.8	4
Coconut milk (Silk)	33	0.4	2.1	2.9	0	19	1.7	4
Hemp milk (Living harvest)	29	1	2.5	0.4	0	13	2.5	4
Hazelnut milk (Ecomil)	52	1	2.5	5.8	/	/	/	/
Multigrain milk (Pacific Organic 7 grain milk)	58	1	0.8	11.3	0.4	15	3.3	6
Cow milk (Amul)	70	3	4.2	4.6	/	141 mg	0.52 µg	70 µg

Note. Adapted from Sethi et al. (2016) with permission.

Table 2.2 *Advantages and limitations of some plant-based alternative milks.*

Plant source	Advantages			Disadvantages		
	Functional components	Health benefits	References	Limitations	Solutions	References
Soymilk	Isoflavones	Protective role against cardiovascular disease, cancer and osteoporosis	(Omoni & Aluko, 2005)	Beany flavour caused by the action of lipoxygenase on unsaturated fatty acids	Hot grinding, alkaline soaking, blanching in boiling water, vacuum treatment at high temperature	(Chiba et al., 1979; Farkas & Goldblith, 1962; Fujimaki et al., 1965)
	Phytosterols	Reducing cholesterol	(Fukui et al., 2002)	Presence of trypsin inhibitors	Inactivation by heating	(Badenhop & Hackler, 1970; Kwok et al., 1993)
Peanut milk	Phenolic compounds	Protective effect against oxidative damage and diseases such as stroke, coronary heart disease and cancer	(Settaluri et al., 2012; Wien et al., 2014)	Beany flavour	Alkaline soaking, roasting, defatting, steaming	(Lee & Beuchat, 1992)
Rice milk	Phytosterols (β-sitosterol and γ-oryzanol)	Reducing cholesterol, hypertension, anti-oxidative, anti-diabetic, and anti-inflammatory effects	(Faccin et al., 2009; Sinchan et al., 2011)	Poor emulsion stability due to high starch content	Degradation of starch by α- and β-amylase or glucosidase	(Mitchell et al., 1988)
Oat milk	β-Glucan	Slowing gastric emptying due to high viscosity, reducing blood glucose level, total and LDL cholesterol	(Truswell, 2002)	Poor emulsion stability due to high starch content	Degradation of starch by α- and β-amylase or glucosidase	(Deswal et al., 2014a)
				Presence of inhibitors phytates	Treatment with phytase to liberate inorganic phosphate from phytic acid	(Zhang et al., 2007)

Sesame milk	Lignans (sesamin, sesamolin, sesaminol)	Anti-oxidative, antitumor, antiviral, hypocholesterolemic, anti-carcinogenic effects	(Namiki, 2007)	Anti-nutritional factors (oxalates)	Decortication	(Kapadia et al., 2002)
				The low solubility of proteins in water	Alkaline soaking, germination, roasting, defatting, microwave heating	(Quasem et al., 2009)
				Bitterness and chalkiness	Roasting, alkaline soaking	(Prakash et al., 1986)
Almond milk	α-Tocopherol	Anti-oxidative properties, protecting against free radical reactions	(Burton & Ingold, 1989; Niki et al., 1989)	/	/	/
	Arabinose	Prebiotic effects	(Mandalari et al., 2008)			
Coconut milk	Lauric acid	Promoting brain development, strengthening the immune system, maintaining the elasticity of the blood vessels	(Seow & Gwee, 1997)	/	/	/
	Vitamin E	Anti-ageing properties				

Note. Adapted from Sethi et al. (2016) with permission.

2.2.2 Formation of plant-based alternative milks

Plant-based alternative milks can be considered as oil-in-water emulsions or colloidal suspensions, consisting of plant proteins, oil bodies and other dissolved or disintegrated materials from plant cells (Yadav et al., 2017). Depending on the plant materials, plant-based alternative milks are mostly similar in appearance and consistency to mammalian milk (Sethi et al., 2016). However, the particle size distribution of plant-based alternative milks is rarely homogeneous. The stability and particle size of the final product depend to a large extent on the nature of raw materials; in addition, processing conditions such as applied disintegration, thermal and homogenization methods may also play a role in the stability of the product (Sethi et al., 2016; Silva et al., 2020).

The general outline of a modern industrial-scale process for plant-based alternative milks manufacture derived from Mäkinen et al. (2016) is shown in Figure 2.1.

2.2.2.1 Almond milk

Almonds are a nutritious food with a good source of protein, high concentrations of unsaturated fatty acids, high amounts of dietary fibre and no cholesterol (Sathe, 1992). Because of their phytochemicals composition and healthy lipid profile, almond consumption has been reported to have positive health benefits, reducing cardiovascular disease, obesity and Type 2 diabetes (Tan & Mattes, 2013). Almond milk is a colloidal dispersion that is produced by the physical disintegration of almond seeds in water. The process used for commercial almond milk preparation is outlined in Figure 2.2. However, many studies investigating the physicochemical and structural properties and digestibility of almond milk used a simplified “home-made” method without subsequent formulation and heat treatment processes (Devnani et al., 2020; Gallier & Singh, 2012a; Vanga et al., 2020).

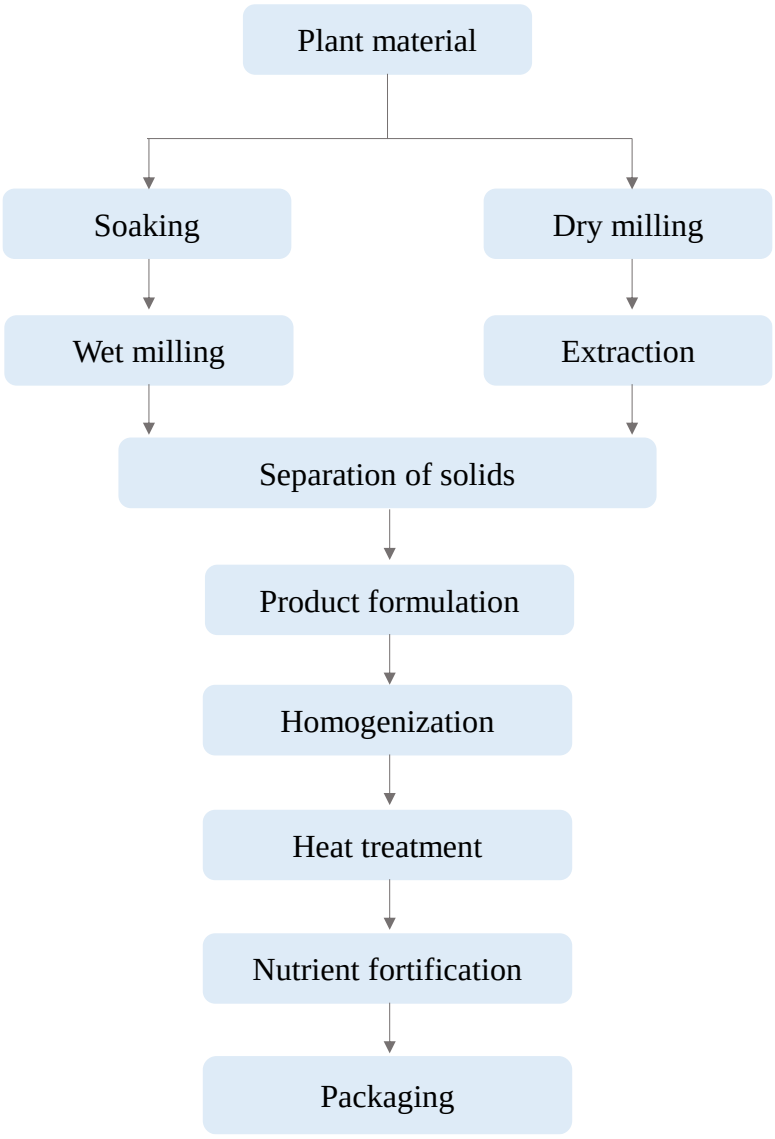

Figure 2.1 *General outline of the manufacturing process of plant-based alternative milks.*

Note. Adapted from Mäkinen et al. (2016) with permission.

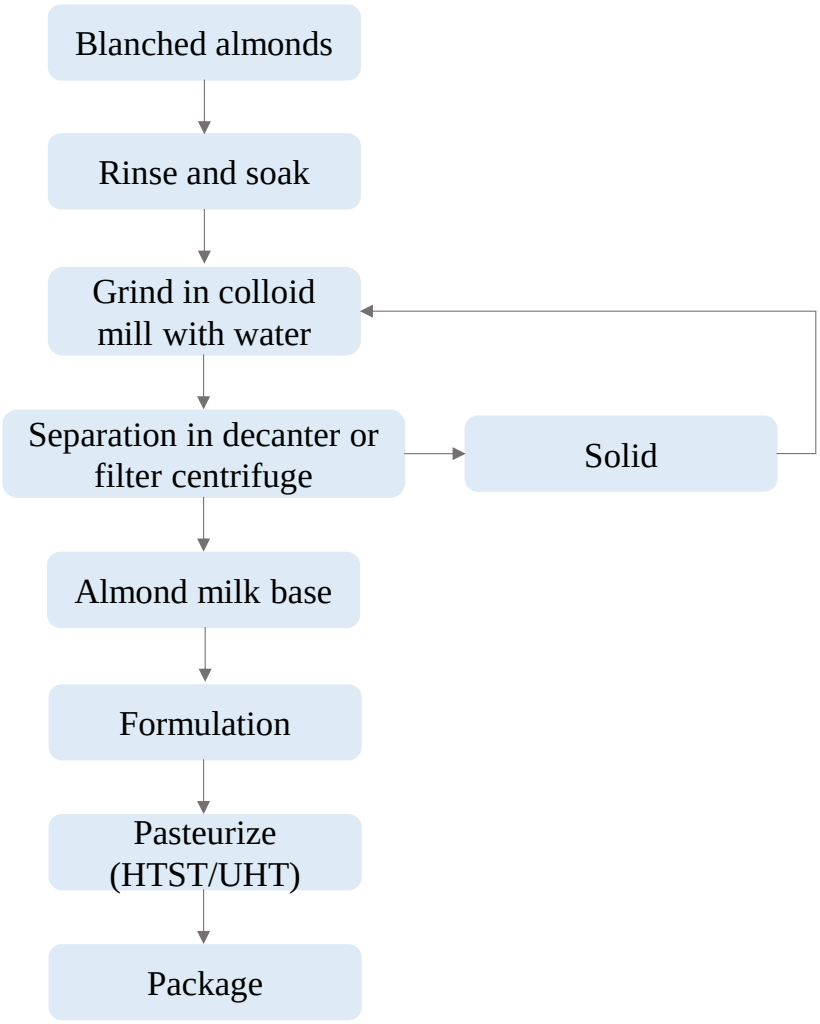

Figure 2.2 *The process used for commercial almond milk preparation.*

Note. Derived from Prosoya (http://www.prosoya.com/almond-milk-machines/).

2.2.2.2 Soymilk

Soymilk is sometimes referred to soy beverage or soy drink. It is a stable oil-in-water emulsion that is similar to cow milk in colour and nutritional composition (Hajirostamloo, 2009; Shurtleff & Aoyagi, 2000). Soymilk is a healthy drink that can be used as an alternative to cow milk added to tea, coffee or cereals. The traditional processing of soymilk includes washing, and soaking of soybeans, followed by wet disintegration, filtration and heating (usually at 93–100 °C for 30 min). The general manufacturing process of soymilk is shown in Figure 2.3.

Heat treatment is an essential process that can destroy or remove spoilage microorganisms and extend the shelf life of the product (Kwok & Niranjan, 1995). Soymilk produced by traditional methods has an unpleasant beany flavour and odour. This is due to the formation of volatile compounds catalysed by lipoxygenase during the wet grinding process (Wilkens et al., 1967). The presence of growth inhibitors in raw soymilk is another concern, such as trypsin inhibitors, lectins, and saponins. Trypsin inhibitors were one of the first reported anti-nutritional factors, which may reduce protein digestibility and cause some health problems, such as pancreatic hypertrophy (Rackis, 1974; Yuan & Chang, 2007). Proper heat treatment can effectively inactivate trypsin inhibitors and lipoxygenase, eliminate the beany flavours and improve the nutritional value and sensory properties of soymilk (Kumar et al., 2003; Lv et al., 2011; Yuan et al., 2008). Heat treatment can also induce protein denaturation and improve its digestibility (Nishinari et al., 2014).

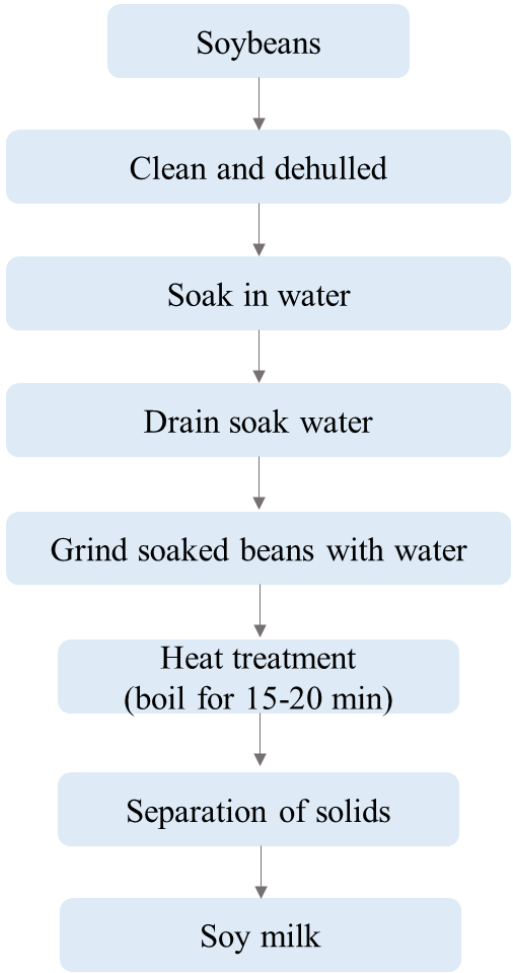

Figure 2.3 *The traditional process for the preparation of soymilk.*

Note. Adapted from Giri and Mangaraj (2012) with permission.

2.2.2.3 Oat milk

Oats are a natural functional food that is highly valued for their dietary fibre, phytochemicals, and high nutritional value (Sethi et al., 2016). Oat lipids have a favourable ratio of polyunsaturated to saturated fatty acids because of the high proportions of oleic acid and linoleic acid (Zhou et al., 1998; Zwer, 2010). Oats have been shown to have many health benefits beyond basic nutrition, such as reducing total cholesterol, lowering low-density lipoprotein blood cholesterol levels, and regulating

blood glucose levels. These effects are largely associated with the presence of soluble dietary fibre (β-glucan) in oats (Braaten et al., 1994; Kapica, 2001; Wood et al., 1994).

Oat milk is one of the newer milk alternatives that has appeared in the global market in recent years. Because of its acceptable taste, nutritional benefits, and low environmental impact, oat milk has become the second most popular plant-based alternative milks, following almond milk (Watson, 2020). The main ingredients of oat milk are water and oat flour. The typical process for producing oat milk includes mixing the oats with water to create a slurry, enzymatic hydrolysis of the starch, and filtration, followed by heat treatment and homogenization. The resulting product is a colloidal suspension of extracted protein particles and oil bodies. The general manufacturing process of oat milk, derived from Deswal et al. (2014b), is shown in Figure 2.4. Enzymatic hydrolysis is an important step in the production of oat milk (Sethi et al., 2016). Oats contain a significant amount of starch (50–60%), which may pose problems during heat treatment, as oat starch forms a viscous gel at temperatures of 44.7–73.7°C (Tester & Karkalas, 1996). Enzymes such as α-amylase and β-amylase are used to maintain the liquid state of oat milk at high temperatures (Deswal et al., 2013). Heating is applied to improve the microbial stability of the product (Mäkinen et al., 2016).

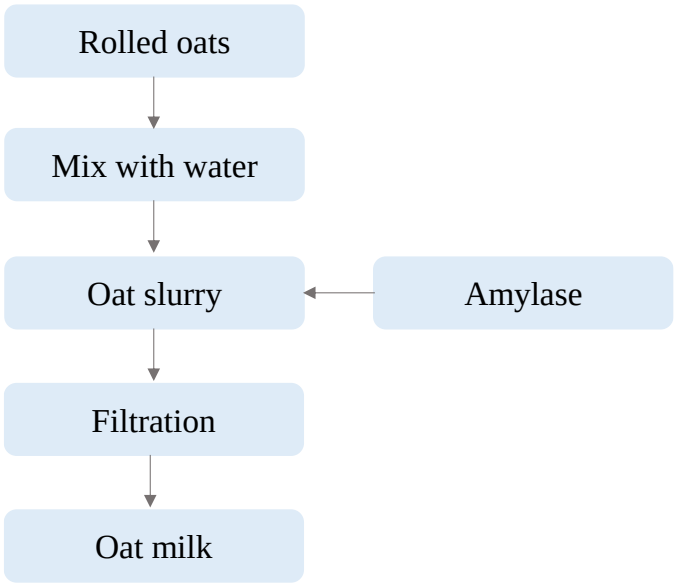

Figure 2.4 *A typical process for producing oat milk.*

Note. Adapted from Deswal et al. (2014b) with permission.

2.2.3 Proteins in plant-based alternative milks

In plant seeds, storage proteins are the main source of nitrogen and amino acids for germinating seeds and embryos.

2.2.3.1 Soy protein

The soybean plant originated in China and has been cultivated for several centuries in Asian countries. Soybean seeds consist of approximately 35–40% protein, 15–20% oil and about 12% carbohydrates on an average dry weight basis, providing the most inexpensive source of edible oils and high-quality proteins (Kwok & Niranjan, 1995; Messina, 1995). Soy protein has been widely recognized as having a higher PDCAAS than other plant proteins (Schaafsma, 2000). Soy proteins contain mainly seed storage proteins (glycinin and β-conglycinin), as well as other minor components such as protease inhibitors (Kunitz and Bowman-Birk), lipoxygenases, urase and lectins (Nielsen, 1996).

The major seed storage protein is salt-soluble globulin, which consists of two main subgroups, including glycinin (11S) and β-conglycinin (7S), accounting for approximately 40% and 25% of the total seed protein, respectively (Derbyshire et al., 1976; Manjaya et al., 2007).

Glycinin, also known as the 11S globulin, is a hexameric protein (360 kDa) assembled from six similar subunits (Figure 2.5) (Badley et al., 1975). Each subunit is composed of an acidic polypeptide (A) and a basic polypeptide (B), with molecular weights of 37–42 kDa and 17–22 kDa, respectively (Kitamura et al., 1976). The acidic and basic polypeptides are covalently linked by a disulfide bond (Kella et al., 1986). The isoelectric point (*pI*) of glycinin is pH 6.4 (Thanh & Shibasaki, 1976). Besides, glycinin has a high denaturation temperature of 84.5°C, probably because it contains a large amount of β-sheet (64%), which contributes to its thermal stability (Damodaran et al., 2007).

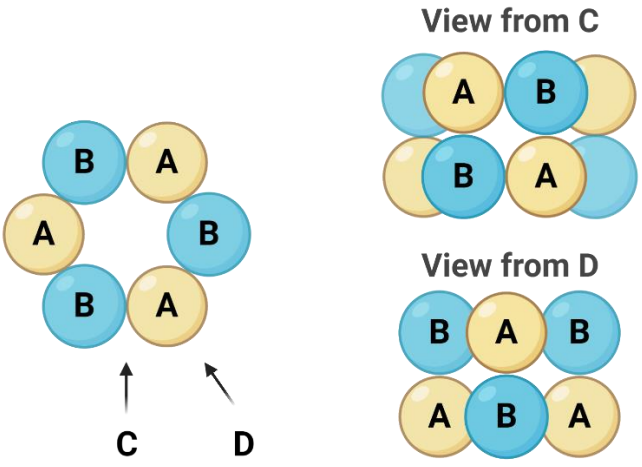

Figure 2.5 *Schematic diagram of glycinin molecule consisting of acidic (A) and basic (B) subunits.*

Note. Reproduced from Tang (2019) with permission.

7S globulins are a complex mixture of β-conglycinin, γ-conglycinin and basic 7S. β-Conglycinin is a trimer protein with an estimated molecular weight of 180–210 kDa (Utsumi et al., 1997). β-Conglycinin is composed of three subunits: α (68 kDa), α′ (72 kDa), and β (52 kDa), linked by hydrogen bonds and hydrophobic interactions (Thanh et al., 1978). 50% of α′ and α subunits of β-conglycinin are disulphide bonds linked, together or with minor protein P34 (Wadahama et al., 2012). 7S has an *pI* of pH 4.9 (Koshiyama, 1968).

Heating is an important processing procedure in the production of soymilk due to the presence of anti-nutritional factors in raw soybeans. Guo et al. (1997) investigated the effect of heat treatment on the interaction of protein and oil bodies in soymilk and they reported that the distribution of both protein and soy oil bodies changed after heating. More detailed information will be documented in section 2.3.4.3.2. Wallace et al. (1971) investigated the impact of heating on the digestion of soy proteins by pepsin. Interestingly, the authors conclude that heating reduced the digestibility of soy protein compared to unheated soy proteins in soymilk.

2.2.3.2 Almond proteins

Almonds are an important source of high-quality proteins. Over 95% of almond protein is soluble in water, salt and buffer solutions (Sathe, 1992). Soluble almond proteins consist of four main fractions, including 2S (18.4%), 9S (10.7%), 14S (65.0%) and 19S (5.9%), which can be separated by ultracentrifugal techniques (Wolf & Sathe, 1998). The 14S fraction corresponds to the major storage protein amandin, which accounts for approximately 65–70% of the total water-soluble proteins (Sathe et al., 2002). Amandin is also classified as a globulin, which consists of two major pairs of polypeptides with estimated molecular weights ranging from 20–22 kDa and 40–42 kDa. These polypeptides correspond to basic and acidic polypeptides linked by disulphide bonds

(Acosta et al., 1999; Albillos et al., 2008; Jin et al., 2009; Sathe et al., 2002). The solubility of almond proteins in defatted almond flour is minimum at pH ≤ 4, regardless of ionic strength (Sathe, 1992).

Sathe (1992) studied the susceptibility of almond proteins to digestive proteases. Pepsin appeared to be the most efficient proteolytic enzyme for almond proteins compared to trypsin and chymotrypsin, regardless of whether almond proteins were heat-denatured or not. The author also reported that heat denaturation of almond proteins at 100°C for 30 min prior to pepsin digestion [denaturation temperatures of isolated almonds protein fractions ranged from ~46–79 °C (Zhang et al., 2017)] did not alter the protein profile to a significant extent. They concluded that the sites in native almond proteins that are susceptible to protein hydrolysis can be accessed by pepsin. However, thermal denaturation improved the extent of hydrolysis of almond proteins by trypsin and chymotrypsin.

2.2.3.3 Oat proteins

Oats contain a higher protein content (15–20%) than other cereals (McMullen, 1991), with PDCAAS ranging from 43 to 69 (Peterson, 1992). The oat storage proteins are primarily distributed in the embryo or germ, the aleurone layer in the bran and the starchy endosperm (Zwer, 2010). Oat proteins exist in the form of protein bodies, which range in size from 0.3–5 μm in diameter (Miller & Fulcher, 2011). The proportions of the different oat protein compositions depend on the extraction conditions. According to Osborne protein solubility, oat proteins can be separated into globulins, albumins, prolamins and glutelins (Lásztity, 1998). Table 2.3 shows the total protein content, molecular weight and *pI* of the protein fractions in oats (Klose & Arendt, 2012).

Table 2.3 *Total protein content, molecular weight and pI of protein fractions in oats.*

Protein fraction	**% Total protein in oats**	**Molecular weights**	***pI*s**
Albumins	1–12	14–17 20–27 36–47	pH 4.0–7.0
Globulins	50–80	20–35 50–60	pH 5.5 and pH 8.0–10.0
Prolamins	4–15	17–34	pH 5.0–9.0
Glutelins	<10	9	–

Note. Adapted from Klose and Arendt (2012) with permission.

The globulin fraction is the storage protein in oats that is soluble in salt solutions. Oat globulins constitute 70–80% of the total oat proteins (Robert et al., 1983). They are a mixture of different polypeptides, as determined by different techniques such as ultracentrifugation, electrophoresis. Burgess et al. (1983) classified the globulins into 12S, 7S, and 3S by ultracentrifugation of oat grain extracts extracted with 1 mol/L NaCl. They reported that 12S globulins have relatively lower glycine content but higher glutamine and glutamic acid content compared to 7S and 3S globulins (Burgess et al., 1983). 12S is the predominant fraction of globulins. It is an oligomeric protein with a quaternary structure very similar to that of legumins. 12S globulins are hexamers, assembled from six nearly identical subunits, each with a molecular weight of 54 kDa. Electrophoresis analysis under reducing conditions showed that each subunit consists of two polypeptides: α-subunit (32 kDa) and β-subunit (22 kDa) (Shotwell et al., 1990). In the native status, the α-and β-subunits form a dimer, linked by disulphide bonds (Burgess et al., 1983). The α-subunit is weakly acidic, with an *pI* between pH 5 and 7, and is referred to as an acidic polypeptide. The β-subunit is basic, with an *pI* of pH 8 to 9, and is referred to as a basic polypeptide. 7S and 3S globulins account for only a small amount of oat proteins. 7S

globulins consist of a polypeptide with a molecular weight of 55 kDa and some minor components with an estimated molecular weight of 65 kDa. 3S fraction contains at least two major components with molecular weights of 15 and 21 kDa, respectively (Klose & Arendt, 2012).

Differential scanning calorimetric (DSC) analysis showed that oat globulins have a thermal denaturation temperature of 110°C (Ma & Harwalkar, 1984), which is higher than that of soy glycinin (about 80°C, at neutral pH and without salt addition) (Hermansson, 1978), almond proteins [46–79°C, (Zhang et al., 2017)] and hemp proteins [95°C, (Tang et al., 2006)]. The higher denaturation temperature may be due to increased hydrophobic interactions between subunits at higher temperatures (Gorinstein et al., 1996).

Prolamins are a group of proteins that are soluble in ethanol-water mixtures. Oat prolamins, also known as avenins, account for approximately 4–15% of total groat proteins (L´asztity, 1998). Oat prolamins are a mixture of polypeptides with molecular weights ranging from 17 to 34 kDa (Shotwell et al., 1990) with *pI*s of pH 5–9 (Klose et al., 2009). Water-soluble albumins account for 1–12% of the total oat proteins. The major albumin components have molecular weights of 14–17 kDa, 20–27kDa and 36–47 kDa (Klose et al., 2009; Ma & Harwalkar, 1984) with *pI*s between pH 4–7.5. DSC analysis showed that the denaturation temperature of oat albumins is about 87°C (Ma & Harwalkar, 1984). After extraction of globulins, albumins and prolamins, residual proteins can be extracted with alkaline or acidic solutions termed glutelins, but these extractions are usually incomplete. Oat glutelins are a minor group of polypeptides with a molecular weight of 9 kDa (Klose et al., 2009) and represent less than 10% of the total oat proteins

(L´asztity, 1998). DSC analysis showed no endothermic transition for oat prolamins and glutelins (Ma & Harwalkar, 1984).

2.2.4 Lipids in plant-based alternative milk

2.2.4.1 Structure of plant oil bodies

In most plants, seeds store lipid as food reserves for germination and subsequent seedling growth (Murphy & Vance 1999). Plant lipids are present in small subcellular particles called oil bodies or oleosomes. The main function of the oil body is to store triacylglycerols (TAG), which are composed of three fatty acyl chains esterified to a glycerol backbone. In general, isolated oil bodies have a spherical shape with a diameter of 0.5–2.5 μm, depending on species, nutritional and environmental factors (Huang, 1992). Oil bodies contain mainly TAGs and small amounts of phospholipids and proteins. The core of TAGs is encapsulated by a monolayer of phospholipids embedded with membrane proteins (Huang, 1992). An illustration of the structure of plant oil body is shown in Figure 2.6.

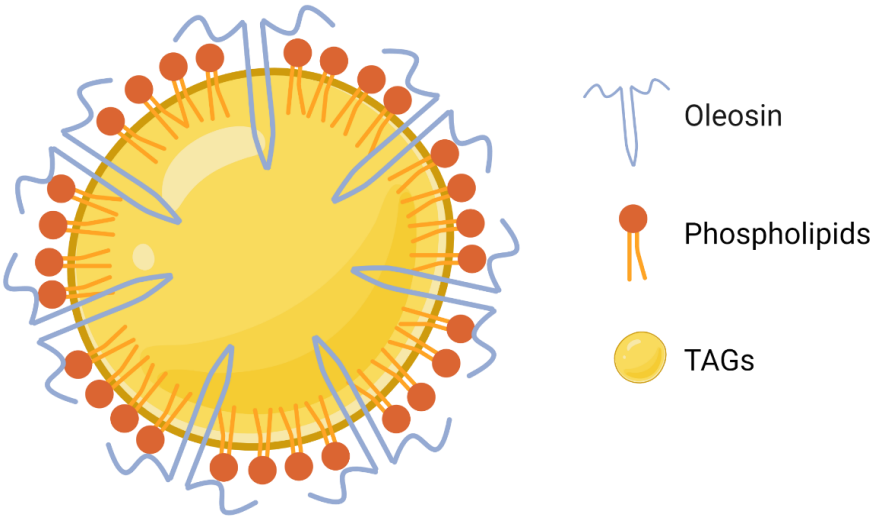

Figure 2.6 *The structure of the plant oil body.*

Note. The illustration is not on-scale. The figure was created with BioRender.com.

2.2.4.2 Oleosins

The oil bodies contain unique proteins, including oleosins, caleosins and steroleosins (Murphy, 2001; Shimada & Hara-Nishimura, 2010), which cover the phospholipid layer on the surface of the oil bodies. Oleosins are the most abundant membrane proteins and play a structural role. They are a group of alkaline proteins specific to oil bodies with a molecular weight of 15–26 kDa (Tzen & Huang, 1992). Oleosins are insoluble in water, regardless of the pH and the ionic strength (Li et al., 1992).

The primary structure of the oleosin contains an amphipathic N-terminal, an amphipathic C-terminal and a central hydrophobic domain (Tzen & Huang, 1992). The N- terminal and C-terminal domains of oleosins remain umbrella-like outside the oil body, interacting with the phospholipids and surrounding water (Huang, 1996). The central hydrophobic domain is pinned in the core of the TAGs. The oleosins and phospholipids form a thickened protective film on the surface of TAGs mass (Tzen et al., 1993).

Oleosins play an important role in stabilizing oil bodies and their synthesis during seed maturation (Wang & Huang, 1987). At neutral pH, oil bodies are highly stable against aggregation or coalescence within mature seeds or after floating centrifugation (Tzen et al., 1992; Tzen & Huang, 1992). The stability is due to the membrane proteins providing a steric barrier and electrostatic repulsion, protecting the phospholipids from phospholipase attack and preventing the oil bodies from coming close to each other (Huang, 1994). It has been shown that the digestion of oil bodies with trypsin causes degradation of oleosins and coalescence of oil bodies. However, phospholipases A2 or C have no effects on the stability of the oil bodies. They are unable to act on interfacial phospholipids without the oleosins being removed (Tzen & Huang, 1992).

2.2.4.3 Oil bodies in the plant-based alternative milk

2.2.4.3.1 Almond oil bodies

Almonds are a rich source of lipids, including unsaturated fatty acids, phytosterols, tocopherol, and phospholipids. Depending on the variety and harvest, almond kernels contain approximately 44–61% of lipids (Maguire et al., 2004; Yada et al., 2011). Compared to other tree nuts, almonds have a higher unsaturated fat content (around 91.6%) (Maguire et al., 2004). The major fatty acids in almonds are oleic (66.6%), linoleic (24.3%) and palmitic (5.8%) acids (Gallier et al., 2012). Like other plants, lipids in almonds are present in the oil bodies, where the oil droplets are surrounded by a monolayer of phospholipids and oleosins (Beisson et al., 2001; Tzen et al., 1993). Almond oil bodies may remain intact during the manufacture of almond milk. Gallier et al. (2014) showed that the microstructure of oil bodies within almond milk was intact and not disrupted after physical disintegration.

Beisson et al. (2001) determined the particle size of almond oil bodies by a light diffraction method on a Mastersizer. They reported that the average particle size ($d_{4,3}$) of the almond oil bodies was 2.6 μm. After water extraction, some almond storage proteins were attached to the surface of the oil bodies via electrostatic interactions (Beisson et al., 2001). After purification, a peptide with a molecular weight of 16 kDa tightly bound to the oil body was identified as almond oleosin. The authors also reported that after digestion of the oil bodies with a protease K, the oleosins degraded to a new 8 kDa polypeptide that resisted protein digestion regardless of the incubation time. Gallier and Singh (2012a) reported that pepsin hydrolysis of oleosins in almond milk may have allowed pancreatic lipase to access the surface of the oil body for more efficient lipolysis, although the part of oleosins (i.e., an 8 kDa polypeptide) resisted attack by the protease because of the insertion of its central hydrophobic domain into the oil core (Beisson et al.,

2001). However, Beisson et al. (2001) reported that recombinant human gastric lipase was able to access the oil core of almond oil bodies and hydrolyze the triacylglycerols without pre-digestion of the protein coat.

2.2.4.3.2 Soy oil bodies

Soy lipids are mainly composed of TAGs and are stored in the oil bodies (Huang, 1992). The structure of soybean oil bodies is similar to that of almond oil bodies. Iwanaga et al. (2007) reported that the average particle size ($d_{3,2}$) of soybean oil bodies extracted at pH 6.8 was 250 nm. Soybean oil bodies are very stable to coalescence in the aqueous medium. They remain largely intact when exposed to high temperatures, high centrifugation speeds (50,000 *g*), or in the presence of urea (9 M) (Chen & Ono, 2010; Wu et al., 2011). Soybean oil bodies have an *pI* around pH 4 and are stable to aggregation or creaming at pH values far from their *pI* (i.e., pH ≤ 2 or pH≥ 6) and at relatively low salt concentrations (i.e., NaCl ≤ 25 mM at pH 7) (Iwanaga et al., 2007).

During the production of soymilk, 11S, 7S and some other minor proteins readily bind to the surface of the oil bodies in raw soymilk. However, most of the 11S and 7S can be removed by washing with a pH 8.0 buffer, while other proteins such as P34 are stable to washing with a pH 9.0–10.0 buffer, but they may be removed after washing at pH 11.0 (Chen & Ono, 2010).

Guo et al. (1997) investigated the interaction between oil bodies and proteins in soymilk before and after thermal treatment. They separated the soymilk into three fractions by ultracentrifugation (156, 000 *g*, 30 min): floating, soluble (< 40 nm) and particulate (> 40 nm) fractions.

In raw soymilk, oil bodies are incorporated into soybean storage proteins (mainly 11S and 7S proteins) and are present in the particulate fraction (> 40 nm in diameter)

(Ono et al., 1991). It is known that heating causes the denaturation of proteins and increases their surface hydrophobicity (Sorgentini et al., 1995). When raw soymilk is heated from 65 to 75 °C, the 7S protein dissociates and some of the oil bodies are released from the particulate fraction (Guo et al., 1997).

With further heating, the 11S protein denatures when the temperature reaches 80 °C, and nearly all of the oil bodies are liberated to the floating fraction at 90 °C (Guo et al., 1997). The dissociated proteins and lipids may form a complex with a characteristic buoyant density during heating, induced by increased surface hydrophobicity (House, 1978). After heat treatment at 75–95°C, the precipitated fraction (particle size > 40 nm) of soymilk consists of protein particles, while the soluble (i.e. supernatant, particle size < 40 nm) fraction consists of soluble proteins (Peng et al., 2016). Soy protein subunits are heterogeneously distributed in the particulate and soluble fractions. The acidic polypeptides of 11S, α′ and α subunits of 7S tend to be present in the soluble form due to their high hydrophilicity, whereas the basic polypeptides of 11S and β subunits of 7S are present in a particulate fraction (Ren et al., 2009). The major (> 90%) component of the floating fraction is triglycerides after centrifugation, which are present as oil bodies and account for approximately 85% of the total lipids in soymilk (Guo et al., 1997). The oil bodies may remain intact during heating. More than 50% of the total protein in the floating fraction is composed of oleosins, the interfacial proteins of the soybean oil bodies (Guo et al., 1997).

2.2.4.3.3 Oat oil bodies

Oat lipids have a favourable ratio of polyunsaturated to saturated fatty acids due to the high proportions of oleic acid and linoleic acid (Zwer, 2010). Oat oil bodies share a similar oil body structure with other plant oil bodies such as wheat, maize, almond and soy. White et al. (2006) isolated and characterized oat oil bodies from oat grains. They

reported that some extraneous proteins, such as storage proteins, were bound to the surface of oil bodies after water extraction. However, these extraneous proteins could be removed through urea washing. After purification, the main protein bound to the oil bodies had a molecular weight of 15–17 kDa and this was identified as oleosins. The particle size of purified oil bodies in buffer at pH 7.5 ranged from 0.23–to 3.87 μm (the median diameter was 1.2 μm)(White et al., 2006). When the pH was reduced to 6.5, close to that of oat milk, the isolated oil bodies tended to aggregate due to reduced electronegative repulsion (White et al., 2006).

2.3 Cow milk

Milk is a complex biological fluid containing a variety of components in a dispersed state. The main components of milk are proteins, lipids and lactose as well as numerous minor components such as minerals, vitamins, immunoglobulins, hormones and enzymes (Fox, 2009). The average composition of whole milk is shown in Table 2.4. Milk fat consists mainly of triglycerides (98%) and is present as an emulsion of fat globules surrounded by a surface membrane of phospholipids and glycoproteins. The proteins in milk can be divided into two categories, casein and whey protein (Fox, 2003; Fox et al., 1998; Wong et al., 1996). Caseins exist in milk as complex particles containing calcium and phosphate in the form of colloidal suspension, while whey proteins are present in soluble form. Lactose is the sugar present in milk and whey, which contains monosaccharides, glucose and galactose. The salts in milk contain mainly cations such as calcium, magnesium, potassium and sodium, and anions such as inorganic phosphate, chloride and citrate (Gaucheron, 2005).

Table 2.4 *Average composition of milk* [a]

Component	The average content in milk (% w/w)	Range (% w/w)	The average content in dry matter (% w/w)
Water	87.1	85.3–88.7	
Solids-not-fat	8.9	7.9–10.0	
Fat in dry matter	31	22–38	
Lactose	4.6	3.8–5.3	36
Fat	4.0	2.5–5.5	31
Protein	3.3	2.3–4.4	25
casein	2.6	1.7–3.5	20
Mineral substances	0.7	0.57–0.83	5.4
Organic acids	0.17	0.12–0.21	1.3
Miscellaneous	0.15		1.2

Note. [a] Typical for the milk of lowland breeds. Adapted from Walstra et al., (1999) with permission.

2.3.1 Milk proteins

Cow milk contains 3–4% protein, consisting mainly of casein and whey protein (Varnam & Sutherland, 2001). Caseins account for approximately 80% of the total protein in milk, which can be precipitated from milk when milk is acidified to pH 4.6 (the isoelectric pH) at around 30 ºC. Whey protein (approximately 20%) is the protein that remains soluble under these conditions (Fox et al., 2015). The principal component of milk proteins is shown in Figure 2.7.

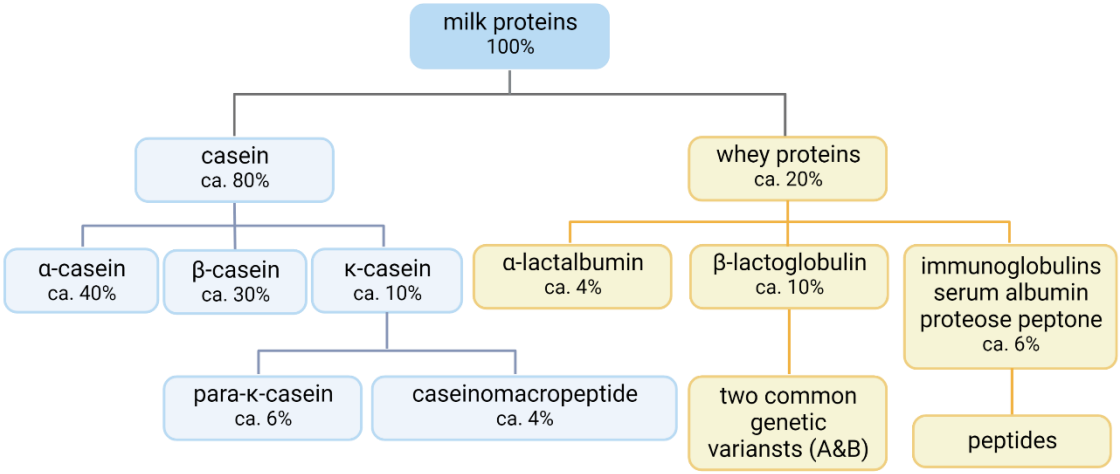

Figure 2.7 *Main components of milk proteins.*

Note. Adapted from Cheison and Kulozik, (2017) with permission.

2.3.1.1 Caseins

Caseins are a group of phosphoproteins that can be subdivided into four individual types: α_{s1}-, α_{s2}-, β- and κ-caseins in a ratio of approximately 4: 1: 3.5: 1.5 (Huppertz, 2013). α_{s1}-Casein is sensitive to calcium because of the presence of phosphate groups and may precipitate in the presence of calcium at around pH 7.0 (Varnam & Sutherland, 2001). β-Casein makes up about 35% of the casein in cow milk (Huppertz, 2013). It contains the most hydrophobic groups and forms aggregates with the inner hydrophobic domain and N-terminal hydrophilic domain exposed to the surrounding serum (Varnam & Sutherland, 2001). Compared to α_{s1}-casein, β-casein is less sensitive to calcium-induced precipitation (Huppertz, 2013). β-Casein contains high levels of phosphate groups and tends to bind with metal ions in the milk. The binding of calcium ions to β-casein decreases with increasing ionic strength but increases with increasing temperature or pH (Baumy & Brule, 1988; Parker & Dalgleish, 1981). κ-Casein is calcium-insensitive and has a low level of phosphorylation (Huppertz, 2013). The κ-casein molecules consist of a relatively

stable polymeric structure linked by disulfide bonds. The Phe_{105}–Met_{106} bond protrudes from its molecular surface, which is the sensitive site to chymosin cleavage. κ-Casein can be cleaved by chymosin into caseinomacropeptide and para-κ-casein (Varnam & Sutherland, 2001).

Caseins in milk exist in the form of micelles, which play a crucial role in the physicochemical stability of mammalian milks (Fox & Brodkorb, 2008). Casein micelles are made up of several thousand individual casein molecules with an average diameter of approximately 200 nm. A typical casein micelle particle comprises over 20,000 individual protein molecules (de Kruif, 1998). The casein micelles are highly hydrated, containing approximately 3 to 4 g of H_2O per g protein (Jeurnink & de Kruif, 1993; Walstra, 1979). The dry matter of casein micelles consists of approximate 92% protein and 8% colloidal calcium phosphate (O'mahony & Fox, 2013).

Casein micelles are essentially complex association colloids, which are assembled by the highly phosphorylated α_{s1}-, α_{s2},- and β-caseins interacting with calcium phosphate and surface-stabilized by κ-casein (Figure 2.8) (Dalgleish, 2011). The κ-casein on the micellar surface stabilizes the particles and prevents aggregation by protruding its macropeptide (residues 106–169 of the protein molecule) into the serum, forming a hairy layer around the particles (de Kruif & Zhulina, 1996; Horne, 1986). This hairy layer is highly hydrophilic and interacts well with water. This extended macropeptide layer, estimated 5–10 nm thick, surrounds the micelles and provides steric stabilization to the micelles (de Kruif & Zhulina, 1996; Horne & Davidson, 1986).

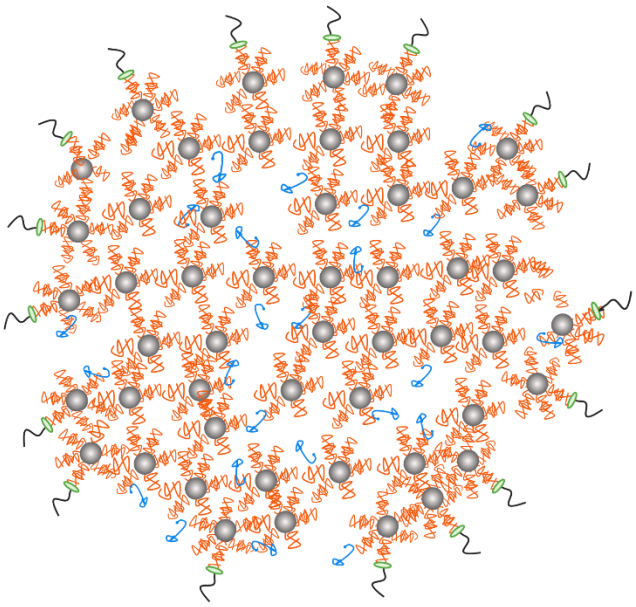

Figure 2.8 *Schematic diagram of casein micelle structure.*

Note. Reproduced from Dalgleish and Corredig (2012) with permission. The α_{s1}-, α_{s2}- and β-caseins (orange) interact with the calcium phosphate nanoclusters (grey spheres). Some β-caseins (blue) hydrophobically bind to other caseins. The κ-caseins (para-κ-caseins in green) and the caseinomacropeptide chains in black) are located on the outside of the surface.

Casein micelles are stable to commercial homogenisation, ultracentrifugation or in the presence of high levels of calcium ions (O'mahony & Fox, 2013). The micelles can be moderately heated without significant disruption to their structures or aggregation. However, by lowing the pH from 6.7 to approximately 5.5, the hairy layer can be easily collapsed (de Kruif & Zhulina, 1996; Donato et al., 2007). This acidification may weaken the steric stabilization barrier and induce aggregation of casein micelles as caseins approach their *pI*. Furthermore, the κ-casein macropeptide layer can be removed by some specific proteinases (chymosin, pepsin), with the result that caseins aggregate (coagulate) or gel in the presence of calcium ions (Lucey, 2011), which are the bases for the production of yoghurt and cheeses products (Dalgleish & Corredig, 2012).

2.3.1.2 Whey proteins

Whey protein is a group of acid-soluble proteins that accounts for approximately 20% of the total protein in cow milk. It comprises two major proteins, β-lactoglobulin (β-lg) and α-lactalbumin (α-la), as well as small amounts of serum albumins and immunoglobulins (Kinsella et al., 1989). Whey proteins are typical compact globular proteins; the protein molecules fold in their native state through the formation of disulfide bonds between cysteinyl residues, in which most of the hydrophobic residues are buried in the interior of the molecule (Fox, 2009).

Whey proteins differ from caseins in their properties. For example, whey proteins can be used over a wider pH range than caseins, because whey proteins are soluble at pH 4.6, whereas caseins are insoluble at pH close to their *pI*. In addition, whey proteins may remain soluble when treated with specific proteolytic enzymes (chymosin or pepsin), while the caseins are coagulable (O'mahony & Fox, 2013). Furthermore, caseins are very stable at temperatures up to 140 °C, whereas whey proteins are relatively heat-labile. Mild heat treatment (< 75 °C) can result in the association of whey proteins with milk fat globule membrane proteins (Ye et al., 2004; Zheng et al., 2013). Under extensive heating, whey proteins denature and interact with themselves and with casein micelles to form aggregates via disulphide bonds and hydrophobic interactions (Donato & Guyomarc'H, 2009; Guyomarc'H et al., 2003).

β-Lg is the major component of bovine whey, accounting for approximately 50% of whey protein or about 12% of the total protein of cow milk. During the heat treatment of milk, β-lg unfolds, penetrates the hairy layer of casein micelles and forms disulphide bonds with the interior of κ-casein, which has a significant effect on the thermal stability of milk and coagulation by rennet (Anema & Li, 2003). The extent of association of β-lg with casein micelle is highly dependent on the heating conditions. When milk is heated

at 75°C to 90°C for 80 min, all β-lg denatures and associates with casein micelles via κ-casein (Corredig & Dalgleish, 1996); however, when milk is heated at 130°C for 100 s, only 50% of β-lg associates with κ-casein (Oldfield et al., 2005). β-Lg in its native state is resistant to hydrolysis by pepsin in the gastric environment (Peram et al., 2013), but the susceptibility of β-lg to pepsin is improved by heat-induced conformational changes (Reddy et al., 1988).

α-La, the other main protein of bovine whey, however, is heat-stable (Nik et al., 2010). Due to its compact globular structure, α-la is relatively resistant to enzymatic proteolysis. Many attempts have been made to increase its susceptibility to proteolysis by modifying its structure under different conditions. It has been reported that hydrolysis of α-la can be improved by lowering the pH to 2.0 when pepsin was present or by esterification when α-la was treated with trypsin at 37°C (El-Zahar et al., 2005; Sitohy et al., 2001). The binding of zinc to α-la also increases its susceptibility to chymotryptic and tryptic hydrolysis (Permyakov et al., 1991). Due to the excellent heat stability of α-la, heating α-la prior to enzymatic hydrolysis may not significantly affect its digestibility, as α-la tends to refold throughout the cooling process if the heating temperature applied below 100°C (i.e., the temperature of irreversible denaturation of α-la) (Schmidt & Poll, 1991).

2.3.2 Milk lipids

Cow milk contains 4–5% fat and can be considered an oil-in-water emulsion. Almost all (> 95%) of the milk fat exists in the form of fat globules with particle sizes of 0.1–10.0 μm (Table 2.5). Each fat globule consists of a triglyceride core surrounded by a protective membrane, named the milk fat globule membrane (Keenan & Dylewski, 1995; Mulder & Walstra, 1974). This membrane is approximately 10 nm in thickness and acts as an emulsifier, protecting the fat globules from aggregation, coalescence, creaming and

enzymatic degradation. The milk fat globule membrane is highly structured and consists of polar lipids and membrane-specific proteins (Dewettinck et al., 2008). Milk fat globules play an important role in determining the properties of milk and cream products, such as viscosity, colour and foaming capacity (Walstra, 1995). There have been several major reviews on milk fat globules and their stability (Dewettinck et al., 2008; Jensen, 2002; MacGibbon, 2020; Walstra, 1995).

Table 2.5 *Properties of milk fat globules.*

Milk fat globules	
Main component	Fat
To be considered as	Emulsion
Content (% dry matter)	4
Volume fraction	0.04
Particle diameter	0.1–10 µm
Number per ml	10^{10}
Surface area (cm^2/ml milk)	700
Density (20°C; kg · m^{-3})	920
Visible with	Microscope
Separate with	Milk separator
Diffusion rate (mm in 1h)	0.0
Isoelectric pH	~3.8

Note. Adapted from Walstra et al., (1999) with permission.

2.4 Gastric digestion of plant-based- and dairy-based milks

Before reviewing the gastric digestion of plant or dairy-based milk, it is important to understand the basic functions of the human stomach.

2.4.1 Functional anatomy of the human stomach, gastric secretion and gastric emptying

The human stomach is essentially a "J" shaped muscular bag comprising the fundus, corpus, antrum and pylorus (Figure 2.9) (Tortora & Derrickson, 2008). The primary functions of the stomach are: (i) to act as a reservoir for ingested foods and to deliver the gastric contents to the duodenum in physiologically appropriate amounts; (ii) to soften and reduce the size of the ingested food, producing a mixture (i.e., chyme) through both secretions of gastric juice and grinding by antral motility; (iii) to secrete enzymes and hydrochloric acid for initiate protein and lipid digestion and inhibit some harmful bacteria (Mahadevan, 2014; van Aken, 2010).

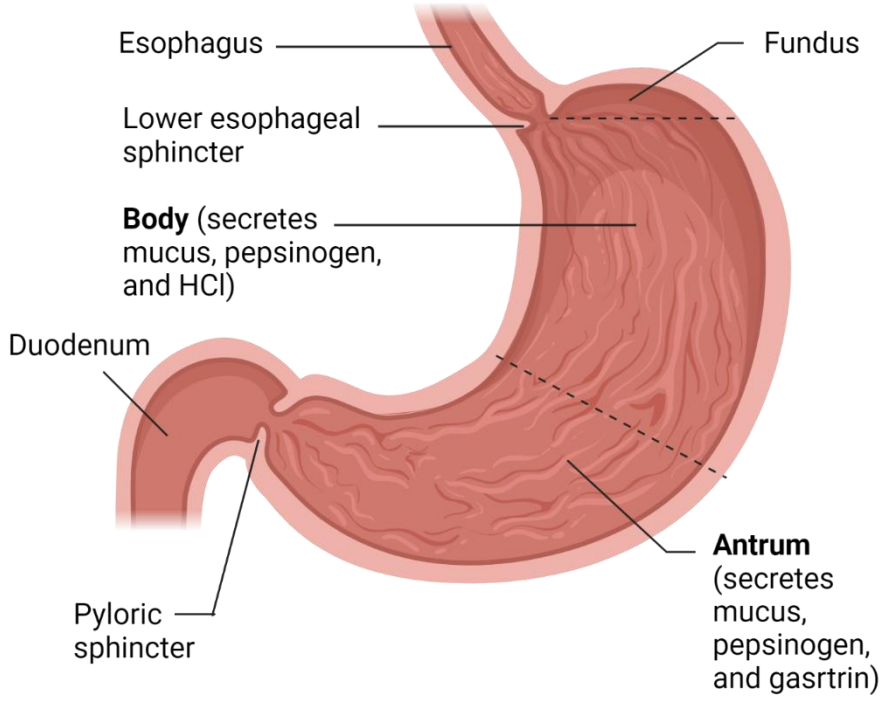

Figure 2.9 *The anatomy of the human stomach.*

Note. Reproduced from Barrett et al. (2010) with permission.

2.4.1.1 Gastric secretion

The gastric mucosa contains many deep glands. The structure of the glands from the fundus and body of the stomach is shown in Figure 2.10. The cells of the gastric gland secrete about 2.5 litres of gastric juice per day (Barrett et al., 2010). The gastric juice in the fasting state mainly contains pepsins, lipase, mucus, intrinsic factor and minerals (i.e., cations: Na^+, K^+, Mg^{2+}, H^+; anions: Cl^-, HPO_4^{2-}, SO_4^{2-}). Hydrochloric acid and intrinsic factors are secreted by the parietal (oxyntic) cells (Barrett et al., 2010). The digestive enzymes, pepsinogens (i.e., the inactive precursors of pepsins) and gastric lipase are produced by the chief (zymogen, peptic) cells (Figure 2.10). The glands in the cardia and the pyloric region secrete mucus. Gastric secretion also contains three major stimuli: gastrin, histamine and acetylcholine, which play important roles in regulating the rate of gastric acid and pepsin secretion (Barrett et al., 2010).

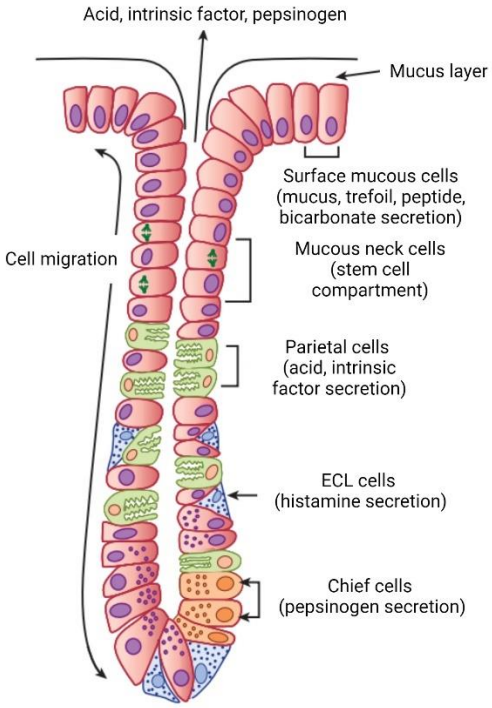

Figure 2.10 *Structure of a gastric gland from the fundus and body of the stomach*

Note. Adapted from Barrett et al. (2010) with permission.

In the fasted state, it is widely accepted that the pH of human gastric juice varies from pH 1 to 3 (Lindahl et al., 1997). Upon consumption of a meal, the pH value of the stomach contents typically increases to 5.5–7, depending on the buffering capacity of ingested foods (Dressman et al., 1990; Roger et al., 2010). With the secretion of hydrochloric acid, the pH of the stomach decreases and returns to its pH level during the fasting state after a few hours (N'Goma et al., 2012). This acidic environment in the stomach prevents microbial growth, provides optimal conditions for the action of pepsin and also has the potential to alter the structure of the food matrix.

In the fasted state, the typical ionic strength of gastric juice is about 100 mM Cl^- is the predominant ion in gastric juice with an average concentration of 100 ± 28 mM The average concentrations of Na^+ and K^+ in gastric juice are 68 ± 29 mM and 13.4 ± 3.0 mM, respectively. The concentration of Ca^{2+} in the stomach is very low and may vary between individuals (Lindahl et al., 1997). The ionic strength may vary further with food intake due to the additional ions from food (Kalantzi et al., 2006).

The stomach secretes two main digestive enzymes including pepsin and gastric lipase. Pepsins is secreted in inactivated precursors and activated by acid. Pepsin is aspartic proteinase, which cleaves the bonds adjacent to aromatic amino acids, phenylalanine, tryptophan, and tyrosine, and it initiates protein digestion in the stomach. Unlike pepsin, gastric lipase is of less quantitative significance (10–30% fat is digested in the stomach) for fat digestion, as most fat digestion begins in the small intestine (Hamosh, 1990).

The concentration of pepsin in the stomach appears to be variable. Schmidt et al. (1970) reported a basal pepsin output of approximately 0.87 mg/ml gastric secretion, which was determined by hydrolysis of haemoglobin at pH 1.7 using porcine pepsin as a standard. Lambert et al. (1968) used a similar analysis method and reported a pepsin

output ranging from 0.83–1.27 mg/ml. Kalantzi et al. (2006) studied the fasting state by administering 250 ml of water to twenty healthy human subjects and aspirating their gastric contents from their antrum. They reported that the pepsin level in the fasted stomach did not differ significantly at different sampling times, with the median values of pepsin concentration ranging from 0.11 to 0.22 mg/ml ($P > 0.05$) from a 20 to 60 min sampling period. However, after 500 ml intake of an Ensure plus meal (750 kcal, 31 g proteins, 24.6 g fat and 101 g carbohydrates), the pepsin concentration was doubled relative to the values determined in the fasted state, which ranged from 0.26 mg/ml at 30 min to 0.58 mg/ml at 210 min.

The pepsin activities reported in the literature also demonstrate large variability, which is probably because different assays and calculations were applied (Armand et al., 1995; DiPalma et al., 1991; Ulleberg et al., 2011). The pepsin activities found in adults are summarized in Table 2.6.

Table 2.6 *Summary of pepsin activities found in adults.*

Study	N	Meal	Activity (U/ml gastric contents)		Unit definition
Armand et al. (1995)	6	Low fat	Basal Stimulated	942 ± 120 (±SE) 718 ± 147	1U=0.1μmol tyrosine-containing peptides in 10 min at pH 1.8 from a 2% haemoglobin substrate at 37°C
		High fat	Basal Stimulated	1333 ± 115 1042 ± 204	
Ulleberg et al. (2011)	18		Stimulated	37.3[a] ± 21.5(±SD) 26.7[a] (pooled)	1U= 1.0 increase at $A_{280\ nm}$ in 10 min at pH 3.0 from a 2% haemoglobin substrate at 37°C
DiPalma et al. (1991)	29		Gastric mucosa biopsy specimens (3 months–26 yrs)	180–780 /mg protein	1U=0.1μmol tyrosine-containing peptides in 10 min at pH 1.8 from a 2% haemoglobin substrate at 37°C

Note. [a] It can be converted to Anson unit (0.001 increase at ΔA280 in 1min) by multiplying the factor of 100: 3730 ± 2150 Anson units/ml, and 2670 Anson units/ml (pooled).

DiPalma et al. (1991) determined the activity of pepsin in the biopsy specimens from the stomachs of 29 human subjects aged from 3 months to 26 years. They reported that the pepsin activity in specimens from the gastric body ranged from 180 to 780 pepsin units/mg protein (tyrosine unit, the unit definition is shown in Table 2.6), which was significantly higher than those from the antrum. They also indicated that there was no statistical difference in the pepsin activity of biopsy specimens among different age groups. Their results provide valuable evidence that the digestive enzyme pepsin has been fully developed shortly after birth.

However, the pepsin activity in the fasted and fed stomach is a different matter from that in the biopsy specimens. Armand et al. (1995) collected the basal and stimulated gastric juices from 6 healthy human subjects after two-week periods of a high-fat diet or a low-fat diet. In the fasted state, the pepsin activity of the basal gastric secretion was 1,333 units/ml and 942 units/ml for the subjects who had the high-fat and low-fat diet period, respectively (tyrosine unit, the unit definition is shown in Table 2.6). After pentagastrin stimulation, they found that pepsin activity was positively related to daily fat intake. They concluded that consumption of a high-fat diet can increase pepsin activity in humans.

Ulleberg et al. (2011) investigated the characteristics of individual human gastric juice. They stimulated gastric acid secretion by continuous instillation of an isotonic solution and collection of the gastric contents through aspiration from 18 healthy human subjects (6 men and 12 women) aged from 20 to 42 years old. Individual pepsin activity of the gastric secretions was found to have substantial differences: there was no detectable pepsin activity from 8 subjects; among the others, the pepsin activities ranged from 7.3 units/ml to 70.5 units/ml, with a mean value of 37.3 units/ml (the unit definition is different from Armand et al. (1995) and DiPalma et al. (1991) and shown in Table 2.6).

They also determined the pepsin activity of pooled gastric juice (n=18) and reported a value of 26.7 units/ml.

Unfortunately, clinical studies that reported the pepsin activity of gastric contents (in the fasted or fed state) in healthy humans are very limited, probably due to ethical reasons. The main challenge to compare the pepsin activity among these studies is the different assay conditions or unit definitions applied. However, using a physiological relevant enzymatic concentration in the *in vitro* digestion models is extremely important. In the paper written by Minekus et al. (2014), the COST infogest network proposed a static *in vitro* digestion model for an international consensus, in which the authors recommended applying a pepsin activity of 4, 000 units/ml secretion and using the Anson unit for pepsin activity determination internationally. The definition of an Anson unit is: "one unit will produce an ΔA_{280} of 0.001 per minute at pH 2.0 and 37°C, measured as trichloroacetic acid-soluble products" (Minekus et al., 2014), which is different from the unit definitions used in the clinical studies that summarized in Table 2.7. However, the pepsin activity reported in Ulleberg et al. (2011) can be roughly converted to the Anson units (an ΔA_{280} of 0.001 increase in one minute) by multiplying a factor of 100. The converted pepsin activity is 3,700 Anson units/ml gastric secretion corresponding to 37.3 units/ml reported by Ulleberg et al. (2011).

2.4.1.2 Gastric emptying

When foods enter the stomach from the oesophagus, the fundus and upper part of the gastric body can accommodate a large quantity of food in a short period without significant increases in intragastric pressure (Goyal et al., 2019). The stomach then secretes the gastric juice to soften the ingested food, grinds the solid food to smaller sizes and forces the chyme towards the pylorus and enters the duodenum through an antral contraction. The antral contraction frequency is around 3.0 ± 0.2/ min (Marciani et al.,

2001). Only liquid and semi-solid gastric contents, as well as chyme with sizes smaller than 1–2 mm, can leave the stomach and enter into the duodenum for further digestion and absorption. This process refers to gastric emptying, which is a complicated and well-regulated process (Hellström et al., 2006; Malagelada & Azpiroz, 2010). The mechanisms and physiology of gastric emptying have been recently reviewed by Liu et al. (2020).

Gastric emptying rate is usually reported as initial lag phase T_{lag} and half-emptying time $T_{1/2}$. T_{lag} is the time between ingestion of food and the beginning of emptying and $T_{1/2}$ is the time at which half of the ingested food is emptied. Generally, the gastric emptying rate of liquid chyme and solid chyme is different and liquid chyme empties faster than solid chyme (Liu et al., 2021) (Figure 2.11). After ingestion, liquid foods are rapidly distributed throughout the entire stomach. The emptying of liquid food follows an exponential time course with a rapid initial emptying. Water empties promptly after ingestion and has a half-emptying time of 15–20 min, driven by the gastro-duodenal pressure gradient (Figure 2.11) (Hellström et al., 2006). In contrast, the emptying of solid meals follows a biphasic pattern. The solid foods are redistributed in the stomach and pulverized to smaller sizes (1–2 mm) in the lag phase, and pass through the pylorus into the duodenum during the linear emptying phase (Figure 2.11) (Meyer et al., 1981; Meyer et al., 1979). Gastric emptying of a solid meal (Western-type meal) usually requires 2 to 4 h after ingestion (Dressman et al., 1990; Gardner et al., 2002).

Gastric emptying is a complex process, which is affected by not only food properties but also biological factors (Acevedo-Fani & Singh, 2022a; Goyal et al., 2019; Liu et al., 2020). The factors affecting gastric emptying are summarized in Table 2.7.

Food characteristics, for example, chemical composition, ingested volume, osmolality, calorie content, viscosity and processing, have been reported to affect food digestibility and gastric emptying rates (Kong & Singh, 2009; Kwiatek et al., 2009;

Lundin et al., 2008; Mazzawi et al., 2019; Zhu et al., 2013). *In vivo*, biological factors such as gastrointestinal peptide hormone regulation, the feedback effect of nutrients and the age, gender and gastric pH, motility and digestive enzyme concentrations of an individual may also play extremely important roles in gastric emptying (Gamble et al., 2013; Hellmig et al., 2006; Karhunen et al., 2008; Tougas et al., 2000). For example, energy-enriched meals are delivered more slowly than non-nutrient liquid meals because of a negative feedback mechanism controlled by duodenal receptors. Delivery of nutrients to the small intestine is maintained at a constant rate through the action of the gut peptide hormone cholecystokinin (Brener et al., 1983; Collins et al., 1984; Hunt & Stubbs, 1975).

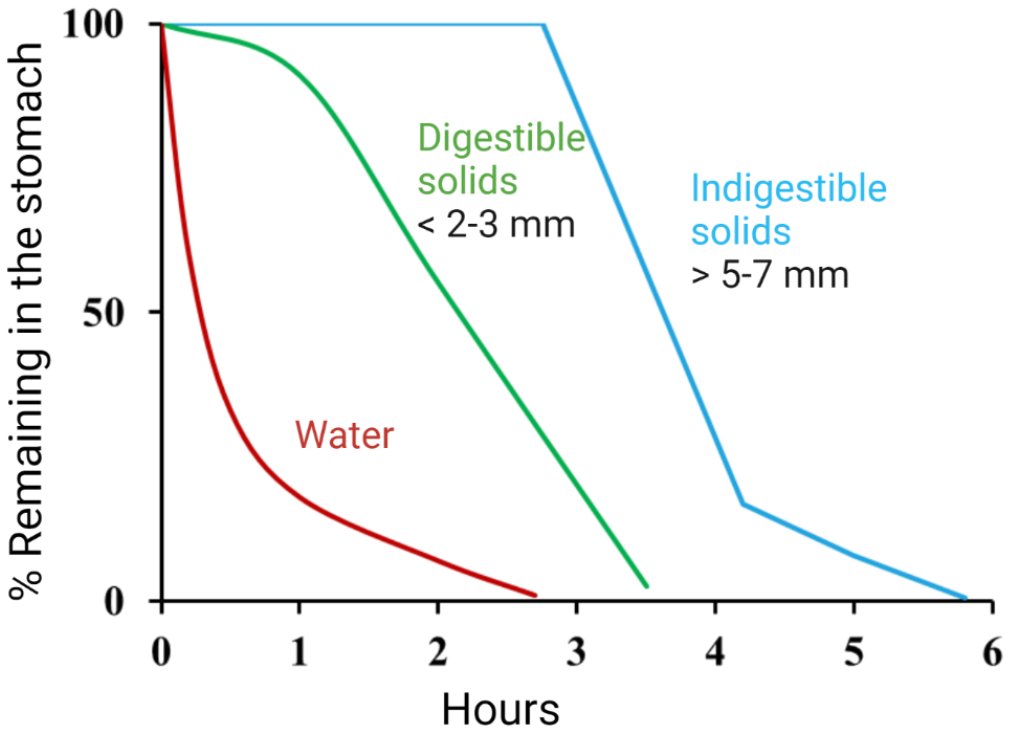

Figure 2.11 *Gastric emptying rates vary with the physical state of foods.*

Note. Adapted from Goyal et al., (2019) with permission.

Table 2.7 *Factors that affect gastric emptying.*

Factors		Relationship with gastric emptying	References
Food properties	Calories	Negative correlation	Mazzawi et al. (2019)
	Viscosity	Negative correlation	Zhu et al. (2013)
	Processing	Related (roasted almond had an increased gastric emptying rate)	Kong and Singh (2009)
	Volume/amount	Positive correlation	Kwiatek et al. (2009)
	Chemical composition	The emptying rate follows: carbohydrates > protein > fat	Lundin et al. (2008); Mackie et al. (2013)
Biological factors	Age	The emptying rate follows: infants > adults = senior	Hellmig et al. (2006); Bonner et al. (2015)
	Gender	The emptying rate follows: males >females on special occasions	Tougas et al. (2000); Camilleri et al. (2012)
	Feedback effects of nutrients	Related to central neurons	Karhunen et al. (2008)
	Gastrointestinal peptide hormone	Promoting hormones (e.g. glucagon-like peptide 1); inhibitory hormones (e.g. cholecystokinin)	Gamble et al. (2013)

Note. Adapted from Liu et al., (2021) with permission.

2.4.2. Gastric digestion of plant-based alternative milks

2.4.2.1 Behaviour of plant-based alternative milk under gastric conditions

There is only limited information in the literature on the behaviour of plant-based alternative milk in the gastrointestinal tract, regarding structural and physiochemical changes in plant-based alternative milk and their consequence on nutrient delivery. Most studies on plant-based alternative milk focused on the bioaccessibility of bioactive compounds due to their proposed health benefits (Rodríguez-Roque et al., 2013; Wu et al., 2012), while other researchers have studied the effect of different processing conditions on physicochemical and sensory properties (Peng et al., 2017).

Gallier and Singh (2012a) studied *in vitro* gastrointestinal digestion of almond milk. The almond milk was prepared by extracting almond proteins and oil bodies in water. In the acidic enzymatic environment of the stomach (pH 1.5), the almond proteins remained almost insoluble and formed large aggregates. The interfacial proteins, peptides and phospholipids covered the almond oil bodies throughout the entire gastric digestion and no coalescence of oil bodies was reported. However, during the subsequent simulated intestinal digestion (pH 7.5), these flocs were disrupted, and the interfacial peptides and phospholipids were replaced by bile salts. The authors concluded that gastric hydrolysis of the interfacial proteins covering the oil bodies played an important role in the subsequent lipid digestion in the duodenum. In a later study, Gallier et al. (2014) investigated the gastric digestion of almond milk in the rat stomach. Flocculation of almond oil bodies in the stomach was confirmed *in vivo*, and coalescence of some oil bodies in the gastric chyme has also been reported.

Gallier et al. (2013) investigated the gastrointestinal digestion of walnut milk using an *in vitro* static model. Walnut milk was obtained after sieving a mixture of

crushed walnuts and water. Similar to almond milk, under acidic gastric conditions (pH 1.5), walnut milk destabilised, aggregated and the oil bodies coalesced. The coalesced walnut oil bodies formed large aggregates up to ~ 275 μm in size in the stomach. Interestingly, the authors reported that in the simulated small intestinal environment, lipid digestion resulted in the formation of a new structure known as "spontaneous biological multiple-phase emulsion", in which the oil-water and water-oil interfaces were stabilized by bile salt crystals.

Soymilk has gained more interest from researchers compared to other alternative milks. Liu et al. (2019b) used the INFOGEST static model to study the microstructural changes and degradation of soymilk colloidal particles during *in vitro* gastrointestinal digestion. They reported that at an acidic gastric pH (pH 3.0), the size of soymilk particles increased due to the formation of many large aggregates. They observed that soybean oil bodies were entrapped in the aggregated protein clusters, but no extensive coalescence was found. However, protein aggregates were hydrolyzed and broken down by pepsin, releasing the entrapped oil bodies and producing peptides and amino acids. Rui et al. (2019) also observed similar aggregation of soymilk under gastric conditions. They reported that gastric digestion induced an increase in the mean particle size of soymilk ($d_{4,3}$ increased from ~ 0.81 to 27 μm), and many large aggregates ($d_{4,3}$ ~ 43 μm) were formed during the subsequent duodenal digestion.

Baglieri et al. (1994) studied the gastric emptying and nitrogen absorption of soymilk in humans. They reported that gastric half-emptying times were 37.74 min and 36.52 min for soymilk and soy protein isolate solution, respectively, and that both samples emptied from the stomach after 140 mins. No significant differences in the gastric emptying rates and gastro-jejunal nitrogen absorption were found between soymilk and

soy protein isolate. The authors also concluded that the true gastro-jejunal digestibility of soy protein was close to that of animal proteins (e.g., milk protein).

Protein digestibility of soymilk was investigated by Rui et al. (2016), who reported that hydrolysis of soy protein by pepsin was rapid as soon as *in vitro* gastric digestion began. The degree of hydrolysis of soluble soy proteins (in the supernatants after centrifugation at 9,800 × *g* for 20 min at 4 °C) of soymilk steadily increased with increasing digestion time. The authors also compared the protein bioaccessibility of soymilk with two soymilk curds produced by fermentation with *Lactobacillus Plantarum* 70810 and *Lactobacillus Plantarum* B1-6, respectively, during digestion. Both curds were less hydrolysed and less soluble than soymilk. The authors explained that the curd matrix hindered enzymatic hydrolysis of soy proteins during digestion.

In a most recent study, Xu et al. (2021) investigated the effects of particle size on the digestibility of soymilk. Soy flour of different particle sizes (> 100 mesh, 50–100 mesh, 28–50 mesh, 14–28 mesh and < 14 mesh) was dispersed in water to reconstitute the soymilk samples. During gastric digestion, the authors observed that the proteins released from the soybean flour particles aggregated, while the lipid droplets disintegrated into smaller colloidal entities. Their interesting finding suggested that protein and lipid were digested more efficiently (i.e., more small molecular degradation products were produced) when the particle size of soybean flour was larger (i.e., >100 mesh or 50–100 mesh).

Overall, among the limited studies on the digestion behaviour of plant-based alternative milks, most have applied static digestion models, which have several advantages, including being fast and simple, with relatively good reproducibility. However, it is less representative of the real gastric environment than that can be achieved in dynamic gastric digestion models, which provide an understanding of changes in food

structures and the rate of release of macronutrients into the small intestine for further digestion and absorption (Lucas-González et al., 2018). To our knowledge, no detailed studies on the digestion profile of plant-based alternative milk in a dynamic digestive environment have been reported, for example, on structural changes in proteins when experiencing changes in pH, or on the spatial distribution of macronutrients in the stomach due to physical instability (e.g., creaming or sedimentation).

2.4.2.2 Effects of gastric processing on the stability of plant-based alternative milk

For many plant-based alternative milks, their natural oil bodies are isolated from plant seeds to form an aqueous suspension with storage proteins during manufacturing and they behave similarly to the protein-stabilised oil-in-water emulsions, for example, almond milk, coconut milk, soymilk, walnut milk or cashew milk (Sethi et al., 2016). After ingestion, the plant-based alternative milk slips through the oesophagus into the stomach after a short period in the mouth. In the stomach, the plant-based alternative milk is exposed to a highly acidic pH and mixes with gastric juices containing hydrochloric acid, digestive enzymes, mucus and minerals. There is also a mechanical shear provided by peristalsis in the stomach to efficiently mix the ingested milk with the gastric juices (Ekmekcioglu, 2002; Kalantzi et al., 2006; Pal et al., 2007). Under these gastric conditions, some changes may occur in the structure and physicochemical stability of the plant-based alternative milk. The major factors that may potentially affect the stability of plant-based alternative milk under gastric conditions are summarised in this section.

2.4.2.2.1 Gastric pH and ionic strength

The initial factor that affects the dispersion status of plant-based alternative milk once it has been exposed to the gastric environment is the pH in the stomach. During meal intake, the gastric pH is not constant at the fasting level of around 1.9. It initially increases in response to the buffering capacity of the food consumed and then gradually decreases

due to the secretion of gastric acid (Dressman et al., 1990; Roger et al., 2010). Plant-based alternative milk is a dispersion of plant storage proteins, membrane protein-stabilized oil bodies and carbohydrates. The lowering of pH due to gastric acid secretion may have an important impact on the stability of storage proteins and membrane protein-stabilized oil bodies.

Most food proteins are acidic proteins with an *pI* at pH 4–5. When the gastric pH drops from neutral, the solubility of these proteins decreases due to the lack of electrostatic repulsion. This may promote aggregation and precipitation through hydrophobic interactions (Damodaran et al., 2007). When the pH of the stomach is close to the isoelectric pH, i.e., where proteins exhibit minimum solubility, instability such as aggregation is expected to occur for storage proteins dispersed in plant-based alternative milk.

In addition, gastric pH may have an effect on the colloidal stability of membrane protein-stabilized oil bodies. At neutral pH (i.e., the pH of most plant-based alternative milk), the negative charge of the oleosin molecule is exposed on the surface of the oil body and the positive charge faces the interior, interacting with the phospholipid layer and free fatty acids (Huang, 1992). Because of the specific orientation of the oleosin molecules, the surfaces of plant seed oil bodies are usually negatively charged at neutral pH. For example, Gallier et al. (2012a) reported a ζ-potential of -29.9 mV for almond milk oil body. Bonsegna et al. (2011) reported a ζ-potential of -30 mV for hazelnut oil bodies at pH 7 and Gallier, Tate, et al. (2013) reported a ζ-potential of -39 mV for native walnut oil bodies at neutral pH. Native coconut oil bodies from coconut milk had a ζ-potential of -13 mV at an extraction pH of 6.1 and purified oil bodies exhibited a ζ-potential of -33.8 at pH 7.5 (Dave et al., 2019). When plant seed oil bodies are subjected to the gastric environment, lowering the gastric pH due to the secretion of hydrochloric

acid results in ionization of side-chain groups of the interfacial proteins. This may induce a reduction of ζ-potential of the oil body, leading to a transition through the *pI*. With a further reduction in the gastric pH, the surface charge of the oil bodies rapidly becomes positive (Gallier & Singh, 2012a). The loss of electrostatic repulsion on the surface of oil bodies creates a net attractive interaction, leading to flocculation (Golding et al., 2011). Flocculation may further enhance creaming and coalescence (Damodaran et al., 2007).

Theoretically, unlike emulsions prepared by a single protein type, the isoelectric pH of plant storage proteins and membrane protein-stabilized oil bodies are usually different in plant-based alternative milk. For example, the *pI* of almond storage protein amandin ranges from 4.55–6.3 (Sathe et al., 2002), while the *pI* of almond oleosin is around pH 4–5 (Bonsegna et al., 2011). Hazelnut proteins have an *pI* of pH 4.5 (Sen & Kahveci, 2020), while hazelnut oleosin has an pI of about pH 5 (Bonsegna et al., 2011). Therefore, the storage proteins and oil bodies may behave slightly differently at different gastric pH values. However, plant milk is a complex system where the surface of plant oil bodies is not only covered by membrane protein (oleosins) but also bound to some storage proteins via weak interactions, for example, as seen in almond oil bodies (Beisson et al., 2001) and coconut oil bodies (Dave et al., 2019). In addition, commercial plant-based alternative milk is often processed by homogenization, which may cause some changes in the composition of interfacial proteins. Thus, aggregation of plant storage proteins and flocculation of oil bodies are likely to occur at similar time scales in the dynamic gastric environment.

In addition, when plant-based alternative milks enter the stomach, there may be important changes in the osmolality and ionic strength of the stomach chyme due to the addition of ions and solutes from the ingested milk. The high ionic strength may also

contribute to electrostatic changes and induce flocculation of plant protein-stabilized emulsions (Zhu et al., 2018).

2.4.2.2.2 Gastric enzyme

Pepsin is the predominant endoproteinase in the human stomach. Because of the action of pepsin, plant proteins are usually partially hydrolysed in the stomach into peptides and a very limited amount of free amino acids (Santos-Hernández et al., 2020). With gastric emptying, the peptides are delivered into the small intestine for further digestion and absorption. Pepsin has a pH optimum of 1.6 to 3.2 and is inactive at pH above 6.5 (Barrett et al., 2010), its action is affected by the gastric pH.

Protein digestion by gastric pepsin has two major potential effects on the structure of plant-based alternative milks. 1) Hydrolysis of storage proteins may lead to degradation of proteins to peptides and/or breakdown of aggregates formed under acidic gastric conditions, thereby releasing the oil bodies from the protein network (Gallier & Singh, 2012a). 2) Hydrolysis of interfacial proteins can alter the interfacial protein composition and surface net charges, which has the potential to considerably alter the structure of the oil bodies. This may, for example, induce flocculation, coalescence of the oil bodies (Gallier et al., 2013).

Protein digestion within the stomach is largely dependent on the susceptibility of plant proteins to pepsin, which is influenced by the tertiary conformation of the protein molecules (Singh & Ye, 2013). The protein digestibility of almond milk has been studied *in vitro* (Gallier & Singh, 2012a). Gallier and Singh (2012a) reported that almond storage proteins were readily hydrolysed by pepsin to low molecular weight peptides after incubation at pH 1.5 for 5 mins. However, some of the peptides generated were observed to be resistant to further digestion by pepsin. Beisson et al. (2001) observed an 8 kDa polypeptide was protected from pepsin hydrolysis in both reconstituted and native almond

oil bodies, which was the central hydrophobic domain of almond oleosins. The pepsin-resistant site of the oleosins was anchored in the triglyceride core of the oil bodies, which was protected from pepsin attack. Nguyen et al. (2015) studied the digestibility of soy proteins under simulated infant gastrointestinal conditions. They reported that 7S and 11S were only partially hydrolyzed (~60–80%) after 1 h in the gastric phase. In contrast, Rui et al. (2016) and Liu et al. (2019) reported that all major fractions of the soy proteins were degraded as soon as gastric digestion began.

Human gastric lipase (HGL) is another enzyme present in the human stomach. HGL is found mainly in the proximal stomach (Moreau et al., 1988). It is stable over a broad pH range (pH 2–7) (Hamosh, 2020) with maximum lipolytic activity at pH 5–5.4 (Sams et al., 2016). HGL is a highly surface-active enzyme that penetrates membranes present at the oil-water interface and accesses the triglyceride core (Singh & Ye, 2013). It has been reported that recombinant human gastric lipase can access the oil core of almond oil bodies and hydrolyze the triacylglycerols without pre-hydrolysis of the protein coat (Beisson et al., 2001). During gastric lipolysis, HGL binds at the oil-water interface and preferentially cleaves the sn-3 position of the glycerol backbone, producing mainly sn-1,2 -diglycerides and free fatty acids (Rogalska et al., 1990). Lipolysis progressively generates protonated free fatty acids that accumulate at the oil-water interface and modify the physicochemical properties of the oil-water interface, particularly the interfacial tension (Gargouri et al., 1987; Miller & Small, 1987; Patton et al., 1982). Gastric lipase is also likely to have a potential effect on the stability of plant-based alternative milks, although this remains to be elucidated, as indicated by the limited publications on plant-based alternative milks. An addition, the gastric lipase has become commercially available for *in vitro* studies very recently.

2.4.3 Behaviour of mammalian milk under gastric conditions

The digestive behaviour of cow milk has been extensively investigated (Roy et al., 2022; Roy et al., 2020; Ye et al., 2016a, 2017). The major proteins in milk, caseins and whey proteins, are known to have different susceptibility to peptic cleavage, which results in different gastric emptying rates. Boirie et al. (1997) proposed the concept of "slow" casein and "fast" whey protein based on the different digestion rates of casein and whey proteins. Casein coagulates in the stomach, which greatly reduces the gastric emptying rate and results in a slow release of amino acids to the small intestine (He & Giuseppin, 2014; Mahe et al., 1992). Casein is therefore classified as a slowly digested protein. In contrast, whey protein has been reported to induce a dramatic but short increase in plasma amino acids after ingestion; it is classified as a fast protein. Whey protein has a faster gastric emptying rate because it remains soluble in the stomach and passes rapidly into the small intestine without being hydrolysed by pepsin (Boirie et al., 1997; He & Giuseppin, 2014).

The different susceptibility of milk proteins to digestive enzymes is dependent on their tertiary conformational properties (Kitabatake & Kinekawa, 1998; Schmidt & van Markwijk, 1993; Tunick et al., 2016). Caseins exhibit a flexible and natively disordered structure (Dickinson, 1989; Gaspar et al., 2008), making them more prone to hydrolysis by pepsin in the stomach (Mahe et al., 1996). Conversely, native β-lg shows some resistance to pepsin hydrolysis due to its highly folded conformation, while α-la is more resistant to trypsin cleavage (Bayram et al., 2008).

In the latest study, Roy et al. (2022) investigated the changes in structure and physicochemical properties that occur in milk during gastric digestion in piglets. The authors studied the similarity and differences in the digestion behaviour of raw cow, goat and sheep milk. They observed that all milk samples were separated into curds and liquid

phase in the piglet's stomach within 30 min of feeding (pH ~5.9). The gastric coagulation of caseins arose from the cleavage of Phe_{105}–Met_{106} peptide bond of κ-casein by pepsin (Tam & Whitaker, 1972), which destabilized the structure of casein micelles (Ye et al., 2016b). The curds (i.e., aggregated caseins) remained longer time in the stomach due to their large size and slow disintegration, whereas the liquid phase (i.e., mostly soluble whey proteins) readily emptied from the stomach. Under microscopy, they observed that the majority of milk fat globules were also involved in the protein matrix of the curds, where extensive coalescence of fat globules was noticed, which could be attributed to hydrolysis by pepsin and gastric lipase. They indicated that the rate of milk fat globules liberated from the curd into the surrounding liquid serum was largely dependent on the rate of disintegration of the surrounded protein network. Interestingly, they observed cow milk curds had a relatively firmer curd consistency and more fused protein matrix compared to goat and sheep milk curds.

The coagulation behaviour and susceptibility to proteases of milk proteins can be modified by processing such as emulsification, high pressure, homogenization or heat processing (Mulet-Cabero et al., 2019a; Tunick et al., 2016; Ye et al., 2016a, 2016b, 2017; Ye et al., 2019; Zeece et al., 2008). The impacts of heat treatment on milk protein digestibility have been well-reviewed recently by Li et al. (2021).

Miranda and Pelissier (1987) investigated the effect of heating on the digestion of skim milk in the rat stomach. They suggested that in raw skim milk, casein coagulation by pepsin causes preferential emptying of whey proteins. However, heat treatment of skim milk [ultra-high temperature processing (UHT), or 120 °C for 20 min] slowed down the gastric emptying of whey proteins but increased the gastric emptying rate and protein hydrolysis of caseins.

Recently, Ye et al. (2016a) offered an insight into the effect of the structure of milk curds formed in the stomach on the rate of hydrolysis of milk proteins. They studied the gastric digestion of raw and heated (90 °C, 20 min) skim milk using a dynamic gastric digestion model, the HGS. Their results showed that raw skim milk formed a cheese-like curd with a firm structure, which slowed down the access of pepsin into the interior of the curd. In contrast, heated skim milk formed fragmented network-structured curds with many larger voids. The whey proteins in the heated samples were denatured and attached to the κ-casein on the surface of the casein micelles (Walstra & Jenness, 1984). This interaction appeared to weaken the protein network of the casein micelles under gastric conditions. The structural changes that occur during gastric digestion resulted in different rates of gastric hydrolysis of casein and whey proteins. Proteolysis of caseins in heated milk appeared to be more pronounced than that in unheated milk, primarily due to the small, fragmented structure of curds, which made caseins in the heated milk more susceptible to pepsin hydrolysis. In the heated milk, denatured whey proteins were more prone to pepsin hydrolysis, while native β-lg and α-la remained intact in the raw milk. These results clearly illustrated how heat treatment can alter the rate of digestibility of milk proteins through structure-based modifications.

Ye et al. (2016b, 2017); Ye et al. (2019) and co-works investigated further the gastric digestion of whole milk, in terms of the effect of pretreatment on the behaviour of proteins and fat globules during dynamic *in vitro* digestion. By comparing the digestion behaviour of raw and heated (90 °C, 20 min) whole milk, Ye et al., (2016b) reported that milk proteins formed different structures of curds in which fat globules appeared to be embedded. After curd formation, the different structures of the curds formed with raw milk and heated milk resulted in different rates of the proteolytic breakdown of the curd structure, leading to different rates of fat globule release from the curds into the

surrounding liquid phase. A faster release of fat globules from the curd was observed in the heated milk sample, due to the looser and more fragmented structure of the curd. Interestingly, in both samples, the rate of fat globules release was linearly related to the breakdown of the protein network in the curds, suggesting that the bioaccessibility of milk fat globules may be governed by alteration of the structures of milk proteins (Ye et al., 2016b).

In recent studies, the digestion behaviour of pasteurized milk, UHT milk and homogenized milk has been investigated (Mulet-Cabero et al., 2019a; Ye et al., 2019). Mulet-Cabero et al. (2019a) studied the structural mechanisms and the kinetics of milk digestion using an INFOGEST semi-dynamic *in vitro* model. In their study, whole milk was homogenised, heat-treated with pasteurisation (72°C for 15 s) or ultra-high temperature treatment (140°C for 3 s). In their gastric digestion model with a transparent glass vessel, they observed creaming in the homogenised samples and precipitation in the non-homogenised samples. The heated samples formed more fragmented curds compared to the unheated samples. They also indicated that the higher temperature used for heat treatment, the softer the curds formed and the higher the rate of proteolysis observed at the end of digestion. The homogenised samples appeared to release more nutrients at the end of gastric digestion. Similarly, Ye et al. (2019) suggested that UHT homogenised whole milk had faster rates of protein hydrolysis and fat globules release in the stomach compared to the pasteurised homogenised milk, due to the former curds having more fragmented and crumbled structures than the latter.

2.5 Conclusions

Plant-based alternative milk products are a fast-growing food market category. Knowledge of the preparation processes of plant-based alternative milks, the

physicochemical properties of the plant proteins and lipids in plant-based alternative milks and the physiological conditions of the stomach are essential for understanding their behaviour of digestion under gastrointestinal conditions. Although many consumers have considered the use of plant-based alternative milks to replace dairy products in their daily diet, the nutrient density of natural plant-based alternative milk and dairy milk is different. Furthermore, the digestion stability and nutrient delivery of plant-based alternative milks remain largely unknown, particularly under dynamic *in vitro* gastric conditions and physiological conditions. To address this issue, the current project aims to investigate the gastric digestion behaviour of selected plant-based alternative milk under dynamic *in vitro* conditions and to compare their digestion behaviour with that of cow milk in an animal model.

Chapter 3 General materials and methods

3.1 Materials

3.1.1 Pepsin for *in vitro* studies

Pepsin from porcine gastric mucosa (EC 3.4.23.1; catalogue No. P7000, 695 units/mg solid) was purchased from Sigma-Aldrich Corporation (St. Louis, MO).

3.1.2 Chemicals

Pepstatin A (77170, Pepstatin A, ≥100,000 units/mg protein) was purchased from Sigma-Aldrich Corporation (St. Louis, MO). All chemicals used were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO) or BDH Chemicals (BDH Ltd., Poole, UK) unless otherwise specified.

3.1.3 Water

Water was purified by treatment with a Milli-Q apparatus (Millipore Corp., Bedford, MA) and was used for all experiments.

3.2 Methods

3.2.1 Pepsin activity assay

Pepsin activity was determined using a spectrophotometric stop reaction method as described in Minekus et al. (2014). In brief, a stock solution of pepsin (1 mg/ml) was prepared by dissolving pepsin in 10 mM Tris buffer, and 150 mM NaCl at pH 6.5 and stored on ice. Before the assay, the pepsin was diluted in 0.01 M HCl to the concentrations of 10, 15, 20, 25, and 30 µg/ml. To determine the pepsin activity, 500 µl of 2% (w/v) hemoglobin solution (pH 2) was added into Eppendorf tubes and incubated in a shaking water bath at 37°C for 5 min. 100 µl of the diluted pepsin solution was then added into

the pre-warmed Eppendorf tubes and incubated at 37°C for 10 min exactly. 1 ml of 5% (w/v) trichloroacetic acid was added into the Eppendorf tubes to stop the reaction. The mixture was then centrifuged at 6,000 g for 30 min. The soluble phase was transferred to quartz cuvettes and the absorbance was measured at 280 nm using a spectrophotometer. A blank test was also conducted following the same procedure, but the pepsin was added after the addition of 5% (w/v) trichloroacetic acid solution. One unit of pepsin is defined as the amount that produces an ΔA280 of 0.001 per minute at pH 2.0 at 37 °C measured as trichloroacetic acid-soluble products using hemoglobin as the substrate.

3.2.2 *In vitro* gastric digestion

A dynamic HGS, designed by Kong and Singh (2010), was used to carry out the gastric phase of *in vitro* digestion. The simulated gastric fluid (SGF, pH 1.5) was prepared as described by Minekus et al. (2014) with slight modifications. The composition of the SGF and the parameters for the *in vitro* gastric digestion are summarized in Table 3.1.

The HGS was prewarmed and maintained at 37 ± 0.5 °C by an internal heater and a thermostat. In each experiment, a 200 g sample of the selected milk was prewarmed to 37 °C and was poured into the latex stomach chamber. The SGF consisted of two separate solutions: SGF-HCl (salts and HCl; pH 1.5); pepsin solution (pepsin and $CaCl_2$). To simulate gastric secretion, the SGF-HCl and the pepsin were pumped gradually into the latex gastric chamber separately at flow rates of 2.0 and 0.5 mL/min, respectively. To mimic human gastric emptying, the gastric digesta were sampled at 15-min intervals from a silicone tube attached to the bottom of the stomach chamber. The digesta sample (50 mL) was filtered through a 1-mm pore mesh, which prevented the emptying of larger particles, in order to simulate the effects of gastric sieving (Meyer et al., 1981). The collected sample was termed the emptied digesta (Lobo et al., 2009); the solids (if any) were returned to the stomach chamber. The gastric contraction frequency of the HGS was

3 contractions/min, which aimed to mimic the natural contraction of the stomach (Marciani et al., 2001). Although the maximum digestion duration was 240 min, individual experiments were run and were terminated at intermediate times, so that the contents inside the stomach chamber, termed the gastric chyme, could be collected for more detailed analysis.

Table 3.1 *Composition of the simulated gastric fluid and the parameters for the in vitro gastric digestion*

Composition of the simulated gastric fluid (SGF)		
SGF-HCl (800 mL) (Minekus et al., 2014)		
	Constituent	mmol
	KCl	6.9
	KH_2PO_4	0.9
	$NaHCO_3$	25
	NaCl	47.2
	$MgCl_2(H_2O)_6$	0.1
	$(NH4)_2CO_3$	0.5
	$CaCl_2$	0.15
	pH	1.5
Pepsin solution (200 mL)		
	Pepsin (porcine)	3.2 g, 2224 U/mL (SGF-HCl + pepsin solution)
	$CaCl_2$	0.15 mmol
Parameters used for the *in vitro* gastric digestion		
Gastric conditions		
Temperature	37 ± 0.5 °C	
Ingested amount	Milk	200 g
Flow rate (1)	SGF-HCl	2 mL/min
Flow rate (2)	Pepsin solution	0.5 mL/min
Gastric emptying rate	50 mL per 15 min	
Gastric contraction rate	3 times/min	
Digestion time	Maximum 240 min	

3.2.3 pH measurement

The initial pH refers to the pH of milk samples before digestion. For the gastric chyme samples, as access into the HGS was prevented by simulated gastric contraction, the digestion experiments were stopped, and the gastric chyme was collected and mixed well using a stirring bar to determine the gastric pH. For the emptied digesta samples, the pH was measured at different gastric emptying time points. The pH of all samples was determined using a CyberScan pH 510 pH/mV/°C Meter (Eutech Instruments, Fisher, Malaysia). The pH meter was calibrated with a standard solution of pH 4.0 and 7.0 before measuring.

3.2.4 Physical stability of gastric chyme

A short-term storage stability analysis was performed on the *in vitro* gastric chyme samples of almond milk, soymilk and oat milk. To obtain direct information on the appearance and the physical stability of the digested plant-based alternative milk in the HGS, the digestion experiments were terminated at selected time points. All digested samples were taken from the inside of the stomach chamber. To stop the digestion by pepsin, an aliquot of pepstatin A (0.5 mg/mL in methanol) was added to the collected chyme at a final concentration of 10 µL/mL and was gently mixed for a few seconds. A 5 mL sample was then transferred to a glass tube that was tightly sealed with a wooden cap. The samples were held in the tubes for 0, 10, and 30 min and were then photographed to record the physical stability of each sample.

3.2.5 Particle size measurements

A laser-light diffraction unit (Masterziser 2000, Malvern Instruments, Malvern, Worcestershire, UK) was used to analyze the average particle size and the particle size distribution of the initial plant-based alternative milk, the gastric chyme, and the gastric

digesta emptied during the *in vitro* gastric digestion. The digested samples were collected and measured immediately. For the gastric chyme samples, experiments were terminated at each selected digestion time point, and samples were collected after the gastric emptying. The refractive index of the dispersed phase was set at 1.466 (Gallier & Singh, 2012a), 1.570 (Chen & Ono, 2014), 1.456 (Ye et al., 2013), and 1.456 (with an absorbance value of 0.001) for almond milk, soymilk, oat milk and oat milk-bovine skim milk blend, respectively, and that of water was set at 1.33. A volume of initial or digested sample was added to the dispersion unit until a laser obscuration range of 10–20% had been reached. To better understand the effect of flocculation, in a separate experiment, volumes of initial and gastric chyme samples were dispersed in 2% (w/v) SDS solution (1:1, v/v) and gently mixed for a few seconds before the particle size measurement. The particle size of the oil bodies was characterized by the volume-weighted average diameter [$d_{4,3}$ ($= \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$), where n_i is the number of particles with diameter d_i]. Mean particle diameters were calculated as the average of triplicate measurements on individual samples.

3.2.6 Confocal laser scanning microscopy (CLSM)

The microstructures of the undigested milk, the gastric chyme samples, and the digesta emptied from the HGS were examined using a confocal laser scanning microscope (Leica SP5 DM6000B, Leica Microsystems, Heidelberg, Germany). The digested samples were stained and observed immediately after collection. The fluorescent dye Nile Red, dissolved in acetone (0.1%, w/v), was used to stain the oil phase (argon laser with excitation at a wavelength of 488 nm). Fast Green (1.0%, w/v) was used to stain protein (He–Ne laser with excitation at 633 nm). Experiments were terminated at each selected time point, and the chyme samples were collected and mixed well after gastric emptying. The emptied digesta samples were collected during gastric digestion with simulated

gastric emptying. Samples (200 µL) of gastric chyme or emptied digesta were transferred into Eppendorf tubes and gently mixed with 5 µL of 1.0% (w/v) Fast Green and 10 µL of 0.1% (w/v) Nile Red. Observations were performed at least 5 min after diffusion of the dyes into the samples. The samples were placed on concave confocal microscope slides (Sail; Sailing Medical-Lab Industries Co. Ltd, Suzhou, China), covered with coverslips and observed using 63× oil immersion lenses (Leica HCX PL APO lambda blue 63.0x1.40 OIL UV, Wetzlar, Germany). Images were stored with 1,024 × 1,024 pixel resolution using the microscope software (Leica).

3.2.7 Crude protein content determination

Total nitrogen contents were performed on the undigested plant-based alternative milk and the digesta that was emptied from the bottom of the HGS during gastric emptying. The pepsin hydrolysis in the emptied digesta was stopped by adjusting its pH to pH 7.5 with 10 M NaOH. The total nitrogen contents were determined by a Kjeldahl method according to AOAC International (2006). A conversion factor of 5.18 (Calixto et al., 1981), 5.71 (Jones, 1931; Maubois & Lorient, 2016), 5.83 (Maclean et al., 2003) and 6.25 (Maclean et al., 2003) for almond milk, soymilk, oat milk and oat milk-bovine skim milk blend was used to obtain the protein content from the total nitrogen content.

3.2.8 Crude lipid determination

To determine the lipid content, digesta samples were emptied from the HGS at selected time points during gastric digestion. The digestion was stopped by adjusting the pH of the emptied digesta with 10 M and 0.1 M NaOH to pH 7.5. The crude lipid contents of the plant-based alternative milk before gastric digestion and of the emptied digesta during gradual gastric emptying were measured according to the Mojonnier ether extraction method (AACC 30-10) (AACC, 2000). In brief, 5 mL of plant-based

alternative milk or 10 mL of emptied digesta was weighed in a beaker and treated with 2 mL of ethanol and 10 mL of HCl to dissolve the proteins and disrupt the oil bodies. Ethanol was added and the sample was transferred from the beaker to a Mojonnier tube. The crude lipid content of cow milk was determined using the ammonia Mojonnier method. In brief, 5 mL of cow milk was weighted in Mojonnier tubes and treated with ammonium hydroxide to disperse casein. 2–4 drops of phenolphthalein were added to the sample to help sharpen the visual appearance of the interface between ether and aqueous layers during extraction. Samples were extracted twice using ethanol, diethyl ether, and petroleum ether and were evaporated to dryness in a preweighed flask using a steam bath. The extracted oil was determined gravimetrically and was corrected by reagent blank determination.

3.2.9 Gas chromatography

The total fat in the gastric chyme collected from rats (Chapter 7) was measured according to the AOAC method 996.06 as described by House and Collaborators (1997) and the method described by Zhu et al. (2013) with slight modifications. Briefly, samples (100 mg of gastric chyme) were added into screw-top glass tubes in which 1 ml of internal standard solution (1 mg/ml methyl tricosanoate in heptane), 0.7 ml of 10 M KOH and 5.3 ml of methanol were added. The tubes were incubated for 90 min at 55°C with vigorous shaking every 20 min. The samples were then cooled to room temperature in a cold-water bath. 0.58 ml of 12 M H_2SO_4 was then carefully added, and the tubes were remixed by inversion and incubated for another 90 min at 55°C with vigorous shaking. After incubation, the tubes were then cooled to room temperature and the fatty acid methyl esters (FAMEs) were extracted by adding 3 ml of H_2O, mixing on a vortex mixer for 5 min and then followed by centrifugation at 3,000 *g* for 10 min. The top layer containing FAMEs was transferred into a glass tube and stored at -18 °C until gas chromatography

analysis. FAMEs were separated on a Supelcowax® 10 (30 m x 0.20 mm x 0.20 μm) fused silica capillary column installed in an Agilent Technologies 7890A gas chromatograph equipped with a flame ionisation detector and split/splitless injector (Agilent Technologies, Victoria, Australia). The individual triglycerides in the test sample were calculated as FAMEs multiplied by their respective conversion factor as described in (House & Collaborators, 1997). Total fat was calculated as the sum of individual fatty acids expressed as triglyceride equivalents. Each sample was prepared and analyzed in triplicate.

Chapter 4 [1]Structural and physicochemical changes in almond milk during *in vitro* gastric digestion: impact on the delivery of protein and lipid

4.1 Abstract

Almond milk (about 3% protein and 7% lipids) was prepared using wet disintegration of raw almonds and then subjected to *in vitro* gastric digestion using an advanced dynamic digestion model (i.e., a human gastric simulator). Microstructural changes, physicochemical behaviour, and protein digestion were examined; the release of lipid and protein during digestion was quantified. Under acidic gastric conditions, almond oil bodies flocculated. Proteolysis by pepsin led to destabilization and coalescence of the oil bodies, resulting in creaming and phase separation. This phase separation significantly delayed the delivery of lipids to the small intestine. After 225 min of digestion, ~ 42% of the lipids remained in the stomach. In contrast, protein release was not significantly affected by the gastric behaviour of the almond oil bodies. This study provides a better understanding of how the digestive system transports plant lipids and may be useful in the microstructural design of foods to achieve a controlled physiological response during digestion.

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4.2 Introduction

Almonds are a nutrient-dense food with a good source of protein, a high concentration of unsaturated fatty acids, a high amount of dietary fibre, and no cholesterol (Sathe, 1992). Because of their phytochemicals and healthy lipid profile, the consumption of almonds has been reported to have positive health benefits, reducing cardiovascular disease, obesity, and type 2 diabetes (Tan & Mattes, 2013). Almond milk is a colloidal dispersion that is produced by the physical disintegration of almond seeds in water. Almond milk is considered to be an appropriate alternative to dairy milk for people with lactose intolerance or a milk allergy because it contains no lactose (Fasoli et al., 2011).

The major storage protein in almonds is amandin, which accounts for approximately 70% of the total soluble proteins (Sathe et al., 2002). Almond lipids are assembled in small discrete intracellular organelles called oil bodies, in which the oil droplets are surrounded by a monolayer of phospholipids and oleosins (Beisson et al., 2001; Tzen et al., 1993). Oleosins are water-insoluble proteins and have a low molecular weight (15–26 kDa) (Huang, 1994). They interact with other components on the surface of oil bodies (Huang, 1996; Murphy & Cummins, 1989), stabilizing their structure and preventing them from coalescing (Beisson et al., 2001). Oleosins can completely cover the phospholipid monolayers on the oil body surface, which may protect the phospholipids from attack by phospholipases via the steric hindrance effect (Huang, 1994). It has been shown that the hydrolysis of oleosins by pepsin may allow lipase to access the oil body surface for more efficient lipolysis (Gallier & Singh, 2012a), although oleosins are partially (i.e., an 8-kDa polypeptide) resistant to protease attack because their central hydrophobic domain is inserted in the oil core (Beisson et al., 2001).

The behaviour of almond components, either incorporated in a food matrix or alone, during digestion in the upper gastrointestinal tract has been investigated. Solid or

semisolid food products, such as almond kernels, almond butter, or almond flour, were used in several studies to investigate their physicochemical behaviour, their microstructure, and their corresponding effects on the bioaccessibility of macronutrients during digestion (Bernat et al., 2015; Bornhorst et al., 2016; Grassby et al., 2017; Grundy et al., 2016; Roman et al., 2012; Sze-Tao & Sathe, 2000). Grundy et al. (2015) showed that the particle size and the microstructure of almonds following mastication had a significant impact on lipid release during *in vitro* digestion. Most almond lipids were still encapsulated in the cell walls after oral processing, reducing their bioaccessibility. More recent studies have demonstrated that almonds consumed as the whole kernel (raw or roasted) may not be fully digested, because the chemical and physical breakdown of the almond cell walls in the mouth, stomach, and small intestine is very limited (Grassby et al., 2014; Grundy et al., 2016; Grundy et al., 2015; Mandalari et al., 2014).

Conversely, almond milk has been shown to be highly digestible, because the oil bodies have been released from the almond cellular structures and dispersed in the aqueous phase (Beisson et al., 2001; Gallier & Singh, 2012a). However, only a few studies have explored the behaviour of almond milk during digestion. The most recent studies were conducted by Gallier and Singh (2012a) and Gallier et al. (2014). Gallier et al. (2014) studied the effect of a food matrix on the gastric emptying rate (using $AlCl_3$–$6H_2O$ as a marker) and the apparent ileal fatty acid digestibility of almond lipids in rats. *In vivo*, the oil bodies in almond milk tended to coalesce upon gastric digestion. Almond milk had a faster gastric emptying rate of Al but a lower apparent fatty acid digestibility than almond oil and almond cream. Gallier and Singh (2012a) investigated the digestion behaviour of almond milk in simulated gastric (pH 1.5) and intestinal (pH 7.5) environments using a static digestion model. They suggested that almond milk behaved similarly to a protein-stabilized emulsion. Almond oil bodies flocculated during gastric

digestion, but no coalescence was observed. They also suggested that gastric digestion of the interfacial proteins, which coat the surface of the oil bodies, is a prerequisite for the subsequent lipid digestion in the duodenum. Their static digestion model has several advantages, such as being rapid and simple and having relatively good reproducibility. However, it is less representative of the real gastric environment than a dynamic gastric digestion model (Lucas-González et al., 2018), which provides a understanding of the changes of food structures and the rate of release of macronutrients into the small intestine for further digestion and absorption. Our recent work on the behaviour of milk has revealed new insights into the coagulation of milk in a dynamic human gastric simulator (HGS) (Ye et al., 2016b) and its consequences on the kinetics of delivery of protein and lipids into the small intestine (Ye et al., 2016a).

To date, no studies have used a dynamic *in vitro* model to assess the behaviour of the protein and lipid components of almond milk. In the present study, HGS was used to examine the dynamic structural changes in almond milk during gastric digestion. The HGS provides a more realistic environment in which to simulate the human gastric digestion process (Kong & Singh, 2010), including gastric peristalsis, gradual secretion of SGF containing pepsin, and gastric emptying.

4.3 Materials and methods

4.3.1. Materials

Raw sliced almonds were obtained from Davis Trading Company, Palmerston North, New Zealand. The nutritional composition as stated on the packaging was: protein 19.5%; fat 54.7% including 3.7% saturated fat; carbohydrates 4.8%.

4.3.2. Preparation of samples

Almonds (500 g) were soaked overnight in 2 L of Milli-Q water at room temperature. The almonds in water were then mixed in a wet disintegrator (Jeffress Bros Ltd, Brisbane, Australia) for 8 min at room temperature and the slurry was filtered twice through a 150 μm sieve to remove any residual almond particles. Almond milk in this study refers to the collected filtrate, i.e., the milky aqueous suspension of almond milk oil bodies. The resulting almond milk contained 4.0% (w/w) protein, as measured by the Kjeldahl method according to AOAC (991.20.II) (AOAC International, 2006). A conversion factor of 5.18 was used to obtain the protein content from the nitrogen content. Water was then added to adjust the final protein content to 3.0% (w/w), which is similar to the protein content of bovine milk. 0.02% (w/v) sodium azide was added into the final almond milk to prevent bacterial growth, and the almond milk was kept at 4 °C for a maximum of 4 days.

4.3.3. Dynamic gastric digestion model

The almond milk then went through a simulated gastric digestion, which was performed using independent samples on different days and a gastric digestion model – the HGS (designed by Kong and Singh (2010)). A 200 g aliquot of almond milk was fed into the HGS and was warmed at 37 °C for 2 min. This is similar to a typical serving size (200 g), based on the nutritional information on a standard commercial almond milk (from approximately 200–250 ml) (FDA, 2019). SGF was prepared according to the protocol described in Minekus et al. (2014) with some slight modifications. The SGF was added at a rate of 2.5 mL/min (Kong & Singh, 2010). In brief, a 1.25× concentrate of SGF (pH 1.5) and a solution containing $CaCl_2$ and pepsin (made with Milli-Q water) were added using two separate pumps: (1) 1.25× concentrated SGF was added at a rate of 2.0 mL/min; (2) the solution consisting of pepsin and $CaCl_2$ was added at a rate of 0.5 mL/min. The

final concentration of $CaCl_2$ and pepsin in the SGF was 0.15 mmol/L and 3.2 g/L (i.e., 2224 units/ml) respectively. In a nonenzymatic digestion of almond milk, the SGF was prepared according to the same method but without the addition of pepsin, i.e., a solution consisting of $CaCl_2$ (made with Milli-Q water) was added at a rate of 0.5 mL/min. The gastric contraction frequency was 3 times/min, to simulate the actual contraction of the stomach (Marciani et al., 2001). The HGS was set and maintained at 37 °C by a heater and a thermostat and the gastric digestion was conducted for 240 min (Gallier et al., 2014). For easier operation, a 50 mL digesta sample was removed from the bottom of the stomach chamber at 15 min intervals and was filtered using a mesh with a pore size diameter of 1 mm, to mimic the gastric emptying (Lobo et al., 2009; Meyer et al., 1981). Thus, after 240 min of digestion, all the gastric material would be emptied from the chamber.

The illustration of sampling scheme during the digestion process that was used for analysis is shown in Figure 4.1. Gastric chyme is referred as the sample taken from inside of the HGS chamber at different times. The emptied digesta is referred to the sample removed from the bottom of the chamber at different times. Only the material less than 1 mm passes through the gastric sieve located at the bottom of HGS.

Due to gradual addition of SGF and gastric emptying, samples were diluted with the progress of the digestion process. Assuming that sample is homogeneously distributed in the stomach throughout the entire digestion process, the theoretical diluted concentration could be calculated, and the calculation process and dilution line are shown in the Appendix 1.

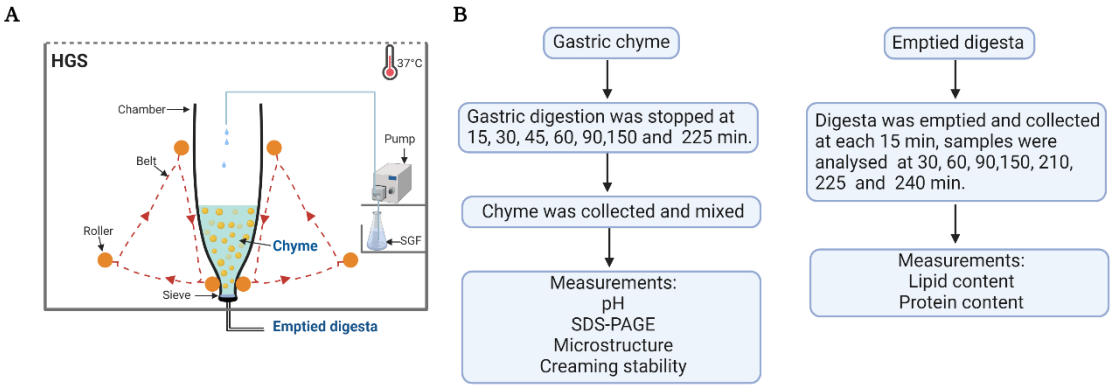


Figure 4.1 *Schematic illustration of an HGS (A) and illustration of sampling scheme used for analysis (B).*

4.3.4. pH Measurement

The initial pH refers to the pH of the almond milk before digestion. As access into the HGS was prevented by the simulated gastric contractions, the digestion experiments were stopped and the gastric chyme was collected and mixed well using a stirring bar to determine the gastric pH.

4.3.5. Protein Hydrolysis

The protein composition of the initial and digested almond milk (i.e. gastric chyme and emptied digesta) during gastric digestion were determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteolysis of digested samples was terminated by adjusting the pH to 7.5 using 10 M and 0.1 M NaOH. A 100 µL aliquot of gastric chyme or emptied digesta was treated with 500 or 600 µL, respectively, of electrophoresis sample buffer [13% (v/v) 0.5 M Tris-HCl buffer, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.04% (w/v) bromophenol blue, β-mercaptoethanol (19: 1, v: v)] and was heated at 95 °C for 5 min. The samples

were centrifuged at 1,000 *g* for 5 min after cooling to room temperature. A 10 μL aliquot of the solution was loaded on to a resolving gel that had been previously prepared on a Mini-PROTEAN II system (Bio-Rad Laboratories, Richmond, CA). The resolving gel contained 16.0% (w/v) acrylamide and the stacking gel was made up of 4.0% (w/v) acrylamide.

The electrophoretic analysis was conducted at 125 V for approximately 60 min. The gel was stained for 40 min under gentle shaking using a Coomassie Brilliant Blue R staining solution [0.003% (w/v) in 10% (v/v) acetic acid (BDH) and 20% (v/v) isopropanol (Merck)]. The gel was destained with a destaining solution of 10% (v/v) acetic acid and 10% (v/v) isopropanol and scanned using a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories). Precision Plus protein unstained standards (Bio-Rad Laboratories) were loaded for estimations of molecular weight. The relative amount of protein was quantified by analyzing the intensity of each band using Image Lab™ software version 5.2 (Bio-Rad Laboratories).

4.3.6. Particle size measurements

A laser-light diffraction unit (Masterziser 2000, Malvern Instruments, Malvern, Worcestershire, UK) was used to analyze the average particle size and the particle size distribution of the initial almond milk, the gastric chyme, and the gastric digesta emptied during the *in vitro* gastric digestion. The digested samples were collected and measured immediately. For the gastric chyme samples, experiments were terminated at each selected digestion time point, and samples were collected after the gastric emptying. The refractive index of almond oil was set at 1.466 (with an absorbance value of 0.001) and that of water was set at 1.33. A volume of initial or digested sample was added to the dispersion unit until a laser obscuration range of 10–20% had been reached. To better understand the effect of flocculation, in a separate experiment, volumes of initial and

gastric chyme samples were dispersed in 2% (w/v) SDS solution (1:1, v/v) and gently mixed for a few seconds before the particle size measurement. The particle size of the almond oil bodies was characterized by the volume-weighted average diameter [$d_{4,3}$ (= $\frac{\sum n_i d_i^4}{\sum n_i d_i^3}$), where n_i is the number of particles with diameter d_i]. Mean particle diameters were calculated as the average of triplicate measurements on individual samples.

4.3.7. Confocal laser scanning microscopy

The microstructures of the initial almond milk, the gastric chyme samples, and the digesta emptied from the HGS were examined using a confocal laser scanning microscope (Leica SP5 DM6000B, Leica Microsystems, Heidelberg, Germany). The digested samples were stained and observed immediately after collection. The fluorescent dye Nile Red, dissolved in acetone (0.1%, w/v), was used to stain the oil phase (argon laser with excitation at a wavelength of 488 nm). Fast Green (1.0%, w/v) was used to stain protein (He–Ne laser with excitation at 633 nm). Experiments were terminated at each selected time point, and the chyme samples were collected and mixed well after gastric emptying. The emptied digesta samples were collected during gastric digestion with simulated gastric emptying. Samples (200 μL) of gastric chyme or emptied digesta were transferred into Eppendorf tubes and gently mixed with 5 μL of 1.0% (w/v) Fast Green and 10 μL of 0.1% (w/v) Nile Red. Observations were performed at least 5 min after diffusion of the dyes into the samples. The samples were placed on concave confocal microscope slides (Sail; Sailing Medical-Lab Industries Co. Ltd, Suzhou, China), covered with cover slips and observed using 63× oil immersion lenses (Leica HCX PL APO lambda blue 63.0x1.40 OIL UV, Wetzlar, Germany). Images were stored with 1,024 × 1,024 pixel resolution using the microscope software (Leica).

4.3.8. Lipid content determination

To determine the lipid content, digesta samples were emptied from the HGS at selected time points during the gastric digestion. The digestion was stopped by adjusting the pH of the emptied digesta with 10 M and 0.1 M NaOH to pH 7.5. The total lipid contents of the almond milk before gastric digestion and of the emptied digesta during gradual gastric emptying were measured according to the Mojonnier ether extraction method (AACC 30-10) (AACC, 2000). In brief, 5 mL of almond milk or 10 mL of emptied digesta was weighed in a beaker and treated with 2 mL of ethanol and 10 mL of HCl to dissolve the proteins and disrupt the oil bodies. Ethanol was added and the sample was transferred from the beaker to a Mojonnier tube. It was extracted twice using ethanol, diethyl ether, and petroleum ether and was evaporated to dryness in a preweighed flask using a steam bath. The extracted oil was determined gravimetrically and was corrected by reagent blank determination. The lipid content was reported as the percentage of grams of lipid per gram of emptied digesta.

4.3.9. Protein content determination

The crude protein contents of the initial almond milk and the emptied digesta during *in vitro* gastric digestion were determined by a Kjeldahl method according to AOAC (991.20.II). A conversion factor of 5.18 was used to obtain the protein content from the nitrogen content. The protein content was reported as the percentage of grams of protein per gram of emptied digesta.

4.3.10. Creaming stability

To observe the creaming behaviour, the experiments were terminated at selected time points and gastric chyme samples were collected from the stomach chamber. Digestion was stopped by adding 10 µL of pepstatin A (0.5 mg/mL in methanol) per

millilitre of gastric chyme. Each gastric chyme sample was mixed for a few seconds and a 5 mL volume was transferred to a glass tube that was tightly sealed with a plastic cap. To record the instability to creaming of each sample, photographs were taken at 0, 10, and 30 min after mixing and storage at room temperature using a digital camera. These photographs gave direct information on the appearance of the gastric chyme in the stomach.

4.3.11. Statistical analysis

A repeated-measures two-factor ANOVA model with the *in vitro* replication as the experimental unit was performed for protein and lipid content using the MIXED model procedure of the statistical software SAS (SAS/STAT version 9.4; SAS Institute Inc.). The statistical linear mixed model included enzyme (with or without), time (0 to 240 min), and their interaction as a fixed effect, whereas replication as a random effect. The most appropriate covariance structure for the mixed models was selected after fitting the models by the Restricted Maximum Likelihood method and comparing them by using the log-likelihood ratio test. For the other response variables (pH and particle size) a two-factor ANOVA without repeated-measures analysis was conducted.

4.4. Results and discussion

4.4.1. pH change during gastric digestion

The simulation of gastric digestion was conducted using an HGS, which provided progressive addition of pepsin and acid and gradual removal of the gastric contents. The changes in the pH of almond milk (i.e., gastric chyme) during gastric digestion are illustrated in Figure 4.2. The pH was significantly influenced by the digestion time and by the interaction of digestion time and treatment (with and without pepsin added) ($P < 0.0001$). The pH of almond milk was approximately 6.37 initially, and it gradually

decreased and reached to ~pH 1.8 in the first 150 min of digestion ($P < 0.0001$), with no further significant change thereafter. The trend was similar in samples with and without pepsin. Changes in pH of the almond milk over time is similar to that of the bovine milk that reported in previous works by Ye et al. (2016a, 2016b), who observed the pH of digested milk reduced to approximately pH 2 after around 140 min of digestion.

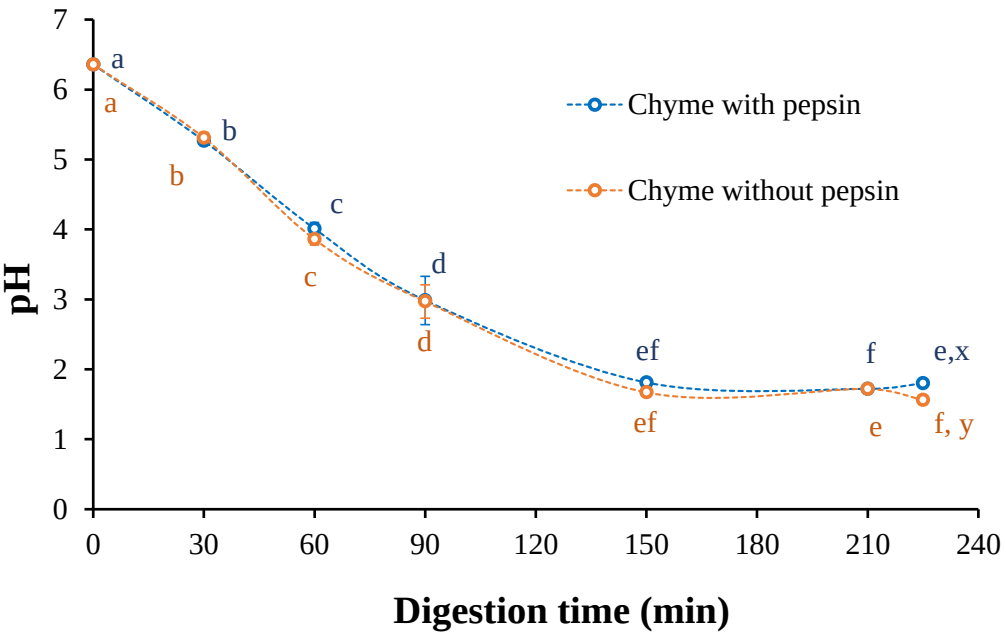


Figure 4.2 *Changes in pH of almond milk during dynamic gastric digestion.*

Note. The pH values refer to the initial (before digestion) almond milk and the gastric chyme samples in the HGS. The measurements were replicated at least two times. Error bars represent standard deviations. Values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) within a certain treatment (with or without pepsin added) across different digestion times. Values with no letter in common (x, y) represent significant different at a certain digestion time across different treatments (with or without pepsin added).

4.4.2. Protein hydrolysis

The extent of proteolysis in the almond milk (i.e., gastric chyme) was determined using SDS-PAGE under reducing conditions (Figure 4.3A). The original almond milk showed several protein bands, including two pairs of major bands, with estimated

molecular weights of 40–42 and 20–22 kDa, which corresponded to the acidic and basic polypeptides of amandin (Garcia-Mas et al., 1995; Sathe, 1992). During gastric digestion by pepsin (Figure 4.3A1), all protein bands gradually became less intense in the first 60 min and several low molecular weight peptides (< 10 kDa) could be seen from 45 min onwards. After 90 min, almost no intact protein bands were detected. The protein bands from the SDS-PAGE gel were quantified (Figure 4.3B1). After 90 min, the intensity of all major protein bands had reduced to almost zero, which was much lower than what would be expected from the hypothetical dilution. This suggests that the disappearance of the majority of the proteins after 90 min was caused by hydrolysis of these proteins by dramatic increase in pepsin activity at this time; the pH at 90 min was 2.99 ± 0.49 which is close to the optimal pH for pepsin activity (Anfinsen et al., 1971).

This proteolysis of the almond proteins (shown in Figure 4.3A1) was substantially slower than that reported by Gallier and Singh (2012a). In their study, all the almond proteins had been hydrolyzed to lower molecular weight peptides within the first 5 min of digestion and no further proteolysis was detected after 15 min. The faster rate of hydrolysis observed in their study was probably due to all the pepsin being added at the beginning of the digestion in their static model and the operation at pH 1.5, which is close to the optimal pH (i.e., pH 2) for pepsin activity(Anfinsen et al., 1971). In contrast, in our study, the pH was around 6.1 at 15 min of dynamic digestion, at which time the activity of the pepsin was probably limited. Moreover, the protein: enzyme ratio (25: 1, w/w) used by Gallier and Singh (2012a) was higher than that applied in our study (50: 1, w/w) in the first 15 min of digestion, because pepsin was gradually added within the SGF to simulate gastric secretion in the present dynamic model. The detailed information regarding calculation of the protein: enzyme ratio in the current study is presented in Appendix 2.

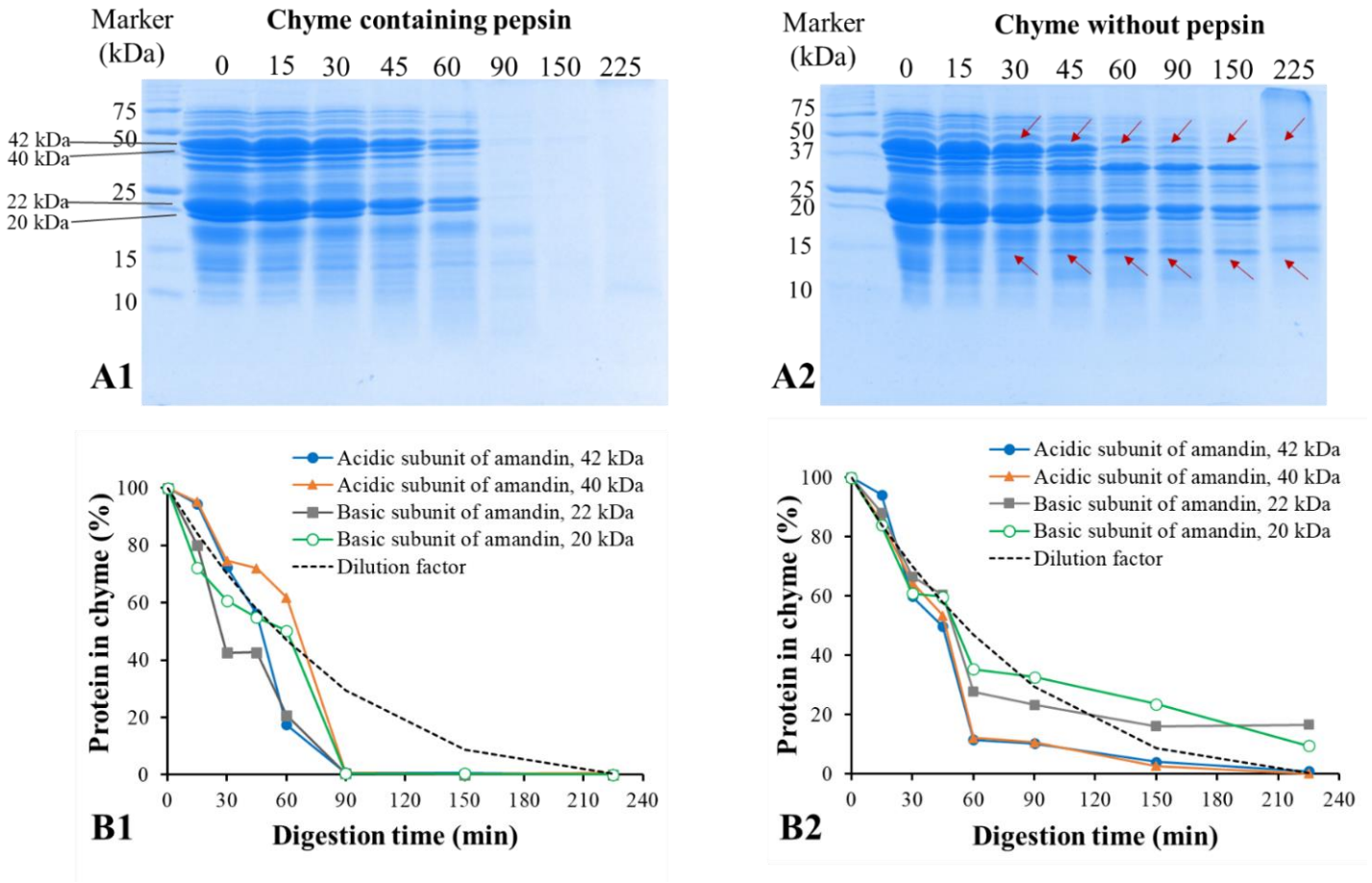


Figure 4.3 *SDS-PAGE (under reducing conditions) (A) and change in content of proteins (B) of the gastric chyme (i.e., digested almond milk samples) with (1) and without (2) pepsin addition during dynamic gastric digestion.*

Note. In figure A, marker refers to a molecular weight marker. The downward-pointing arrows indicate the acidic polypeptide of amandin (~42 kDa), the upward-pointing arrows indicate a new unknown band (~15 kDa). In figure B, the dilution of protein by SGF during dynamic digestion in HGS is shown in the dashed line.

When pepsin was excluded, the protein bands in the gastric chyme samples remained mostly intact (Figure 4.3A2), although they gradually became less intense with an increase in the digestion time because of dilution effects. Interestingly, after 30 min, a new band was observed at around 15 kDa (shown by upward-pointing arrows in Figure 4.3A2) and was accompanied by a decrease in the intensity of the band at 42 kDa (i.e., the acidic polypeptide of amandin, shown by downward-pointing arrows in Figure 4.3A2); it was probably an acid degradation product. This was consistent with the earlier work of Tiwari et al. (2010) who reported that some denaturation or degradation of amandin occurs at acidic pH. Similarly, De Angelis et al. (2018) reported some new protein bands (molecular weights around 20, 15, and 10 kDa) on SDS-PAGE after subjecting raw almonds to simulated oral, gastric, and intestinal environments without added pepsin. These bands originated from amandin, which was confirmed by high performance liquid chromatography–tandem mass spectrometry analysis and bioinformatics database searches. They suggested that the new bands were probably the results of spontaneous protein hydrolysis because of the dramatic change in pH from the neutral environment of simulated salivary fluid (pH 7) to the acidic environment of SGF (pH 3).

4.4.3. Physical changes

4.4.3.1. Creaming stability

The creaming stability of the almond milk in the stomach at different times was determined by monitoring the appearance of the gastric chyme, which provided direct information on how the almond milk oil bodies behaved in the stomach (Figure 4.4). In the chyme samples with added pepsin, creaming was observed visually at 60 min of digestion (Figure 4.4C). The gastric chyme separated into a transparent ‘serum’ layer at the bottom and a white ‘cream’ layer at the top, which probably resulted from extensive coalescence and flocculation. After 150 min of hydrolysis by pepsin, a layer of yellowish

free oil was observed on the top of the aqueous phase of the chyme samples (Figure 4.4C, highlighted with red arrow), indicating that phase separation had occurred. In contrast, when pepsin was excluded from the SGF, no obvious creaming and phase separation were observed in the stomach during the entire digestion process.

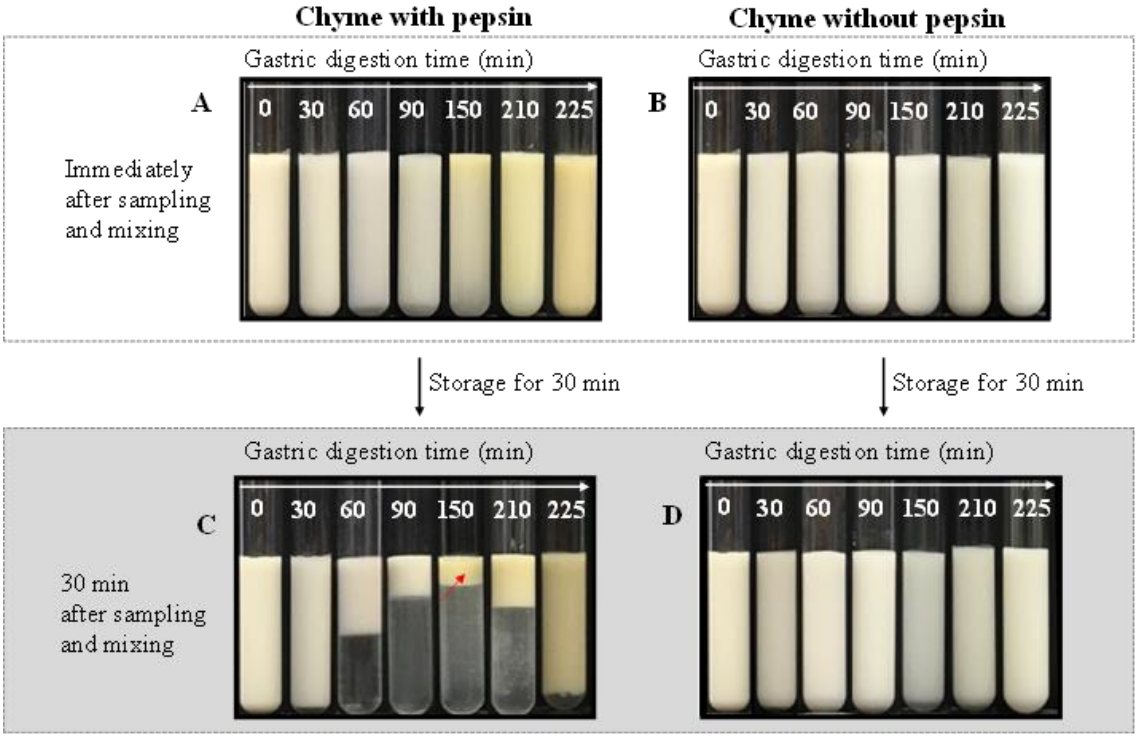


Figure 4.4 *Appearance of the gastric chyme taken from almond milk in the HGS at different digestion times and creaming stability during storage.*

Note. The upward-pointing arrow indicates the cream layer.

4.4.3.2. Particle size

Changes in the particle size distribution of the almond milk (i.e., gastric chyme) during the simulated gastric digestion are shown in Figure 4.5. The volume-weighted mean diameter ($d_{4,3}$) of the initial almond milk was 2.6 μm and the corresponding particle size distribution was monomodal (Figure 4.5A1 and 4.5B1). However, exposing the almond milk to SGF, with and without pepsin addition, led to considerable structural changes.

The average particle size of almond milk was significantly influenced by the treatment (with and without pepsin added), digestion time and their interactions ($P < 0.001$). With gastric digestion by pepsin, the chyme samples steadily shifted towards larger particle sizes within the first 60 min of digestion (Figures 4.5A2 and 4.5A3, 30 min and 60 min), with an increase from 2.6 to 54 μm ($P < 0.001$), indicating flocculation and coalescence of the oil bodies in the gastric environment. To further investigate the coalescence of the oil bodies, the droplet size was determined in an additional experiment, using the same samples but diluted in a 2% SDS solution. This surfactant was used to dissociates protein aggregates and liberate oil droplets (Dave et al., 2019). In the first 30 min of digestion, the size distribution of the samples with 2% SDS showed monomodal peaks, reflecting that only flocculation had occurred (Figure 4.5A2, 30 min in SDS). However, the size distribution profile moved towards larger sizes at 60 min (Figure 4.5B3, 60 min in SDS), indicating coalescence of the oil bodies. As the digestion progressed (with the addition of SDS), the size distribution of the almond oil bodies increased significantly at 150 min, with no further increase thereafter (Figure 4.5A6, 225 min in SDS).

When pepsin was absent, the gastric chyme samples gradually moved to larger particle sizes in the first 60 min of digestion ($P < 0.001$) (Figure 4.5B2 and B3, 30 min

and 60 min), with no further changes thereafter. However, after dilution in SDS solution, a monomodal size distribution (Figures 4.5B1-B6, in SDS) was observed and all samples had similar distributions compared to the undigested sample. This suggests that the increase in the particle size was due to oil body flocculation rather than coalescence in the samples without added pepsin. Indeed, under acidic stomach conditions, many storage proteins may remain attached to the surface of almond oil bodies (Gallier & Singh, 2012a), and the acidic pH may contribute to protein–protein interactions and the formation of aggregates and flocs (Gallier & Singh, 2012a; Sathe, 1992).

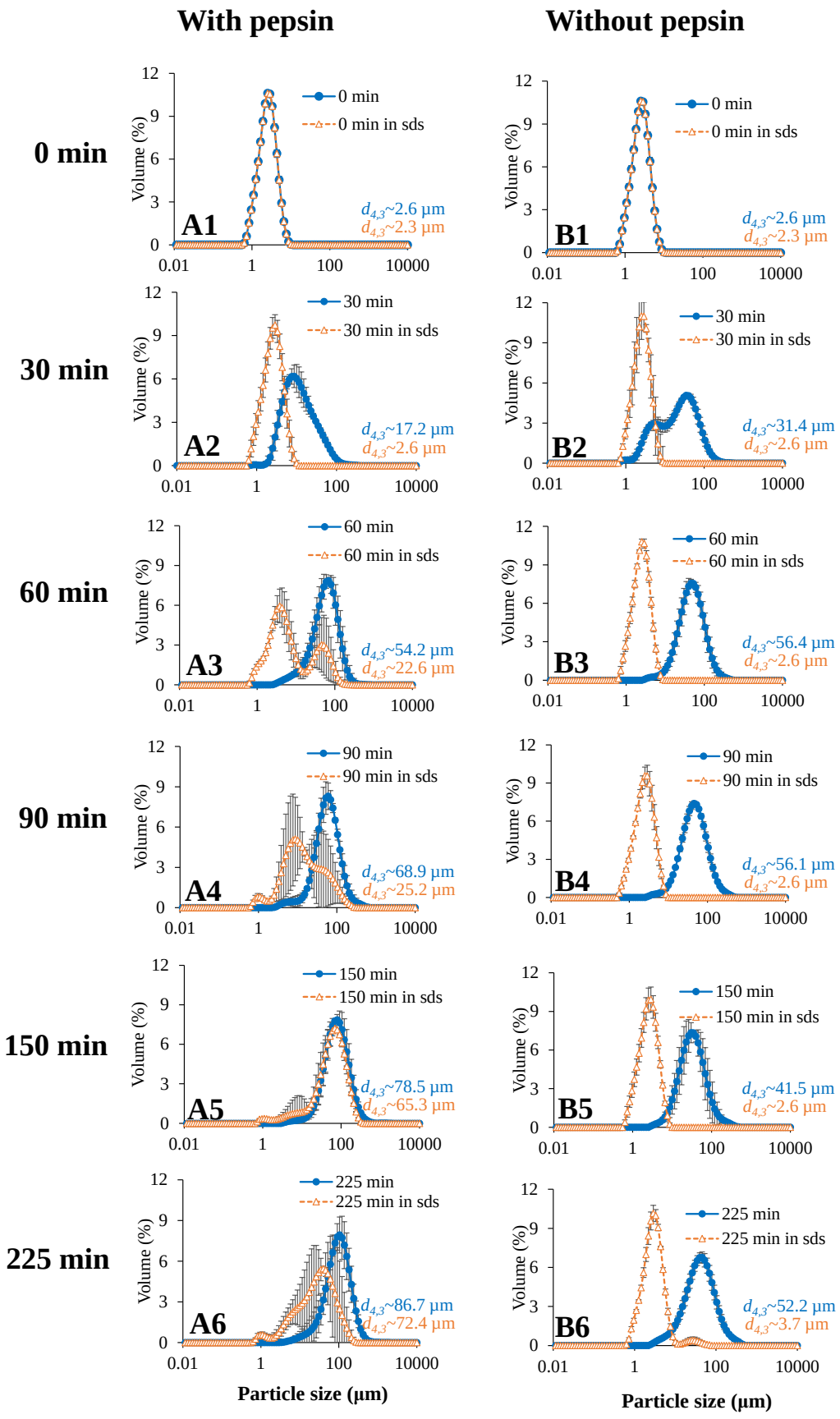


Figure 4.5 *Change in particle size distribution of almond milk samples (i.e., gastric chyme) with (A) and without (B) pepsin addition during gastric digestion in the HGS.*

4.4.3.3. Microstructure

The changes in the microstructure of the almond milk (i.e., gastric chyme) during the transition from an initial neutral pH to the acidic pH of the simulated gastric environment were followed using confocal laser scanning microscopy (Figure 4.6). After 30 min of digestion by pepsin, large amounts of oil bodies flocculated, and were entrapped within the protein matrix (Figure 4.6A2), which is in agreement with the results of particle size distributions (Figure 4.5A2). With further digestion, coalesced oil bodies with larger sizes were observed between 60 and 225 min, which confirmed the particle size measurements (Figures 4.5A3-4.5A6, in SDS) that showed the particle size increased after dilution in SDS solution due to coalescence. At the end of the digestion, many larger coalesced oil droplets with irregular structures were observed in the gastric chyme samples (Figure 4.6A6). In contrast, when pepsin was absent, the confocal micrographs showed that, after 30 min of digestion, the almond oil bodies flocculated, as did a number of small protein aggregates (Figure 4.6B2). At 60 min of digestion, many aggregated protein networks as well as flocculated oil bodies were present (Figure 4.6B3). With a further increase in the digestion time, the aggregated protein matrix networks did not disappear, but remained in the stomach, together with the flocculated oil bodies, until the end of digestion. These microscopic observations are consistent with the particle size distributions (Figures 4.5B1-4.5B6) that suggested that only flocculation occurred in the sample without pepsin added.

We observed that the almond milk was significantly less stable during the simulated gastric digestion in the presence of pepsin than in the absence of pepsin. This instability was almost certainly caused by the proteolytic action of pepsin. It is worth noting that, after 150 min of digestion by pepsin, there was a significant increase in particle size (Figure 4.5A5) and a layer of yellowish free oil on the top of the aqueous

phase (Figure 4.4C). Both were consistent with the observations from SDS-PAGE (Figure 4.3A1), which showed almost no protein bands at 150 min of digestion, suggesting the possibility of the breakdown of the oil bodies. Oleosins, as the interfacial proteins of almond oil bodies, have surfactant properties that limit the tendency of oil bodies to coalesce (Bhatla et al., 2010). Hydrolysis of the interfacial proteins might be one of the main reasons for the behaviour of almond milk during gastric digestion. Under the gastric conditions, the almond proteins coated on the oil body surface were degraded to low molecular weight peptides and amino acids, which were probably not sufficiently surface active to remain at the oil–water interface (Gallier & Singh, 2012b). This resulted in the destabilization of the almond milk oil bodies by flocculation, coalescence, and creaming, ultimately leading to phase separation.

In the absence of pepsin, flocculation and aggregation were the main mechanisms for the destabilization of the almond milk. As the oil body surface is covered entirely by oleosins, almond milk is a protein-stabilized emulsion (Huang, 1996). As the pH decreased from neutral to acidic, i.e., to below the *pI* of the oleosins (pH 4–5)(Bonsegna et al., 2011), interfacial oleosins interacted with each other, resulting in flocculation of the oil bodies (Gallier & Singh, 2012a). The *pI* of the almond storage protein amandin is known to range from 4.55 to 6.3 (Sathe et al., 2002). As amandin was affected by this pH reduction across the *pI* in the simulated gastric environment, aggregation of the proteins was observed.

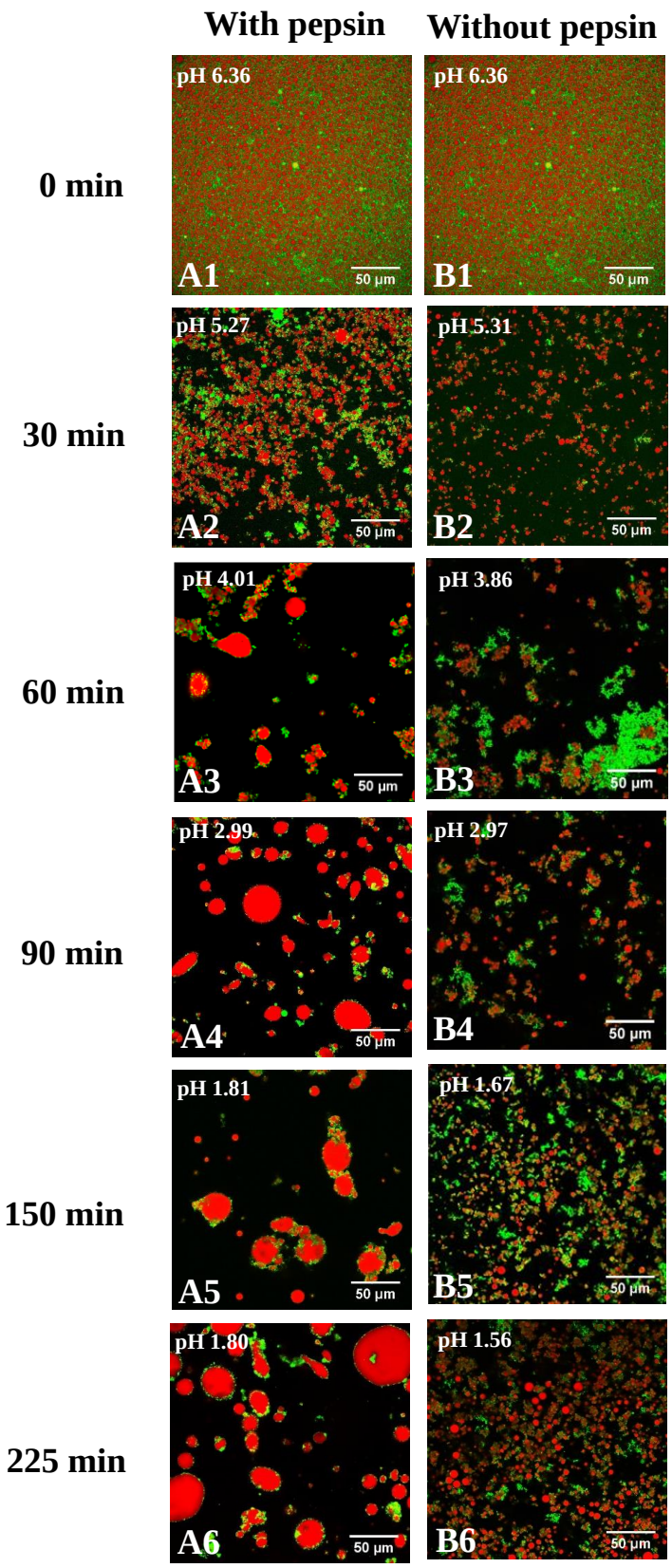


Figure 4.6 *Change in microstructure of almond milk samples (i.e., gastric chyme) with (A) and without (B) pepsin addition during gastric digestion in the HGS.*

Note. The scale bar in all images is 50 µm.

Previous *in vitro* studies on the digestion of almond milk have used static digestion models, which may not provide comparable results with respect to the colloidal behaviour of almond oil bodies under dynamic conditions. Nevertheless, our results were in agreement with those previously obtained by Gallier and Singh (2012a) who observed flocculation and aggregation of almond milk in the gastric phase using a static model. However, the phenomena of phase separation and coalescence were not reported in their study, suggesting that the almond milk may have been digested to a greater extent in the HGS model than in the static digestion model. This probably occurred because static models do not normally mimic mechanical processes, such as gastric peristaltic movements, that occur in the stomach (Oomen et al., 2002). The HGS model has been reported to be more efficient in sample mixing during gastric digestion than the shaking water bath model because of its simulated peristalsis, which allows for better diffusion of pepsin into the food matrix and faster sample breakdown (Roman et al., 2012). However, the extensive coalescence of almond milk observed in the present study was in accordance with an *in vivo* study conducted by Gallier et al. (2014) who clearly observed coalesced oil bodies in the rat stomach using confocal differential interference contrast microscopy. Indeed, natural plant-derived oil bodies tend to coalesce and form larger droplets under gastric conditions. White et al. (2009) reported that sunflower oil body suspensions coalesced with a significant mean particle size increase from 2.3 to 22.5 μm after 30 min of digestion by pepsin. Similarly, Gallier et al. (2013) showed that a walnut oil body dispersion (named walnut milk in their study) underwent coalescence in a simulated gastric environment. The coalesced oil bodies aggregated with an average diameter ($d_{4,3}$) of up to 275 μm after 1 h of digestion.

4.4.4. Lipid delivery

The gastric emptying of lipids (i.e., lipid content in the emptied digesta) during the digestion process was quantified, as shown in Figure 4.7, which illustrates the total lipid content delivered to the small intestine at different time points. The broken line indicates the calculated lipid content of the gastric digesta emptied at each time point, assuming that the oil bodies were homogeneously distributed in the almond milk throughout the entire digestion process. The decrease in the calculated lipid content was due to dilution by the gradual gastric emptying. The original almond milk contained 7.1% oil, i.e., 14 g of oil in 200 g of almond milk prior to digestion. The lipid content of the emptied digesta was significantly influenced by digestion time and the interaction of digestion time and enzyme (with and without pepsin added) ($P < 0.0001$). During the first hour of digestion with pepsin, the oil content of the emptied digesta decreased considerably ($P < 0.0001$), nearly reaching zero and remaining almost unchanged until 225 min. This suggested that only a limited amount of lipids was delivered to the small intestine. This marked delay in the delivery of lipids to the small intestine was due to the creaming and phase separation of the almond milk in the stomach. There was a dramatic increase from 0.3% at 225 min to 12% at 240 min, $P < 0.0001$. Therefore, up to 6 g of oil was emptied at the last emptying point, accounting for approximately 42% of the initial oil content of the almond milk sample (i.e., 14.16 g). This can be explained by the fact that the oil bodies in the stomach underwent extensive coalescence and creaming and floated to the top of the aqueous phase. The lower aqueous phase was emptied first and then the remaining cream layer was emptied. This is similar to that previously reported by Marciani et al. (2007) who observed that a gastric-unstable emulsion separated in the stomach using magnetic resonance imaging in humans. The lower aqueous phase

contained a small amount of the oil (30% in their case) of the original emulsion and was emptied first.

In the absence of pepsin, the oil content of the emptied digesta showed a significant decrease at 60 min ($P < 0.0001$), when the pH of the almond milk was approximately 5.1, which was close to the *pI* of the almond proteins (*pI* 4.55–6.3). This probably occurred because almond oil bodies tend to cream as a result of extensive flocculation at the *pI*, leading to a limited amount of oil bodies being emptied into the small intestine. However, as digestion progressed, the oil contents of the emptied digesta samples without added pepsin were significantly higher than those with added pepsin, which indicated more lipids being emptied in the absence of pepsin, with the exception of the last gastric emptying time point (240 min).

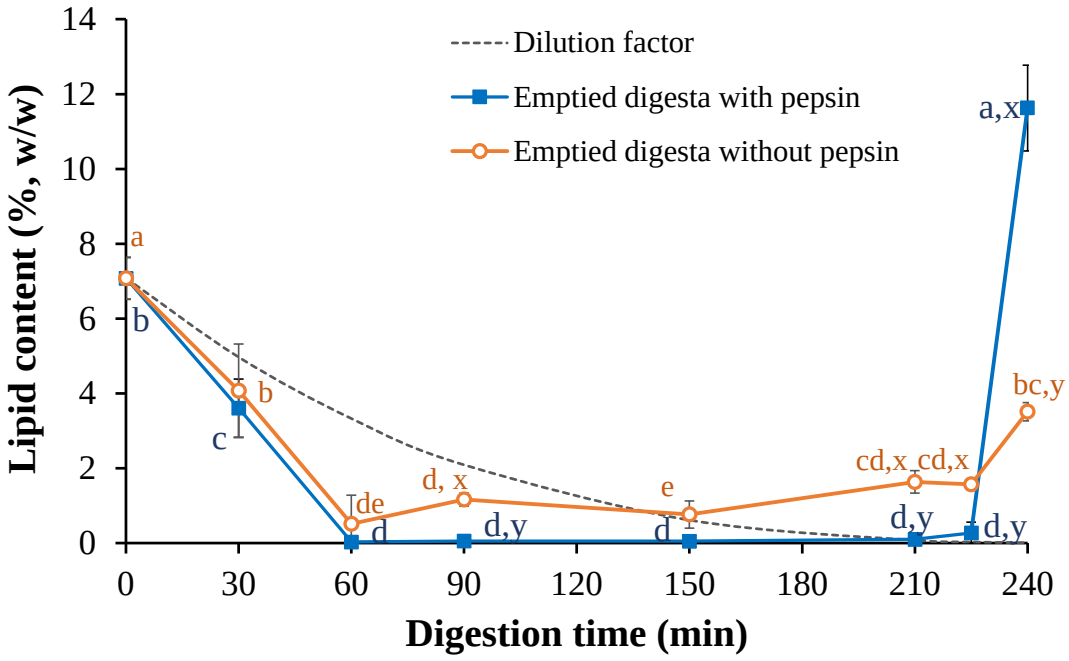


Figure 4.7 *Lipid content (w/w, %) of emptied digesta during gastric digestion in the HGS.*

Note. Values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) within a certain treatment (with or without pepsin added) across different digestion times. Values with no letter in common (x, y) represent significant different at a certain digestion time across different treatments (with or without pepsin added). If no letter is listed, there were no significant differences.

4.4.5. Protein delivery

The protein contents of the gastric digesta emptied from the HGS into the small intestine during the digestion process are shown in Figure 4.8. As above, the broken line (i.e., the dilution factor curve) indicates the calculated protein content of the gastric digesta emptied at different time points. The calculation was based on the hypothesis that the proteins were uniformly distributed in the almond milk. The protein content of the emptied digesta was significantly influenced by the digestion time and the interaction of digestion time and enzyme (with and without pepsin added) ($P < 0.001$). Regardless of whether or not pepsin was present, the protein content of the emptied digesta gradually decreased over digestion time in the first 150 min ($P < 0.001$) and remained unchanged at the late stage of digestion. Moreover, the protein content curves in both samples were close to the hypothetical dilution values. However, the almond proteins that were digested by pepsin seemed to be closer to the dilution factor curve. This was probably because most proteins were digested by pepsin to low molecular weight peptides within the first 90 min in the HGS (Figure 4.3A1). These peptic proteolysis products seemed to be homogeneously distributed in the aqueous phase. This suggested that the almond proteins were delivered to the small intestine with the gradual gastric emptying and were not affected significantly by the destabilization of the almond oil bodies.

In comparison, the protein content of the emptied digesta without added pepsin showed a slightly slower delivery rate of proteins into the small intestine. This probably resulted from the flocculation of the oil bodies and the aggregation of the almond proteins under acidic conditions.

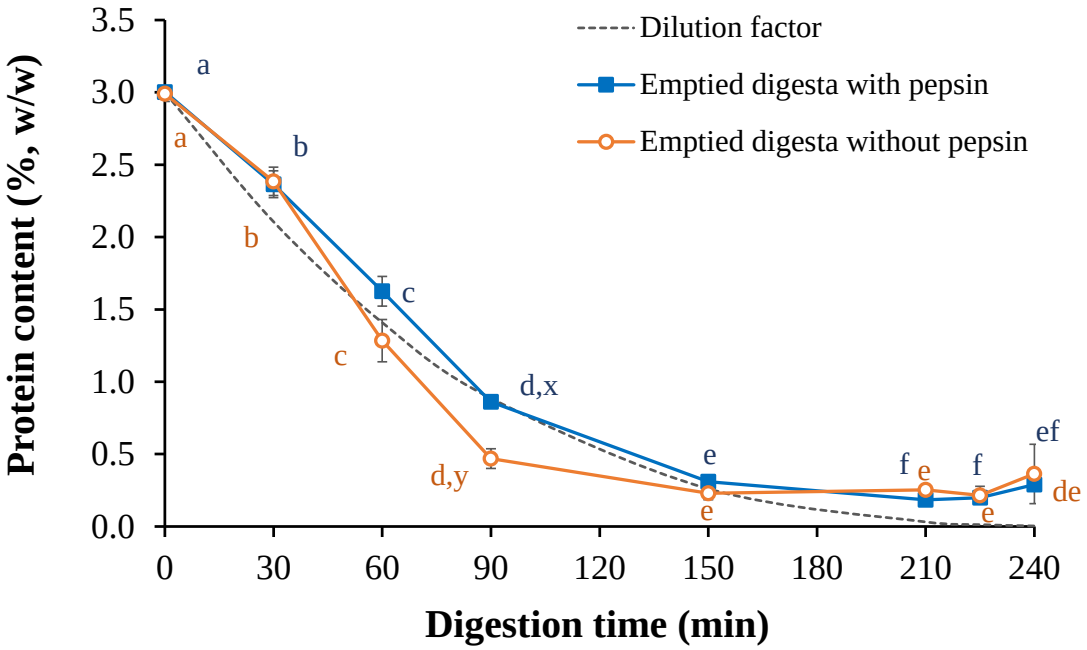


Figure 4.8 *Protein content (w/w, %) of emptied digesta during gastric digestion in the HGS.*

Note. Values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) within a certain treatment (with or without pepsin added) across different digestion times. Values with no letter in common (x, y) represent significant different at a certain digestion time across different treatments (with or without pepsin added). If no letter is listed, there were no significant differences.

Overall, the formation of a cream layer in almond milk under gastric conditions altered the delivery of nutrients; this was due to the proteolytic action of pepsin. Indeed, hydrolysis of the adsorbed protein layers by pepsin plays the most important role in determining the gastric stability of protein-stabilized emulsions (Sarkar et al., 2009). It is interesting to compare the gastric digestion behaviour of almond milk with that of cow milk. Within 10 min during the dynamic *in vitro* gastric digestion of raw whole milk, the milk proteins formed firm curds with a smooth surface, in which the fat globules appeared to be embedded (Marciani et al., 2001; Ye et al., 2016b). After curd formation, proteolysis of the curds by gastric pepsin resulted in liberation of the fat globules from the curds into the surrounding digesta, with the release rate of the fat globules being linearly dependent

on the breakdown of the protein in the curds. Coalescence of the fat globules was observed both in the gastric digesta and within the curd (Ye et al., 2016b).

The stability of food emulsions during gastric digestion may be linked to some physiological responses. The behaviour of gastric digestion has been reported to affect the satiety responses, which are associated with gastric emptying and the release of gut hormones such as cholecystokinin. Lipids, in particular, and proteins are considered to be the most satiating macronutrients (Fiszman & Varela, 2013). Compared with an emulsion that remained homogeneous in the stomach, Marciani et al. (2008) demonstrated that an acid-unstable emulsion broke and formed a fat layer floating on the top of the stomach, led to faster gastric emptying, and increased hunger. In a clinical study, Mackie et al. (2013) also showed that gastric layering of a liquid emulsion resulted in a more rapid gastric emptying rate and increased hunger compared with an isocaloric semisolid diet that sedimented in the stomach. They explored the possible reasons behind the physiological response using an *in vitro* model. They indicated that the release of nutrients was delayed in the liquid matrix, probably because of a lower nutrient concentration in the lower aqueous phase, which entered into the small intestine first. In contrast, the sedimentation in a semisolid diet led to a higher concentration of nutrients emptying from the stomach at an earlier time (Mackie et al., 2013). The formation of the cream layer in the almond milk clearly affected the rate of delivery of lipids to the small intestine for further digestion. This may have some physiological implications in controlling hunger and satiety.

4.5. Conclusions

The present study investigated the *in vitro* digestion of almond milk using a dynamic gastric digestion model (i.e., an HGS). The digestion of almond proteins by pepsin led to the destabilization and coalescence of almond oil bodies, ultimately resulting

in creaming and phase separation of the almond milk, with a lipid layer floating on the top of the aqueous phase. This structural destabilization of the almond oil bodies significantly slowed the gastric emptying of the oil; however, it did not significantly affect the rate of protein delivery to the small intestine.

Overall, the acidic gastric environment and, in particular, the pepsin hydrolysis modified the spatial distribution of the lipids in almond milk and, in turn, affected the rate of energy delivery to the small intestine.

Chapter 5 [2]*In vitro* digestion of soymilk using a human gastric simulator: impact of structural changes on kinetics of release of proteins and lipids

5.1 Abstract

Soymilk (about 3% protein and 1.8% lipids), prepared by wet disintegration of soaked soybeans, was heated at 108 °C for 15 min and then subjected to *in vitro* gastric digestion using an advanced dynamic digestion model (i.e., a human gastric simulator). Microstructural changes, physicochemical stability and protein digestibility were studied; the release of protein and lipid during digestion was also quantified. Gastric digestion significantly influenced the colloidal stability of soymilk, resulting in coagulation of the soy proteins because of the action of pepsin and the acidic pH. The soymilk oil bodies appeared to be entrapped within the coagulated protein particles. With continued digestion, protein hydrolysis by pepsin resulted in the disruption of the coagulum structure, leading to an accelerated gastric emptying of both protein and lipid in the first 45 min. Gastric-induced coagulation did not have a significant impact on either protein or lipid emptying, except for an initial delay of lipid emptying in the first 15 min. No extensive coalescence of soymilk oil bodies was observed under a confocal microscope. This study provides further understanding of the fate of soymilk in the digestive tract and may be useful in the microstructural design of foods to achieve a controlled physiological response during digestion.

[2] This chapter has been published as **Wang, X**., Ye, A., Dave, A., & Singh, H. (2021). *In vitro* digestion of soymilk using a human gastric simulator: Impact of structural changes on kinetics of release of proteins and lipids. *Food Hydrocolloids, 111*, 106235. https://doi.org/10.1016/ j.foodhyd. 2020.106235

5.2 Introduction

Soybeans have been part of the East Asian diet for centuries. Soybean seeds contain approximately 20% fat and 40% protein on a dry weight basis, providing the most inexpensive source of edible oils and high quality proteins (Kwok & Niranjan, 1995). Soymilk is a traditional beverage that is produced by grinding soybeans soaked in water, followed by cooking and removal of the insoluble material by filtration or centrifugation (Chen, 1988). Soymilk contains a variety of nutrients, such as high quality proteins and isoflavones; the consumption of soya-based foods has been shown to have beneficial effects in lowering blood pressure and improving lipid profile (Bricarello et al., 2004; Fukui et al., 2002; Rivas et al., 2002).

During the manufacture of soymilk, heating is an essential processing step to denature anti-nutritional compounds, such as trypsin inhibitors and lipoxygenase, and to modify the structure of the soymilk proteins to improve their colloidal stability (Nik et al., 2009). Soy proteins are known to denature, dissociate and subsequently aggregate to form particles during heating (Guo et al., 1997; Ono et al., 1991), which can influence the stability and the digestibility of soymilk products (Guo et al., 2006; Wallace et al., 1971). Soy lipids are contained in oil bodies, which have an average size of 300–400 nm (Ono, 2008). The surface of soybean oil bodies is made up of a monolayer of phospholipids, embedded with intrinsic proteins, called oleosins (Zhao et al., 2013). Grinding of soybeans in the presence of water leads to the dispersion of oil bodies and extrinsic proteins, but some of these extrinsic proteins remain associated with the oil bodies. After heating (70–100 °C), some (but not all) of the extrinsic proteins dissociated from the oil bodies (Guo et al., 1997; Yan et al., 2016) and a few large oil droplets or oil body-protein aggregates (>1 μm) were also observed (Chen et al., 2014; Cruz et al., 2007). The surfaces of oil bodies in heated soymilk appeared to be coated by oleosins, phospholipids and some

extrinsic proteins (Yan et al., 2016). Ding et al. (2020) reported that the oil bodies isolated from heated soymilk retained the oleosins at their surface.

Because of its proposed nutritional and health benefits, the digestibility of soymilk has been evaluated both *in vivo* and *in vitro* (Baglieri et al., 1994; Liu et al., 2019; Rui et al., 2016, 2019). Most current studies on the digestion of soymilk are focused on the bioaccessibility of bioactive compounds (Rodríguez-Roque et al., 2013; Wu et al., 2012). There is a lack of knowledge on the changes in colloidal behaviour and macronutrient delivery during gastrointestinal digestion. Moreover, no detailed studies on the digestion profile of soymilk in a dynamic digestive environment have been reported; for example, there have been no studies on the spatial distribution of macronutrients because of physical instability (e.g., coalescence or phase separation) in the stomach, or on structural changes of the proteins when experiencing pH changes. Using a dynamic human gastric simulator (HGS), our recent work on the behaviour of milk has revealed new insights into its coagulation (Ye et al., 2016b) and the consequences of this coagulation on the kinetics of the delivery of proteins and lipids to the small intestine (Ye et al., 2016a). Thus, the purpose of this study was to evaluate the structural changes during the gastric digestion of soymilk using a dynamic gastric digestion model.

5.3 Materials and methods

5.3.1 Materials

Commercial dried soybeans were purchased from local grocery stores in Palmerston North, New Zealand. The chemical composition (per 100 g) as determined by standard methods was: protein (AOAC 991.20.II), 32.7 g; fat (AACC 30–10), 14.7 g; moisture (AOAC International, 2000), 10 g; ash (AOAC International, 2002), 4.4 g and total carbohydrate (by difference to achieve 100% of total content), 38.2 g.

5.3.2 Preparation of samples

Dried soybeans (600 g) were rinsed and soaked overnight in 4.2 L of deionized water at room temperature. The soaked soybeans were rinsed again, drained with cold water several times and ground in a seven-fold volume of deionized water (dried beans:water = 1:7, w/w) in a wet disintegrator (Jeffress Bros Ltd, Brisbane, Australia) for 8 min at room temperature. The slurry was filtered twice through a 38 μm sieve to remove any residual soybean particles (okara). The filtrate, termed raw soymilk, was sealed in 400 mL tin cans. The cans were heated in a pilot-plant-scale retort sterilizer (Steriflow batch retort, Roanne, France). The heating process consisted of a linear increase in retort temperature from room temperature to a constant processing temperature of 108 °C in 6 min, followed by a holding period of 15 min at 108 °C. Cooling was performed as quickly as possible. The resulting soymilk contained an average protein content of 3.82% (w/w), determined by a Kjeldahl method according to AOAC method 991.20.II (AOAC International, 2006). A Kjeldahl conversion factor of 5.71 was used to obtain the soy protein content from the nitrogen content (Jones, 1931; Maubois & Lorient, 2016). The soymilk was stored at 4 °C and water was added to adjust the final protein content to 3% (w/w) prior to the digestion study.

5.3.3 Dynamic gastric digestion model and dilution effects

The simulation of the gastric digestion of soymilk was carried out using an HGS [designed by Kong and Singh (2010), Figure 5.1A]. The preparation of the simulated gastric fluid (SGF) was according to the formulation described in Minekus et al. (2014) with some slight modifications and followed the procedure previously reported (Wang et al., 2019). The procedures of digestion have been described by Ye et al. (2016a). The composition of the SGF and the parameters for the *in vitro* gastric digestion are summarized in section 3.2.2.

In brief, a 200 g aliquot of soymilk was warmed in a 37 °C water bath for 5 min and then poured into the HGS. To simulate human gastric secretion, the 1.25 × concentrated SGF and the pepsin solution were pumped into the HGS separately at flow rates of 2.0 and 0.5 mL/min respectively. To simulate human gastric emptying, digesta samples (50 mL), termed emptied digesta, were removed from the bottom of the HGS at 15 min intervals (Lobo et al., 2009). A mesh with a pore size diameter of 1 mm was placed inside the bottom of the HGS to simulate gastric sieving (Meyer et al., 1981). The gastric contraction frequency was 3 times/min, to simulate the actual contraction of the stomach (Marciani et al., 2001). The HGS was set and maintained at 37 °C by a heater and a thermostat. The maximum digestion time was 240 min, but the experiments were terminated at selected time points (15, 30, 45, 60, 90, 150 and 225 min) so that the contents of the HGS, i.e., the gastric chyme, could be removed for further analysis. Control experiments were also conducted with SGF only (without the addition of pepsin).

The sampling scheme during the digestion process that was used for analysis is shown in Figure 5.1B. Gastric chyme was the sample taken from inside the HGS chamber at different times. Emptied digesta was the sample removed from the bottom of the chamber at different times. Only the material with a diameter of <1 mm passed through the gastric sieve located at the bottom of the HGS.

Because of the gradual addition of SGF, samples were diluted continuously with the progression of the digestion process. Assuming that the sample was distributed homogeneously in the stomach throughout the entire digestion process, the diluted concentration could be calculated; the calculation and the dilution line are shown in the Appendix 1 (Table. A1).

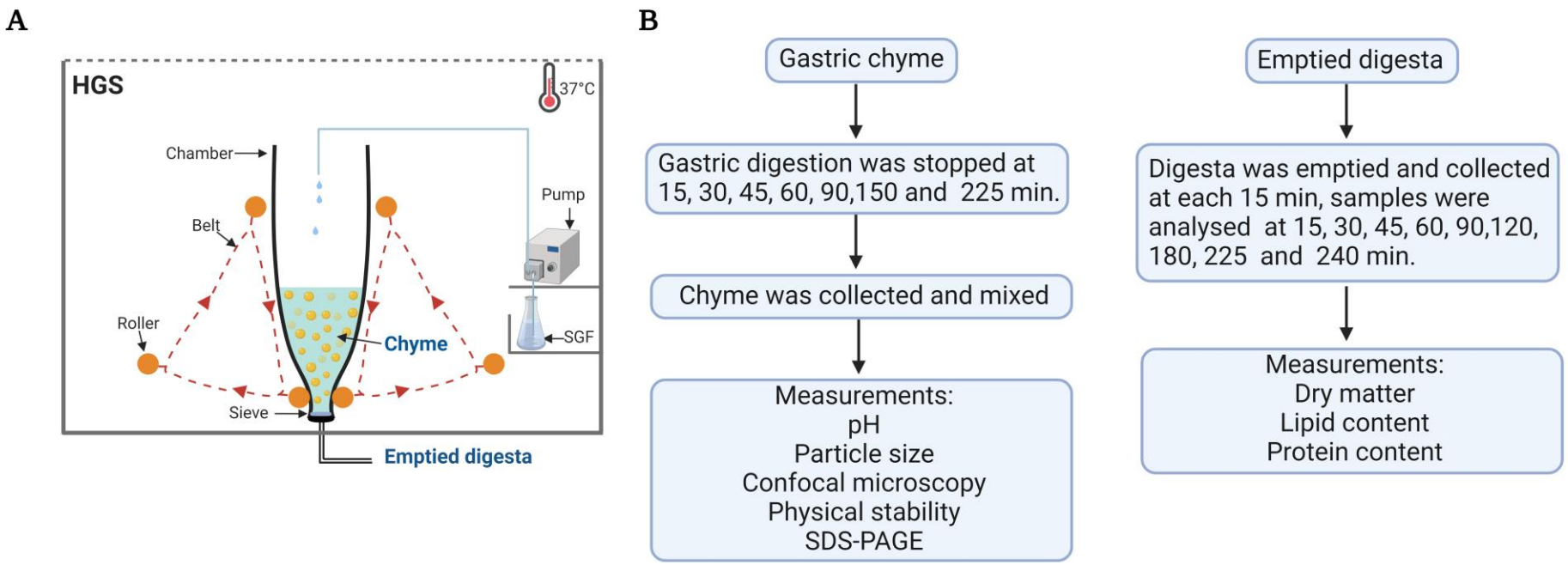


Figure 5.1 *Schematic illustration of an HGS (A) and illustration of the sampling scheme used for analysis (B).*

5.3.4 Chemical analyses

Chemical analyses, including dry matter, lipid and total nitrogen contents, were performed on the undigested soymilk and the digesta that was emptied from the bottom of the HGS during gastric emptying. The pepsin hydrolysis in the emptied digesta was stopped by adjusting its pH to pH 7.5 with 10 M NaOH. The dry matter was determined by a forced air oven method, by placing the samples in dry aluminium dishes and drying at 105 °C until constant weight. The total nitrogen contents of the initial soymilk and the emptied digesta during the simulated gastric digestion were determined by a Kjeldahl method according to AOAC International (2006). A conversion factor of 5.71 for soymilk (Jones, 1931; Maubois & Lorient, 2016) was used to obtain the protein content from the total nitrogen content. Crude oil was measured according to the Mojonnier ether extraction method (AACC method 30–10; AACC, 2000) as previously described in section 3.2.8. All measurements were carried out in at least three replicates.

5.3.5 pH measurement

The initial pH refers to the soymilk before digestion, which was measured by a pH meter prior to digestion. As access into the HGS was prevented by the contractions of the latex stomach bag, to measure the pH at different gastric digestion time points, the digestion experiments were terminated at these respective time points. The gastric chyme was collected and mixed well using a stirring bar and the gastric pH was measured. Each measurement was carried out in triplicate.

5.3.6 Protein hydrolysis

The time-dependent hydrolysis by pepsin of the proteins in the initial and digested soymilk samples in the HGS (i.e., the gastric chyme) was determined by analysing the protein composition of the samples as a function of the digestion time, using sodium

dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. To stop the enzymatic digestion, the gastric chyme samples were raised immediately to pH 7.5, using 10 M NaOH. The protein content of each gastric chyme sample was determined by a Kjeldahl method according to AOAC International (2006). To eliminate the effect of dilution by simulated gastric secretion, 100 μL of each sample was mixed with an electrophoresis sample buffer to obtain an identical protein content of 0.2% (w/v) (Guo et al., 1999). The sample buffer contained 13% (v/v) 0.5 M Tris-HCl buffer, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.04% (w/v) bromophenol blue and β-mercaptoethanol (19:1, v: v). The samples were cooled to room temperature and an 8 μL aliquot of the solution was loaded on to a resolving gel previously prepared on a Mini-PROTEAN II system. The resolving gel contained 14.5% (w/v) acrylamide and the stacking gel was made up of 4.0% (w/v) acrylamide.

The electrophoretic analysis was conducted at 125 V for approximately 80 min. The gel was stained for 40 min under gentle shaking using a Coomassie Brilliant Blue R staining solution [0.003% (w/v) in 10% (v/v) acetic acid (BDH) and 20% (v/v) isopropanol (Merck)]. The gel was destained with a destaining solution of 10% (v/v) acetic acid and 10% (v/v) isopropanol and scanned using a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA, USA). Precision Plus protein unstained standards (Bio-Rad Laboratories) were loaded for estimations of molecular weight. The protein composition from the SDS-PAGE gel was quantified by densitometry using three replicates and Image Lab™ software version 5.2 (Bio-Rad Laboratories).

5.3.7 Particle size measurements

A laser-light diffraction unit (Masterziser 2000, Malvern Instruments, Malvern, Worcestershire, UK) was used to analyse the average particle size and the particle size distribution of the initial soymilk and the gastric chyme as described in section 3.2.5. The

refractive index of soybean oil was set at 1.570 (with an absorbance value of 0.001) (Chen & Ono, 2014) and that of water was set at 1.33. Mean particle diameters were calculated as the average of triplicate measurements on individual samples. All measurements were carried out in three replicates.

5.3.8 Confocal laser scanning microscopy

The microstructures of the initial soymilk and the gastric chyme samples during dynamic gastric digestion were investigated using a confocal laser scanning microscope (Leica SP5 DM6000B, Leica Microsystems, Heidelberg, Germany) as described in section 3.2.6.

5.3.9 Physical stability

To observe the physical state and the stability of the soymilk during digestion, the experiments were terminated at selected time points, because the latex stomach bag prevented easy observation of the gastric chyme inside the HGS. The gastric chyme was collected from the inside of the stomach chamber for a short-term storage stability analysis as described in section 3.2.4. To record the instability to sedimentation of each sample, photographs were taken at 0, 1, 10, 30, 60 and 120 min after mixing and holding at room temperature using a digital camera. The initial changes during storage provided direct information on the appearance of the gastric chyme in the stomach.

5.3.10 Statistical analysis

The *in vitro* digestion experiments were repeated at least three times for each sample and the results were expressed as the mean ± standard deviation of three replicates. A repeated-measures two-factor analysis of variance (ANOVA) model, with the *in vitro* replication as the experimental unit, was performed for protein and lipid contents using the MIXED model procedure of the statistical software R (version 3.6.1, R Foundation

for Statistical Computing, Vienna, Austria). The statistical linear mixed model included enzyme (with or without), time (0–240 min), and their interaction as a fixed effect, but with replication as a random effect. For the other response variables (pH and particle size), a two-factor ANOVA without repeated-measures analysis was conducted. For SDS-PAGE quantification, a single-factor ANOVA was applied to test the significance of variations within groups at a certain digestion time point. If the F value of the overall model was significant ($P < 0.05$), post hoc tests were conducted using Tukey's range test and significance was taken at $P < 0.05$.

5.4 Results

5.4.1 Changes in intragastric pH during dynamic digestion

The undigested soymilk contained 3.0% protein, 1.8% fat and 6.93% dry matter. The gastric digestion was performed using a dynamic *in vitro* digestion model, by the gradual addition of acid and enzyme to approximate the secretion process occurring in the stomach *in vivo*. The changes in the pH profile of soymilk during gastric digestion are shown in Figure 5.2. The pH of the soymilk was significantly influenced by the digestion time ($P < 0.001$). It was approximately 6.65 initially, reduced to pH 4.5 after around 60 min and progressively decreased to pH 2 after 150 min of digestion, with no further significant difference observed thereafter. No significant variations in the pH profiles of the soymilk samples with and without the addition of pepsin were observed.

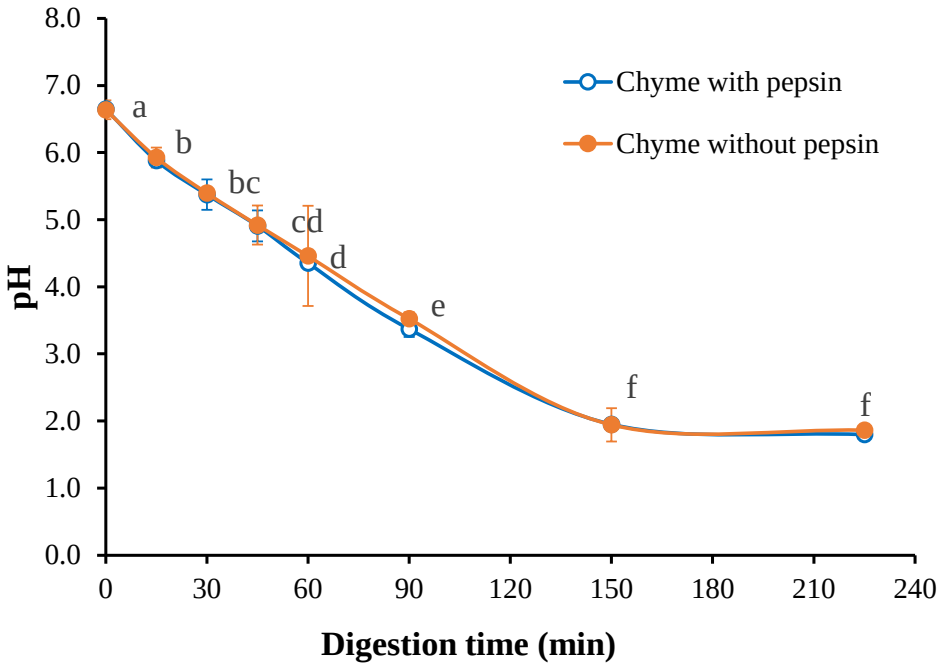


Figure 5.2 *Changes in the pH of soymilk samples during dynamic gastric digestion.*

Note. The pH values refer to the initial (before digestion) soymilk and the gastric chyme samples in the HGS. Values are reported as mean ± standard deviations. Values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) within a certain treatment (with or without added pepsin) across different digestion times.

5.4.2 Characterization of physical changes occurring during gastric digestion

5.4.2.1. Gastric behaviour of soymilk

The changes in the physical stability of the soymilk samples during digestion are shown in Figure 5.3. The soymilk coagulated after approximately 7 min of digestion in the presence of pepsin, with the formation of soft curd particles observed from the top of the HGS. As the digestion progressed, the coagulation became more extensive and many small curd particles tended to aggregate in the HGS. Sedimentation was observed visually after 30 min of digestion (Figure 5.3, chyme with pepsin, 30 min). The gastric chyme separated into a white "sediment" layer at the bottom (shown as the red arrows in Figure

5.3, chyme with pepsin, 30 min) and a transparent "serum" layer at the top, and some white curd particles appeared to float at the top of the serum layer. These curd particles were soft, creamy and fragile. With further digestion, both the precipitated curd particles and the floating curd particles became smaller and the majority of the sediment was emptied from the bottom of the HGS after around 90 min of digestion (Figure 5.3, chyme with pepsin, 90 min).

In the absence of pepsin, protein aggregation and coagulation were observed at around 15 min of digestion, which was later than for the sample with pepsin addition. Sedimentation was clearly observed after 45 min of digestion, with a significant amount of precipitation/aggregation of protein curd particles at the bottom and a transparent serum phase at the top (Figure 5.3, chyme without pepsin, 45 min). These curd particles were larger and firmer than those observed in the sample with added pepsin. With further digestion, these curd particles appeared to become smaller slowly and were gradually emptied.

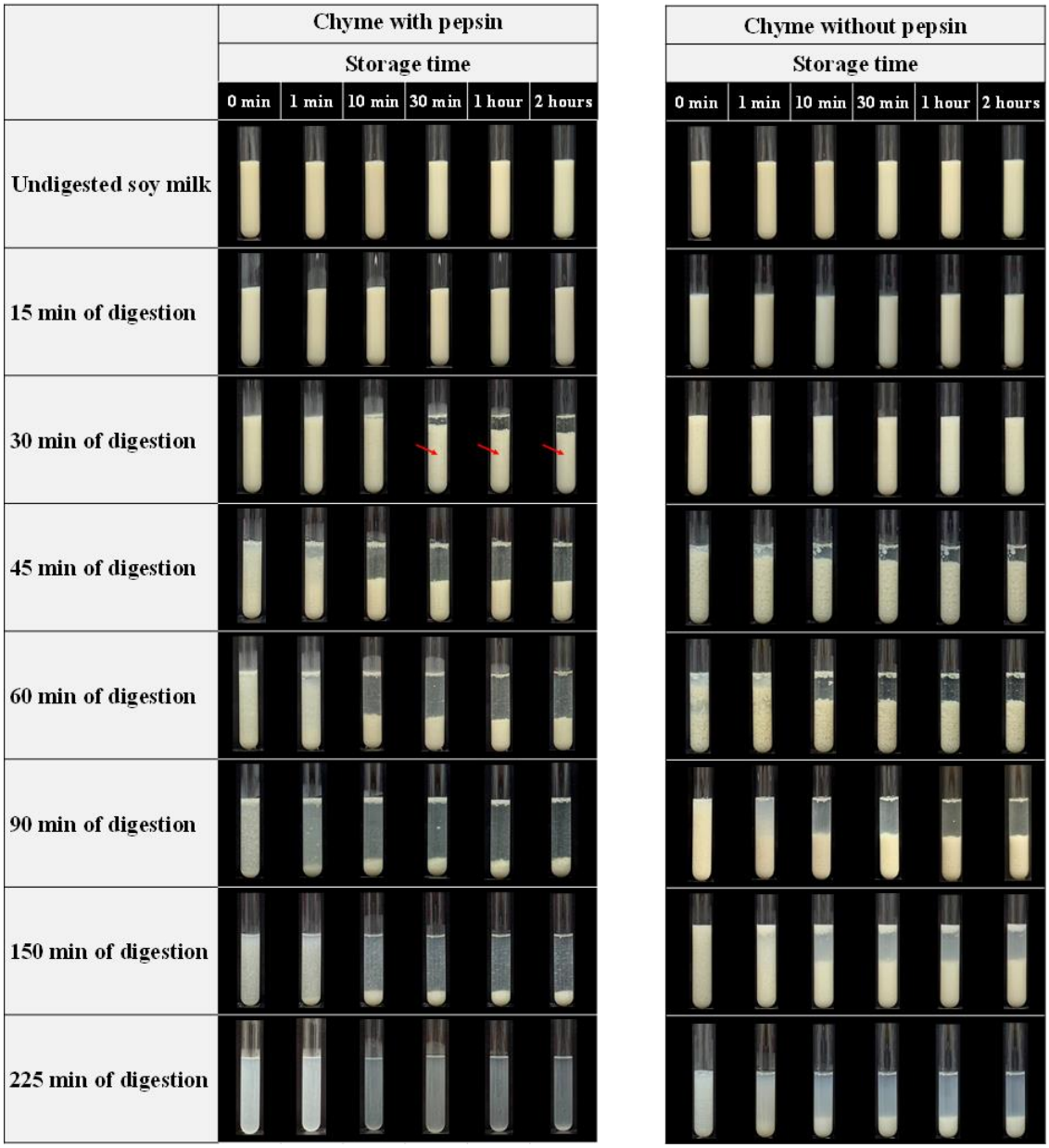


Figure 5.3 *Changes in the physical state of the gastric chyme taken from soymilk at different digestion times in the HGS and sedimentation stability during storage.*

Note. The downward-pointing arrows indicate sedimentation of the gastric chyme.

5.4.2.2. Particle size

The changes in the particle size and the particle size distribution of the gastric chyme samples during the simulated gastric digestion are shown in Figure 5.4. The particle size was significantly influenced by the digestion time, the presence or absence of pepsin and their interaction ($P < 0.001$). Initially, the average volume-weighted mean diameter ($d_{4,3}$) of the soymilk was about 0.56 μm. The soymilk presented a bimodal distribution, with two populations at approximately 0.36 and 5 μm (Figure 5.4B and C, 0 min) respectively. After 10 min of digestion with pepsin, the $d_{4,3}$ was 21 μm and the corresponding particle size distribution remained bimodal, with a peak at about 4 μm and a second peak at 30 μm (Figure 5.4B and 10 min). The $d_{4,3}$ increased up to 95 μm at 60 min of digestion, with no further significant change thereafter. In the absence of pepsin, the $d_{4,3}$ of the soymilk was 1.2 μm after 10 min of digestion. However, it increased rapidly up to ~300 μm at 60 min and then reduced gradually after 90 min of digestion. The $d_{4,3}$ of the soymilk without added pepsin was significantly larger than that of the soymilk that was digested by pepsin between 45 and 90 min of digestion.

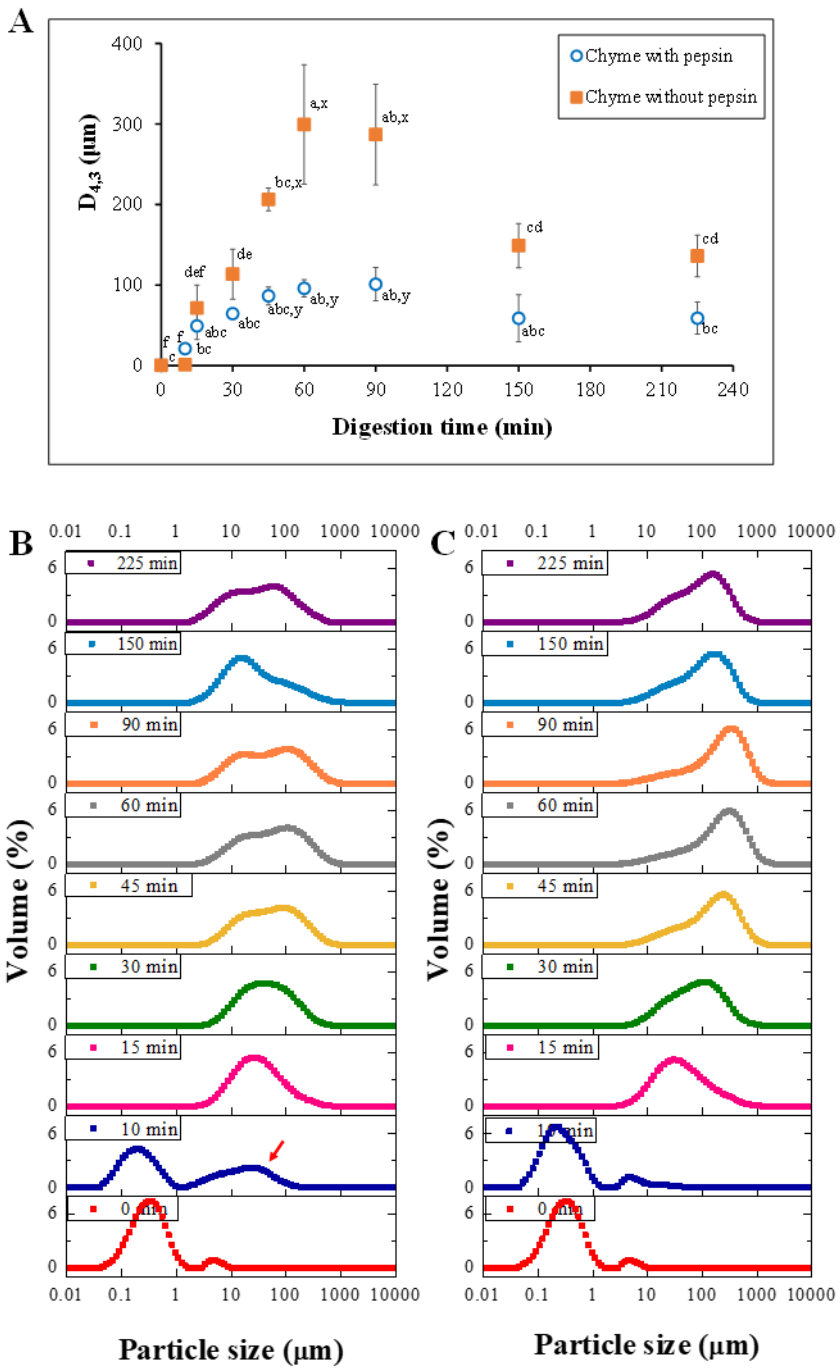


Figure 5.4 *Changes in volume-weighted average diameter $d_{4,3}$ (A) and particle size distribution of soymilk samples (i.e., gastric chyme) with (B) and without (C) pepsin during gastric digestion in the HGS.*

Note. The red arrow indicates protein coagulation by pepsin. The measurements were replicated at least three times. Error bars represent standard deviations. In Figure A, values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) within a certain treatment (with or without added pepsin) across different digestion times. Values with no letter in common (x, y) represent significant differences at a certain digestion time across different treatments (with or without added pepsin). If no letter is listed, there were no significant differences.

5.4.2.3. Microstructure

Confocal laser scanning microscopy provided additional information on the microstructural changes in the soymilk inside the HGS during the simulated gastric digestion (Figure 5.5). Undigested soymilk is essentially an oil-in-water emulsion, with oil bodies dispersed in the aqueous phase. The coagulation of the soy proteins after 10 min of peptic digestion was reflected in the microstructure of the gastric chyme (Figures 5.5B1-5.5B2), seen as large clusters of protein particles. The soybean oil bodies appeared to be evenly distributed throughout the protein clusters and some free oil bodies were also observed in the aqueous phase. With further digestion, larger protein clusters and entrapped lipid droplets were observed (Figures 5.5D1-5.5D2, 5.5F1-5.5F2 and 5.5H1-5.5H2). At the end of the simulated gastric digestion by pepsin, only some tiny protein particles could be observed (Figure 5.5L2). It is worth noting that no significant coalescence of the oil bodies was observed over 4 h of gastric digestion (Figure 5.5L1).

In contrast, no coagulation was observed in the first 10 min in the sample without pepsin addition (Figure 5.5C2), in accordance with the visual observation from the top of the HGS. However, the microstructure imaging showed that protein coagulation occurred after 15 min, with the entrapment of oil bodies within the coagulated protein clusters. After 150 min of digestion, the protein clusters appeared to be smaller but did not disappear until the end of digestion (Figures 5.5K2 and 5.5M2).

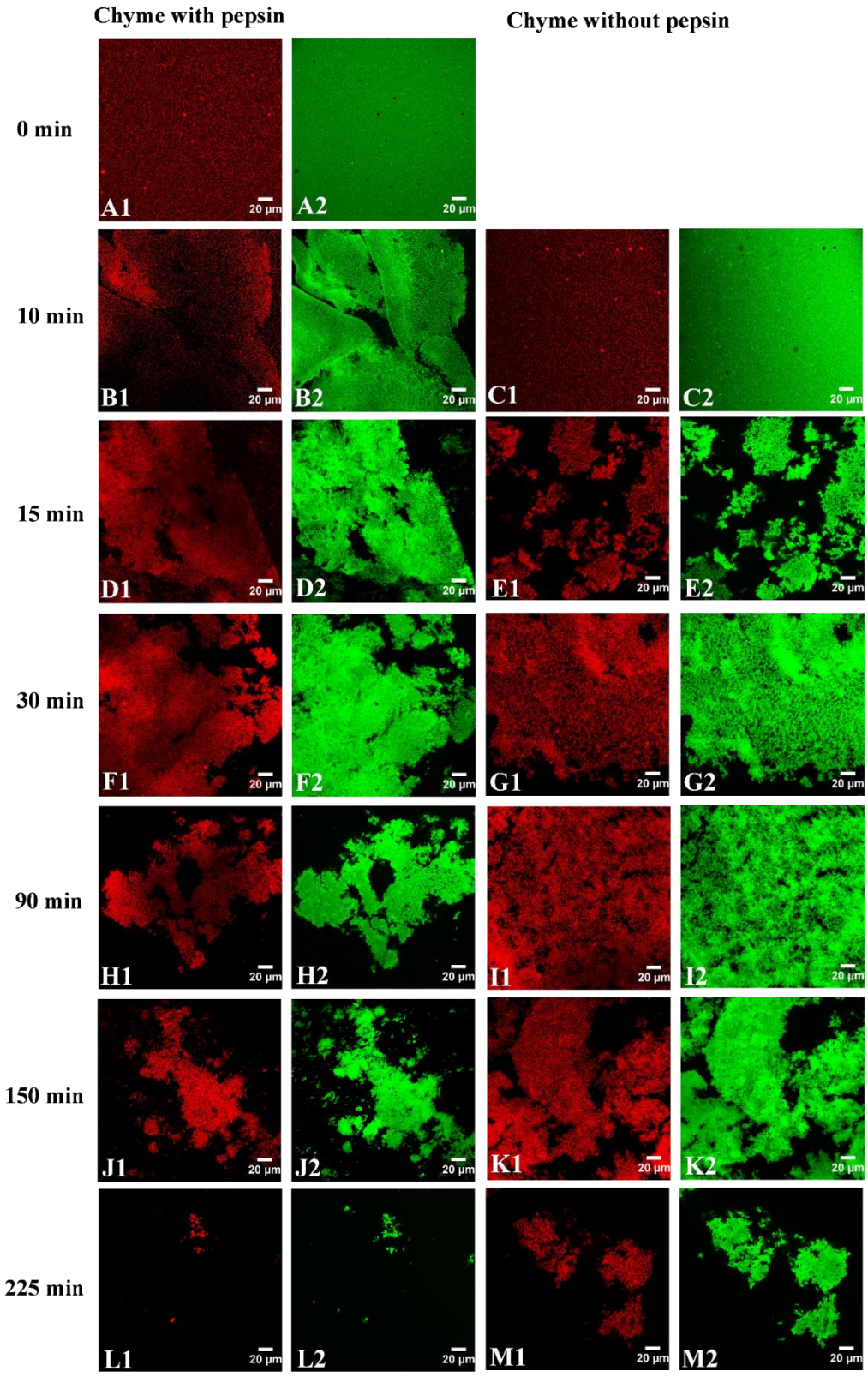


Figure 5.5 *Confocal micrographs showing the microstructural changes in soymilk that occurred at different times of simulated gastric digestion, with and without the addition of pepsin, in the HGS.*

Note. The scale bar in all images is 20 µm.

5.4.3 Protein hydrolysis in the HGS

SDS-PAGE patterns of the protein profiles of the gastric chyme are shown in Figures 5.6A–5.6B. The original soymilk sample (0 min) showed several major polypeptide bands, including β-conglycinin with three subunits (7S α', 72 kDa; 7S α, 68 kDa; 7S β, 52 kDa) and glycinin with acidic (11S A) polypeptide (31–45 kDa) and basic (11S B) polypeptide (18–20 kDa) (Brooks & Morr, 1985; Nishinari et al., 2014; Shuttuck-Eidens & Beachy, 1985; Thanh & Shibasaki, 1977).

To eliminate the effect of dilution by the gradual addition of SGF during digestion, samples were adjusted to the same total nitrogen concentration, prior to loading into each lane of the SDS-PAGE gel. During gastric digestion by pepsin (Figure 5.6A), all protein bands became less intense within the first 15 min. Many low molecular weight peptides (<15 kDa) could be seen from 30 min onwards. After 60 min, the bands corresponding to the major soy proteins almost disappeared; only low molecular weight peptides could be seen.

The proportions of intact major proteins remaining in the gastric chyme as a function of the digestion time were quantified (Figure 5.6C). The rate of reduction of 7S β in the gastric chyme was apparently slower than that of the other proteins. In particular, the protein concentration (relative to the initial input) of the 7S β subunit was significantly higher than that of the other proteins at 30 and 45 min of digestion ($P < 0.05$). In the absence of pepsin, as expected, the protein bands remained intact and no degradation products were observed throughout the entire digestion.

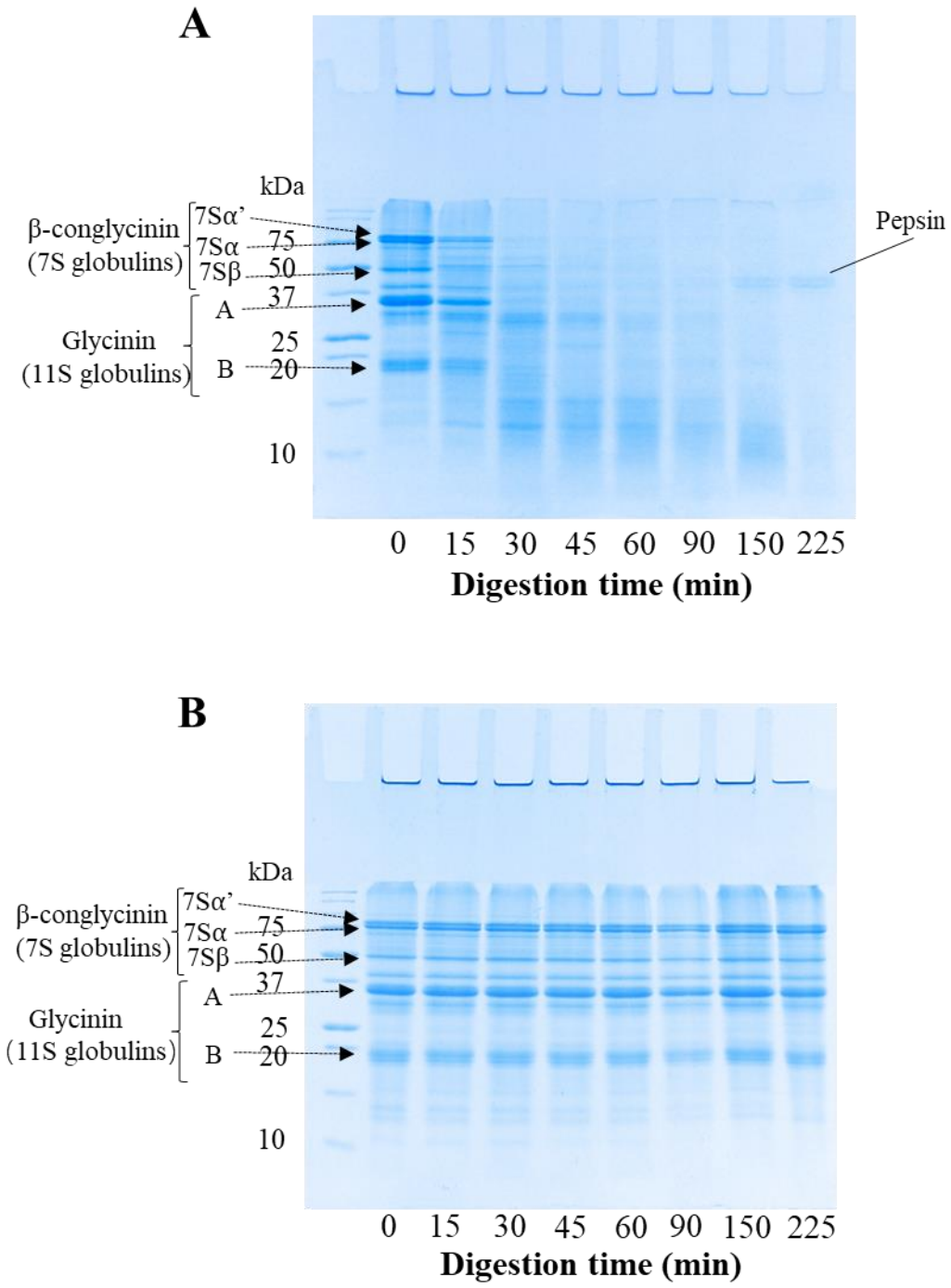


Figure 5.6 *SDS-PAGE (under reducing conditions) of initial (before digestion) and digested (i.e. gastric chyme) soymilk samples with (A) and without (B) pepsin addition at different time points during gastric digestion in the HGS, and relative protein contents of the gastric chyme samples digested with pepsin (C).*

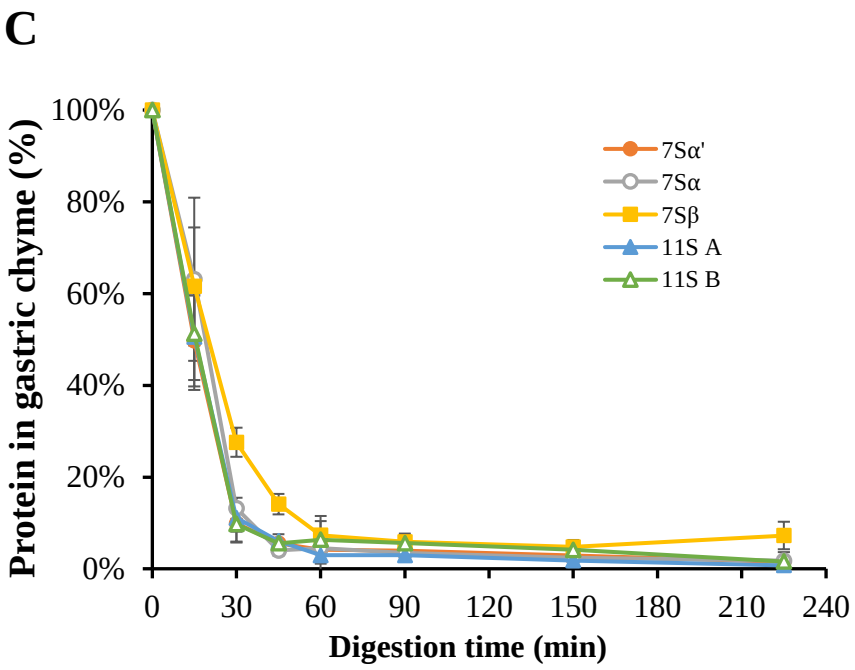


Figure 5.6 *SDS-PAGE (under reducing conditions) of initial (before digestion) and digested (i.e. gastric chyme) soymilk samples with (A) and without (B) pepsin addition at different time points during gastric digestion in the HGS, and relative protein contents of the gastric chyme samples digested with pepsin (C).*

5.4.4 Delivery of macronutrients to the small intestine

The dry matter, total nitrogen and lipid contents of the digesta emptied out of the HGS at different time points during the gastric digestion process were determined (Figure 5.7). The grey broken lines shown in Figures 5.7A, 5.7B and 5.7C indicate the calculated dry matter, protein and crude lipid concentrations in the emptied digesta at each time point. The calculation was based on the assumption that lipid and protein were uniformly distributed in the soymilk during the entire digestion process, as shown in the Appendix 1. The decrease in the calculated values was due to the dilution effect by gradual gastric emptying.

The changes in the dry matter, protein and lipid contents in the emptied digesta during gastric digestion were significantly influenced by the presence or absence of

pepsin, the digestion time and their interaction ($P < 0.001$, Figure 5.7). In the presence of pepsin, the gastric emptying of dry matter and protein (Figures 5.7A–5.7B, ED with pepsin) showed similar patterns: a faster emptying in the first 45 min, compared with the dilution curve, and then almost following the expected dilution curve. In contrast, when pepsin was absent, a slower gastric emptying of dry matter and protein was noticed at 45 and 60 min. Both the dry matter content and the protein content of the emptied digesta in the sample without added pepsin were significantly lower than those in the sample with added pepsin at these time points ($P < 0.001$).

The gastric emptying of lipids generally showed a similar pattern to that of the dry matter and protein, except for the first emptying point, i.e., at 15 min. In the presence of pepsin, gastric emptying of lipids at 15 min was significantly delayed, compared with the sample in the absence of pepsin ($P < 0.001$). However, faster emptying was observed at 45 min and then the gastric emptying pattern almost followed the dilution curve thereafter. When pepsin was absent, a delay in the delivery of lipids was observed between 45 and 60 min, which was significantly slower than that of the sample with added pepsin; however, a slightly faster emptying was observed at 120 min compared with the sample with added pepsin ($P < 0.001$).

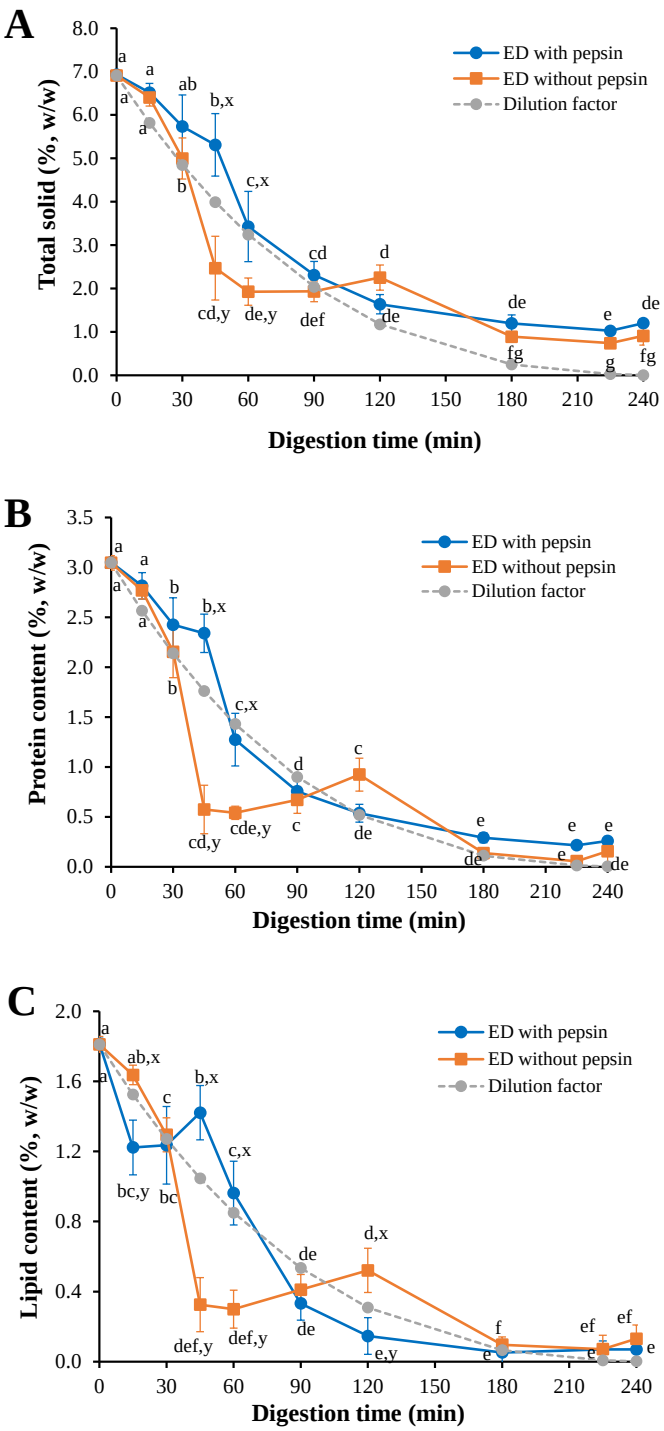


Figure 5.7 *Changes in total solids (A), protein (B) and lipid (C) contents (w/w, %) of the emptied digesta during gastric digestion in the HGS.*

Note. ED, emptied digesta. Values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) within a certain treatment (with or without added pepsin) across different digestion times. Values with no letter in common (x, y) represent significant differences at a certain digestion time across different treatments (with or without added pepsin). If no letter is listed, there were no significant differences. Values are reported as mean ± standard deviations.

5.5 Discussion

Soymilk is a turbid colloidal dispersion with majority of the particles between 50 and 1500 nm. (Figures 5.4B and 5.4C, 0 min), representing protein particles and oil bodies. It is known that protein particles of greater than 40 nm in diameter constitute about 50% (including 14% of more than 100 nm) of the total protein in soymilk (Ono et al., 1991). Soybean oil bodies have an average particle size of 300–400 nm (Iwanaga et al., 2007; Ono, 2008).

5.5.1 Gastric behaviour of soymilk

5.5.1.1. Gastric pH

The gradual decrease in the gastric pH during digestion resulted from the dosing of hydrochloric acid and the buffering effect of the soymilk (Reynaud et al., 2020). Interestingly, the gastric pH profile of the soymilk was similar to those of bovine milk (Ye et al., 2019) and almond milk (Wang et al., 2020), previously obtained using the same HGS system. Notably, a previous *in vivo* study in piglets (Moughan et al., 1991) has shown that there was no significant difference in the changes of gastric pH in the soybean protein-based infant formulas and bovine milk-based infant formulas during gastric digestion. Reynaud et al. (2020) studied the changes in the pH of soymilk in the stomach of miniature pigs and compared different measurement methods. They observed a gradual decrease in pH of the soymilk using an ex vivo method, which was similar to the results in the current study, although the reduction in pH was faster than that measured by the *in situ* method.

5.5.1.2. Coagulation and destabilization of soy proteins in the HGS

The soymilk coagulated within 10 min of digestion by pepsin, at which time the pH was around 6.59. At this time point, tofu-like curd particles with an average particle

size of ~20 μm were formed (Figures 5.4B and 5.4C, 10 min). It is worth noting that, in our study, the coagulation pH (6.59) was much higher than the pH at which acid-induced coagulation of soymilk takes place (pH 5.9), as reported by Fujii (2017). This suggests that the initial coagulation must have been induced by the action of pepsin. However, the molecular mechanism of this early coagulation is not clear at present, as this effect has never been reported. Previous studies have shown that commercial pepsin does not coagulate soy proteins (Fuke & Matsuoka, 1980; Murata et al., 1987; van den Braak et al., 2013). A number of studies conducted by Stanojević and co-workers used a commercial chymosin–pepsin combination to coagulate soy proteins to create a tofu gel (Stanojević et al., 2010; Stanojević et al., 2011, 2017). However, Stanojević et al. (2011) suggested that chymosin was the principal contributor to protein curd formation, and that pepsin played only a minor role in the coagulation, because the pH of soymilk is closer to the optimal pH range for chymosin activity (pH 5.5–6.2) than for pepsin activity (pH 2). Nevertheless, it has been shown that the casein micelles in milk are coagulated by pepsin at a pH of around 6.2–6.3 (Ye et al., 2017; Ye et al., 2016a, b), which is similar to the coagulation pH observed in the current study on soymilk.

Once the curd particles had been formed, they tended to grow in size (Figure 5.4A, chyme with pepsin) and precipitate in the HGS (Figure 5.3). The gastric pH was approximately 5.9 at 15 min, which is close to the *pI* of the β-subunits of β-conglycinin (7S) [pH 5.7–6.0 (Thanh & Shibasaki, 1977)]. As the digestion progressed, the pH further reduced to 4.5 at 60 min, which is close to the isoelectric point of acidic subunits of of glycinin (11S) [i.e., pH 4.2-4.8 (Badley et al., 1975)]. Glycinin and β-conglycinin are the major storage proteins of soybean, and account for 80% of the total soybean proteins (Utsumi et al., 1993). Under these conditions, the protein particles have reduced electrostatic repulsion and the hydrophobic residues of denatured proteins exposed to the

solvent provide the driving force for aggregation (Nik et al., 2011; Nik et al, 2008). The precipitation seen in the HGS therefore reflects the extensive aggregation of the soy proteins.

When pepsin was absent, there was a similar but much more pronounced trend in the change in the particle size of soymilk during gastric digestion (Figure 5.4A). As above, the sharp increases ($P < 0.05$) in the particle size in the first 60 min were caused by protein aggregation, which was induced by the acidic pH, as expected. With further digestion, the gastric pH reduced to ~2 at 150 min. The significant reduction ($P < 0.05$) in the particle size at this time point was attributed to the further decrease in the gastric pH. When the pH was far from the *pI* of the soy proteins, the increase in electrostatic repulsion prevented the association of the proteins (Vetri et al., 2011), and resulted in the breakdown of the protein curds because of the mechanical/shearing action of the HGS.

5.5.1.3. Hydrolysis of soy proteins in the HGS

The SDS-PAGE pattern (Figure 5.6A) suggested that the soy proteins were rapidly hydrolysed by pepsin, with significant differences in the rates of hydrolysis among the different proteins. The β-subunit of β-conglycinin (7S β) appeared to be less susceptible to pepsin hydrolysis, in agreement with previous studies (Astwood et al., 1996; Fu et al., 2002). The other major proteins, i.e., 7S α, 7S α' and 11S A and B fractions, were relatively rapidly hydrolysed by pepsin, as shown in earlier reports (Adachi et al., 2009; Baglieri et al., 1994; Liu et al., 2019; Rui et al., 2019). Nguyen et al. (2015) studied the digestibility of soy proteins under simulated infant gastrointestinal conditions. They reported only partial hydrolysis (~60–80%) of 7S and 11S after 1 h in the gastric phase, which was slower than that observed in the current study. In contrast, Rui et al. (2016) and Liu et al. (2019) reported faster protein hydrolysis than observed in the current study; all major fractions of the soy proteins were degraded as soon as the gastric digestion began.

These variations can be attributed to the differences in the experimental conditions applied in each study. For example, in the studies of Rui et al. (2016) and Liu et al. (2019), all the pepsin was added at the beginning of the digestion in their static models operating at pH 2 and pH 3 respectively, i.e., pHs close to the optimal pH for pepsin activity (Anfinsen et al., 1971). In contrast, in the current study, the pH was around 6.6 in the first few minutes of digestion, at which time both the amount and the activity of pepsin were limited. Besides, Rui et al. (2016) used a shaking waterbath at 55 rpm to simulate gastric mixing in their static digestion model, which might provide a more rapid mixing at the beginning of digestion than that in the HGS used in the current study.

5.5.1.4. Changes in soybean oil bodies during digestion

Using soymilk and the dynamic gastric digestion model, our studies clearly showed that the oil bodies became trapped within the protein curd particles generated during digestion. As digestion progressed, the oil bodies were gradually liberated from the curd particles, as the protein particle structure was dissociated because of hydrolysis by pepsin. It was expected that the hydrolysis of the interfacial protein would cause destabilization and coalescence of the oil bodies, but no obvious coalescence of the oil droplets was noticed over 4 h of gastric digestion (Figure 5.5, with pepsin). Similar results have also been reported by Nguyen et al. (2015) and Liu et al. (2019). It appears that phospholipids and the peptides remaining on the surface were able to protect the oil bodies against coalescence. No gastric lipase was applied in our study. It has been reported that some oil body coalescence was observed after 1 h of simulated gastric digestion of a soy-protein-stabilized emulsion in the presence of gastric lipase (Nguyen et al., 2018).

5.5.2 Effects of gastric digestion on macronutrient delivery

The emptying of gastric contents from the HGS in the presence of pepsin was mainly driven by three critical factors: (1) dilution effects because of the gradual addition of the SGF and progressive removal of the gastric contents; (2) protein hydrolysis by pepsin; (3) the physical state and spatial distribution of macronutrients between the coagulated and serum phases in the HGS. However, the initial coagulation caused by pepsin action did not affect the emptying of gastric contents over 4 h of gastric digestion, except for an initial delay in lipid delivery (at 15 min). This significant delay in lipid emptying ($P < 0.001$) at 15 min, compared with the sample without added pepsin, appeared to be caused by the effect of the initial coagulation due to pepsin action at relatively high pH (pH 6.6); this initial coagulated protein material appeared to contain a large proportion of oil bodies, possibly because of interactions of oleosins at the oil body surface with the aqueous phase proteins, as well as interactions between oil bodies through the surface proteins or peptides. As a result, this coagulated material tended to float upward in the HGS because of its lower density compared with that of the aqueous phase. Consequently, there was a delay in the gastric emptying of lipids from the bottom of the HGS.

The rapid gastric emptying of both protein and lipid that was observed at 45 min can be associated with the combined effects of precipitation of the curd particles and pepsin hydrolysis of the proteins. At this time, the gastric pH was approximately 4.9, and extensive aggregation and precipitation took place because of the isoelectric coagulation of the soy proteins. Meanwhile, protein hydrolysis (Figure 5.6A) contributed to the breakdown of the curd particles (Figure 5.4A), resulting in an accelerated gastric emptying of both the proteins and the lipids from the bottom of the HGS. Protein hydrolysis combined with mechanical shear in the HGS significantly accelerated the

breakdown of the curd particles (Figure 5.4A), which were emptied from the bottom of the HGS when the curd particle size became smaller than 1 mm, because of the simulated gastric sieving function of the HGS. Some oil bodies were released with the disintegration of the protein curds, but microscopic observation (shown in the Appendix 3, Figure A1) suggested that the majority of the oil bodies were still entrapped and were emptied with the protein curd particles. However, after 45 min, the gastric emptying of both protein and lipid from the HGS followed the same trend.

When pepsin was absent, the emptying of gastric contents from the HGS was modulated mainly by the dilution effects and the physical state and spatial distribution of the macronutrients. The significant delay in gastric emptying of both protein and lipid at 45–60 min (Figure 5.7) was driven simply by protein coagulation and precipitation induced by low pH at the isoelectric point of soy proteins. Around this time point, the soybean curds were too large to be transited to the small intestine because of the gastric sieving effect, thereby leading to a delay in the delivery of both protein and lipid. However, when the gastric pH further reduced from pH 4.5 at 60 min to pH 1.9 at 150 min, the protein matrix was gradually dissociated because of the increased repulsion between protein molecules and the shear in the HGS (Vetri et al., 2011). These curd particles became smaller in size and were thus emptied with gradual gastric emptying, which almost followed the hypothetical dilution curve.

Baglieri et al. (1994) studied gastro-jejunal digestion of soymilk in humans. They observed that the majority of the nitrogen was transited into the small intestine in the first 60 min, which supported the results obtained in the present study (Figure 5.7B). They suggested that the gastric emptying of the liquid phase of soymilk followed a power exponential curve and that the half-emptying time of soymilk (37.7 min) was similar to that of skim milk [25 min (Mahe et al., 1992)].

As a traditional drink, soymilk has served as an acceptable substitute for cow milk for many decades because of its nutritional and health benefits (Friedman & Brandon, 2001); however, their behaviours are clearly different under acidic gastric digestion conditions. For cow milk, gastric emptying of caseins was significantly delayed because of the formation of clots by the milk-clotting enzyme, pepsin, which resulted in slow and steady delivery of nitrogen into the small intestine over several hours (Mulet-Cabero et al., 2019; Ye et al., 2016a). In comparison, soy proteins were also susceptible to coagulation by pepsin, but this coagulation behaviour did not have an important impact on gastric emptying of the proteins. The majority of the proteins were transited into the small intestine in the first hour of gastric digestion. In addition, soymilk has shown different digestion behaviour compared with other plant-based natural drinks. Almond milk is highly prone to coalescence of oil droplets and creaming upon gastric digestion, which delays gastric emptying of almond lipids (Wang et al., 2020). Unlike other plant-based beverages, soymilk was more stable to oil droplet coalescence under gastric conditions; instead, soybean oil bodies were entrapped into the protein network and precipitated in the stomach in the present study. These different digestion behaviours in each natural food system may induce different physiological and metabolic effects in the gastrointestinal tract, which requires further investigation.

5.6 Conclusions

The coagulation of soy proteins induced by both the action of pepsin and the acidic gastric pH was observed during dynamic *in vitro* gastric digestion. This resulted in a layer of coagulated protein particles at the bottom of the HGS. Because of relatively small size of these coagulated particles, the overall gastric emptying of proteins from the HGS to the next digestion step was not affected to a substantial extent, although an accelerated emptying was seen in the early stage of gastric digestion. This observation is in stark

contrast to the behaviour of cow milk where coagulation of casein micelle markedly deceases the rate the gastric emptying of casein proteins, due mainly to the large size of the curd particles. These different rates of protein emptying may lead to a different uptake and absorption of amino acid but this requires further investigation, possibly in human clinal trials.

Chapter 6 [3]Structural changes in oat milk and an oat milk–bovine skim milk blend during dynamic *in vitro* gastric digestion

6.1 Abstract

In recent years, plant-based milk alternatives have become increasingly desirable because of their sustainability and proposed health benefits. There is a need to understand the behaviour of plant-based milks during gastric digestion, including protein digestibility, structural changes, and macronutrient accessibility. The aim of this study was to investigate the gastric digestive behaviour s of oat milk and an oat milk–bovine skim milk blend (1:1, v/v), using an *in vitro* dynamic human gastric simulator. During gastric digestion with pepsin, the oat milk did not show significant changes in structure and physical stability. In contrast, the oat milk–bovine skim milk blend formed coagulated, curd-like particles, which were induced by the action of pepsin on the milk proteins. The oat oil bodies were incorporated into the curd particles. This difference in gastric digestive behaviour significantly altered the rate of gastric emptying. Coagulation of the oat milk–bovine skim milk blend slowed the gastric emptying of macronutrients, leading to a delay in the release of protein and lipids into the small intestine compared with the oat milk. This study provides insight into the way the food structure modification during gastric digestion impacts nutrient delivery.

[3] This chapter has been published as **Wang, X**., Ye, A., Dave, A., & Singh, H. (2022). Structural changes in oat milk and an oat milk–bovine skim milk blend during dynamic *in vitro* gastric digestion. *Food Hydrocolloids*, 124, 107311. https://doi.org/10.1016/j.foodhyd.2021. 107311

6.2 Introduction

Oats are a natural functional food that is highly valued for its dietary fiber, phytochemicals, and high nutritional value (Sethi et al., 2016). Oats have a higher protein content (15–20%) than other cereals (McMullen, 1991) and a protein digestibility corrected amino acid score (PDCAAS) ranging from 43 to 69 (Peterson & Brinegar, 1986). Oat lipids have a favourable ratio of polyunsaturated to saturated fatty acids because of high proportions of oleic acid and linoleic acid (Zhou et al., 1998; Zwer, 2010). Oats have been shown to have a number of health benefits beyond basic nutrition, such as reducing total cholesterol, lowering low-density lipoprotein blood cholesterol levels, and regulating blood glucose levels. These effects are largely associated with the presence of soluble dietary fibre (β-glucan) in oats (Braaten et al., 1994; Kapica, 2001; Wood et al., 1994).

Oat milk/beverage is one of the newer milk alternatives that have appeared in the global market in recent years. Because of its acceptable taste, nutritional benefits, and low environmental impact, oat milk has become the second most popular plant-based milk alternative, following almond milk (Watson, 2020).

The main ingredients of oat milk are water and oat flour. The typical process for producing oat milk includes mixing the oat flour with water to create a slurry, enzymatic hydrolysis of the starch, and filtration, followed by heat treatment and homogenization. Heating is applied to improve the microbial stability of the product (Mäkinen et al., 2016). The resulting product is a colloidal suspension of extracted protein particles and oil bodies.

Oat milk has a low PDCAAS because of its low lysine content (Pedo et al., 1999), but this can be overcome by combining oats with bovine milk because bovine milk proteins have relatively high contents of lysine (Manson, 1975). Consequently, attempts

have been made to improve the nutritional value of oat-based beverages by developing novel blended products containing oats and cow milk (Amarnath, 2017; Brückner-Gühmann et al., 2019; Pallavi & Madhusudan, 2016).

Cow milk contains 3–4% protein, which consists mainly of caseins (approximately 80%) and whey proteins (approximately 20%). The caseins exist in milk in the form of micelles, which play a crucial role in the physicochemical stability of mammalian milks. The digestive behaviour of cow milk has been investigated extensively (Mulet-Cabero et al., 2019; Ye et al., 2016a). Casein micelles are known to coagulate in the stomach, which is induced by the cleavage of κ-casein by pepsin and augmented by the acidic conditions in the stomach, whereas whey proteins remain soluble and empty from the stomach more rapidly (Boirie et al., 1997). During the gastric digestion of milk, coagulation and the resulting curd structure have been reported to affect the rates of protein hydrolysis and the release of fat globules in the stomach (Ye et al., 2016a, b).

Currently, little information on the physicochemical behaviour of oat milk in the gastrointestinal environment, especially in the presence of bovine milk proteins, is available. Although oat milk has attracted increasing research interest recently, this has been focused mainly on optimizing oat milk production (Deswal et al., 2014a, b) and the health benefits associated with β-glucan (Önning et al., 1999; Önning et al., 1998). Moreover, it is not known how the blending of oat proteins and milk proteins affects their digestibility. Therefore, the aim of the present study was to investigate the gastric digestive behaviours of oat milk and an oat milk–bovine skim milk blend and how material structures in the stomach impact on the delivery of macronutrients, using an *in vitro* dynamic human gastric simulator (HGS).

6.3 Materials and methods

6.3.1 Materials

Commercial oat flour was obtained from Harraways (Dunedin, New Zealand) and bovine skim milk was purchased from a local supermarket in Palmerston North, New Zealand. α-Amylase from Bacillius amyloliquefaciens (EC 3.2.1.1; 437 units/ml) was kindly provided by Novozymes (Canberra, ACT, Australia).

6.3.2 Preparation of oat milk and oat milk–bovine skim milk blend

The protein contents of the oat flour and the bovine skim milk were determined using the Kjeldahl method according to AOAC International (2006) with total nitrogen conversion factors of 5.83 for oat protein (Maclean et al., 2003) and 6.38 for milk protein (AOAC International, 2006). The protein contents were 12.8% (w/w, as-is basis) for oat flour and 3.84% (w/v) for bovine skim milk. The oat milk was prepared according to the methods described by Deswal et al. (2014b) and Lindahl et al. (1997) with some modifications. Briefly, 500 g of oat flour was soaked and premixed with 1250 g of water using an overhead mixer for 30 min at room temperature. The mixture was then heated at 65 °C for 30 min in a water bath, 1.5 mL of α-amylase was added, and the slurry was incubated at 65 °C for 1 h with continuous agitation. The degradation of starch was confirmed with iodine–KI solution by observing that the color of the KI solution remained yellowish, indicating that the amylose had been eliminated (Laine et al., 2011). The slurry was then filtered through a 100-μm mesh bag to remove any residual oat particles. The filtrate was heated at 100 °C for 10 min to inactivate the α-amylase. Finally, the slurry was cooled to room temperature and the suspension was homogenized at 30/5 MPa using a two-stage valve homogenizer (APV 2000, Copenhagen, Denmark). The resulting oat preparation was termed “oat milk”.

An oat milk–bovine skim milk blend was prepared by mixing 50% of oat slurry and 50% of bovine skim milk on a volume basis. Oat slurry was prepared using the same procedure as described above for the enzymatic incubation, filtration, and heating process. After heating and cooling, the oat slurry and the bovine skim milk were mixed and homogenized at 30/5 MPa using a two-stage valve homogenizer (APV 2000, Copenhagen, Denmark). The resulting mixture was termed "oat milk–bovine skim milk blend".

To prevent undesirable microbial growth, 0.02% (w/v) sodium azide was added to the oat milk and oat milk–bovine skim milk blend samples. All samples were stored at 4 °C for a maximum of 4 days and any unused material was discarded. Water was added to adjust the final protein content to 3% (w/v) prior to the digestion study. A 200 mL oat milk–bovine skim milk blend sample with 3% (w/v) protein content contained ~ 0.52 g of nitrogen from oat protein and ~0.46 g of nitrogen from milk protein.

6.3.3 *In vitro* gastric digestion

A dynamic HGS, designed by Kong and Singh (2010), was used to carry out the gastric phase of *in vitro* digestion. The simulated gastric fluid (SGF, pH 1.5) was prepared as described by Minekus et al. (2014) with slight modifications. Gastric digestion of the oat milk and the oat milk–bovine skim milk blend followed a procedure described in section 3.2.2. The composition of the SGF and the parameters for the *in vitro* gastric digestion are summarized in section 3.2.2. The sampling scheme is illustrated in Figure 6.1.

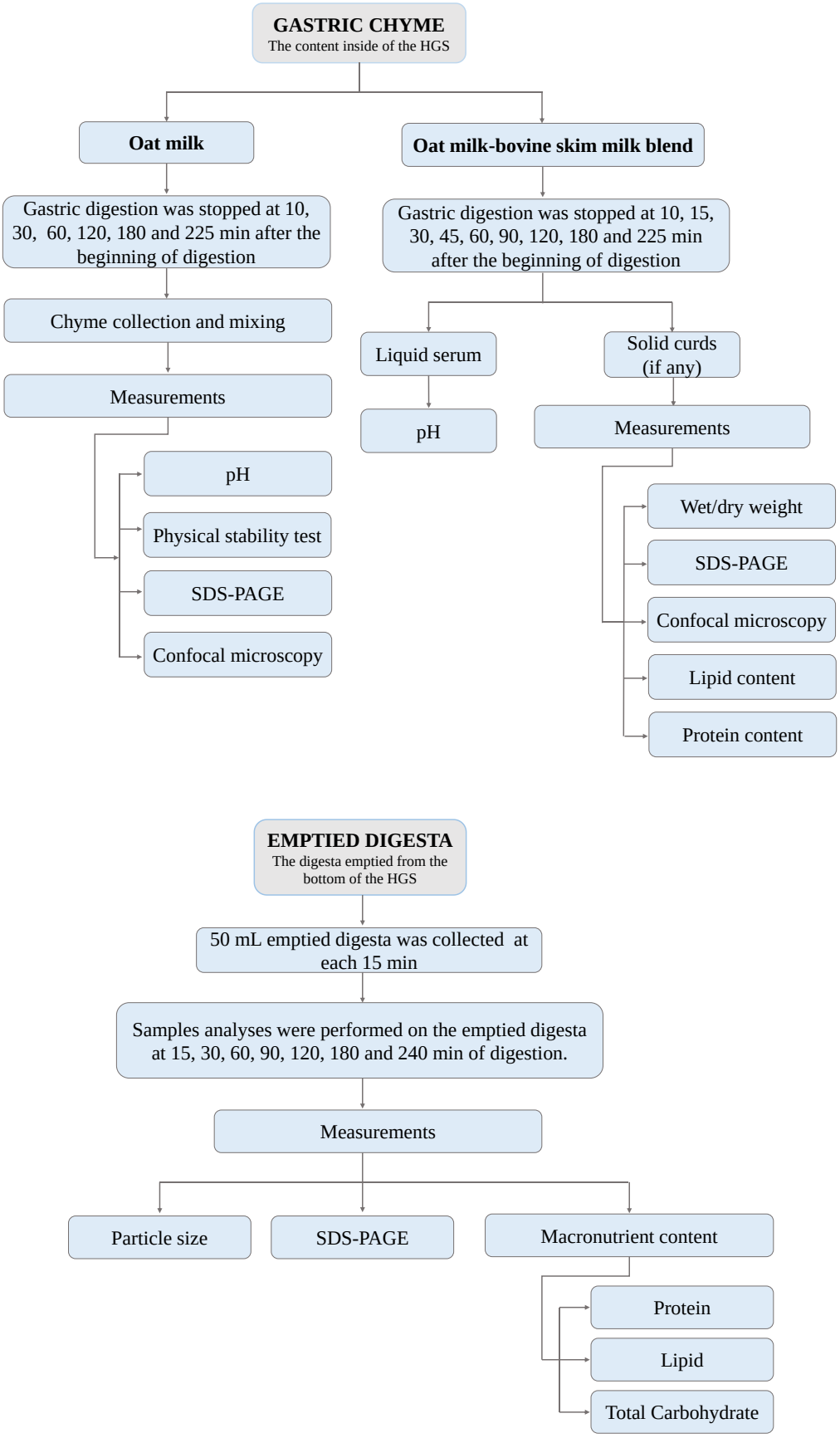


Figure 6.1 *Illustration of the sampling scheme used for analysis.*

6.3.4 Weight of curds

At selected digestion times (see Figure 6.1), the inside contents of the HGS were collected and the gastric chyme was passed through a mesh with a 1-mm pore size to separate the curd/coagulated mass from the aqueous phase. The curd was weighed immediately to obtain a wet weight. The dry weight was determined by drying an aliquot of wet curd sample in a dry aluminium dish at 105 °C in a forced air oven until constant weight.

6.3.5. Chemical analyses

6.3.5.1 Emptied digesta

For the undigested oat milk and oat milk–bovine skim milk blend and their emptied digesta, total solids, lipids, total nitrogen, and ash were determined using an air oven drying method (AOAC International, 2000), the Mojonnier method (AACC method 30–10; AACC, 2000), the Kjeldahl method (AOAC International, 2006), and a gravimetric method (AOAC International, 2002), respectively. The total carbohydrate content was calculated by difference: 100% − (% moisture + % total protein + % lipid + % ash).

6.3.5.2 Curd samples

For chemical analyses, the collected curds were heated at 100 °C for 5 min to stop further enzymatic digestion. The curd samples were freeze dried and then ground into powders using a mortar and pestle. The total protein contents of the curd samples were determined by the Kjeldahl method (AOAC International, 2006). A conversion factor of 6.25 for the digesta and curd samples from oat milk–bovine skim milk blend was used to obtain the protein content from the total nitrogen content (Maclean et al., 2003). The lipid

content of the curds was measured using the Mojonnier method as described in section 3.2.7.

6.3.6 pH measurement

The initial pH refers to the pH of the oat milk and the oat milk–bovine skim milk blend before digestion. To measure the gastric pH at selected times, the digestion experiments were terminated and the gastric content (i.e., chyme) was collected from the inside of the HGS and mixed well using a stirring bar. For the oat milk–bovine skim milk blend samples, the pH values correspond to the pH of the serum phase of the chyme.

6.3.7 Physical stability of oat milk

A short-term storage stability analysis was performed on the oat milk samples only as described in section 3.2.4 as coagulation occurred in the oat milk–bovine skim milk blend.

6.3.8 Identification of proteins by SDS-PAGE

The protein fractions of chyme samples of the oat milk, curd samples of the oat milk–bovine skim milk blend, and the emptied digesta samples of both were characterized by SDS-PAGE under reducing conditions. To stop the enzymatic digestion by pepsin, the liquid chyme and emptied digesta samples were raised immediately to pH 7.5 using 10 M NaOH and the solid curd samples were heated at 100 °C for 5 min after collection. The solid curd samples were then freeze dried and ground. To eliminate the effect of dilution by the simulated gastric secretion, the digesta samples were diluted with different amounts of sample buffer to achieve equal protein concentrations. The ground curd samples were dissolved in the sample buffer overnight under gentle shaking. The sample buffer consisted of 13% (v/v) 0.5 Mol/L Tris-HCl buffer, 10% (v/v) glycerol, 2% (w/v) SDS, 0.04% (w/v) bromophenol blue, and β-mercaptoethanol (19:1, v: v), at pH 6.8. The

buffered samples were heated in a boiling water bath for 5 min and cooled to room temperature. An 8 μL aliquot of the solutions was loaded on to SDS-PAGE gels previously prepared on a Mini-PROTEAN II system. The gels were prepared at 15% (w/v) acrylamide concentration for the resolving gel and at 4.0% (w/v) acrylamide concentration for the stacking gel. The electrophoresis was performed at a constant voltage (110 V) for approximately 80 min. After staining and destaining, the gels were scanned using a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA, USA). Bio-Rad Dual Xtra protein standards (Bio-Rad Laboratories) were loaded for estimation of the molecular weight. The relative amount of protein was quantified for the curd samples by analysing the intensity of each band using Image Lab™ software version 5.2 (Bio-Rad Laboratories).

6.3.9 Confocal laser scanning microscopy

A confocal laser scanning microscope (Leica SP5 DM6000B, Leica Microsystems, Heidelberg, Germany), fitted with a 63 × oil immersion lens, was used to investigate the microstructure of the undigested samples and to follow the changes that occurred during gastric digestion. For liquid samples (from oat milk), the gastric contents in the HGS (i.e., the chyme) were examined as the method prescribed in section 3.2.6. For curd samples (from the oat milk–bovine skim milk blend), the curd fractions were transferred to an Eppendorf tube after sieving using a spatula and mixed with staining solutions. The staining procedure as described in section 3.2.6 was followed. A solution of 1.0% (w/v) Fast Green was used to stain protein (helium–neon laser with excitation at a wavelength of 633 nm). Nile Red (0.1% w/v in acetone) was used to stain the oil phase (argon laser with excitation at a wavelength of 488 nm). The stained samples were then placed on double concave microscope slides. Multiple fields were viewed, and images were stored with 1024 × 1024-pixel resolution using the microscope's software.

6.3.10 Particle size measurements

Particle size distributions of the undigested samples and the emptied digesta were measured using a Masterziser 2000 (Malvern Instruments, Malvern, Worcestershire, UK). A refractive index of 1.456 (with an absorbance value of 0.001) was used for the dispersed phase and of 1.33 for water. The particle size values were characterized by the volume-weighted average diameter ($d_{4,3}$), which is sensitive to the presence of large particles. Mean particle diameters and standard deviations were calculated as the average of triplicate measurements on individual samples.

6.3.11 Statistical analysis

The results were expressed as the mean ± standard deviation of at least three digestion experiments performed independently. A repeated-measures two-factor analysis of variance (ANOVA) model, with the *in vitro* replication as the experimental unit, was performed for the protein, lipid, and total carbohydrate contents of the emptied digesta using the MIXED model procedure of the statistical software R (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria). The statistical linear mixed model included different samples (oat milk or oat milk–bovine skim milk blend), time (0–240 min), and their interaction as a fixed effect, but with replication as a random effect. For the other response variables (pH and particle size), a two-factor ANOVA without repeated-measures analysis was conducted. For the wet weight, dry weight, and lipid content of the curds, and SDS-PAGE quantification, a single-factor ANOVA was performed. The correlations between protein and lipid in the curds were also determined. If the F value of the overall model was significant ($P < 0.05$), post-hoc tests were conducted using Tukey's range test and significance was taken at $P < 0.05$.

6.4 Results and discussion

6.4.1 Gastric pH profile

The undigested oat milk contained 3.0% (w/v) protein and 1.5% (w/v) lipid, and the undigested oat milk–bovine skim milk blend contained 3.0% (w/v) protein and 0.76% (w/v) lipid. The initial pHs of the undigested oat milk and the undigested oat milk–bovine skim milk blend were 6.26 and 6.62, respectively. The changes in the gastric pH during digestion were significantly affected by the digestion time ($P < 0.001$), the type of milk system (i.e., oat milk or oat milk–bovine skim milk blend, $P < 0.001$), and their interaction ($P < 0.001$) (Figure 6.2). The pH of the oat milk samples declined more rapidly than that of the oat milk–bovine skim milk blend samples, particularly over the pH range 5–3. This difference was attributed to the buffering capacity of milk proteins, especially casein micelles (Brückner-Gühmann et al., 2019). After about 180 min of digestion, the final pH was about 2 for both milk samples. The profile of the gastric pH changes with the digestion time of the oat milk–bovine skim milk blend was similar to that of soymilk – bovine milk blends (1:1, v/v) reported previously by Wegrzyn et al. (2021).

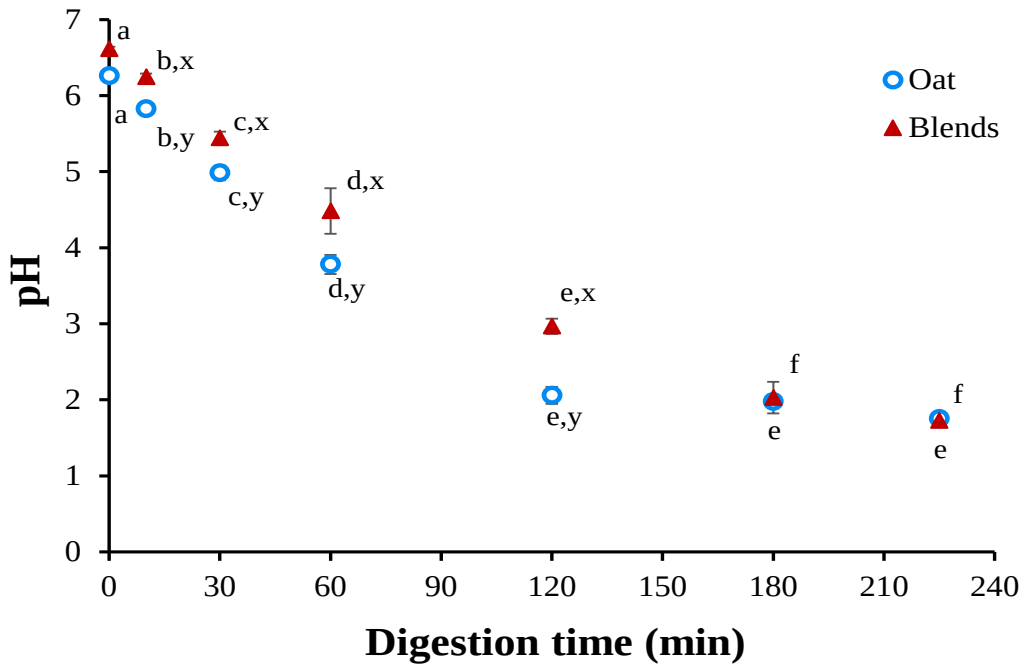


Figure 6.2 *Changes in the pH of oat milk and oat milk–bovine skim milk blend samples during dynamic gastric digestion.*

Note. The pH values refer to the initial (before digestion) sample and the gastric chyme samples in the HGS. The measurements were replicated at least three times. Error bars represent standard deviations. Values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) within a certain sample (oat milk or oat milk–bovine skim milk blend) across different digestion times. Values with no letter in common (x, y) represent significant differences at a certain digestion time across different samples (oat milk or oat milk–bovine skim milk blend). If no letter is listed, there were no significant differences.

6.4.2 Structural changes in the oat milk and the oat milk–bovine skim milk blend during gastric digestion

The gastric digestive behaviour s of the oat milk and the oat milk–bovine skim milk blend differed considerably. During the entire 4 h of gastric digestion, the oat milk sample remained liquid, and there was no visible destabilization at the top of the HGS. To further understand the physical stability of oat milk during gastric digestion, a short-term storage stability experiment was conducted at room temperature (Figure 6.3). No significant creaming or coagulation was observed at different digestion time points,

except that the chyme sample collected at 120 min of digestion tended to sediment after being held for 30 min. It was also noticed that only transparent liquid remained in the HGS after 120 min of digestion, indicating that the majority of the oat milk had been emptied from the HGS.

The oat milk–bovine skim milk blend produced a visible curd and a liquid fraction after about 10 min of digestion when the pH was about 6.2. The curd samples removed from the HGS at different times were filtered through a sieve with a 1-mm pore size; the appearance of the retained curds is shown in Figure 6.4. These curds consisted initially of many loose small soft particles (Figure 6.4, 10 and 15 min). After 15 min of digestion, the wet weight and the dry weight of the curds achieved maximum values of about 27 and 7 g, respectively (Figure 6.5A and 6.5B). To understand the factors responsible for the initial coagulation at around pH 6.2, two control experiments were conducted. Firstly, a digestion experiment without added pepsin was performed on the same blend sample. Protein coagulation was observed at 60 min when the gastric pH was approximately 4.7 (Appendix 4, Figure A2), which is close to the isoelectric point of casein. This indicated that the initial coagulation at pH 6.2 was the result of the action of pepsin. In another control experiment, the bovine skim milk was diluted to the same protein concentration as that of the milk protein in the blend sample (i.e., 1.45%). This diluted bovine skim milk was subjected to digestion under the same conditions (in the presence of pepsin) to examine the coagulation ability of milk protein at this concentration. The results showed that curd formed after 10 min (see Appendix 5, Figure A3) when the pH was around 6.35. These results confirmed that the initial destabilization of the blend samples that occurred at about pH 6.2 was induced by coagulation of the casein micelles because of the action of pepsin (Ye et al., 2016a). With further digestion, the coagulum presented a denser and more compact structure (Figure 6.4, 30–120 min), but the weight declined progressively

(Figures 6.5A and 6.5B). This was due to the combined effects of pH decrease, pepsin hydrolysis and the mechanical mixing in the HGS. After 180 min, no curd particles with size >1 mm were obtained.

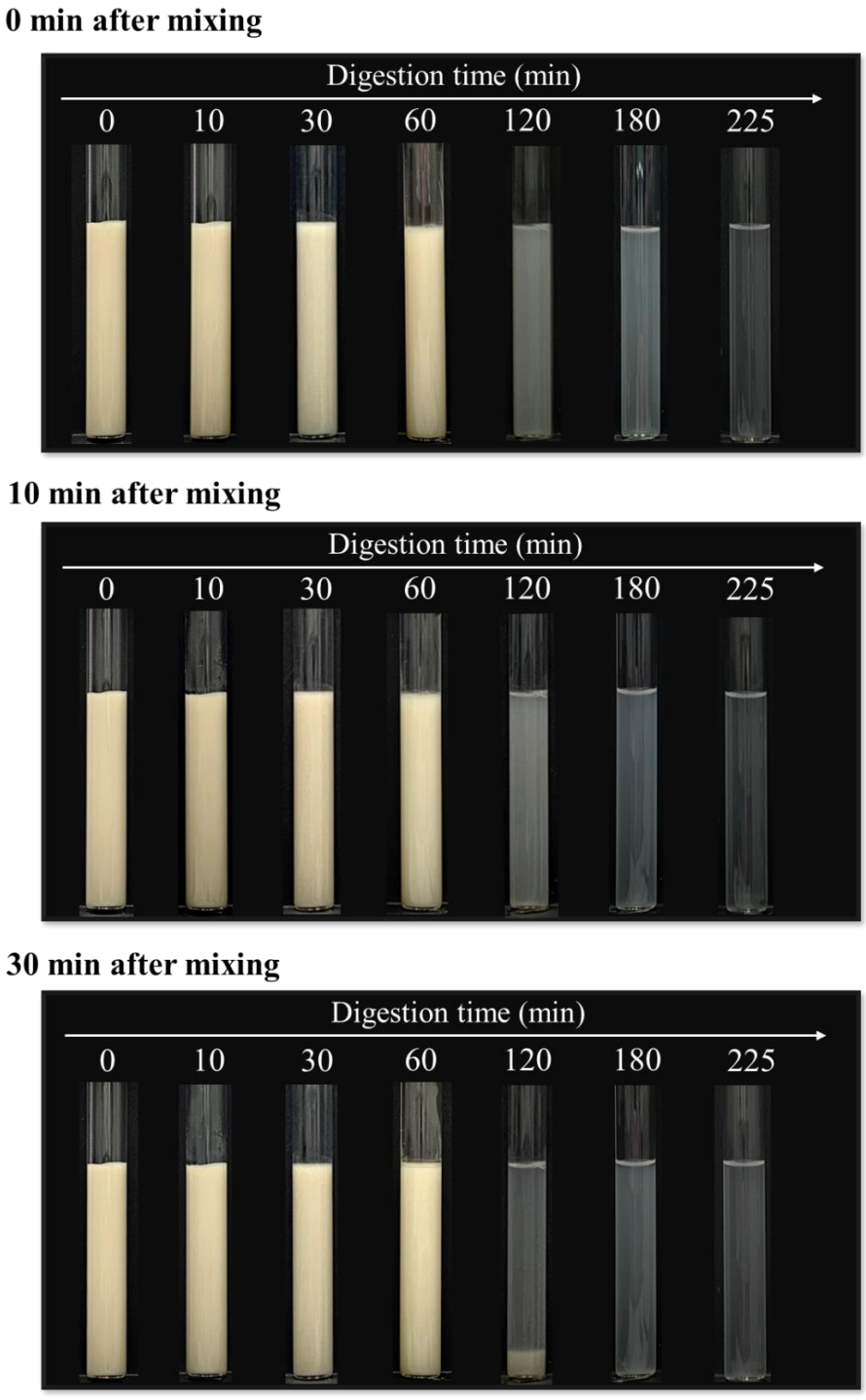


Figure 6.3 *Changes in the physical state of the gastric chyme taken from oat milk in the HGS at different digestion times and sedimentation stability during storage.*

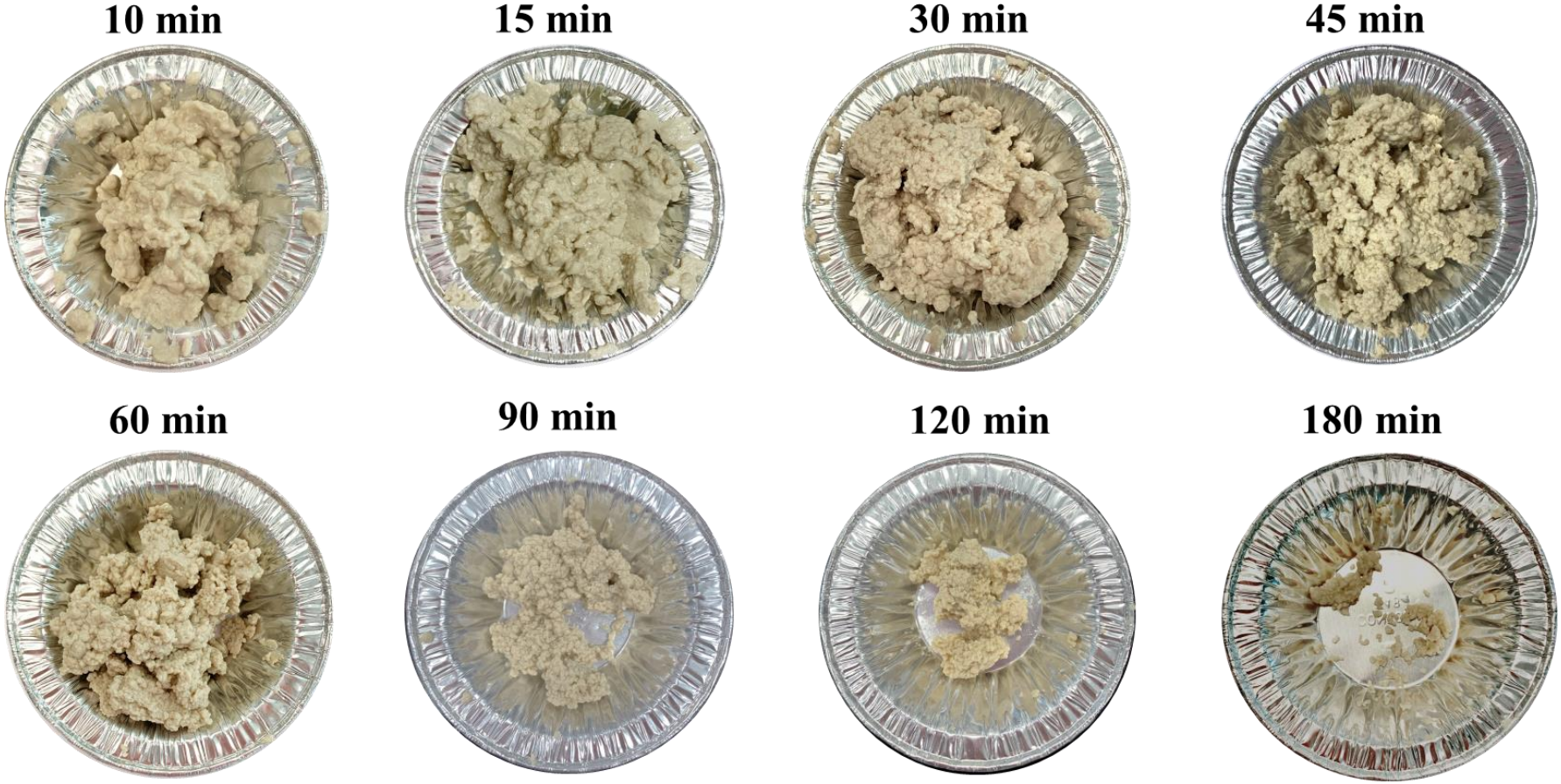


Figure 6.4 *Photographs of the curds formed in the oat milk–bovine skim milk blend during gastric digestion. The diameter of the aluminium tray is 7.3 cm.*

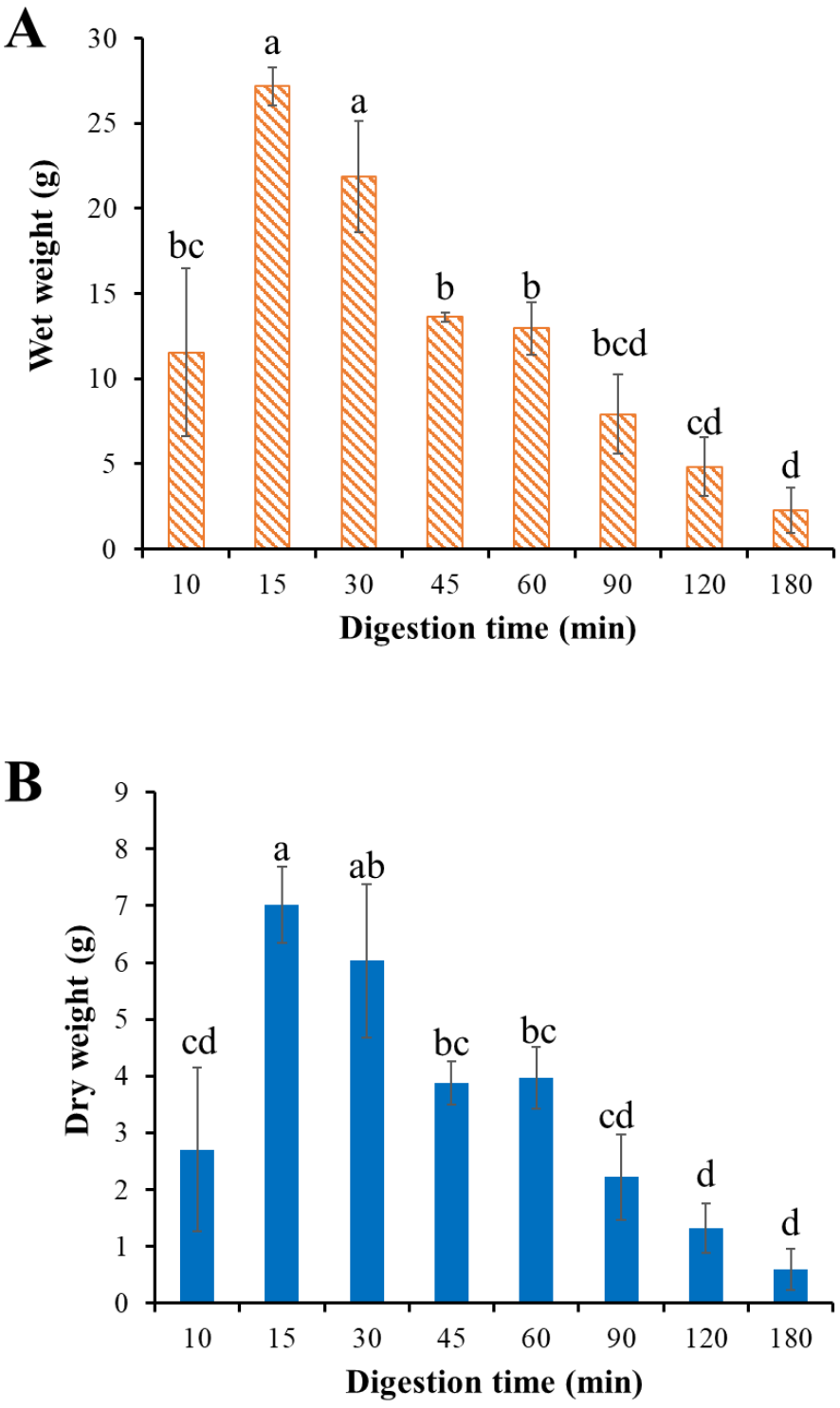


Figure 6.5 *(A) the wet weight and (B) the dry weight of the curds formed in the oat milk–bovine skim milk blend during gastric digestion at different time points.*

6.4.3 Protein hydrolysis

The protein hydrolysis during digestion was monitored using SDS-PAGE under reducing conditions (Figures 6.6 and 6.7). Samples in each lane were adjusted to the same protein concentration to eliminate the effect of dilution because of the gradual dosing of SGF during the digestion. Figure 6.6 revealed bands corresponding to several polypeptides in the undigested oat milk. Two major bands with molecular weights of 32 and 22 kDa were acidic (12S-A) and basic (12S-B) polypeptides of 12S globulin, respectively, which are the major storage protein fractions in oats (Burgess et al., 1983). The 12S-A and 12S-B subunits are considered to be linked via disulfide bonds in the native state and to form a dimer with a molecular weight of 54 kDa (Burgess et al., 1983). The faint bands around 65 kDa and below 15 kDa corresponded to the 7S and 3S fractions, respectively, which are two minor protein fractions in oats (Klose & Arendt, 2012). During the first 60 min of digestion, the 7S, 12S-A, and 12S-B bands became slightly less intense, indicating that protein hydrolysis by pepsin was slow during this period. From 120 min onwards, oat protein bands were mostly absent, indicating that the oat proteins had been digested by pepsin. It is noted that the optimum acidity for pepsin activity is about pH 2 (Axelsson et al., 1983). At an acidic pH, the oat proteins 12S-A and 12S-B would be partially unfolded and readily susceptible to pepsin hydrolysis (Brinegar & Peterson, 1982; Burgess et al., 1983). Rapid proteolysis of oat proteins by pepsin at pH 2 has also been observed previously by Nieto-Nieto et al. (2014).

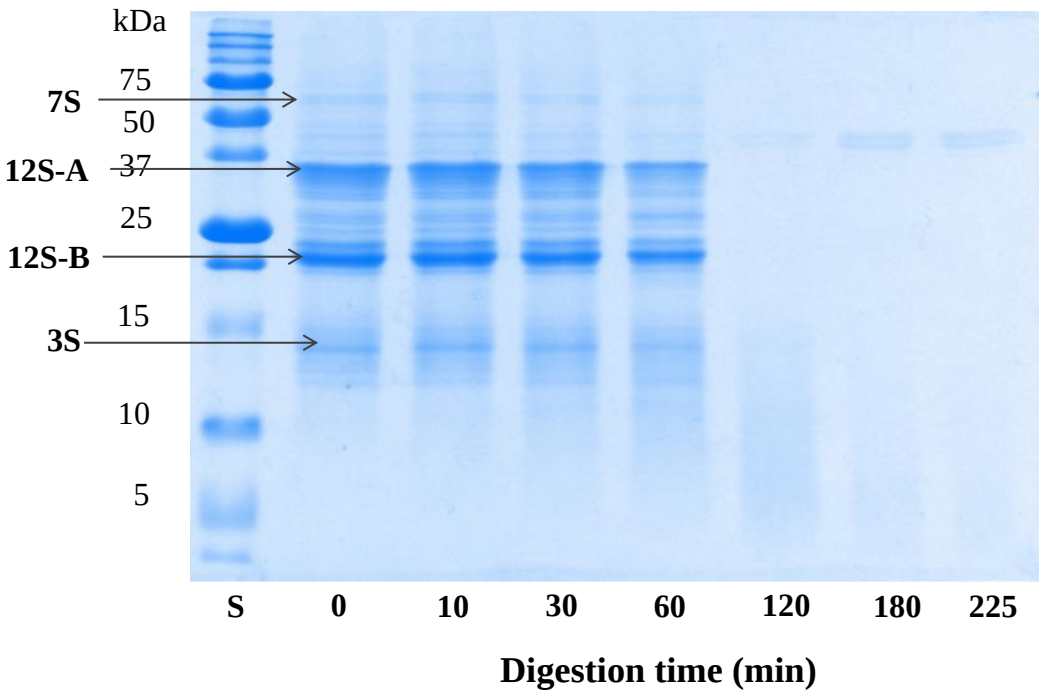


Figure 6.6 *SDS-PAGE (under reducing conditions) of initial (before digestion) and digested (i.e., gastric chyme) of oat milk samples at different time points during gastric digestion in the HGS.*

Note. S = molecular weight standard.

The curd samples from the oat milk–bovine skim milk blend were similarly analyzed using SDS-PAGE (Figure 6.7A) and the proportions of some major proteins in the curds were quantified as a function of the digestion time (Figure 6.7B). The undigested blend sample (0 min) had several major bands corresponding to oat proteins 12S-A and 12S-B and milk proteins, αs1-casein, αs2-casein (αs-CNs), β-CN, κ-CN, β-lactoglobulin (β-lg), and α-lactalbumin (α-la). After 10 min of digestion, the κ-CN band was very faint and a new band at around 15 kDa that corresponded to para-κ-CN was present, which confirmed that the early coagulation was induced by the hydrolysis of κ-CN. In the first 30 min of digestion, the protein profiles of the curds were found to be similar (Figure 6.7A). However, at 60 and 90 min, there was a relatively faster decrease in the band intensities of the αs-CNs and β-CN than of 12S-A and 12S-B ($P < 0.05$, Figure

6.7B), indicating a more rapid hydrolysis of the milk proteins than of the oat proteins at pHs between 4.5 and 3.5. After 120 min, the intensities of all protein bands diminished and there were many low molecular weight peptides (<15 kDa), suggesting extensive proteolysis of both milk proteins and oat proteins by pepsin at about pH 2.

Interestingly, the curd sample contained not only milk protein (i.e., αs-CNs, β-CN, and para-κ-CN) bands but also oat protein (12S-A and 12S-B) bands (Figure 6.7A, 10 min). This suggested that specific oat proteins were also incorporated into the curd particles, which may have been due to the interactions between milk proteins and oat proteins, including the oil body membrane proteins. Some of these interactions may have occurred during homogenization of the blend. Homogenization reduces the size of oil bodies and consequently increases the surface area of the droplets (Gallier et al., 2017). Consequently, both milk proteins (caseins and whey proteins) and oat proteins can adsorb on to the surface of emulsified droplets due to the protein amphipathicity (Dalgleish, 1996; Jiang et al., 2015; Srinivasan, 2007), although oat proteins have been reported to have poor emulsifying properties at neutral pH (Ma, 1983; Ma & Harwalkar, 1984; Zhang et al., 2015). It was also noticeable that the incorporation of the oat proteins in the casein matrix (i.e., curds) (Figure 6.7A) delayed the breakdown of the oat protein compared with that of the protein in the oat milk alone (Figure 6.6), implying that the oat proteins in the curds were protected from attack by the added pepsin, which essentially can act only on the surface of the particles.

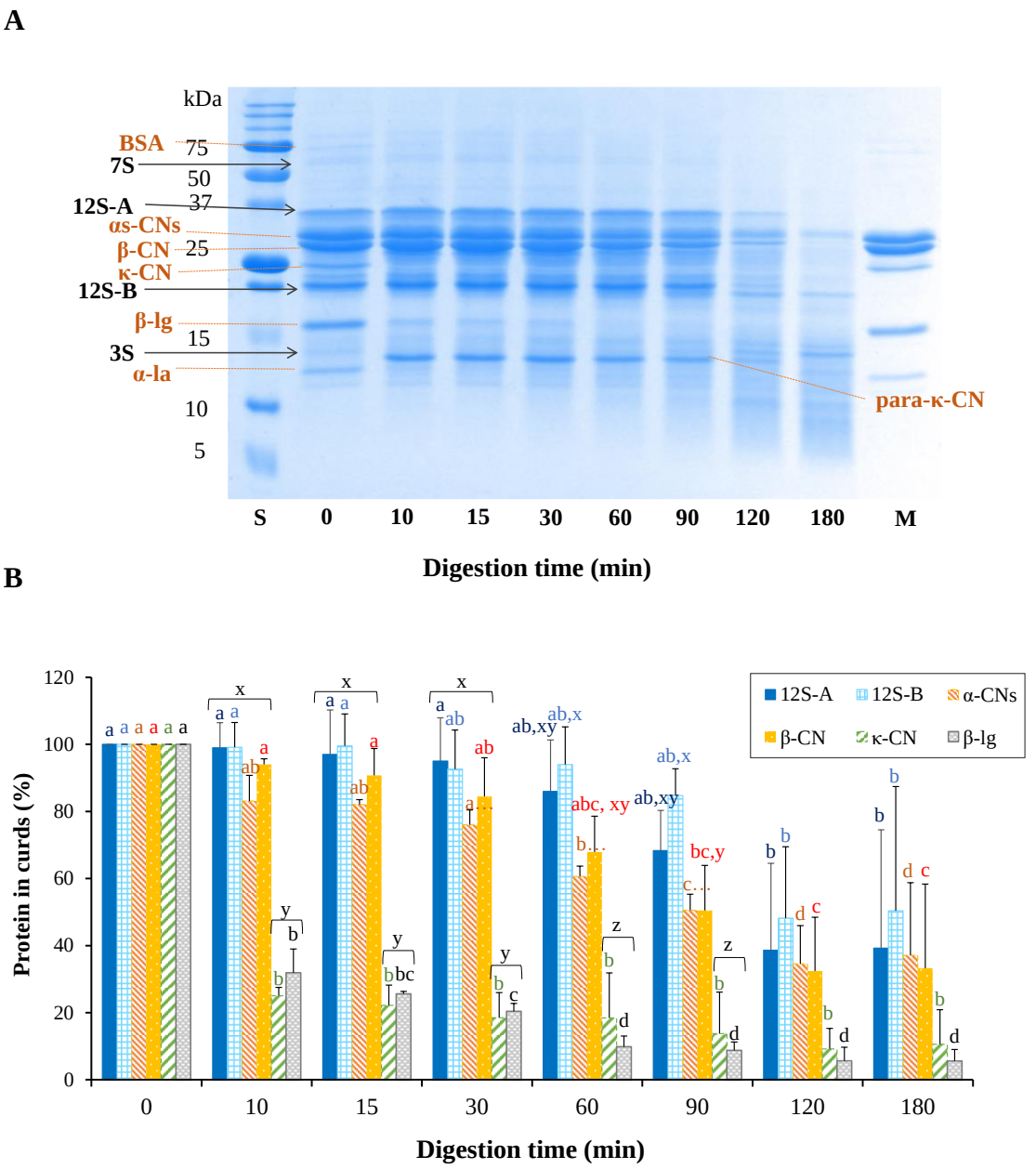


Figure 6.7 *(A) SDS-PAGE (under reducing conditions) of initial (before digestion) and curd samples formed in the oat milk–bovine skim milk blend at different time points during gastric digestion in the HGS, and (B) relative protein contents of the curd samples in the oat milk–bovine skim milk blend.*

Note. S = molecular weight standard; M = trim milk; CN = casein; lg = lactoglobulin. Values with no letter in common (a, b, c, d) represent significant differences ($P < 0.05$) within a certain protein (12S-A, 12S-B, αs-CNs, β-CN, κ-CN, β-lg) across different digestion times. Values with no letter in common (x, y) represent significant differences ($P < 0.05$) at a certain digestion time across different proteins (12S-A, 12S-B, αs -CNs, β-CN, κ-CN, β-lg). If no letter is listed, there were no significant differences.

6.4.4 Microstructural changes occurring during gastric digestion

Confocal microscopy was used to monitor the microstructural changes in the gastric chyme samples during gastric digestion. The undigested oat milk consists of many free oil bodies and large protein–oil body aggregates in the aqueous phase (Figure 6.8A1, Peter & Ton, 2007), which is consistent with previous observations by Mäkinen et al. (2015). No significant structural changes were observed over 4 h of digestion, but the relative proportions of protein aggregates and oil bodies observed decreased over time, particularly after 120 min. This is in agreement with visual observation (Figure 6.3) and SDS-PAGE results (Figure 6.6), where only transparent liquid remained in the HGS and no protein bands were observed in the chyme.

Similarly for the oat milk–bovine skim milk blend, the undigested samples contained large protein–oil body aggregates and many free oil bodies (Figure 6.8B1). Figures 6.8B2–6.8B6 reveal the microstructures of the curd samples generated in the blend. Protein coagulation was observed at 10 min (Figure 6.8B2), which was consistent with the curd formation in the HGS that was observed visually (Figure 6.4). The curd network consisted of a loose, irregular protein matrix with many tiny oil bodies attaching to the surface of or being embedded in the protein matrix (Figure 6.8B2). Figures 6.8B2–6.8B6 indicate little change in the microstructures of the curd samples, except that the protein networks seemed to be more open and looser at 180 min (Figure 6.8B6). This observation of the loose structure at 180 min agrees with the SDS-PAGE results (Figure 6.7A), in which most proteins have been hydrolyzed to peptides, contributing the breakdown of protein network.

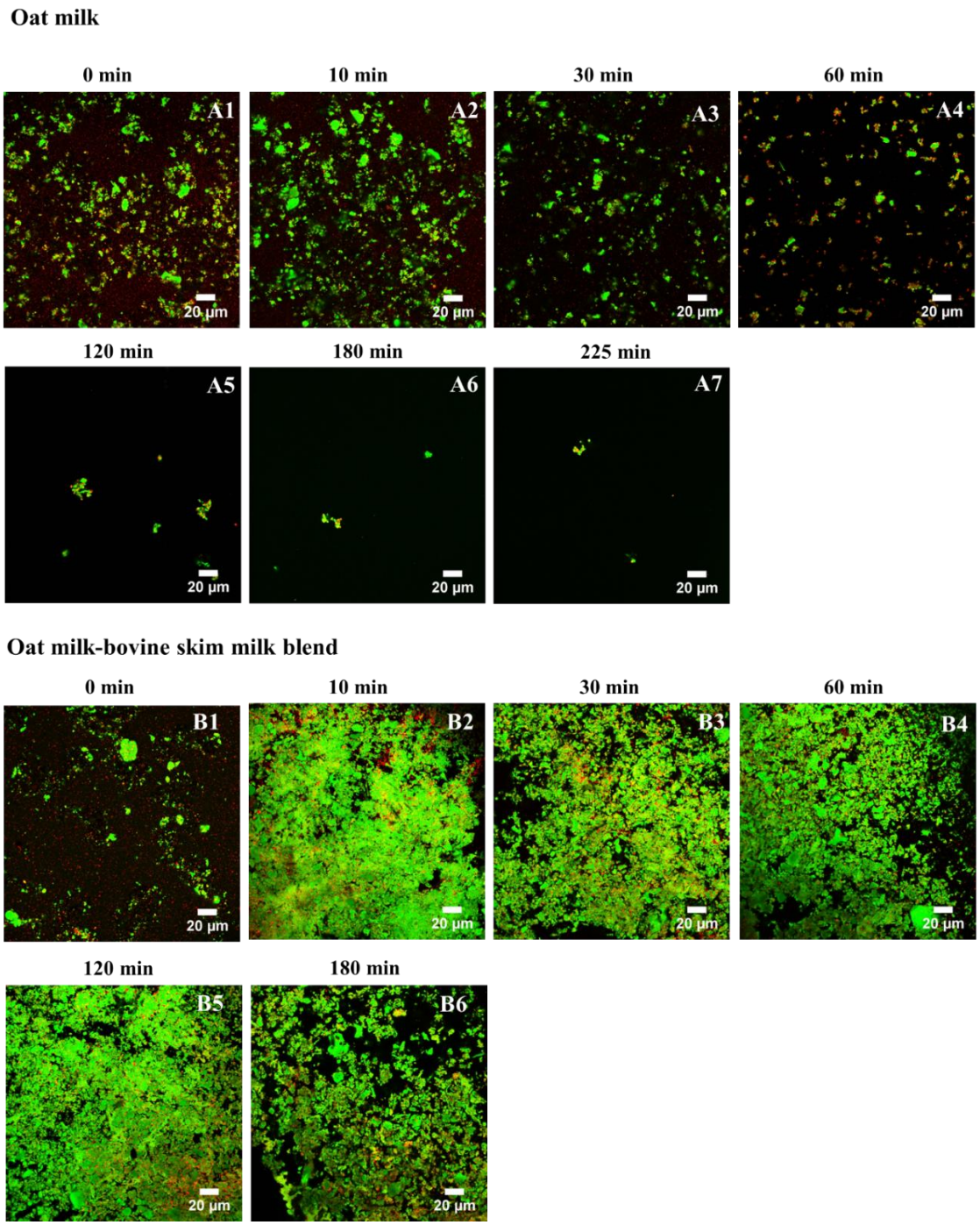


Figure 6.8 *Confocal micrographs showing the microstructural changes in (A) oat milk samples and (B) oat milk–bovine skim milk blend samples that occurred at different times of simulated gastric digestion in the HGS.*

Note. Samples were stained with Nile Red (for lipid) and Fast Green (for protein). The scale bar in all images is 20 μm.

6.4.5 Curd structure and release of oil bodies during digestion of the oat milk–bovine skim milk blend

As shown in Figure 6.8B, the majority of the oil bodies were embedded within the curd structure. To understand the influence of the curd structure on the behaviour of the oil bodies, the lipid content of the curds was determined as a function of the digestion time (Figure 6.9A). The undigested (initial) blend sample contained around 3% protein and 0.76% lipid (i.e., ~6 g of protein and 1.53 g of lipid in a 200 mL sample). The changes in the lipid content of the curd over the digestion time followed a similar pattern to that of the dry weight (Figure 6.5B). At 15 min of digestion, the curds contained a maximum of 0.66 g of lipid, which accounted for approximately 43% of the total lipid content in the blend. This confirmed that many, but not all, oil bodies were incorporated in the curd. As the digestion progressed, the lipid content of the curds decreased ($P < 0.05$), along with the reduction in the weight of the curds (Figures 6.5A and 6.5B), suggesting that the migration of lipids from the curds into the continuous phase was governed by curd breakdown processes.

The relationship between the lipid and protein contents remaining in the curds at different digestion times is shown in Figure 6.9B. A linear regression model was used to fit the protein and lipid contents of all curd samples at all digestion time points. The regression equation obtained was: predicted lipid content = −0.44 + 0.96x (protein content) (Figure 6.9B, $R^2 = 0.9747$, $P < 0.001$). The Pearson correlation test indicated that the decrease in the lipid content of the curds was strongly correlated with a decrease in the protein content remaining in the curds, $r = 0.99$, $P < 0.001$. This result indicated that the rate of release of the oil bodies was positively linked to the rate of curd breakdown, which in turn was determined by the extent of protein hydrolysis. These results are consistent with previous studies (Roy et al., 2021; Ye et al., 2017). Although no information on the

gastric coagulation of oat milk–bovine skim milk blends is currently available, our results indicate that there is a strong relationship between lipid release and curd breakdown, which is similar to the behaviour of bovine whole milk.

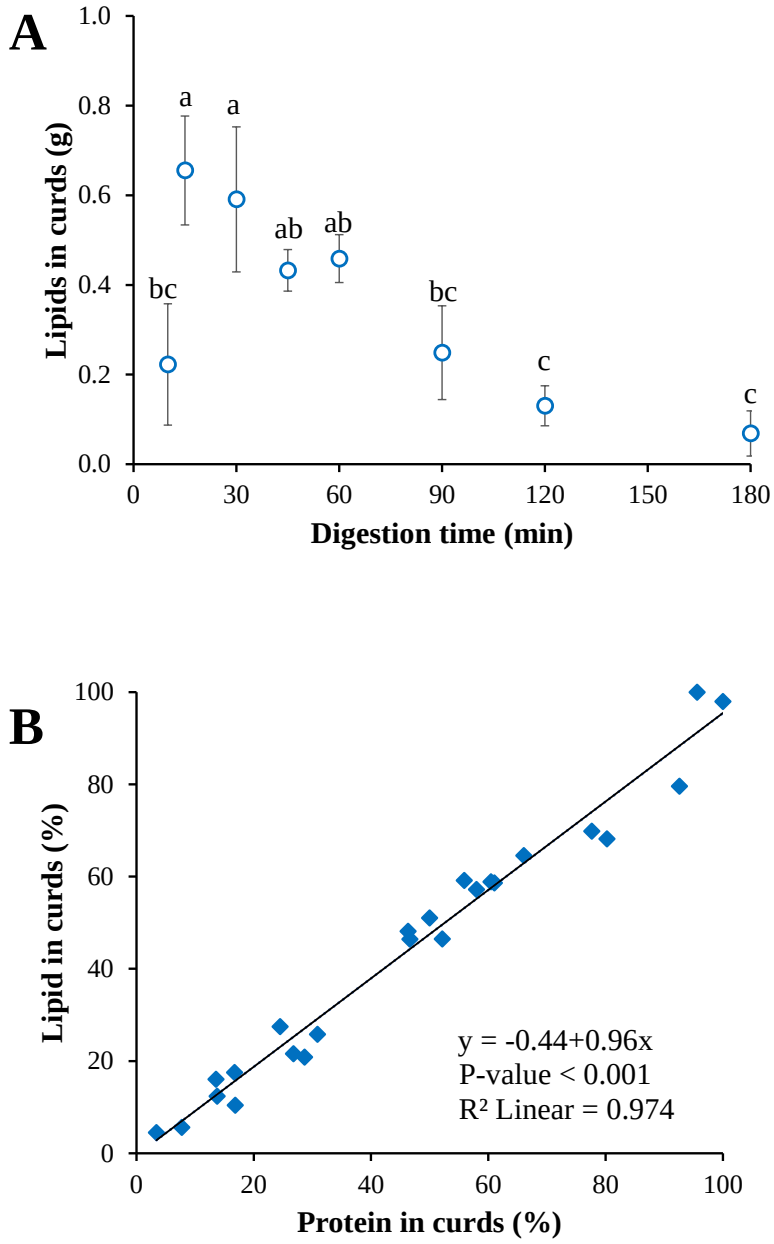


Figure 6.9 (A) *Changes in the weight of lipid retained in the curds during gastric digestion in the HGS and (B) relationship between the lipid and protein contents retained in the curds during gastric digestion in the HGS from 10 to 180 min.*

Note. Values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) in the weight of lipids across different digestion times.

6.4.6 Physicochemical characteristics of emptied gastric digesta

The gastric contents were emptied from the bottom of the HGS at different digestion times and collected after sieving. The emptied digesta represented the material that enters the next digestion step (i.e., the intestinal phase). The gastric environment had significant impacts on: (1) the particle size of the emptied digesta; (2) the protein composition of the emptied digesta; (3), the rate of delivery of proteins, lipids, and carbohydrates.

6.4.6.1. Particle size of the emptied digesta

Before digestion, the volume mean particle diameter ($d_{4,3}$) of the oat milk was about 8.3 μm (Figure 6.10A). Over 4 h of gastric digestion, $d_{4,3}$ did not show any significant variation ($P > 0.05$, Figure 6.10A). In contrast, the $d_{4,3}$ of the oat milk–bovine skim milk blend increased markedly, from 7.1 μm initially to 80 μm after 15 min of digestion (Figure 6.10B). The corresponding particle size distribution shifted from 0.04–158 μm at 0 min to a much larger size range of 0.8–1000 μm at 15 min (Figure 6.10B, 15 min). With further increases in the digestion time, the $d_{4,3}$ consistently increased and reached around 208 μm at 90 min but it gradually decreased thereafter (Figure 6.10B). The considerable volume of large particles observed in the oat milk–bovine skim milk blend was due to the presence of protein aggregates/curd particles that were small enough to pass through the sieve. The confocal microscope images also confirmed that curd particles/protein aggregates were present in the emptied digesta of the blend sample (shown in Appendix 6, Figure A4). Significant differences in the particle sizes of the emptied digesta from the oat milk samples and the oat milk–bovine skim milk blend samples could have potential implications for their subsequent digestion in the small intestine. It has been reported that the rate and the extent of *in vitro* intestinal lipid digestion is influenced by the structure and the size of the gastric digesta entering the

small intestine. Emptied digesta with larger particle sizes have a slower rate and a lesser extent of lipid hydrolysis than those with smaller particle sizes (Wang et al., 2019).

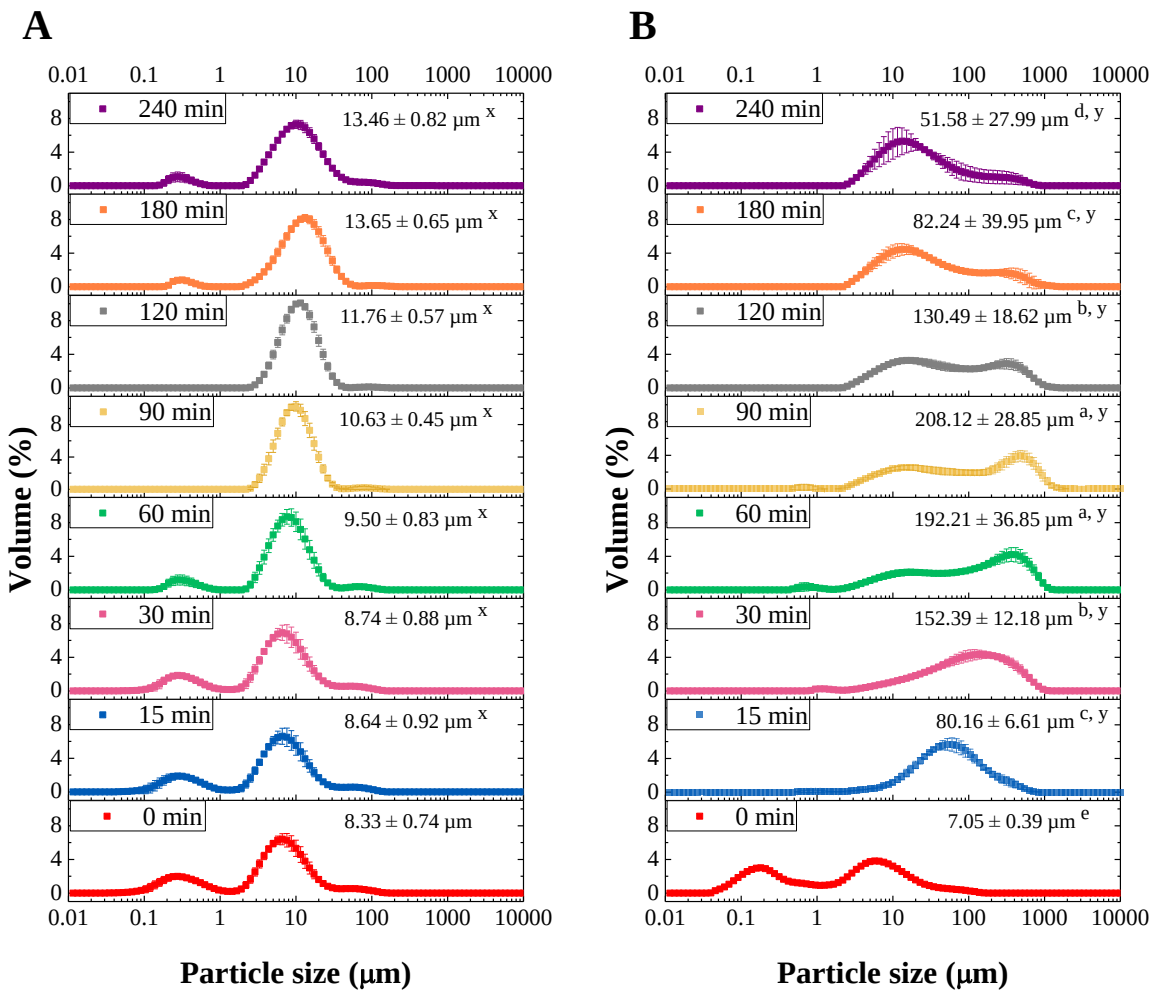


Figure 6.10 *Changes in particle size distribution of the emptied digesta collected from (A) oat milk samples and (B) oat milk–bovine skim milk blend samples during gastric digestion in the HGS.*

Note. Values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) within a certain sample (oat milk or oat milk–bovine skim milk blend) across different digestion times. Values with no letter in common (x, y) represent significant differences at a certain digestion time across different samples (oat milk or oat milk–bovine skim milk blend). If no letter is listed, there were no significant differences.

6.4.6.2. Protein composition of the emptied digesta

The protein compositions of the digesta emptied from the HGS at different times were determined using SDS-PAGE (Figures 6.11 and 6.12). To observe the protein composition without the effect of gastric dilution, the proteins in each lane in Figures 6.11 were standardized to equal concentrations. In the oat milk samples, the major protein bands did not change during the first 30 min, indicating that little hydrolysis occurred at high pH (pH > 5.5) (Figure 6.11A). After 90 min of digestion, no intact protein bands could be observed; instead many small molecular weight peptides were present. This indicated that rapid protein hydrolysis by pepsin occurred during this period, because of an increase in pepsin concentration and a decrease in pH, which is consistent with the protein composition of the gastric chyme in the HGS (Figure 6.6).

For the emptied digesta of the blend samples, intact β-lg and α-la were present in the early stages of digestion (Figure 6.11B). From 90 min onwards, α-la was no longer detectable, probably because it had been hydrolyzed by pepsin (Miranda et al., 1989). β-lg was retained for a longer digestion time, although the band became less intense in the later stages of digestion, which was probably due to its resistance to pepsin hydrolysis. The disappearance of the κ-CN band along with the presence of the para-κ-CN band was observed at 15 min; this was responsible for the formation of curds in the HGS (Ye et al., 2016a). There was a considerable decrease in the band intensities of both αs-CNs and β-CN in the emptied digesta at 15 min, probably because the formation of curds in the HGS had slowed down the gastric emptying of the caseins. Nevertheless, faint casein bands were still observed in the early stages of digestion, probably because of the specific structure of the curd particles (Figure 6.4). A few smaller curd particles (diameter <1 mm) in the liquid phase also escaped from the HGS (Appendix 6, Figure A4). Bands corresponding to the major oat storage proteins 12S-A and 12S-B were observed in the

first 90 min but were no longer detectable thereafter, indicating that any oat 12S in the emptied digesta had been hydrolyzed by pepsin.

The SDS-PAGE gels without standardization of the protein concentrations in each lane are shown in Figures 6.12A and B. The relative concentrations of the oat proteins 12S-A and 12S-B were quantified (Figure 6.12C). Interestingly, we found that the involvement of milk proteins in the oat-based milk sample (Figure 6.12B) modified the gastric emptying rate of the oat proteins relative to that in the oat milk sample (Figure 6.12A). It was noticeable that, at 15 min, 12S-A and 12S-B in the oat milk–bovine skim milk blend had a delayed emptying ($P < 0.05$) compared with those in the oat milk, implying that the oat protein was indeed interacting with the casein matrix. Blending bovine skim milk with oat milk not only modified the protein composition of the emptied digesta but also controlled the rate of delivery of some specific proteins.

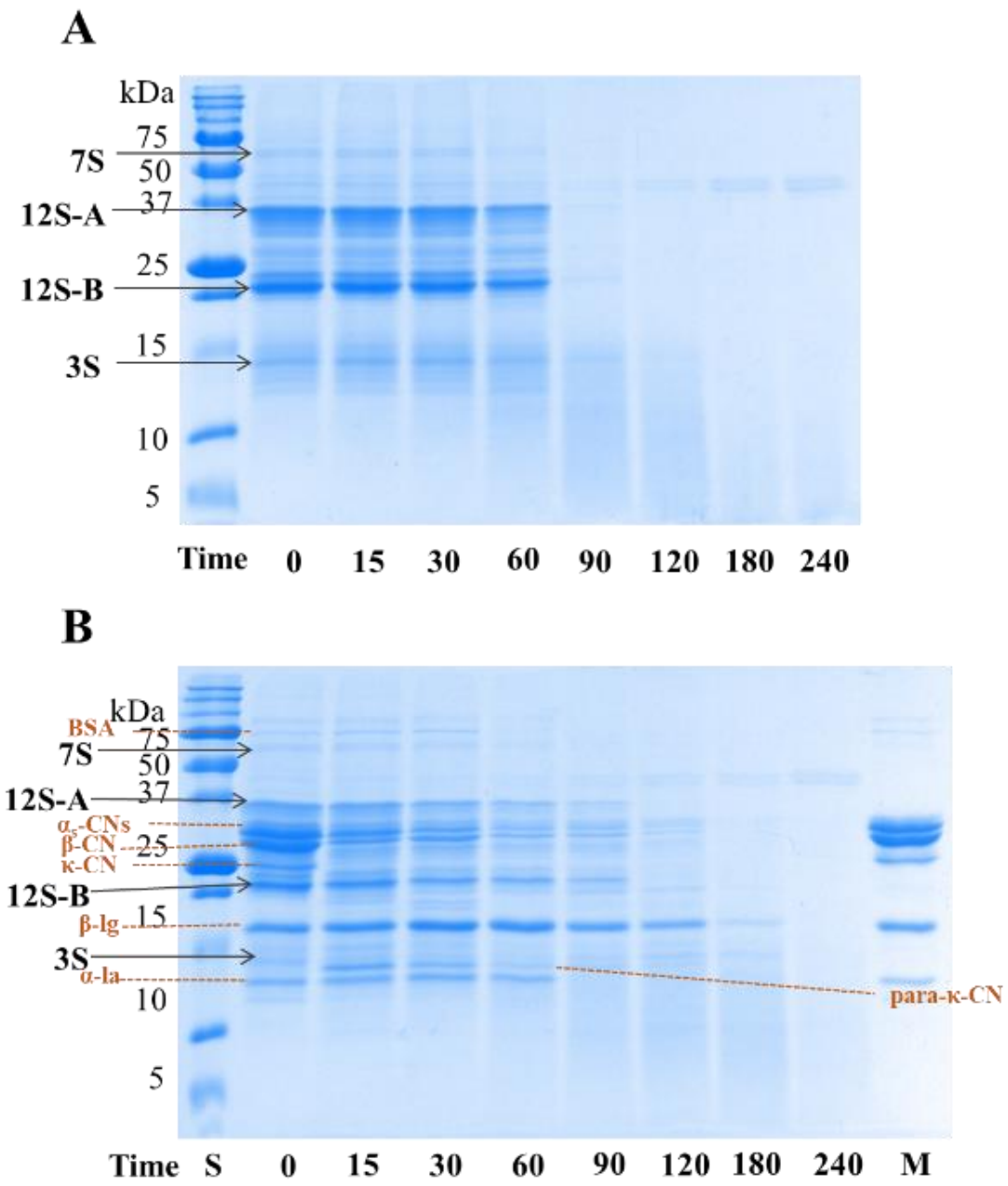


Figure 6.11 *SDS-PAGE (under reducing conditions) of the initial (before digestion) and emptied digesta collected from (A) oat milk samples and (B) oat milk–bovine skim milk blend samples at different time points during gastric digestion in the HGS with standardization of the protein concentration to be constant between each lane.*

Note. M, trim milk.

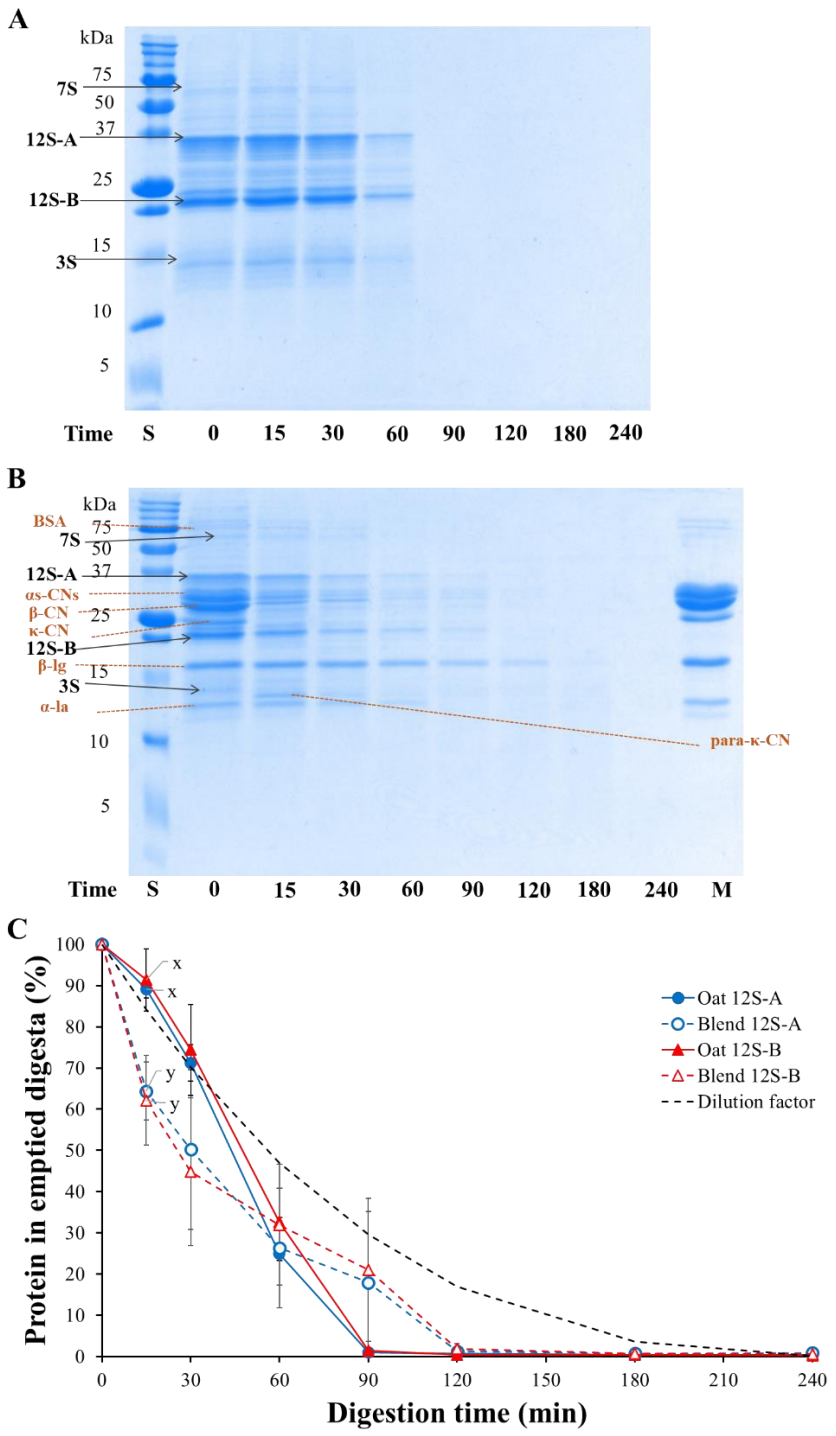


Figure 6.12 *SDS-PAGE (under reducing conditions) of the initial (before digestion) and emptied digesta collected from (A) oat milk samples and (B) oat milk–bovine skim milk blend samples at different time points during gastric digestion in the HGS without standardization of the protein concentration in each lane, and (C) changes in the relative protein contents of oat proteins 12S-A and 12S-B in the oat milk (A) and the oat milk–bovine skim milk blend (B) as a function of digestion time.*

Note. Values with no letter in common (x, y) represent significant differences ($P < 0.05$) at a certain digestion time across different samples (12S in oat milk or 12S in oat milk–bovine skim milk blend). If no letter is listed, there were no significant differences.

6.4.6.3. Macronutrient delivery to the small intestine

The protein, lipid, and total carbohydrate contents of the digesta emptied out of the HGS during digestion are shown in Figure 6.13. The values shown as grey dashed lines in Figures 6.13A1, 6.13B1, and 6.13C1 are expected trends based on dilution because of the gradual addition of SGF. Because the oat milk–bovine skim milk blend was made by mixing oat milk and bovine skim milk (1: 1, v: v; see section 6.3.2), the blended milk and oat milk samples contained 3% (w/v) protein but different amount of lipids and total carbohydrates. The macronutrient concentrations shown in Figures 6.13A2, 6.13B2, and 6.13C2 were corrected for the effect of gastric dilution and different initial macronutrient contents. The corrected macronutrient delivery was calculated as the difference between measured values of the emptied digesta and the calculated dilution values. They were expressed as the percentage of the difference against the quantity of macronutrients in the sample before digestion (Figures 6.13A2, 6.13B2, and 6.13C2). Taking the lipid content as an example,

$$Percent\ difference\ in\ lipid\ (\%) = \frac{measured\ value\ of\ lipid\ in\ digesta(g) - expected\ value\ of\ lipid\ based\ on\ dilution\ (g)}{amount\ of\ lipid\ in\ the\ sample\ before\ digestion\ (g)} \times 100$$

The changes in the protein, lipid, and total carbohydrate concentrations of the emptied digesta during digestion were significantly influenced by the different samples, the digestion time, and their interaction ($P < 0.001$). For oat milk, the delivery of protein, lipid, and total carbohydrates almost followed the dilution curve, except that a slightly faster delivery of protein and carbohydrates was observed in the first 30 min ($P < 0.05$) (Figures 6.13A1, 6.13A2 and 6.13A3). This suggests that the delivery of protein, lipid, and total carbohydrates in the oat milk was dominated by the gastric dilution effect.

In contrast, the pattern of gastric emptying was significantly different in the blend sample (Figure 6.13). The delivery of protein and lipid showed a considerable delay in the first 60 min, with the lowest values observed at emptying points of 15 and 30 min (Figures 6.13A and 6.13B). This delay was caused by the formation of large curd particles, which prevented the protein emptying from the HGS. The delivery of lipids was also affected (Figures 6.13B) because the oil bodies were incorporated into the curd matrix (Figure 6.8B). These larger curd particles were retained in the stomach until they were broken down to sizes smaller than 1 mm before being discharged into the duodenum (Meyer, 1980). With further digestion, the delivery of lipid was less delayed. The emptying of protein was considerably more rapid at 120 and 180 min and the digesta had higher protein contents than those of the oat milk sample ($P < 0.05$). This was attributed to the breakdown of the curds into smaller sizes because of protein hydrolysis by pepsin (Figure 6.11B) and the mechanical mixing effect of the HGS. This allowed more protein and lipids to be evacuated. In particular, it was noticed that no curd particles larger than 1 mm were collected after 180 min of digestion (Figure 6.4). However, it was noted that carbohydrate delivery followed the dilution curve throughout the entire digestion process (Figure 6.13C1), suggesting that carbohydrate was not immobilized by the curd formation in the HGS.

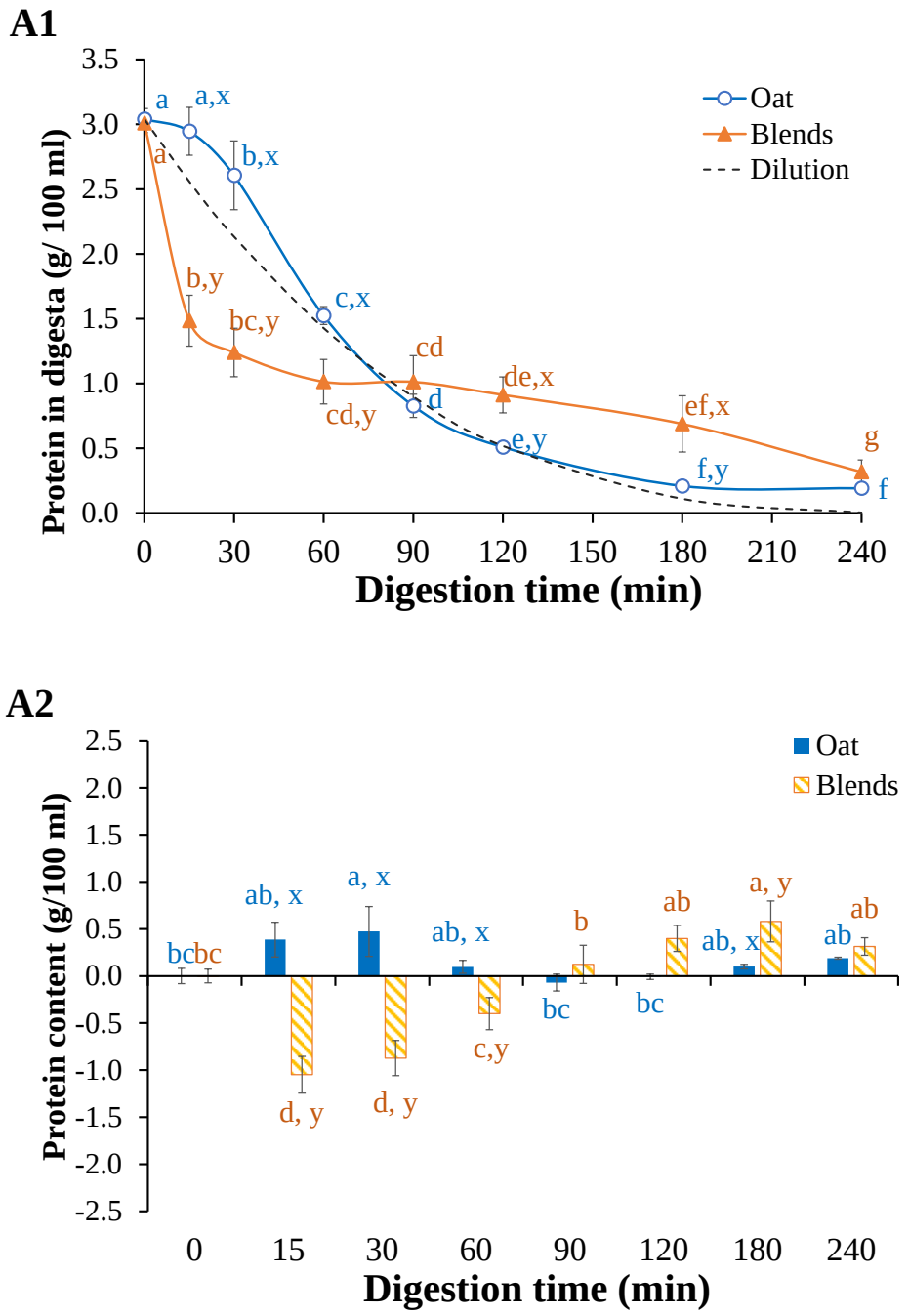


Figure 6.13 *Changes in (A) protein, (B) lipid, and (C) total carbohydrate contents (g/100 mL) of the emptied digesta taken from oat milk samples and oat milk–bovine skim milk blend samples (1) during gastric digestion in the HGS, and (2) corrected protein (A), lipid (B), and total carbohydrate (C) contents after deducting dilution effects.*

Note. Values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) within a certain sample (oat milk or oat milk–bovine skim milk blend) across different digestion times. Values with no letter in common (x, y) represent significant differences at a certain digestion time across different samples (oat milk or oat milk–bovine skim milk blend). If no letter is listed, there were no significant differences. The measurements were replicated at least three times. Error bars represent standard deviations.

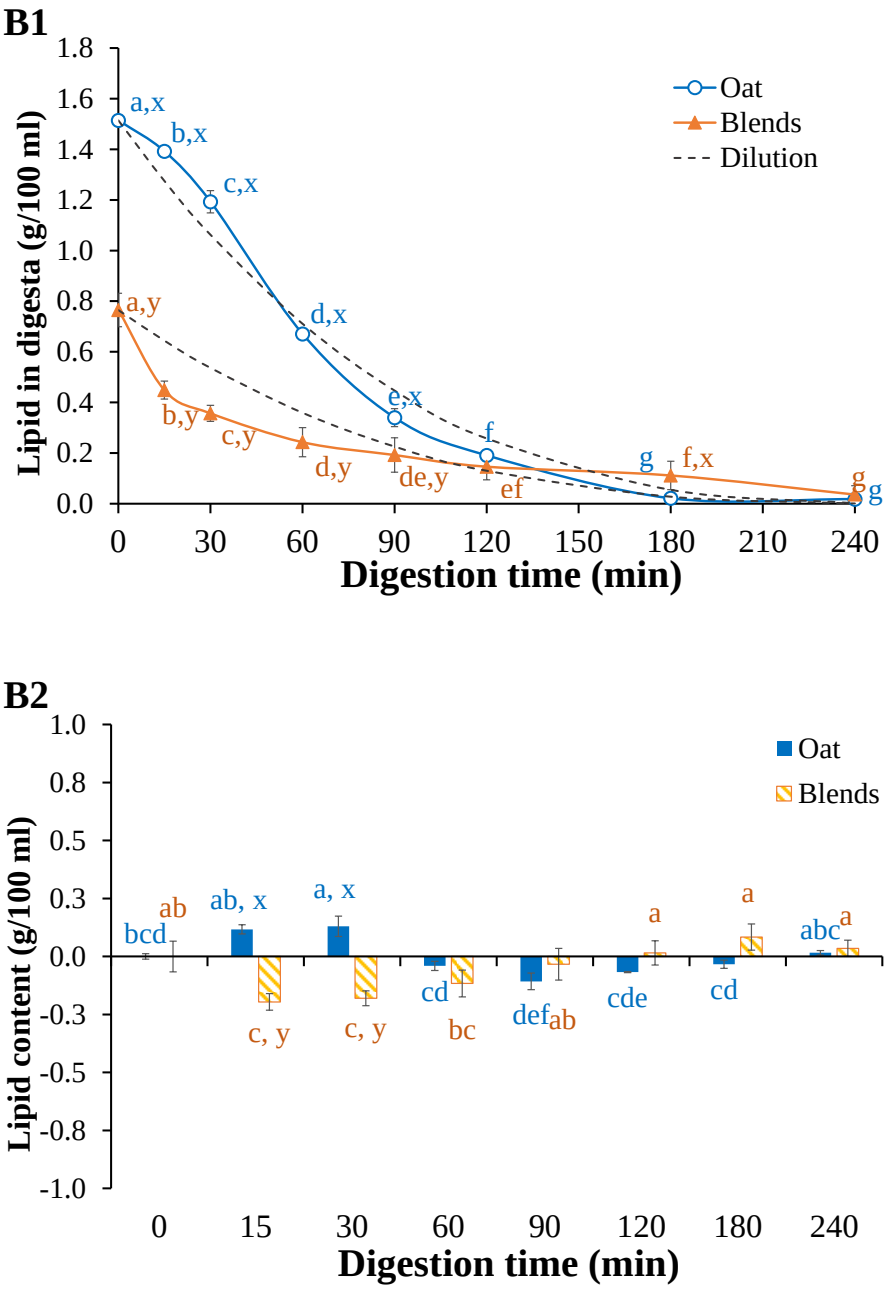


Figure 6.13 *Changes in (A) protein, (B) lipid, and (C) total carbohydrate contents (g/100 mL) of the emptied digesta taken from oat milk samples and oat milk–bovine skim milk blend samples (1) during gastric digestion in the HGS, and (2) corrected protein (A), lipid (B), and total carbohydrate (C) contents after deducting dilution effects.*

Note. Values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) within a certain sample (oat milk or oat milk–bovine skim milk blend) across different digestion times. Values with no letter in common (x, y) represent significant differences at a certain digestion time across different samples (oat milk or oat milk–bovine skim milk blend). If no letter is listed, there were no significant differences. The measurements were replicated at least three times. Error bars represent standard deviations.

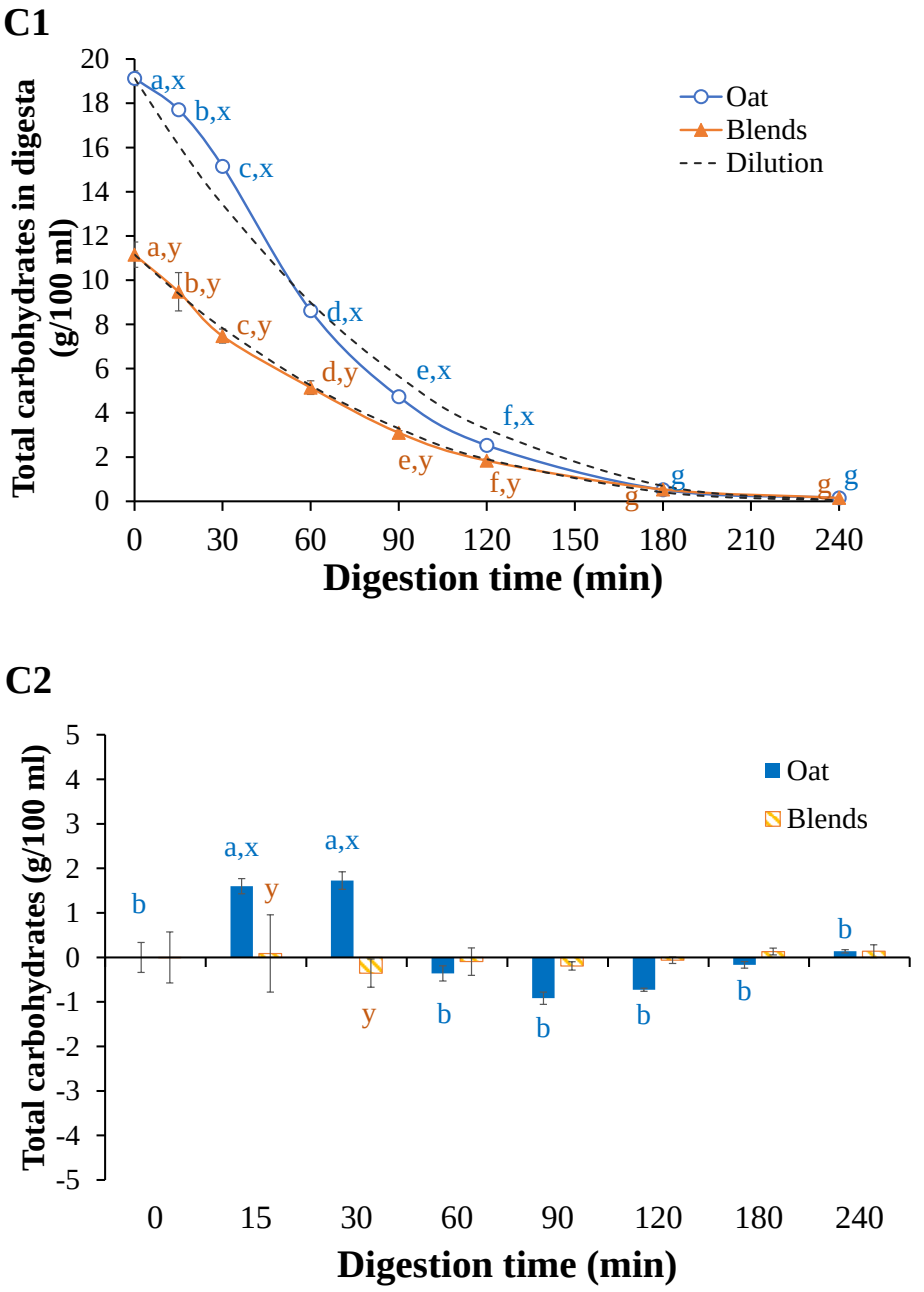


Figure 6.13 *Changes in (A) protein, (B) lipid, and (C) total carbohydrate contents (g/100 mL) of the emptied digesta taken from oat milk samples and oat milk–bovine skim milk blend samples (1) during gastric digestion in the HGS, and (2) corrected protein (A), lipid (B), and total carbohydrate (C) contents after deducting dilution effects.*

Note. Values with no letter in common (a, b, c) represent significant differences ($P < 0.05$) within a certain sample (oat milk or oat milk–bovine skim milk blend) across different digestion times. Values with no letter in common (x, y) represent significant differences at a certain digestion time across different samples (oat milk or oat milk–bovine skim milk blend). If no letter is listed, there were no significant differences. The measurements were replicated at least three times. Error bars represent standard deviations.

The HGS digestion model revealed a considerably different behaviour for the digestion of oat milk than for the digestion of the oat milk–bovine skim milk blend. The bovine milk proteins contained in the oat milk–bovine skim milk blend played an important role in altering the gastric digestive behaviour and the consequent kinetics of macronutrient delivery. Interestingly, although protein coagulation in the oat milk–bovine skim milk blend samples was induced by bovine milk proteins, the structure of the curd was significantly different from that of milk curd obtained under gastric conditions (Appendix 5, Figure A3; Ye et al., 2019). The oat milk–bovine skim milk blend samples generated loose curd particles, whereas the skim milk had a more intact curd structure with strong firmness. It has been demonstrated that the structure and the properties of milk curds formed under gastric conditions can be influenced by many factors such as pretreatment (e.g., heating or homogenization), composition, and fat content (Bergeim et al., 1919; Mulet-Cabero et al., 2019; Ye et al., 2016a, 2017). In addition, it has been reported that replacing 50% of the milk protein in milk substitutes with plant protein (e.g., soybean protein) can prevent caseins from clotting during gastric digestion *in vivo* and *in vitro* (Caugant et al., 1994). Wegrzyn et al. (2021) reported that the addition of soy proteins to milk protein beverages interfered with the formation of the milk curds. The homogenization procedure used for sample preparation and the presence of oat protein in the present study may have been responsible for the different structure of the curd formed in the blend sample.

The data discussed above is summarized in Figure 6.14.

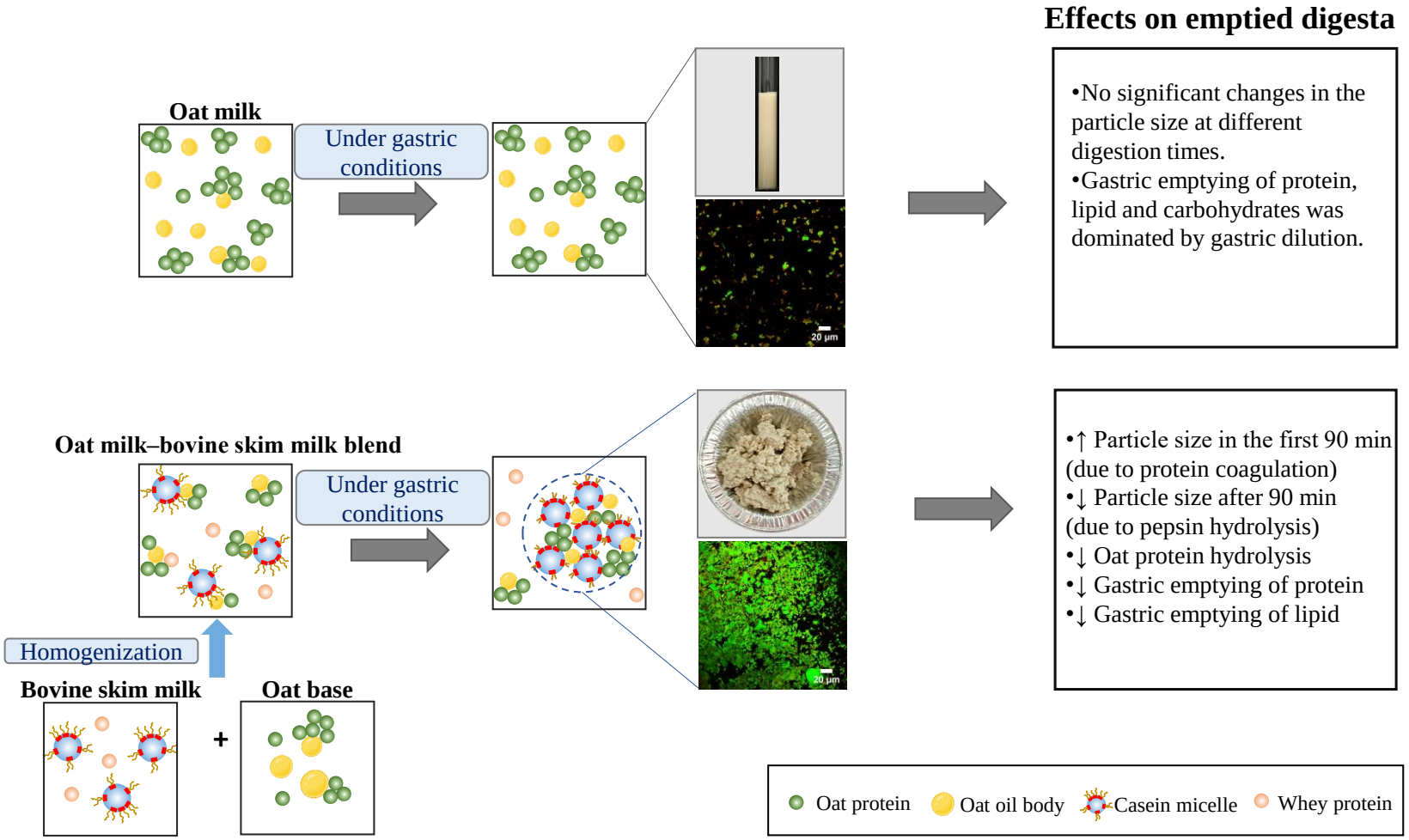


Figure 6.14 *A schematic diagram of the gastric behaviour of oat milk and oat milk-bovine skim milk blend as discussed in detail in Chapter 6.*

These differences between the oat milk and the oat milk–bovine skim milk blend during the gastric stage of digestion are likely to have physiological significance for humans. Oat milk remained essentially in the liquid phase and emptied relatively faster from the simulated stomach chamber. In contrast, like soymilk–bovine skim milk blended drinks (Wegrzyn et al., 2021), the macronutrients of the oat milk–bovine skim milk blend transformed into solid particles and were retained longer in the stomach. Delayed gastric emptying and the associated gastric retention have been identified as important causes of upper gastrointestinal intolerance, with symptoms such as nausea, reflux, and vomiting, and often prevent nutritional targets from being achieved (Landzinski et al., 2008; Ridley & Davies, 2011). Data from *in vivo* and *in vitro* studies strongly suggest a link between the food structure and the gastric emptying rate (Bornhorst et al., 2013; Mackie et al., 2013; Ye et al., 2019). Previous studies in our laboratory have shown that almond oil bodies creamed during *in vitro* gastric digestion and caused a delayed gastric emptying of the lipids, whereas soymilk formed small-sized curds and sedimented, which induced a faster emptying of both soy proteins and lipids (Wang et al., 2020, 2021). Moreover, it is well known that milk curd stays in the stomach for several hours and provides a slow release of amino acids into the bloodstream (Boirie et al., 1997). Our results suggest that the specific loose structure of the curds in the blend milk may potentially improve gastric emptying compared with that of cow's milk; however, further research is required.

6.5 Conclusions

The gastric digestive behaviour and the macronutrient delivery of oat milk and an oat milk–bovine skim milk blend were investigated *in vitro*. The findings from this study suggest that oat milk remained as a liquid and was relatively gastric stable, which resulted in steady emptying from the stomach. In contrast, the oat milk–bovine skim milk blend formed loose curd particles in the HGS. The milk caseins in the oat milk–bovine skim

milk blend dominated its coagulation behaviour and led to a delayed gastric emptying of both proteins and lipids. This study provides an insight into the possibility of modifying the structure of food by designing the food composition and thus obtaining the desired gastric digestive behaviour and gastric emptying rate.

Chapter 7 [4]Gastric digestion of cow milk, almond milk and oat milk in the rat

7.1 Abstract

In this study, the gastric digestions of isocaloric and iso-macronutrient cow milk, almond milk and oat milk were compared in rats euthanized at different post-feeding times. The cow milk separated into a curd phase and a liquid phase in the rat stomach. This coagulation of the cow milk led to higher ($P < 0.05$) protein and lipid retention in the stomach compared with almond milk and oat milk. Almond milk oil bodies aggregated, creamed and rapidly layered in the stomach. This induced a faster ($P < 0.05$) gastric emptying of proteins compared with cow milk and oat milk, and a slower gastric emptying of almond lipids than of almond proteins. In contrast, no significant physical change during the digestion of oat milk was found, with both the proteins and the lipids being steadily emptied from the stomach. This *in vivo* study provides information on the gastric digestion and emptying of plant-based milks, compared with animal-based milks, that will be useful for the design of novel plant-based drinks.

[4] This chapter has been published as **Wang, X.,** Wolber, F. M., Ye, A., Stroebinger, N., Hamlin, A., Zhu, X., Montoya, C., & Singh, H. (2022) Gastric digestion of cow milk, almond milk and oat milk in the rat. *Food & Function, 13*(21),10981-10993.167

7.2 Introduction

In recent years, there has been growing consumer interest in replacing cow milk with plant-based milks in the diet for a variety of reasons, including lactose intolerance, cow milk allergy, ethical considerations, environmental issues and lifestyle choices (Sethi et al., 2016; Valencia-Flores et al., 2013). Moreover, plant-based milks have been shown to have several health benefits, such as cholesterol reduction and regulation of blood glucose levels, which may appeal to health-conscious consumers (Sethi et al., 2016).

Plant-based milks are water extracts of nuts, cereals, legumes, pseudocereals and oil seeds (Mäkinen et al., 2016). Unlike cow milk, plant-based milks vary widely in nutritional quality and most have a very low protein content (Jeske et al., 2017). The development of plant-based milks with nutritional values that are comparable with those of cow milk has been attempted (Vogelsang-O'Dwyer et al., 2021). The gastric digestion behaviour of cow milk has been studied extensively, both *in vivo* and *in vitro* (Boirie et al., 1997; Mulet-Cabero et al., 2019; Roy et al., 2022; Tunick et al., 2016; Ye et al., 2019). In cow milk, the caseins exist in the form of casein micelles, which are known to coagulate in the human stomach, whereas the whey proteins remain soluble and pass into the small intestine more rapidly (Boirie et al., 1997; He & Giuseppin, 2014; Mahe et al., 1992). During the gastric digestion of cow milk, coagulation and the resulting curd structure have been reported to influence the rates of protein hydrolysis and gastric emptying of both proteins and lipids (Roy et al., 2022; Ye et al., 2016b). However, little is known about the gastric digestion behaviour of plant-based milks, and how their gastric digestion compares with that of cow milk.

The gastric digestions of some plant-based milks were recently studied *in vitro* (Gallier, Tate, et al., 2013; Xu et al., 2021). For example, using a dynamic human gastric

simulator (HGS), we showed that almond milk (3% protein and 7% lipid) destabilized quickly under the simulated gastric conditions (i.e., acidic pH and pepsin) (Wang et al., 2020). The initial reduction in the gastric pH induced the aggregation of soluble almond proteins and the flocculation of natural oil bodies. With further digestion, hydrolysis of the interfacial proteins led to the coalescence of the oil bodies because of the increased pepsin activity at the lower pH. In the HGS, almond milk was eventually separated into a cream layer, consisting of coalesced oil bodies, and a transparent lower aqueous phase, containing soluble proteins. This phase separation significantly delayed the *in vitro* gastric emptying of the almond lipids, whereas the almond proteins and peptides that were distributed in the aqueous phase were emptied first. In our recent work, using the HGS, we found no visible physical destabilization, such as coagulation or phase separation, in oat milk under the simulated gastric conditions. As the oat milk remained as a liquid and was relatively gastric stable, the oat proteins and lipids emptied from the HGS at similar rates (Wang et al., 2022).

To our knowledge, no *in vivo* study has investigated the physicochemical and structural changes in plant-based milks during gastric digestion and their effects on the retention of macronutrients, and no comparisons between plant-based milks and animal milks are available. Thus, in this study using the rat model, the gastric digestions of cow milk, almond milk and oat milk over time were compared. The macrostructure and the microstructure of the gastric chymes were assessed (visually via photographs and microscopically), and the gastric emptyings of the proteins and lipids were determined.

7.3 Materials and methods

7.3.1 Materials

Pasteurized, unhomogenized cow whole milk was purchased from a local supermarket in Palmerston North, New Zealand. Raw sliced almonds and cow skim milk powder were obtained from Davis Trading Company (Palmerston North, New Zealand). Commercial oat flour was obtained from Harraways & Sons Ltd (Dunedin, New Zealand). Oat oil was a gift from Givaudan (Dubendorf, Switzerland). α-Amylase from *Bacillus amyloliquefaciens* (EC 3.2.1.1; 437 units/mL) was kindly provided by Novozymes (Canberra, ACT, Australia). All other reagents used were of analytical grade and were purchased from Sigma-Aldrich Co. (St. Louis, MO) or BDH Chemicals (BDH Ltd, Poole, UK) unless otherwise specified.

7.3.2 Preparation of experimental milks

Three isocaloric "milks" were formulated to contain equal quantities of protein, fat and carbohydrate. The cow milk was prepared by mixing 80.5% (w/w) pasteurized, unhomogenized cow whole milk, 3% (w/w) cow skim milk powder and 16.5% (w/w) sucrose, based on the final product.

The almond milk was composed of 27% (w/w) almond whole base, 53% (w/w) almond skim base and 20% (w/w) sucrose, based on the final product. The almond whole base was prepared as described in section 4.3.2. Briefly, raw sliced almonds (500 g) were soaked overnight in 2 L of deionized water at room temperature. The almonds in water were then mixed in a wet disintegrator (Jeffress Bros Ltd, Brisbane, Australia) for 8 min at room temperature and the slurry was filtered twice through a 50-μm mesh bag (Filtercorp International Ltd, Auckland, New Zealand) to remove any residual almond particles and to obtain full-fat whole almond base. The skim almond base was prepared

following a previous method (Devnani et al. (2020). In brief, the whole almond base was centrifuged (Sorvall WX Ultra 100; Thermo Scientific, Asheville, NC) twice at 15,000 *g* and 4 °C for 30 min and fat was removed manually from the top to obtain the skim almond base.

The oat milk consisted of 98.5% oat base and 1.5% oat oil, based on the weight of the final product. The oat base was prepared according to the method described in section 6.3.2. Briefly, oat flour (500 g) was soaked and premixed with deionized water (1250 g) using an overhead mixer for 30 min at room temperature. The mixture was then heated at 65 °C for 30 min in a water bath, 1.5 mL of α-amylase was added and the slurry was incubated at 65 °C for 1 h with continuous agitation. The slurry was filtered through a 100-μm mesh bag (Filtercorp International Ltd) to remove any residual oat particles and was then heated at 100 °C for 10 min to inactivate the α-amylase to obtain the oat base. After cooling to room temperature, oat oil was added to the oat base; this was homogenized at 30/5 MPa using a two-stage valve homogenizer (APV 2000; APV, Copenhagen, Denmark) to obtain the oat milk.

The dry matter of the experimental milk preparations was determined gravimetrically using an air-oven-drying method (AOAC International, 2000a). The total lipid contents of the experimental milks were determined using the Mojonnier method (AACC, 2000) (ammonia Mojonnier for cow milk, and acid Mojonnier for almond milk and oat milk) as described in section 3.2.8. The protein contents of the experimental milks were calculated as nitrogen multiplied by: 6.38 for cow milk (AOAC International, 2006); 5.18 for almond milk (Calixto et al., 1981); 5.83 for oat milk (Maclean et al., 2003). The nitrogen contents were determined using the Dumas method (AOAC International, 2005) by Nutrition Laboratory (Massey University, Palmerston North, New Zealand). The ash content was determined using a gravimetric method (AOAC International, 2002). The

total dietary fiber contents of the almond milk and the oat milk were determined using an enzymatic-gravimetric method (AOAC International, 2000b) by Nutrition Laboratory (Massey University, Palmerston North, New Zealand). The available carbohydrate content was calculated by difference: 100% – (%moisture + %total protein + %fat + %ash + %fiber). The gross energy contents of the experimental milks were determined using a LECO AC500 bomb calorimeter (LECO Corporation, St. Joseph, MI) by Nutrition Laboratory (Massey University, Palmerston North, New Zealand). The particle sizes of the experimental milks were measured using a Mastersizer 2000, as described in section 3.2.5.

7.3.3 *In vivo* study

Ethics approval for this study (MUAEC protocol 21/43) was granted by the Animal Ethics Committee, Massey University, Palmerston North, New Zealand.

Forty adult male Sprague-Dawley rats (mean body weight 647 g) were obtained from AgResearch (Hamilton, New Zealand) and housed in a temperature-controlled room at 22 °C with a 12-h light and 12-h dark cycle. During acclimatization, the rats were pair-housed for socialization in standard cages with aspen chip bedding, ad libitum chow and water; environmental enrichment, such as stainless-steel toys and aspen chew sticks, was also provided. The rats were randomly allocated to the three experimental milk groups (12 animals per experimental milk) and a baseline group (n = 4) receiving a solution containing 10% sucrose solution only. The rats were trained to drink their respective milk or the 10% sucrose solution out of a syringe twice daily until they voluntarily consumed the milk.

Before the test day, the rats were weighed, then singly housed in metabolic cages and fasted overnight (the minimum fasting time was 16 h) to reduce their stomach

contents and prevent coprophagia. During this period, the rats had unrestricted access to a 10% sucrose solution. On the test day, the sucrose solution was removed 3 h before feeding. The rats were moved to larger, wire-topped cages with no bedding. They were observed constantly and the cage was cleaned immediately when a faecal pellet was produced. Each rat was fed a single serving of the respective experimental milk or the 10% sucrose solution at a dose rate of 10 mL/kg body weight using syringes. At selected sampling time points (30, 60 or 120 min after feeding), the rats in the experimental groups were humanely euthanized by gas anaesthesia (n = 4 rats per time point and milk type). The rats in the baseline group (n = 4) were humanely euthanized immediately (i.e., at time 0 min) after feeding with the 10% sucrose solution. The baseline group allowed the determination and correction of both the nitrogen of endogenous origin and the nitrogen remaining in the stomach from previous meals.

The rats were then dissected open, and their stomachs were carefully removed, rinsed with reverse osmosis water and gently dried with a cotton pad. The stomach was dissected open, and the gastric chyme was gently collected into a container, such that the chyme underwent minimal disturbance, and the weight of the gastric chyme was recorded. A portion (< 100 μL) of the fresh sample was stored on ice and the microstructure was examined by confocal laser scanning microscopy within 1 h. The remaining sample was measured for pH value, then heated in a boiling water bath for 10 min to inactivate enzymes and stored at –20 °C until analysis. The sample was thawed and mixed thoroughly; aliquots were used to determine the protein content using the Dumas method (AOAC International, 2005) and the lipid content using gas chromatography as described in section 3.2.9.

7.3.4 pH measurements

The pHs of the experimental milks and the gastric chymes collected at each digestion time point were measured using a pH meter equipped with a glass microelectrode (HI1083B, Hanna Instruments). For the gastric chymes from the cow milk group, which consisted of liquid contents and solid curds, the pH values correspond to the pH of the liquid chyme.

7.3.5 Confocal laser scanning microscopy

The microstructural characteristics of the experimental milks and the gastric chymes were examined using a confocal laser scanning microscope (Leica SP5 DM6000B; Leica Microsystems, Heidelberg, Germany) with a 63× oil immersion lens. The fresh gastric chyme samples were stored in an ice bath and examined as soon as possible. Nile Red (0.1% in acetone, w/v), excited with an argon laser at a wavelength of 488 nm, was used to stain the oil phase. Fast Green (1.0%, w/v), excited with a helium–neon laser at 633 nm, was used to stain protein. Images were stored with 1024 × 1024-pixel resolution using the microscope software (Leica).

7.3.6 Identification of proteins using SDS-PAGE

Reducing glycine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to study the protein compositions of the experimental milks and the gastric chymes. The gastric chymes were mixed with different volumes of sample buffer to achieve equal protein concentrations. The sample buffer consisted of 13% (v/v) 0.5 M Tris-HCl buffer, 10% (v/v) glycerol, 2% (w/v) SDS, 0.04% (w/v) bromophenol blue and β-mercaptoethanol (19:1, v: v), at pH 6.8. The mixture was heated in a boiling water bath for 10 min and then cooled to room temperature. An 8 μL aliquot of the buffered gastric chyme was loaded on to an SDS gel previously prepared on a Mini-

PROTEAN II system. The gels were prepared at 16% (w/v) acrylamide concentration for the resolving gel and at 4.0% (w/v) acrylamide concentration for the stacking gel. The electrophoresis was run at a constant voltage of 110 V for approximately 90 min. After staining and destaining, the gels were scanned using a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA). Bio-Rad Dual Xtra protein standards (Bio-Rad Laboratories) were applied for the estimation of molecular weights. The SDS-PAGE analysis was performed on the gastric chyme samples from all rats, but only representative data are shown. The relative amount of protein in each gel was quantified by analysing the intensity of each band using Image Lab™ software version 5.2 (Bio-Rad Laboratories).

7.3.7 Calculations and statistical analyses

The gastric emptying profile was derived based on the determined relative retention of protein and lipid in the stomach as a function of time. The relative protein retention was calculated as follows:

$$\text{Relative protein retention (\%)} = \frac{\text{protein in the gastric chyme(mg)} - \text{baseline protein (mg)}}{\text{protein in the ingested milk (mg)}} \times 100$$

where the baseline protein was considered to be the protein content of the stomach chyme collected from the rats receiving sucrose solution only (i.e., baseline values).

The lipid content in the baseline samples was negligible. Thus, the relative lipid retention was calculated as follows:

$$\text{Relative lipid retention (\%)} = \frac{\text{lipids in the gastric chyme (mg)}}{\text{lipids in the ingested milk (mg)}} \times 100$$

The effect of the experimental milks on the relative retentions of proteins and lipids in the stomach over time was determined after fitting a power exponential model (Elashoff et al. (1982) using the Proc NLIN procedure of SAS:

$$\text{Relative retention}_{\text{Time}} = \alpha_0 \exp - (\kappa \times \text{time})^{\beta}$$

where α_0 is the relative amount of protein or lipid at time 0 (i.e., 100%), κ is the slope of the curve and β is an index for the shape of the curve. The fitted relative protein and lipid retention curves of the experimental milks were then compared using the *F* test by considering whether the data for all the experimental milks were better explained by a single non-linear model (i.e., no difference between the milks; reduced model) or by individual non-linear models (i.e., full model). As the full model better explained the data for the relative protein and lipid retentions, the estimated parameters across the experimental milks were compared using a t-test. For the analysed parameters, the normal distribution and the homogeneity of variance for the residuals were evaluated using PROC UNIVARIATE and the ODS GRAPHICS of SAS.

$T_{1/2}$ (i.e., the time at which half the protein or lipid present at time 0 had been emptied from the stomach) values were also estimated and compared. The parameters κ and β were used to determine the $T_{1/2}$ using the following equation: (Odunsi et al. (2009).

$$T_{1/2} = (1/\kappa) \times [\text{Log}\,(1/0.5)(1/\beta)]$$

The number of replications (n = 4) required to determine statistical significance at each time point per experimental milk was estimated (power = 0.8) based on data reported in previous studies (Dalziel et al., 2020; Leray et al., 2003; G. Miranda & J. P. Pelissier, 1987; Ye et al., 2019). A one-factor analysis of variance was performed for the chemical compositions (protein, lipid, carbohydrate, dry matter and gross energy contents) of three

different batches of each experimental milk and the SDS-PAGE protein profile. For the pH of the gastric chyme, a two-factor analysis of variance, with different experimental milks, post-feeding times and their interaction, was conducted using the statistical software SPSS (version 28.0.1.1(15)). The normal distribution and the homogeneity of variance for the residuals were examined using the Shapiro–Wilk test and the Levene test. Variances that lacked normality or homogeneity of variance were evaluated using the Kruskal–Wallis test. When the F value of the overall model was significant ($P < 0.05$), posthoc tests were conducted using Tukey's range test and significance was taken at $P < 0.05$. The results are reported as mean ± standard error of the mean.

7.4 Results and discussion

7.4.1 Experimental milks

The proximate chemical compositions and the particle sizes of the experimental milks are shown in Table 7.1 and Figure 7.1. The cow milk, almond milk and oat milk were formulated to contain the same levels of protein, lipid and carbohydrate, as well as similar energy contents ($P > 0.05$). The almond milk and oat milk naturally contained fibre, and the oat milk had a higher fibre content than the almond milk ($P < 0.05$). The mean particle size ($d_{4,3}$) of the oat milk (~ 6.6 μm) was larger than those of the cow milk (~ 3.2 μm) and the almond milk (~ 2.4 μm). The intake of milk by the rats on the test day did not differ across the three groups (mean voluntary feed intake of 6.4 ± 0.3 g/meal; $P > 0.05$). On the test day, the actual weight of milk ingested by the rats was recorded and all rats consumed no less than 99% of their full allotted portion (10 mL/kg body weight) of milk.

Table 7.1 *Chemical compositions and particle sizes of experimental milks*[a]

Milk	Cow	Almond	Oat
Protein, g/100 g	3.38 ± 0.02	3.24 ± 0.09	3.31 ± 0.00
Fat, g/100 g	3.13 ± 0.04	3.20 ± 0.04	3.14 ± 0.02
Dry matter, g/100 g	28.9 ± 0.17	28.9 ± 0.36	28.9 ± 0.14
Ash, g/100 g	0.76 ± 0.01 [a]	0.43 ± 0.04 [b]	0.37 ± 0.03 [b]
Fibre, g/100 g	ND	0.27 ± 0.05 [b]	0.49 ± 0.02 [a]
Carbohydrate, g/100 g	21.6 ± 0.19	21.8 ± 0.23	21.6 ± 0.17
Gross energy, kcal/100 g	130.1 ± 0.38	130.6 ± 1.11	133.9 ± 0.77
$d_{4,3}$, µm	3.17 ± 0.16 [b]	2.36 ± 0.06 [b]	6.58 ± 0.28 [a]

Note. [a]Values in the same row with different superscript letters (a, b, c) differ ($P < 0.05$). ND, Not determined.

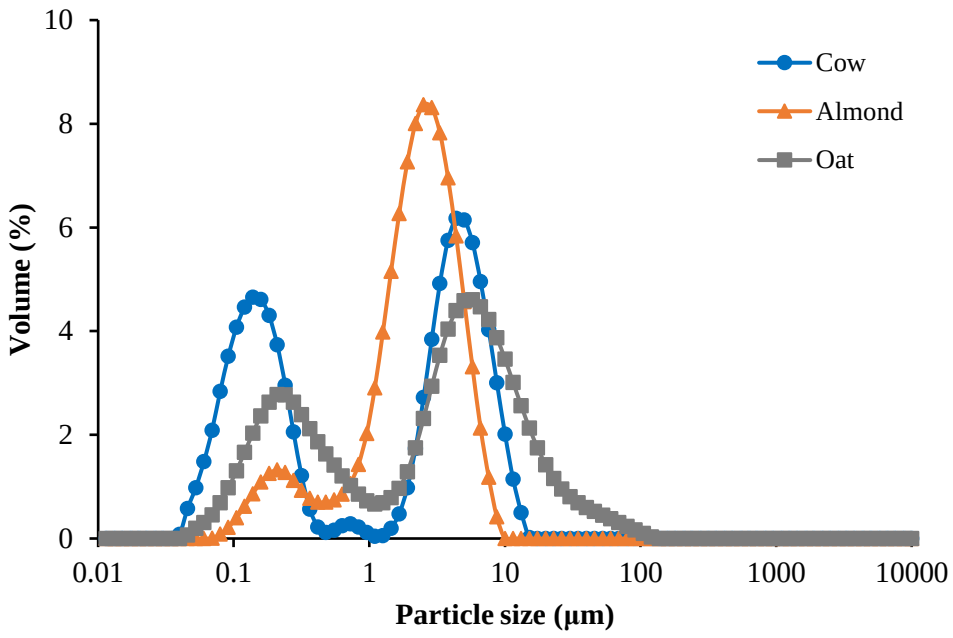


Figure 7.1 *Particle size distributions of cow milk, almond milk and oat milk freshly prepared before each test day.*

7.4.2 Gastric pH

The pH values at time 0 were 6.52, 6.22 and 6.27 for cow milk, almond milk and oat milk respectively (Figure 7.2). The gastric pH was significantly affected by the digestion time ($P < 0.001$) and the milk type ($P < 0.001$), but not by their interaction ($P > 0.05$). The fasting pH of the rat stomach has been reported to be ~ pH 2 (Lubach et al., 2013). In the current study, the fasting pH was not determined; however, the pH of the gastric chyme collected from the baseline rats (i.e., the rats fed 10% sucrose solution with a pH of 8.4 and sacrificed immediately after feed intake) was approximately pH 3.1 (data not shown).

After ingestion, as expected, the pH of the gastric contents decreased over the digestion time for all milks. At 30 min, the gastric pH values were 5.73, 5.78 and 5.08 for cow milk, almond milk and oat milk respectively (Figure 7.2). These gastric pH values were much higher than the reported fasting gastric pH (Lubach et al., 2013), which was due to the high initial pH of the experimental milks and their buffering effect (Figure 7.2). The gastric pH for the rats fed oat milk was lower than those for the other two experimental groups over the digestion time. In particular, after 120 min, the gastric pH for the oat milk had reached the reported fasting pH of 2, whereas the pH values for the cow milk and the almond milk were ~ 3.5 and ~ 3 respectively. These differences in the gastric pH profiles can be ascribed mainly to the buffering capacity of each milk. For example, compared with oat milk, the higher gastric pH for cow milk can probably be attributed to the high buffering capacity of the milk proteins, soluble minerals and colloidal calcium phosphate (Salaün et al., 2005).

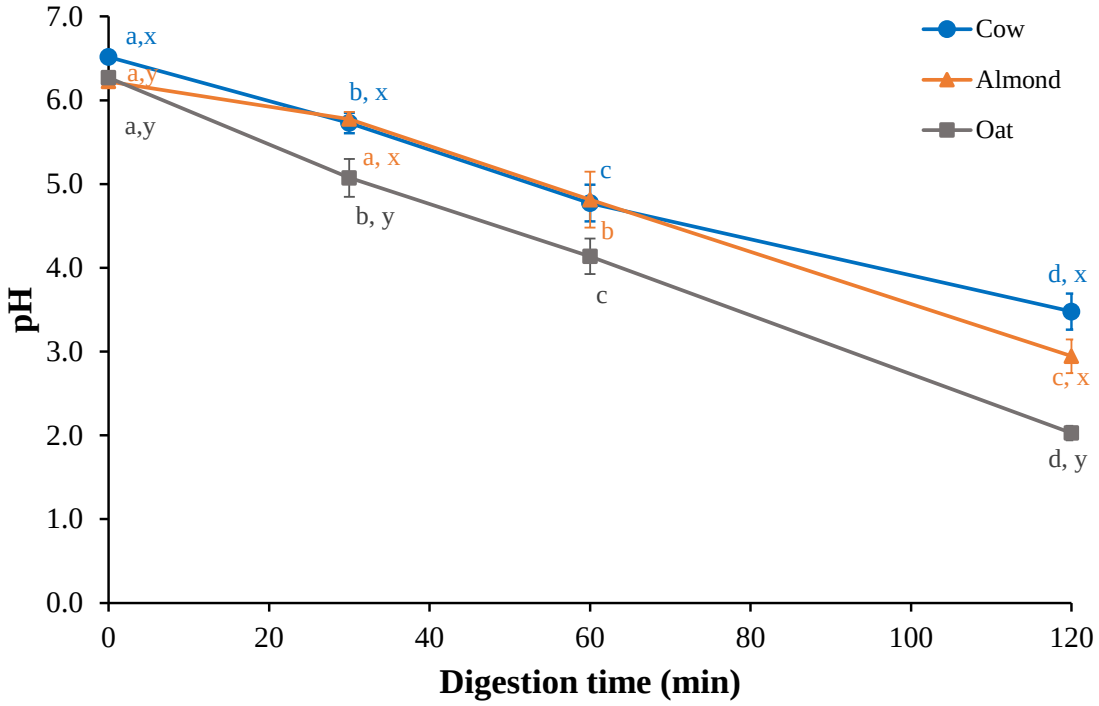


Figure 7.2 *Changes over time in the pH of the chyme of rats fed cow milk, almond milk and oat milk.*

Note. The pH values (mean ± standard error of the mean) refer to the initial (before digestion) experimental milks and the gastric chymes in the rat stomach. Values with different letters (a, b, c, d) differ ($P < 0.05$) within a milk type (cow milk, almond milk and oat milk) across different digestion times. Values with different letters (x, y) differ ($P < 0.05$) at a certain digestion time across milk types (cow milk, almond milk and oat milk).

7.4.3 Structural changes during gastric digestion

The general structure of the gastric chyme samples was examined visually (Figure 7.3) and using confocal laser scanning microscopy (Figure 7.4). After 30 min, when the gastric pH was ~5.7, the gastric chyme of the rats fed cow milk had separated into a curd (coagulum) fraction and a liquid serum fraction (Figure 7.3A1). The coagulated protein network was clearly seen using confocal microscopy, with many milk fat globules incorporated into the protein matrix (Figure 7.4A2). The formation of the curd has been attributed to the coagulation of casein micelles that is induced by the proteolytic action

of pepsin (Ye et al., 2016a); the liquid fraction consists mostly of soluble whey proteins (Roy et al., 2022; Ye et al., 2016a). With further digestion, the curd appeared to become more compact (Figure 7.3A2). At 120 min, most of the curd remained in the stomach, whereas the serum fraction was emptied out from the stomach (Figure 7.3A3). Some of the fat globules in the cow milk (Figure 7.4A1) coalesced after 30 min of digestion and remained aggregated for the subsequent 90 min (Figures 7.4A2–7.4A4). This disruption of the milk fat globules was caused by the proteolytic and lipolytic action of pepsin and digestive lipase on the milk fat globule membrane (Levy et al., 1982; Roy et al., 2022). Gastric pepsin is able to hydrolyze the milk fat globule membrane proteins under gastric conditions (Ye et al., 2011), which may weaken the interfacial structure, leading to coalescence of the fat globules, especially under gastric shear forces. This facilitates the action of digestive lipase on the triacylglycerol core and further accelerates the release of free fatty acids (Gallier et al., 2013). It is worth mentioning that both lingual and gastric lipases are present in the rat stomach and contribute to the gastric digestion of dietary lipids (Hamosh & Scow, 1973; Levy et al., 1982).

	30 min	60 min	120 min
Cow			
Almond			
Oat			

Figure 7.3 *Photographs of the gastric contents (chymes) collected over time from rats fed cow milk, almond milk and oat milk. The diameter of the 5 ml-tube is 16mm.*

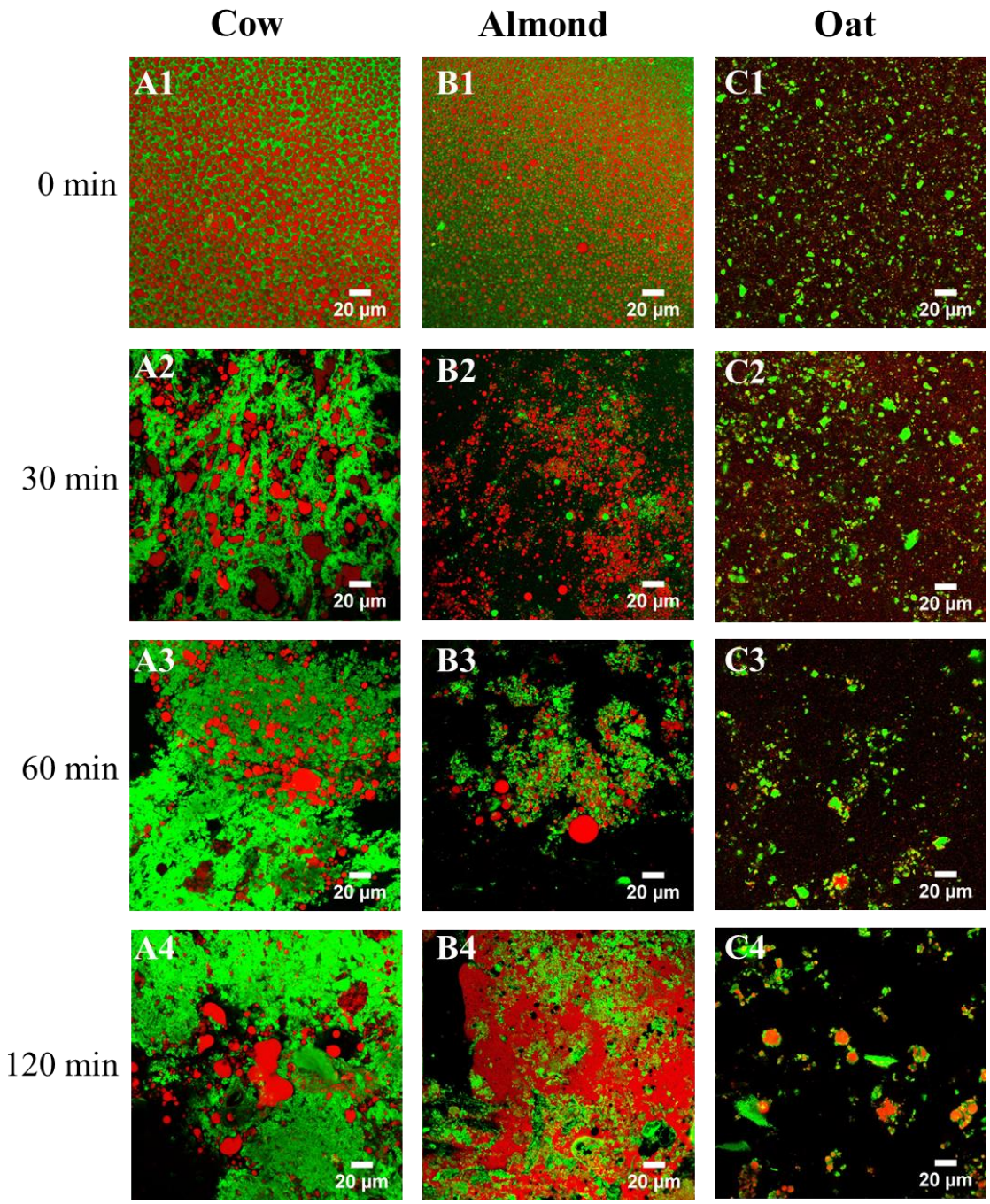


Figure 7.4 *Confocal micrographs of cow milk, almond milk and oat milk remaining in the rat stomach at different digestion times.*

Note. Samples were stained with Nile Red (for lipid) and Fast Green (for protein). The scale bar in all images is 20 μm.

The undigested almond milk had oil bodies dispersed uniformly in an aqueous phase containing protein (Figure 7.4B1). Almond oil bodies are stabilized by a monolayer of phospholipids embedding oleosin proteins (Beisson et al., 2001). After 30 min of digestion, the almond milk quickly destabilized, as illustrated in Figure 7.3B1, with some flocs floating in the tube. Under confocal microscopy, notable flocculation of almond oil bodies and clusters of protein aggregates were clearly seen (Figure 7.4B2). Droplet flocculation is a common phenomenon that occurs in food protein-stabilized emulsions under gastric conditions (Gallier et al., 2014; Gallier et al., 2013; Golding et al., 2011; Wang et al., 2019). This flocculation was probably induced by the loss of electrostatic charges on the surface of the almond oil bodies because of acidification (Gallier & Singh, 2012a). The low gastric pH results in the ionization of side-chain groups of the interfacial proteins. When the gastric pH is close to the isoelectric point of the interfacial proteins, the electrostatic repulsion between oil bodies is reduced, contributing to flocculation (Golding et al., 2011). Likewise, the presence of gastric mucins may also induce weak flocculation via depletion interactions (Sarkar et al., 2009). After further digestion (60 min, Figure 7.3B2), there was a thick cream layer floating on the top of the cloudy aqueous phase. Under confocal microscopy, the formation of protein aggregates and a detectable amount of coalesced oil bodies were also observed (Figure 7.4B3). The almond storage protein, amandin, has an isoelectric point ranging from 4.55 to 6.3 (Sathe et al., 2002). When the pH of the stomach slowly declined from a neutral pH to a more acidic pH (i.e., near the isoelectric pH), the solubility of the proteins decreased, primarily because of a lack of electrostatic repulsions, which promoted aggregation of the almond proteins via hydrophobic interactions (Damodaran et al., 2007; Sathe, 1992). Coalescence of oil bodies is a consequence of extensive flocculation and enzymatic hydrolysis (pepsin and digestive lipase in this case) (Wang et al., 2020). It has been reported that recombinant

human gastric lipase is able to access the oil core of almond oil bodies and hydrolyze the triacylglycerols without pre-hydrolysis of the protein coat (Beisson et al., 2001). The tendency to form a creaming layer at the top of the stomach can be explained by the fact that the poor mixing within the fundus is not strong enough to redisperse the lipids (Marciani et al., 2007). At 120 min of gastric digestion, the chyme formed a layer of yellowish oil along with some flocs (Figure 7.3B3). Figure 7.4B4 reveals that the structure of the majority of the almond oil bodies had been disrupted and that the entire microstructure appeared to be a pool of free oil trapped within the aggregated protein matrix.

The undigested oat milk consisted of large protein aggregates and oil bodies (Figure 7.4C1), which is consistent with previous reports (Mäkinen et al., 2015). The major storage proteins of oat (i.e. globulins) have isoelectric points of approximately 5.9–7.2 and 8.7–9.2 for its acidic and basic polypeptides respectively (Brinegar & Peterson, 1982). Oat globulins have limited solubility at a neutral and slightly acidic pH and exist as aggregates, as shown in Figure 7.4C1 (Ercili-Cura et al., 2015; Ma & Harwalkar, 1984). During the 120 min of gastric digestion, no significant change, such as coagulation or creaming, was observed in the gastric chyme of the rats fed with oat milk (Figures 7.3C1–7.3C3). This finding is in line with previous *in vitro* studies conducted by our laboratory (Wang et al., 2022), in which no physical destabilization phenomena were detected throughout the entire gastric digestion. It is worth noting that, in the current study, oat oil bodies appeared to coalesce after 60 min of digestion and several large, coalesced oil bodies appeared at the end of the digestion (Figures 7.4C3 and 7.4C4). This was not previously observed in our *in vitro* study (Wang et al., 2022), probably because gastric lipase was not used in the *in vitro* model. The action of gastric lipase in the rat stomach seems to be the main factor that contributes to the coalescence of oat oil bodies.

7.4.4 Protein hydrolysis in the gastric chyme

The protein compositions of cow milk, almond milk and oat milk and their respective gastric chymes, as determined by SDS-PAGE, are shown in Figures 7.5-7.7. In each gel, the samples were normalized to contain equivalent protein concentrations in each lane, in order to eliminate the effects of different amounts of protein remaining in the stomach because of different gastric emptying and dilution from the continuous secretion of gastric juice.

The undigested cow milk (time 0) gel was composed mainly of bands corresponding to α_{s1}-casein, α_{s2}-casein (α_s-CNs), β-CN, κ-CN, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) (Figure 7.5A). After 30 min of digestion (pH ~ 5.7, the κ-CN band became faint, with less than 35% intact protein remaining in the chyme (Figure 7.5B; $P < 0.05$). At the same time, a new band at around 15 kDa that corresponded to para-κ-CN was clearly observed; this is the hydrolysis product of κ-CN (Miranda & Pelissier, 1983). Pepsin is able to rapidly cleave the Phe_{105}–Met_{106} bond in κ-CN at a low enzyme concentration and a relatively high pH (i.e. pH 6) (Tam & Whitaker, 1972; Yang et al., 2022). This confirmed that the early coagulation of the milk proteins was induced by the hydrolysis of κ-CN by gastric pepsin, which destabilized the structural stability of the casein micelles (Dalgleish & Corredig, 2012a). The α_s-CNs and β-CN bands were present during the entire digestion period and showed little degradation, even after 120 min of gastric digestion. This result is in line with previous *in vitro* and *in vivo* studies of the gastric digestion of cow milk (Roy et al., 2022; Ye et al., 2016a; Ye et al., 2019). For instance, in piglets, casein bands were the predominant fraction in the gastric curd formed when feeding cow milk and remained little digested during the gastric digestion time, even up to 210 min (Roy et al. (2022).

In the case of the whey proteins, the β-Lg and α-La bands became less intense over time ($P < 0.05$). This was mainly because the majority of the whey proteins remained soluble in the aqueous phase and gradually emptied from the stomach over time (Ye et al., 2019). After 120 min of digestion, approximately 20% of the β-Lg remained in the gastric chyme although the liquid fraction had been completely emptied at this time point (shown in Figure 7.3A3). This suggests that a portion of the whey proteins was also in the curd, probably because of the liquid phase entrapped in the curds; this was also observed in the piglet study (Roy et al., 2022). It is also likely that some whey proteins had been associated within the curd, possibly because of the pasteurization process used in the preparation of the experimental milk (Anema & Li, 2003; Ye et al., 2019).

Figure 7.6A revealed several bands corresponding to polypeptides of the major storage protein, amandin, in almond milk (time 0). The two pairs of major bands with molecular weights of 40–42 and 20–22 kDa were acidic and basic polypeptides of amandin (Sathe, 1992). There was no significant degradation of the basic polypeptides (22–20 kDa) at 30 min of gastric digestion, whereas the acidic polypeptide (42–40 kDa) bands became less intense (Figure 7.6B; $P < 0.05$). Similarly, a more efficient hydrolysis of acidic polypeptides than of basic polypeptides after incubation with pepsin has been observed (Sze-Tao and Sathe (2000). At 60 min of digestion, all polypeptide bands became less intense, with only less than half of the proteins remaining intact in the chyme ($P < 0.05$). After 120 min of digestion, no intact protein bands could be seen. The increase in the extent of proteolysis was due not only to the increase in the digestion time, but also to the reduction in pH of the gastric chyme towards values near the optimum pH for pepsin activity (Anfinsen et al., 1971) and the high susceptibility of almond proteins to pepsin proteolysis (Gallier & Singh, 2012a; Sathe, 1992). This observation is consistent

with previous results, which showed that the majority of almond proteins were hydrolyzed at pH 3 during *in vitro* dynamic gastric digestion (Wang et al. (2020).

The gel for the undigested oat milk was composed of two major protein bands with molecular weights of 32 and 22 kDa, corresponding to acidic (12S-A) and basic (12S-B) polypeptides of 12S globulin respectively (Figure 7.7A) (Burgess et al., 1983). The faint bands around 65 kDa and below 15 kDa were the 7S and 3S fractions respectively (Klose & Arendt, 2012). The extent of hydrolysis for the 7S, 12S-A and 12S-B protein bands in the gastric chyme gradually increased ($P < 0.05$) within the first 1 h, whereas the 3S band did not change over this time (Figures 7.7A and 7.7B). At 120 min of digestion (when the pH reached 2.0), all intact oat protein bands were absent; the proteins had been hydrolyzed into low molecular weight (< 15 kDa) peptides. This is in agreement with previous *in vitro* studies (Nieto-Nieto et al., 2014; Wang et al., 2022). At such an acidic pH, the oat proteins 12S-A and 12S-B are partially unfolded and readily susceptible to proteolysis by pepsin (Brinegar & Peterson, 1982; Burgess et al., 1983; Nieto-Nieto et al., 2014).

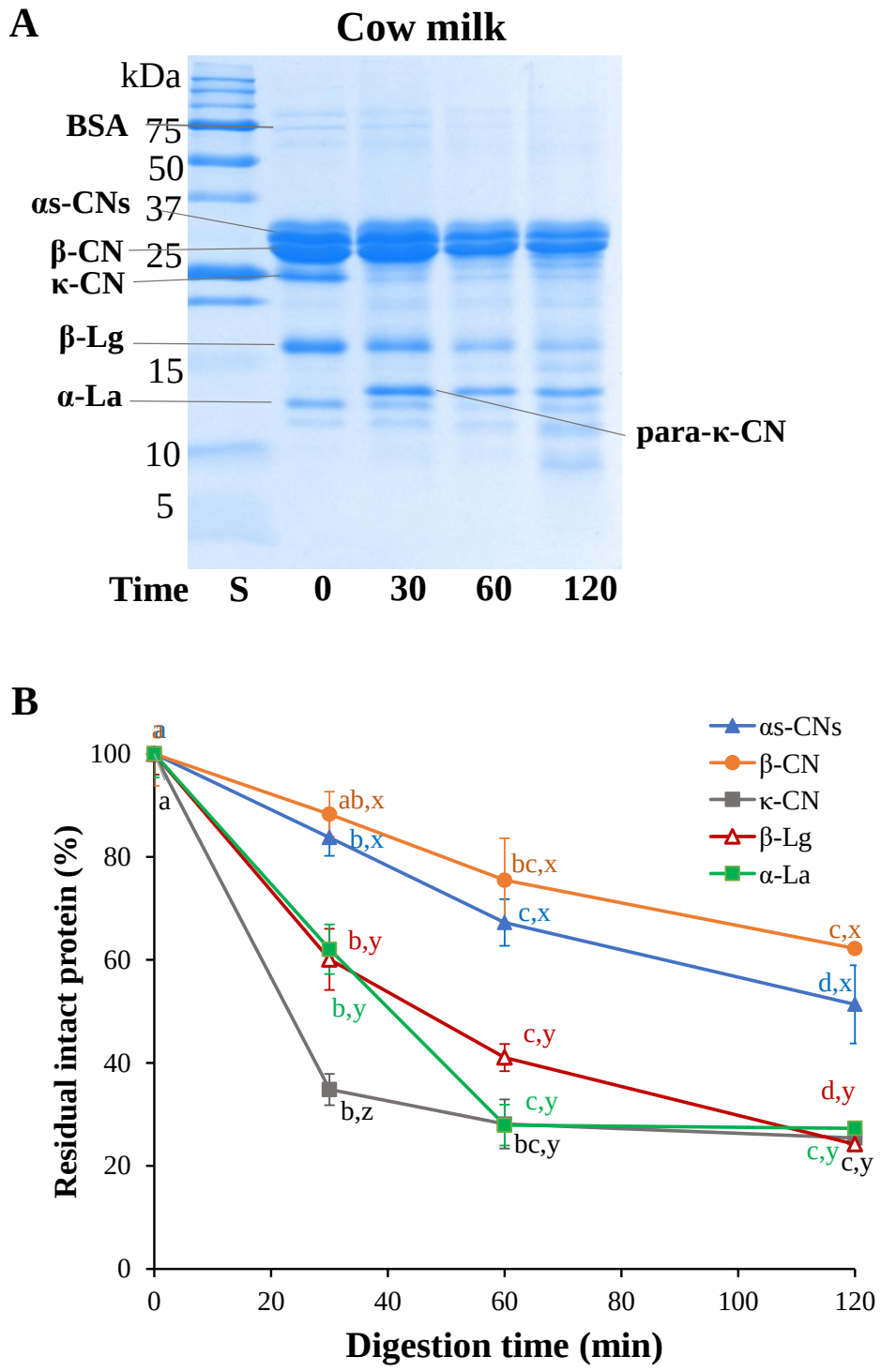


Figure 7.5 *SDS-PAGE patterns (under reducing conditions) of cow milk before (time 0 min) or after (30–120 min) gastric digestion in rats (A) and relative amounts of selected proteins in each gastric chyme sample (B).*

Note. S = molecular weight standard; BSA = bovine serum albumin; CN = casein; Lg = lactoglobulin; La = lactalbumin. Values with different letters (a, b, c, d) differ ($P < 0.05$) within the individual proteins across different digestion times. Values with different letters (x, y, z) differ ($P < 0.05$) at a specific digestion time across different proteins. Error bars represent the standard error of the mean for $n = 4$.

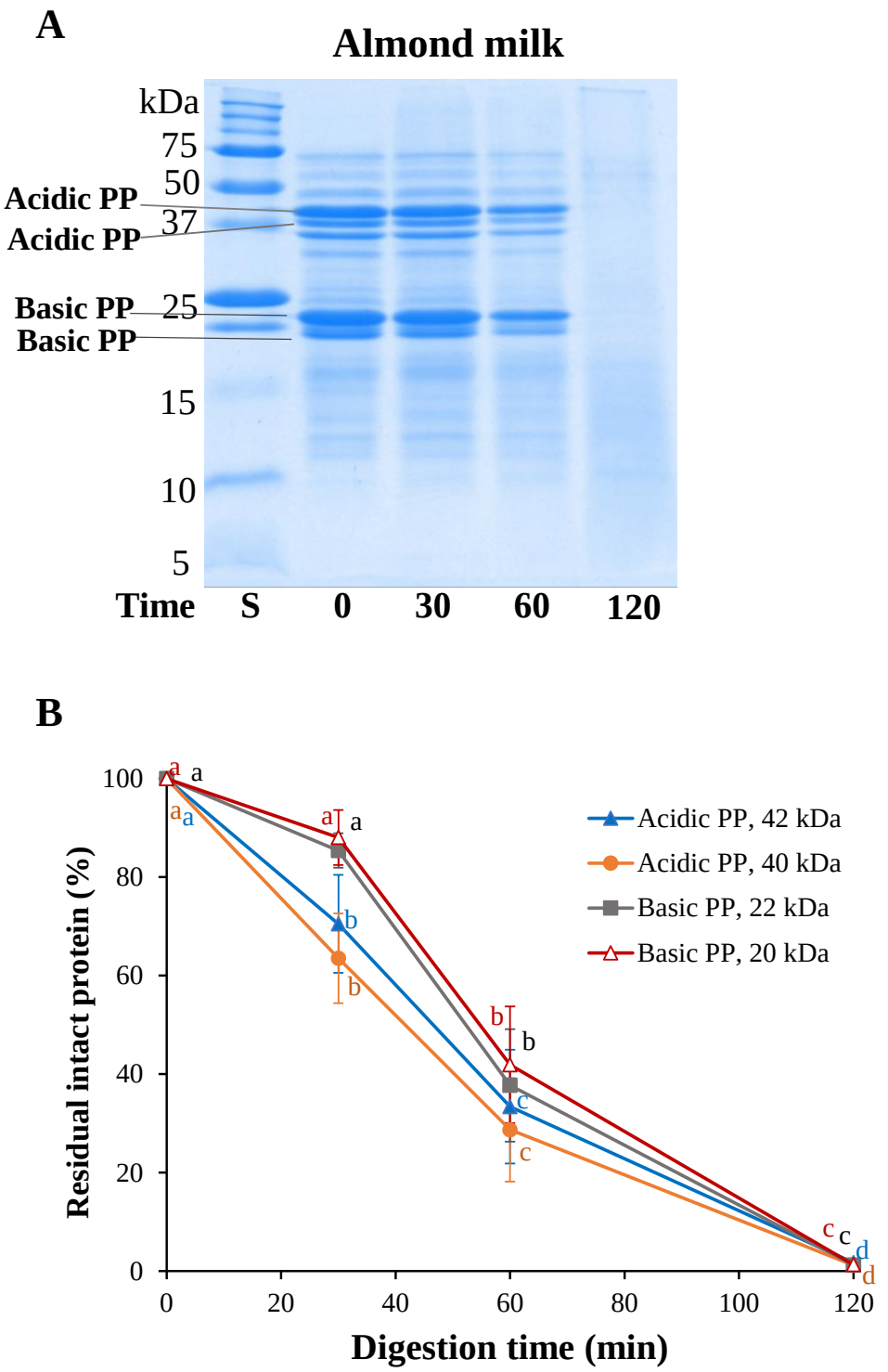


Figure 7.6 *SDS-PAGE pattern (under reducing conditions) of almond milk before (time 0 min) or after (30–120 min) gastric digestion in rats (A) and relative amounts of selected proteins in each gastric chyme sample (B).*

Note. S = molecular weight standard; PP = polypeptide. Values with different letters (a, b, c, d) differ ($P < 0.05$) within the individual proteins across different digestion times. Values with different letters (x, y, z) differ ($P < 0.05$) at a specific digestion time across different proteins. Error bars represent the standard error of the mean for $n = 4$.

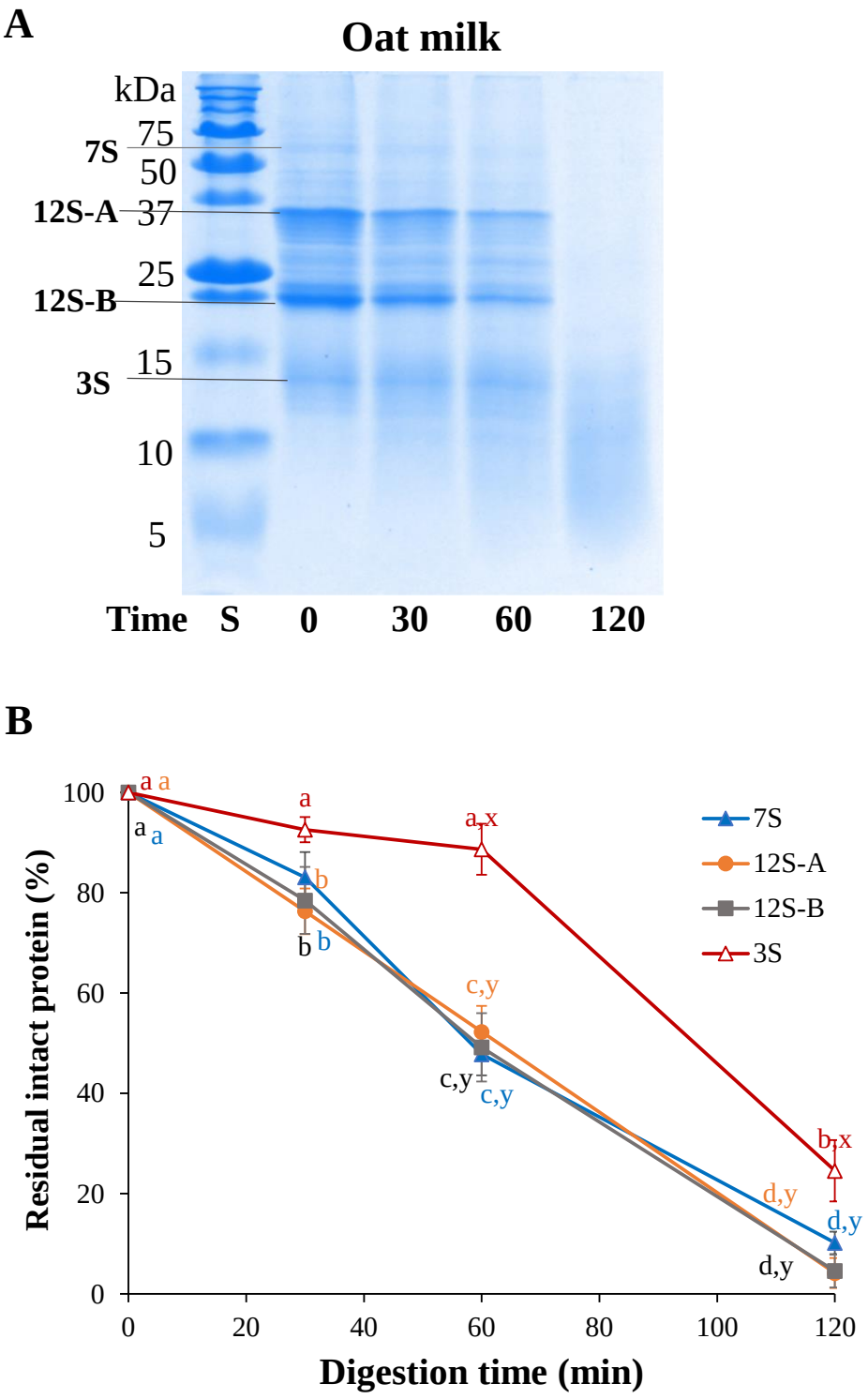


Figure 7.7 *SDS-PAGE pattern (under reducing conditions) of oat milk before (time 0 min) or after (30–120 min) gastric digestion in rats (A) and relative amounts of selected proteins in each gastric chyme sample (B).*

Note. S = molecular weight standard. Values with different letters (a, b, c, d) differ ($P < 0.05$) within the individual proteins across different digestion times. Values with different letters (x, y, z) differ ($P < 0.05$) at a specific digestion time across different proteins. Error bars represent the standard error of the mean for $n = 4$.

7.4.5 Gastric emptying rate of macronutrients

The gastric emptying profiles of the proteins and lipids were investigated. The relative retention of protein in the stomach differed across the experimental milks (Figure 7.8; $P < 0.001$). In general, the proteins in cow milk emptied more slowly ($P < 0.05$) than those in almond milk and oat milk. The gastric emptying rate of protein (i.e., κ values in the insets in Figures 7.8 and 7.9) followed the order: almond milk > oat milk > cow milk. Accordingly, the predicted $T_{1/2}$ (the time required to empty half the total protein content of the milk) followed the order: cow milk (89 min) > oat milk (55 min) > almond milk (36 min).

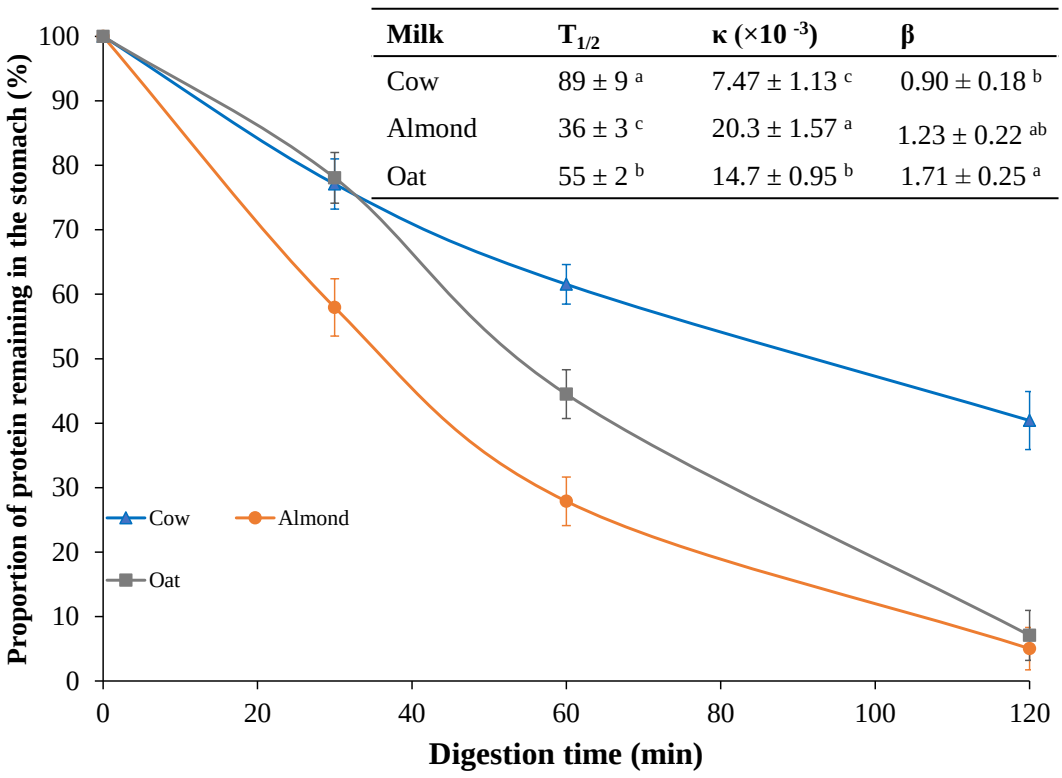
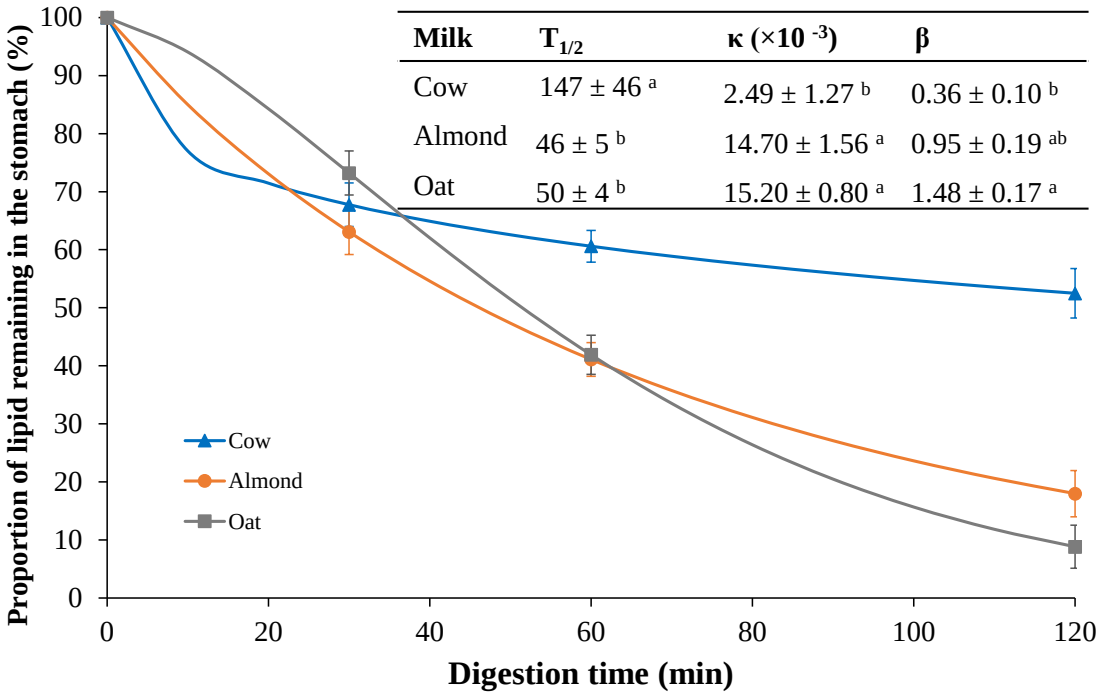


Figure 7.8 *Changes in protein in the rat stomach at different digestion times after feeding with cow milk, almond milk and oat milk.*

Note. Values are reported as mean ± standard error of the mean; n = 4 rats per experimental milk and time combination. κ is the slope (%/min × 10^{-3}) of the curve and $T_{1/2}$ (min) is the half gastric emptying time. Mean values with different superscript letters (a, b, c) differ ($P < 0.05$).

The relative retention of lipid in the stomach also differed across the experimental milks ($P < 0.001$) (Figure 7.9). Similarly, the lipids in cow milk had the slowest gastric emptying ($P < 0.05$) compared with those in almond milk and oat milk. The gastric emptying rate of lipid followed the order: almond milk = oat milk > cow milk. The predicted $T_{1/2}$ (the time required to empty half the total lipid content of the milk) followed the order: cow milk (147 min) > oat milk (50 min) = almond milk (46 min).



Milk	$T_{1/2}$	κ (×10 $^{-3}$)	β
Cow	147 ± 46 ^{a}	2.49 ± 1.27 ^{b}	0.36 ± 0.10 ^{b}
Almond	46 ± 5 ^{b}	14.70 ± 1.56 ^{a}	0.95 ± 0.19 ^{ab}
Oat	50 ± 4 ^{b}	15.20 ± 0.80 ^{a}	1.48 ± 0.17 ^{a}

Figure 7.9 *Changes in lipid in the rat stomach at different digestion times after feeding with cow milk, almond milk and oat milk.*

Note. Values are reported as mean ± standard error of the mean; n = 4 rats per experimental milk and time combination. κ is the slope (%/min × 10^{-3}) of the curve and $T_{1/2}$ (min) is the half gastric emptying time. Mean values with different superscript letters (a, b, c) differ ($P < 0.05$).

The slowest gastric emptying rate for cow milk of both protein and lipid can be attributed to the curd formation in the stomach (see Figure 7.3). The curd is a coagulated protein matrix, with a large number of fat globules entrapped into the protein network (see Figure 7.4). Thus, most of the protein (mainly caseins) and lipid had migrated into the curd structure during gastric digestion and only a small proportion of protein and lipid remained in the liquid fraction (Roy et al., 2022). In humans, foods with particle size smaller than ~ 1–2 mm (< 1.5–2 mm for rats (Jang et al., 2013)) can pass through the pylorus into the duodenum (Meyer et al., 1981; van Aken, 2010). Thus, because of their large particle size, the curd particles were retained in the stomach and were released slowly as they were degraded during digestion. This observation on the emptying of both protein and lipid in cow milk is in agreement with the study conducted on piglets (Roy et al., 2022).

It was noted that almond milk had a more rapid gastric emptying of protein than oat milk, but there was no difference in the gastric emptying rate of lipid between these two milks. To further understand this variation in the gastric emptying rate of protein and lipid, the lipid/protein (w/w) ratio of the gastric chyme was determined for both plant-based milks (Figure 7.10). For oat milk, the lipid/protein ratio was close to 1 throughout the 120 min of digestion, indicating that protein and lipid emptied from the stomach at similar rates. In contrast, the lipid/protein ratio for almond milk gradually increased with the digestion time, but the variations between individual rats at 120 min were too large to reach a definite conclusion. The large variation may have been caused by the small sample size (n = 4) in the present study, different activities of digestive enzymes (pepsin and lipase) and differences in the gastric motility and mixing patterns in individual rats. However, the mean lipid/protein ratio at 120 min for almond milk was 2.9:1. These results indicate that almond lipids required a longer time to empty from the stomach relative to

almond proteins. This was probably because the almond oil bodies flocculated and coalesced upon gastric digestion, and the almond milk separated rapidly into a lipid-rich upper layer and a lower aqueous phase (Figures 7.3 and 7.4). This would lead to more rapid emptying of proteins from the aqueous phase whereas the lipid layer would be expected to have somewhat delayed emptying. Our previous *in vitro* study on almond milk using a dynamic gastric simulator showed a more rapid emptying of almond proteins than of almond lipids (Wang et al., 2020). Once the almond milk had layered in the stomach chamber, all the almond lipids floated on top of the chyme and no detectable lipids were emptied until the lower aqueous phase had completely emptied from the stomach (Wang et al., 2020). In the present *in vivo* study, this trend was not clearly visible, although the almond lipids tended to empty slowly over time. This may have been because the almond milk had a higher lipid content (7% lipids) in the *in vitro* study, whereas the almond milk in the present *in vivo* study contained 3% lipids.

In comparison with almond milk, oat milk is a relatively more homogeneous and gastric-stable emulsion (Figures 7.3 and 7.10). The protein fractions in oat milk had a slightly delayed gastric emptying compared with those in almond milk. The soluble dietary fibre β-glucan might play a role in the delayed emptying of oat milk. Oat β-glucan has been shown to be able to delay gastric emptying (Begin et al., 1989; Mälkki & Virtanen, 2001).

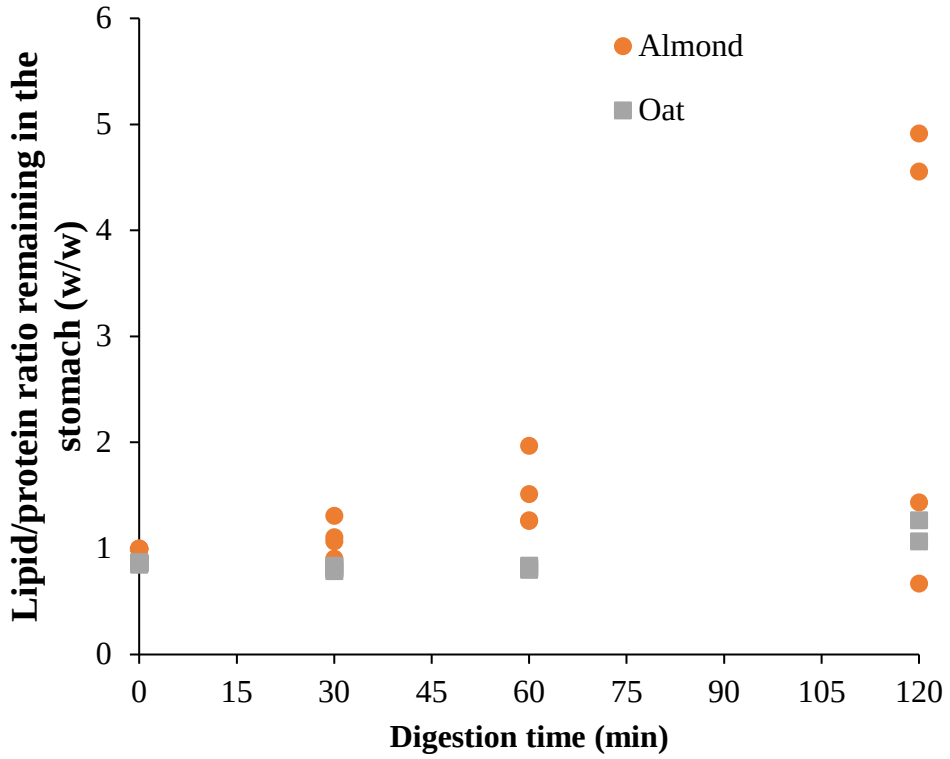


Figure 7.10 *The ratio of lipid to protein of the almond milk and the oat milk in the rat stomach at different times.*

Gastric emptying is a complex process that depends on both food properties and biological factors (Acevedo-Fani & Singh, 2022; Liu et al., 2020). Food characteristics, for example, nutritional composition, ingested volume, calorie content, viscosity and processing, have been reported to have an effect on food digestion and gastric emptying rates (Kong & Singh, 2009; Kwiatek et al., 2009; Lundin et al., 2008; Mazzawi et al., 2019; Zhu et al., 2013). *In vivo*, biological factors such as gastrointestinal peptide hormone regulation, the feedback effect of nutrients and the age, gender, gastric pH, gastric motility and digestive enzyme concentrations of an individual may also play extremely important roles in gastric emptying (Gamble et al., 2013; Hellmig et al., 2006; Karhunen et al., 2008; Tougas et al., 2000). The gastric emptying rate of liquid is determined by the pressure difference between the stomach and the small intestine.

Overall, in the present study, cow milk behaved significantly differently from the other two liquid milks because of its distinctive feature of casein micelles. It was ingested as a liquid food but then formed a solid curd in the stomach, with a size much larger than the size of food particles that can pass through the pyloric valve (i.e., 1–2 mm) to the duodenum (Meyer et al., 1981). The macronutrients that were entrapped into the curd phase were physically retained in the stomach and thus induced delayed gastric emptying. Almond milk separated into a floating cream layer and a lower aqueous layer in the stomach, and the proteins in the lower aqueous phase appeared to empty at a more rapid rate than the lipids in the cream layer. In contrast, oat milk remained homogeneous and the proteins and lipids emptied gradually. Considering the physiological conditions, in addition to the different dietary fiber contents, the different types of protein, lipid and carbohydrate in the almond milk and oat milk, the gastrointestinal hormone response and individual biological properties such as gastric pH, gastric motility and digestive enzyme concentration may also have affected the gastric emptying rates, especially when the sample size was small (Liu et al., 2020).

7.5 Conclusions

This study provided information about the gastric digestion of proteins, and the intragastric stability and gastric emptying profile of plant-based milks in the rat model. The results were compared with those from a dairy-based milk. They highlight intragastric stability and food structure/matrix changes during the gastric digestions of the different experimental milks, which had important effects on the gastric emptying rate of proteins and lipids. The satiety response, small intestinal digestion and absorption of different plant-based milks will require further studies. This study may have implications for designing novel plant-based products that most closely mimic the beneficial properties of dairy milk.

Chapter 8 General discussion and future recommendations

8.1 General discussion

Plant-based alternative milk is a fast-growing food category worldwide. There is a growing number of consumers using plant-based drinks to replace cow milk. However, the scientific knowledge about digestion and absorption of plant-based alternative milks is largely unknown. The understanding of their digestion behaviour lays the foundation for the successful development of novel products for specific consumer needs. In particular, gastric digestion plays a critical role in controlling the digestion kinetics. This project investigated the digestive behaviour of selected plant-based alternative milks under gastric conditions, with the main focus on the changes in the structures and physicochemical properties during dynamic gastric digestion, and the consequences on the macronutrient release for the next step of digestion.

Firstly, the *in vitro* gastric digestion of three kinds of the most popular plant-based alternative milks, i.e., almond milk (Chapter 4), soymilk (Chapter 5), and oat milk (Chapter 6), was investigated using an HGS. The HGS has been widely recognized as a useful tool to study the physical and chemical properties of food materials in the simulated gastric environment. Using this model, the simulated gastric secretion (dynamic pH and enzyme concentration) and gastric emptying can be precisely controlled.

Plant-based alternative milk is a natural, complex oil-in-water emulsion system that is similar to cow milk. In plant-based alternative milk, lipids exist in the formation of oil bodies that are stabilized by specific membrane proteins (mainly oleosins). The plant storage proteins are mostly dispersed in the aqueous phase, and a small number of storage proteins may also weakly interact with the membrane proteins. Under dynamic

simulated gastric conditions, the HGS model revealed the considerable difference in the digestion behaviour, physical stability, and microstructure of almond milk, soymilk and oat milk. Almond milk was a typical gastric-unstable emulsion (Chapter 4). Upon gastric acidification, almond oil bodies flocculated, and proteins aggregated rapidly and formed large aggregates (Figure 8.1). The oil bodies coalesced quickly due to the pepsin hydrolysis of the interfacial proteins, and this accelerated the creaming process in the HGS, with an upper layer rich in lipids and a lower aqueous phase consisting of proteins. This layering substantially delayed gastric emptying of lipids, whereas protein was emptied gradually (Figures 8.2 and 8.3). The results of gastric digestion of almond milk highlight the role of the interfacial properties of oil bodies in controlling the gastric colloidal stability and food structure of plant-based alternative milk.

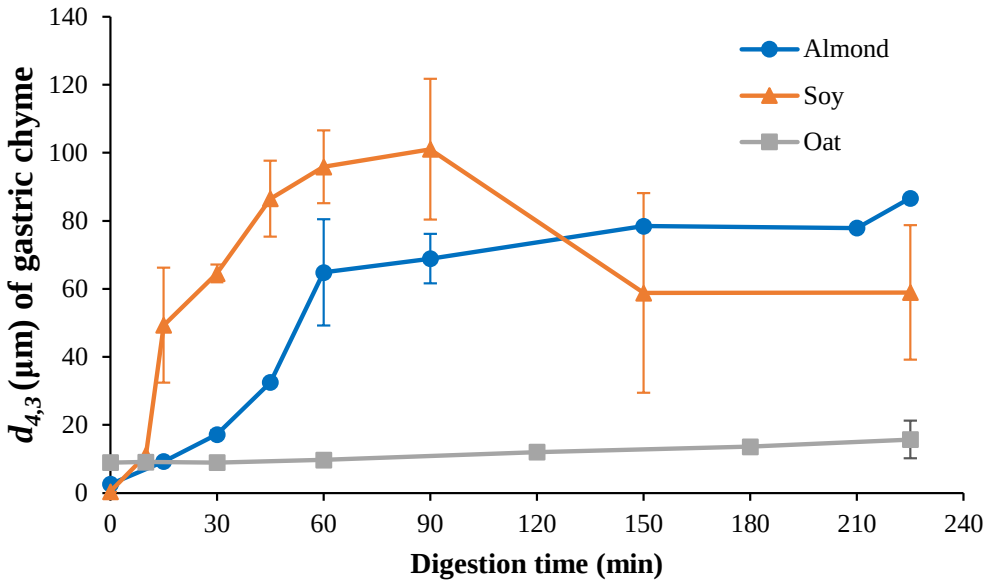


Figure 8.1 *Changes in volume-weighted average diameter $d_{4,3}$ of gastric chyme of almond milk, soymilk, and oat milk during gastric digestion in the HGS.*

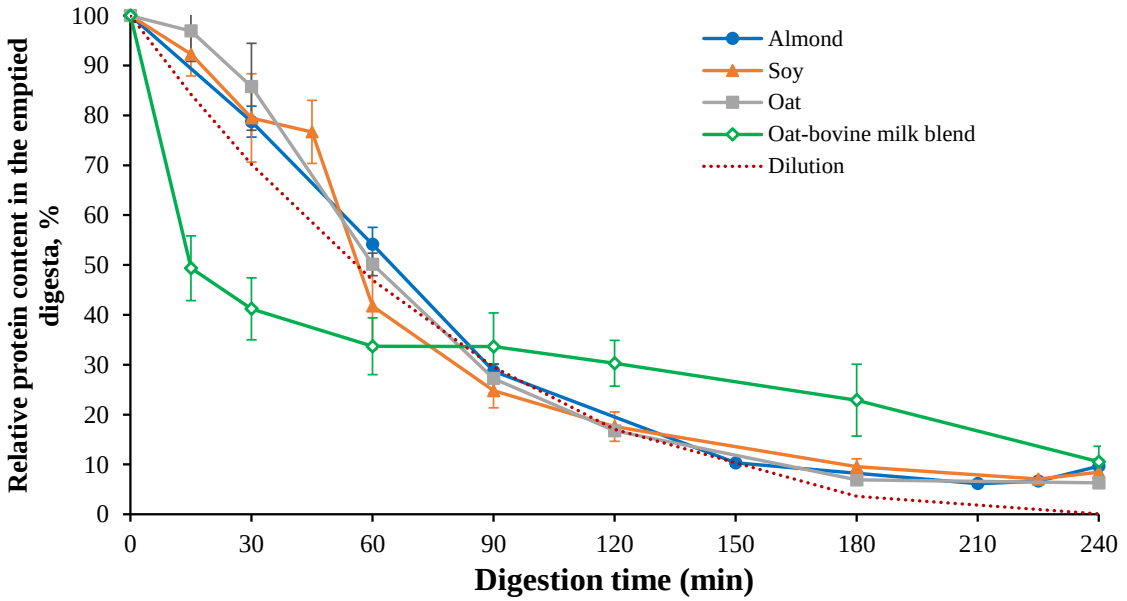


Figure 8.2 *Relative protein content in the emptied digesta collected from almond milk, soymilk, oat milk and oat-bovine milk blend during gastric digestion.*

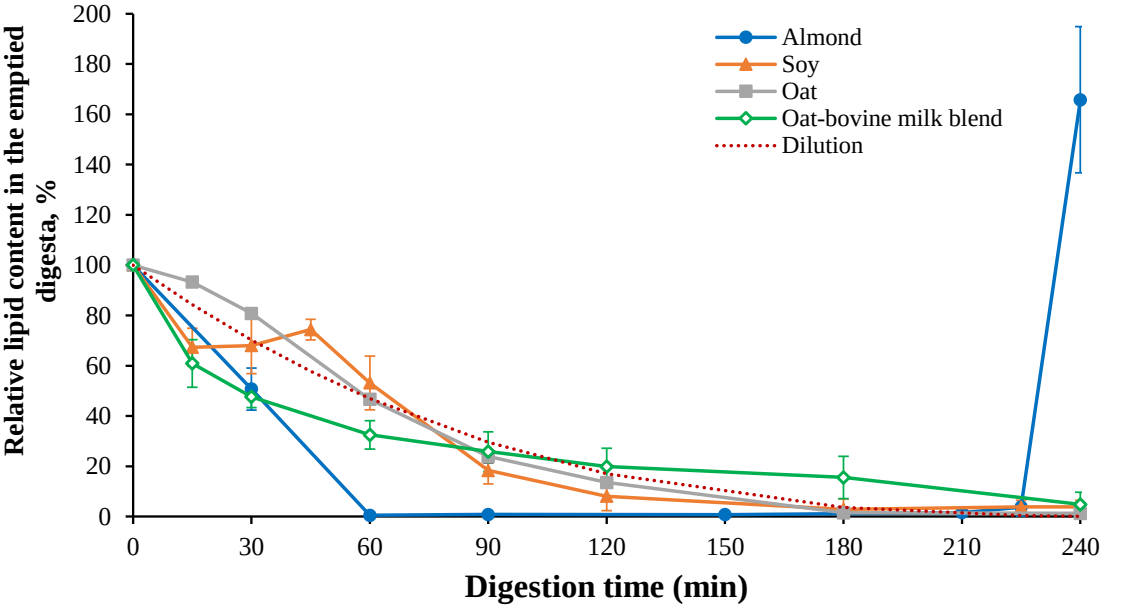


Figure 8.3 *Relative lipid content in the emptied digesta collected from almond milk, soymilk, oat milk and oat-bovine milk blend during gastric digestion.*

Under the same conditions, soymilk coagulated and formed small tofu-like gel particles after a few minutes of gastric digestion, when the gastric pH was around 6.59 in the HGS (Chapter 5). One interesting finding in this study is that this initial coagulation was induced by the action of pepsin rather than isoelectric pH, although the molecular mechanism is not clear, as this has not been reported before. Once the coagulated gel particles developed, the particle size of soymilk increased rapidly and then decreased subsequently (Figure 8.1), which reflects its microstructural evolution in the HGS. The microstructural changes were affected by gastric pH and pepsin in two aspects: 1) pepsin induces protein coagulation (increase in particle size), probably through hydrolysis of some specific polypeptides, 2) pepsin hydrolyses and breaks down the formed curd particles (decrease in particle size) at acidic pH. The coagulated network comprising protein and oil bodies quickly precipitated in the HGS, and the soymilk separated into a sediment phase (mainly soy protein and lipids) and an upper clear aqueous phase. Interestingly, this coagulation did not significantly affect the gastric emptying of both protein and lipid (Figures 8.2 and 8.3).

Surprisingly, the overall delivery of macronutrients was neither significantly delayed by the protein coagulation nor accelerated to a substantial extent by the breakdown of small sedimented curd particles. This indicates the dynamic balance between the formation and the breakdown of coagulated particles in the complex chemical and biochemical environment of the stomach is an important factor that must be considered when designing food emulsions involving coagulable proteins. However, one limitation here is that the digesta was emptied from the bottom of the HGS because of the design of the HGS, not from the end of a J-shape stomach. The real gastric emptying kinetics of soymilk under physiological conditions may need to investigate in *in vivo* models.

The study in Chapter 6 demonstrated that oat milk was a relatively stable emulsion system, as no significant physical destabilization was observed under dynamic pH changes and no considerable changes in particle size of oat milk over digestion time (Figure 8.1). The protein and lipid were relatively homogeneously dispersed in the oat milk throughout the entire gastric digestion, and both were emptied gradually (Figures 8.2 and 8.3).

One remarkable difference in the intragastric microstructures between the three kinds of plant-based alternative milk is that extensive coalescence was found in almond milk, but no notable coalescence was seen in soymilk and oat milk. One possible explanation is that the curd particles in soymilk and the protein aggregates in oat milk play a role as a physical barrier protecting plant oil bodies from the attack of pepsin. However, it is more likely that the interfacial membrane of soy oil bodies and oat oil bodies have superior stability against protein hydrolysis by pepsin.

In Chapter 6, the role of dairy protein in the gastric digestion behaviour of plant- and dairy-based milk blends was also investigated. The milk blend was prepared by mixing oat milk with bovine skim milk (1: 1, v: v). As expected, the milk blend showed a considerably different digestion behaviour compared to oat milk itself. The milk blend coagulated in the first few minutes of gastric digestion, which was induced by coagulation of the casein micelles due to the hydrolysis of κ-casein by pepsin. The proximate analysis and SDS-PAGE analysis indicated that the curd particles consisted of not only dairy proteins (mainly caseins), but also some specific oat proteins and oat lipids. The coagulation remarkably delayed gastric emptying of both proteins and lipids due to the curds having a larger size than the sizes required to empty from the HGS (Figures 8.2 and 8.3). It was also found that the oat proteins in the curds were protected from attack by the added pepsin. This study clearly showed that the addition of dairy protein into plant-based

milk can significantly modify the gastric structure, protein hydrolysis and macronutrient delivery of plant-based milk. On the other hand, plant components had also altered the structure of coagulated dairy protein networks. This study provides insights into the use of dairy protein as an effective approach to designing food structure, manipulating gastric colloidal stability and digestion kinetics of plant-based alternative milks.

The gastric behaviours of almond milk, soymilk, oat milk and oat milk-bovine skim milk blend in the HGS were summarized in Table 8.1

Table 8.1 *Summary of gastric behaviour of almond milk, soymilk, oat milk and oat milk-bovine skin milk blend in the HGS.*

	Almond milk	Soymilk	Oat milk	Oat milk-bovine skim milk blend
Protein content	3% (w/w)	3% (w/w)	3% (w/v)	3% (w/v)
Lipid content	7.08% (w/w)	1.81% (w/w)	1.40% (w/v)	0.73% (w/v)
Colloidal stability	Flocculation, coalescence, creaming, phase separation.	Coagulation, sedimentation.	No significant change.	Coagulation, sedimentation.
Oil bodies distribution	Oil bodies floated on the top of the aqueous phase.	Most oil bodies were incorporated into the particles.	Oil bodies were dispersed in the oat milk.	Most oil bodies were incorporated into the curd and released gradually with the breakdown of the curd structure.
Oil bodies microstructure	Oil bodies coalesced and formed free oil.	No coalescence was found.	No microstructural change was found.	No coalescence was found.
Storage protein distribution	Storage protein homogenously dispersed in the HGS.	Protein coagulated and sedimented at the bottom of the HGS.	Proteins were dispersed in the oat milk.	Casein formed curd and sedimented in the HGS, some oat proteins were incorporated into the curd, while the others were dispersed in the serum phase with whey proteins.
Protein microstructure	Protein aggregated at isoelectric pHs.	Storage proteins coagulated and formed small-sized curd particles.	No microstructural change was found.	Dairy protein coagulated and some of the oat proteins were incorporated into the curds.
Protein hydrolysis	Most proteins have been hydrolyzed when pH dropped to ~3 at 90 min.	The major soy proteins hydrolyzed when pH dropped to 4.5 at 60 min	Oat proteins were hydrolyzed rapidly when pH was 2 at 120 min.	Both dairy proteins and oat proteins were hydrolyzed extensively when pH was 2 at 120 min, but the incorporation of the oat proteins in the casein matrix delayed the breakdown of the oat proteins compared with that of the proteins in the oat milk alone.
Gastric emptying	Lipid emptying was significantly delayed	Gastric-induced coagulation did not have a significant impact on either protein or lipid emptying, except for an initial delay of lipid emptying in the first 15 min.	Gradual emptying of protein and lipid	Both protein and lipid emptying were significantly delayed.

The findings in Chapter 7 provide an understanding of the difference between *in vivo* digestion behaviour of plant-based alternative milk and cow milk. Almond milk and oat milk were selected for the reason that almond milk was a typical gastric-unstable sample and oat milk showed superior physical stability in the HGS. The adult rats were selected as *in vivo* models due to their larger stomach volume compared to the growing rats. The cow milk, almond milk and oat milk were formulated to contain an equal quantity of protein, fat and carbohydrates and similar gross energy in order to better understand the role of food structure in controlling gastric emptying of protein and lipids. In this *in vivo* study, many changes in the macrostructure, microstructure, colloidal stability and protein hydrolysis that was observed in the HGS were confirmed, although the dynamic pH profile and gastric emptying profile were slightly different between the *in vitro* and *in vivo* system. The difference was possibly caused by the different food compositions of milks used in different systems, as the milks tested for *in vivo* study have been formulated. The results demonstrated different gastric retention profiles of almond milk, oat milk and cow milk due to their different structural properties in the stomach. In the rat stomach, almond milk aggregated, creamed and rapidly layered, whereas there were no physical changes in oat milk. Cow milk coagulated and formed a curd phase and a liquid phase. This coagulation led to higher retention of protein and fat in the stomach compared to almond milk and oat milk. Almond milk had the fastest gastric emptying of protein compared to cow milk and oat milk and tended to have a slower gastric emptying of lipids when compared to almond protein due to the creaming.

These findings highlight intragastric stability and food structure/matrix changes during gastric digestion of the different milk types, which play a part in the gastric emptying rate of protein and lipids. A summary of the comparisons of the changes during *in vitro* and *in vivo* gastric digestion is shown in Table 8.2.

Table 8.2 *Summary of the difference and similarities of the changes of almond milk and oat milk during in vitro and in vivo gastric digestion.*

Properties	Almond milk		Oat milk	
	In vitro (Chapter 4)	*In vivo* (Chapter 7)	*In vitro* (Chapter 6)	*In vivo* (Chapter 7)
Protein content	3.0% (w/w)	3.24% (w/w)	3.0% (w/v)	3.31% (w/w)
Lipid content	7.08% (w/w)	3.20% (w/w)	1.40% (w/v)	3.14% (w/w)
Targeted digestion time	240 min	120 min	240 min	120 min
Digestion temperature	37 ± 0.5 °C	37.5-37.8°C (rats' normal body temperature)	37 ± 0.5 °C	37.5-37.8°C (rats' normal body temperature)
Digestive enzymes	Pepsin	Rats' pepsin and lipase	Pepsin	Rats' pepsin and lipase
Gastric pH changes	The pH was 4 at 60 min and 1.8 at 150 min in the HGS.	The gastric pH was 4.8 at 60 min and 3 at 120 min in rats.	The pH was 3.8 at 60 min and 2 at 120 min in the HGS.	The gastric pH was 4.1 at 60 min and 2 at 120 min in rats.
Gastric colloidal stability	Flocculation, coalescence creaming, phase separation.	Flocculation, coalescence creaming, phase separation.	No significant changes were observed	Coalescence of oil bodies was observed, probably due to the presence of the lipase.
Protein hydrolysis	Extensive hydrolysis was observed at pH 3.	Extensive hydrolysis was observed at pH 3.	Extensive hydrolysis was observed at pH 2.	Extensive hydrolysis was observed at pH 2.
Gastric emptying	After creaming, no detectable lipids emptied until the lower aqueous phase had completely emptied from the HGS.	The trend observed in the HGS was not clearly visible here, although lipids tended to empty slower than proteins, probably due to a reduced lipid content (55% reduced) of almond milk used here.	Both protein and lipid were steadily emptied from the HGS.	Both protein and lipid were steadily emptied from the stomach.

Finally, the findings presented in this thesis provide the latest information about the gastric digestion behaviour of plant-based alternative milk and how their behaviour is different compared to cow milk. The main innovative results from this project highlight the impact of structural properties and the modification of protein composition of plant-based alternative milk on controlling their physicochemical and structural changes during gastric digestion, and the direct consequences on alteration of gastric delivery kinetics. From an application point of view, the knowledge gained from this PhD project may provide new insights into the tailored design of novel plant-based alternative milk products or milk blend products for specific consumer needs.

8.2 Suggestions for future research

Influence of processing on digestion behaviour

This thesis provides an understanding of the gastric digestion behaviour of almond milk in its natural state. However, in commercial manufacturing, heating (pasteurization or UHT) and homogenization are often applied. The influence of commercial processing on the changes in physical and chemical properties and structural stability under gastric conditions is not clear, this needs to be investigated.

Inclusion of gastric lipase in in-vitro digestion models

The digestion behaviour of plant-based milks was studied in the HGS in the presence of pepsin only and gastric lipase was not applied, as protein digestion is the major digestion behaviour in the stomach and the current study focused on protein digestion. However, it would be interesting to include gastric lipase in future gastric digestion investigations such as the influence of gastric lipolysis on the structural changes and digestion behaviour.

Investigation of plant oil body membrane proteins properties

This thesis highlights the interfacial stability of oil bodies as a determining factor in the gastric colloidal stability of the whole plant-based milk system. Although protein hydrolysis of plant-based alternative milk was monitored in the current thesis, the digestion properties of oil body membrane protein (mainly oleosins) require further investigation. The information on the physicochemical and functional properties of oil body membrane proteins will be valuable in designing the interfacial composition of artificial emulsion systems.

Investigation of protein blends-stabilized emulsion system

The digestion behaviour of the oat milk-cow skim milk blend was studied in detail in the current thesis. However, due to the nature of oat milk, i.e., the oil bodies are dispersed within aggregated protein particles, after the centrifugation, the oil bodies precipitated with the protein aggregates. Therefore, the interfacial composition of the milk blend was not studied. It would be interesting to investigate the interfacial composition of the emulsion made with isolated plant protein and dairy protein, and their interfacial properties during gastric digestion. In addition, it will be desirable to study the blends of other plant-dairy combinations, e.g., almond and cow milk.

Investigation of intestinal digestion

The findings from this study demonstrate different gastric stability between different plant-based alternative milks and compared to cow milk. It would be interesting to further investigate the influence of the different gastric digestion behaviours on the subsequent small intestinal digestion of released lipids and proteins.

Suggestions for future gastric digestion studies in rats

In this study, the baseline and postprandial blood sugar and blood triglycerides of rats were analyzed to understand nutrient absorption. However, no clear trends between different milks over digestion time were found (Appendix 8 and 9) due to the large variations between individual rats even in the baseline group. This suggests that the baseline and postprandial blood samples should be collected from the same individual to reduce the variation between individuals. In the current study, blood samples were collected from rats' hearts after they were fully anesthetised, and the rats were euthanised after blood sampling. Cardiac puncture allows the collection of a sufficient amount of blood from a single animal (5-10 ml) for analysis. However, if monitoring changes in the preprandial and postprandial blood samples from the same rat, the cardiac puncture method might be not an appropriate technique, applying other blood collection techniques will need to be considered.

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Appendices

Appendix 1: Diluted concentration calculation

The calculated values are based on assuming that sample is homogeneously distributed in the stomach throughout the entire digestion process.

Emptying time /min	**0**	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**	**135**	**150**	**165**	**180**	**195**	**210**	**225**	**240**
Emptying volume /ml		50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
SGF addition volume /ml	/	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5
C_{before}, concentration before dilution		100%	84%	70%	58%	47%	38%	30%	23%	17%	12%	9%	6%	4%	2%	1%	0%
V_{before} volume before dilution /ml		200	188	175	163	150	138	125	113	100	87.5	75	62.5	50	37.5	25	12.5
V_{after} volume after dilution /ml	200	237.5	225	213	200	188	175	163	150	138	125	113	100	87.5	75	62.5	50
C_{after}, concentration after dilution	100%	84%	70%	58%	47%	38%	30%	23%	17%	12%	9%	6%	4%	2%	1%	0%	0%

$$C_{after} = C_{before} \times \frac{V_{before}}{V_{after}}$$

Where C_{after} is the concentration of sample after dilution; C_{before} is the concentration of sample before dilution, it equals to C_{after} at the previous emptying time point, e.g. C_{before} at 30 min =C_{after} at 15 min; V_{before} is the volume of sample inside of the stomach before dilution, V_{before} equals to V_{after} at the previous time point minus the gastric emptying volume, e.g. V_{before} at 30 min=V_{after} at 15 min-50 ml; V_{after} is the volume of sample inside of the stomach after dilution, i.e. V_{after} =V_{before} + SGF addition volum

Appendix 2 The protein/enzyme ratio at 15 min in the HGS

In the chapter 4	
At 15 min, before emptying	
Pepsin used	P7000, Sigma -Aldrich
Protein content in the HGS (g)	200 g×3%=6 g
SGF added at 15 min (volume, ml)	2.5 ml/min × 15 min=37.5 ml
Pepsin concentration in SGF	3.2 g/L SGF
Pepsin content in the HGS at 15 min	3.2 g/L × 10^{-3} × 37.5 ml=0.12 g
Protein/enzyme ratio	50: 1
In the Gailler et al 2012' study	
Pepsin used	P7000, Sigma -Aldrich
Protein/enzyme ratio	25: 1

Appendix 3: Microstructural changes in the digesta emptied of soymilk at different times of *in vitro* gastric digestion

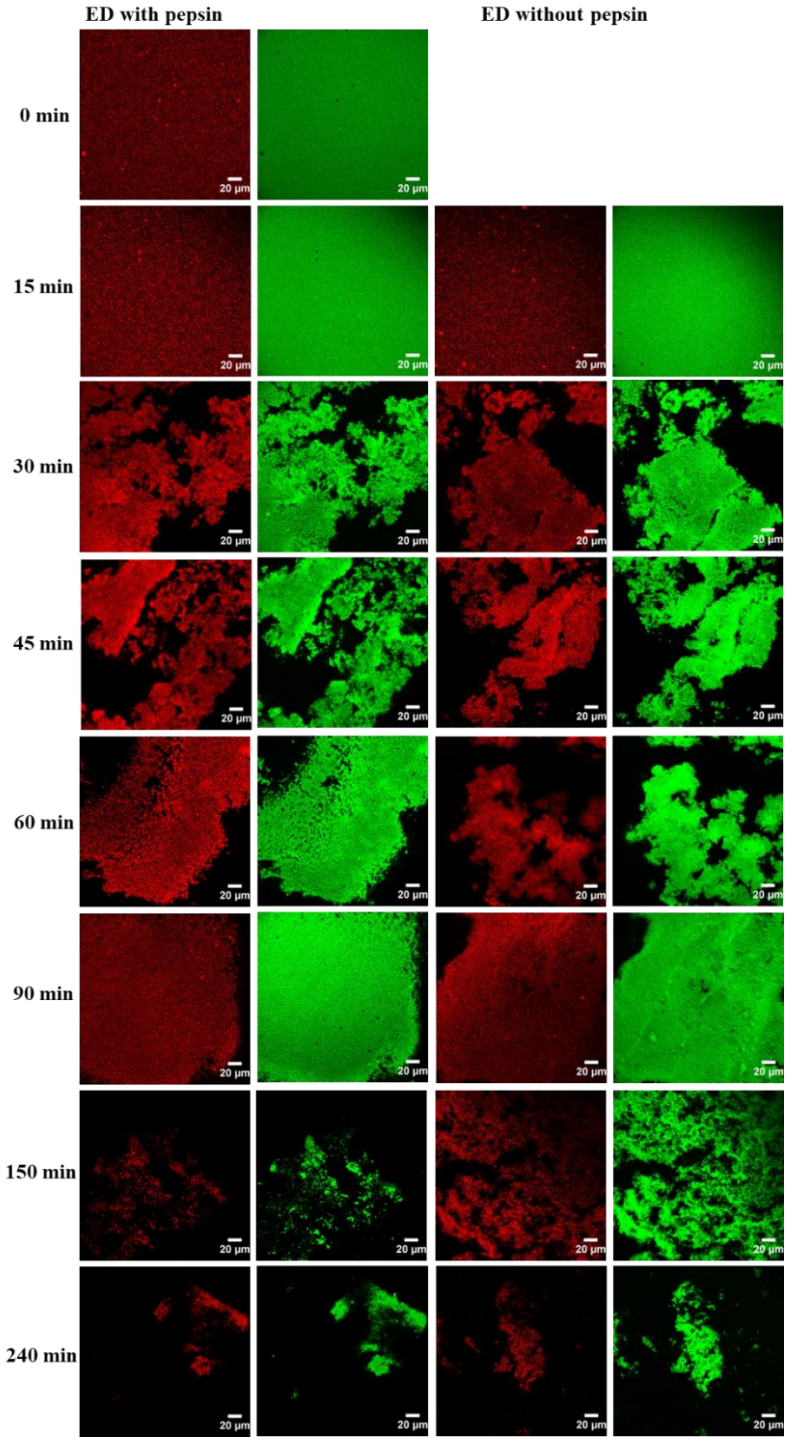

Figure A1 *Confocal micrographs showing the microstructural changes in the digesta emptied of soymilk at different times of simulated gastric digestion, with and without the addition of pepsin. Note.* Samples were stained with Nile Red (for lipid) and Fast Green (for protein). The scale bar in all images is 20 µm. ED, emptied digesta.

Appendix 4: The photograph of curd particles formed in the oat milk–bovine skim milk blend during *in vitro* gastric digestion in the absence of pepsin

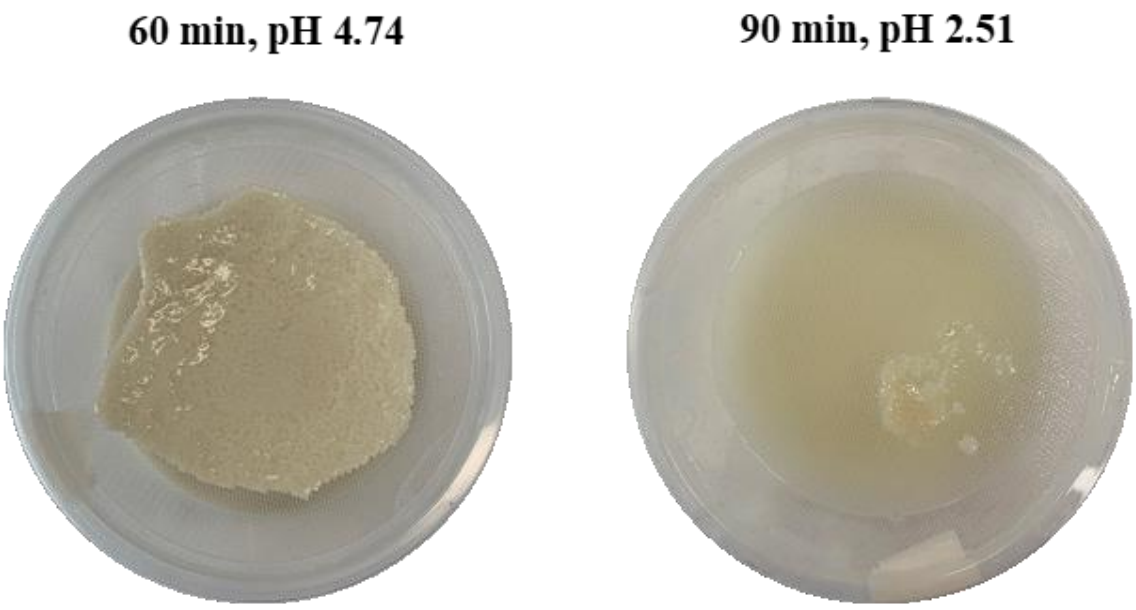

Figure A2 *Curd particles formed in the oat milk–bovine skim milk blend during in vitro gastric digestion in the absence of pepsin.*

Appendix 5: The photograph of curd formed from 1.45% (w/v) skim milk during *in vitro* gastric digestion

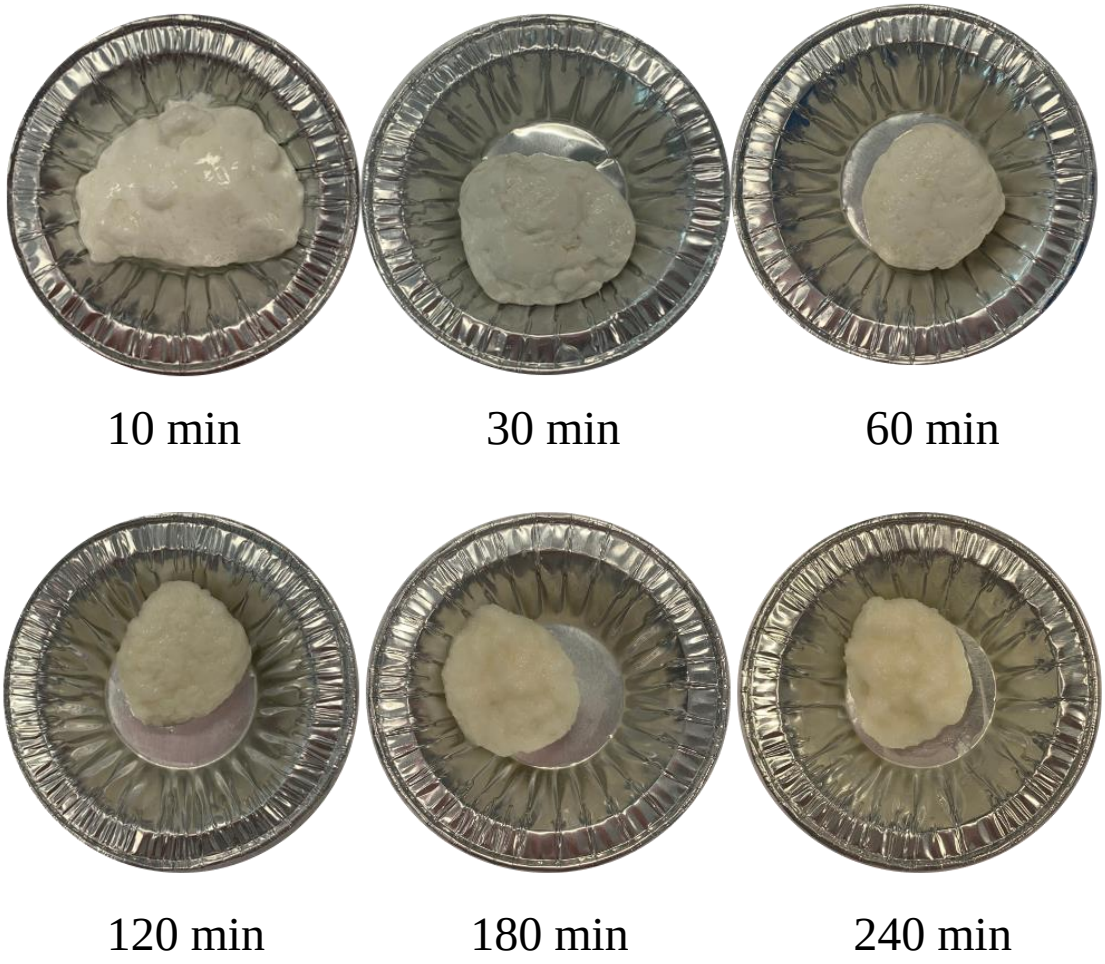

Figure A3 *Curd formed from 1.45% (w/v) skim milk during in vitro gastric digestion.*

Appendix 6: The microstructural changes in the emptied digesta of oat milk samples and oat milk–bovine skim milk blend samples collected at different gastric emptying points of *in vitro* gastric digestion

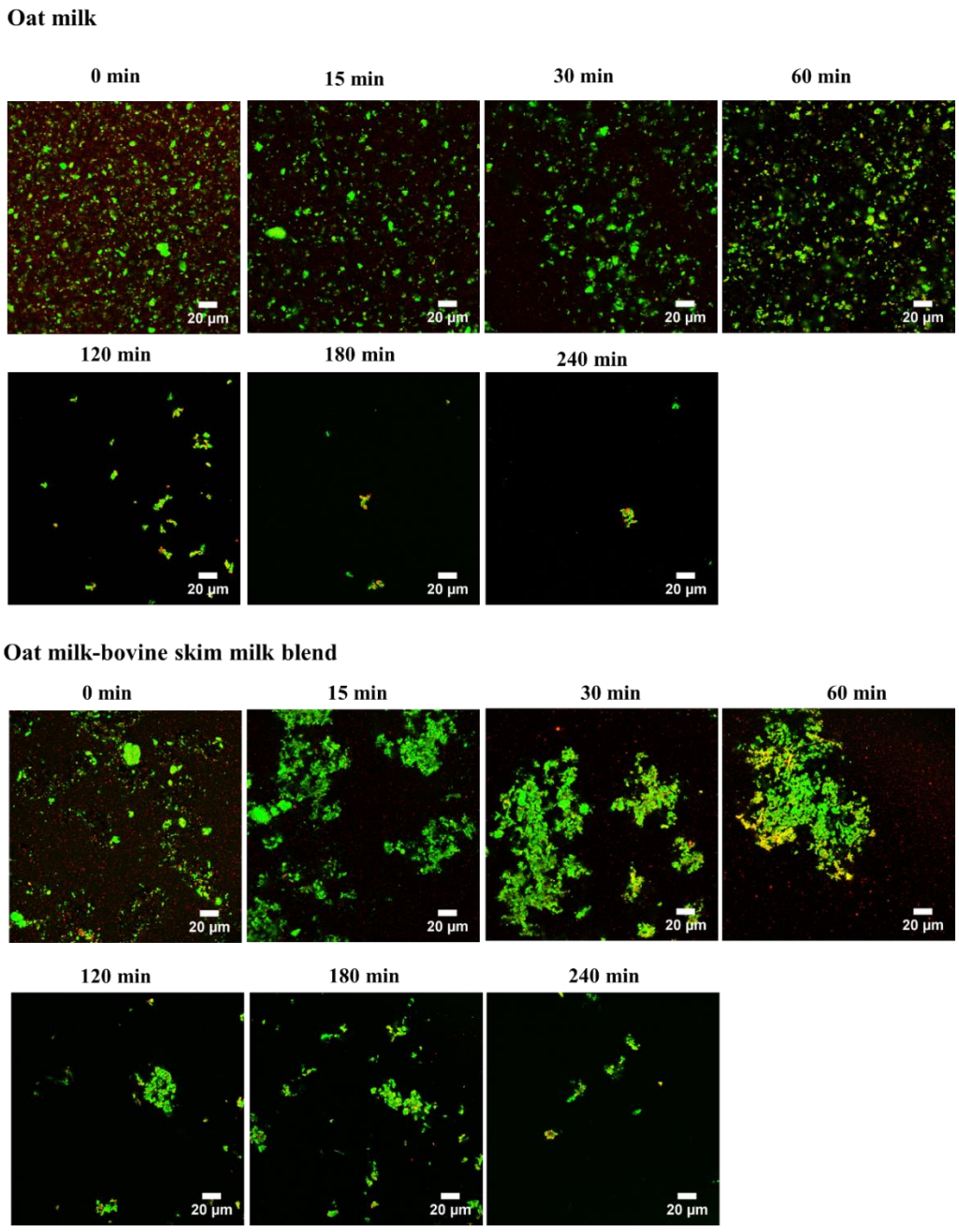

Figure A4 *Confocal micrographs showing the microstructural changes in the emptied digesta of oat milk samples and oat milk–bovine skim milk blend samples that collected at different gastric emptying points of simulated gastric digestion in the HGS.*

Note. Samples were stained with Nile Red (for lipid) and Fast Green (for protein). The scale bar in all images is 20 μm.

Appendix 7: Confocal micrographs of cow milk, almond milk and oat milk in the rat small intestine at different digestion times.

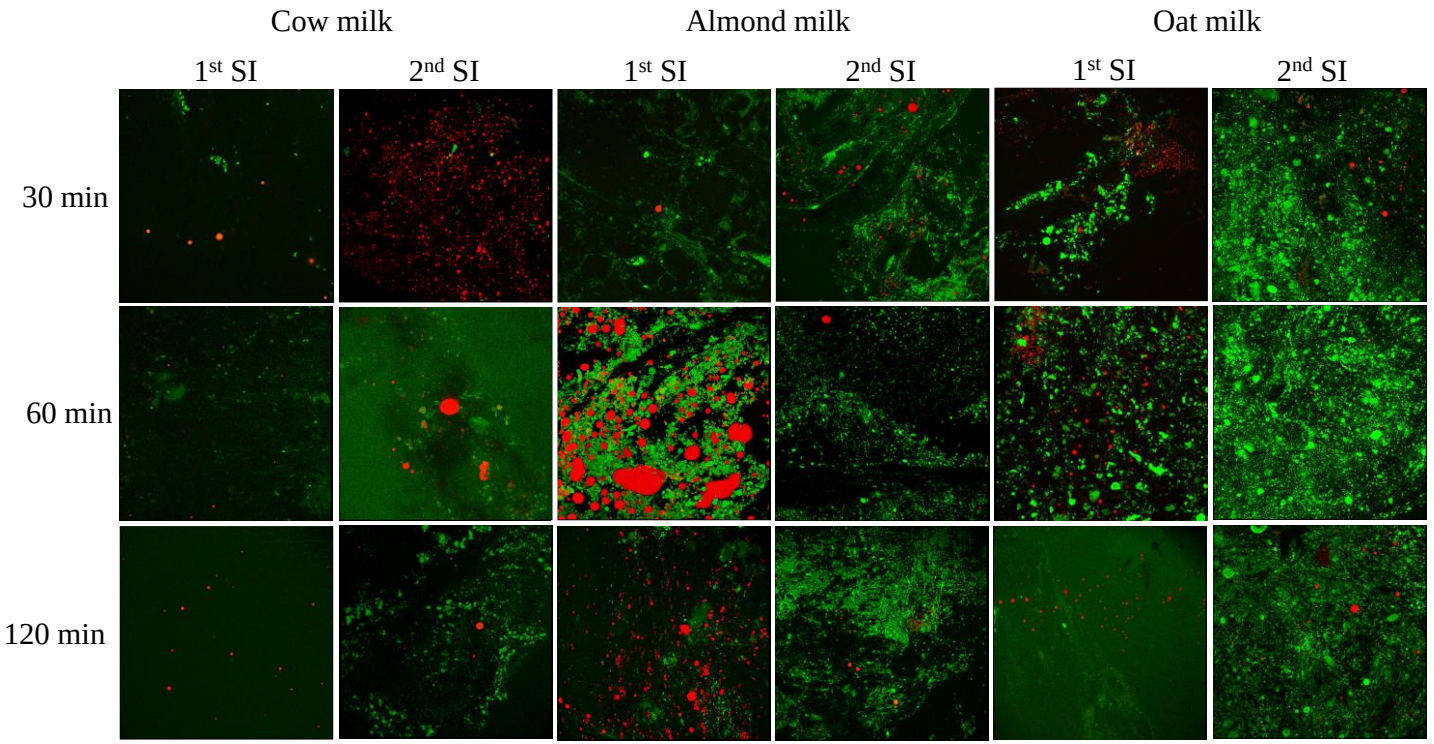

Figure A5 *Confocal micrographs of cow milk, almond milk and oat milk in the rat small intestine at different digestion times.*

Note. Samples were stained with Nile Red (for lipid) and Fast Green (for protein). 1st SI, the upper part of the small intestine (jejunum); 2nd SI, the lower part of small intestine (ileum).

Appendix 8: The serum triglyceride in rat fed with cow milk, almond milk and oat milk

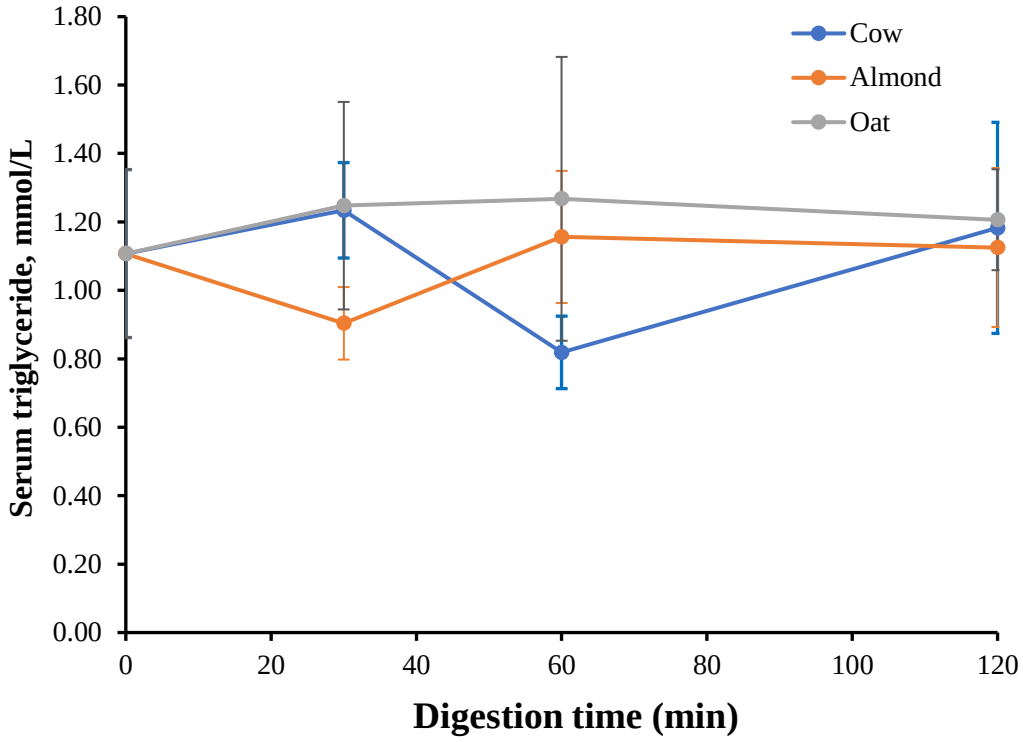

Figure A6 *Changes over time in the serum triglycerides (mean ± standard error of the mean) of rat fed with cow milk, almond milk and oat milk.*

Note. The serum triglycerides were determined using GPO-PAP (Lipase/ Glycerol kinase) method by the Nutrition laboratory, Massey University. A two-way ANOVA was performed to analyze the effect of digestion time and milk type on the level of serum triglycerides. The results showed that no significant effect on the serum triglyceride of either the digestion time or the milk types or their interaction.

Appendix 9: The Blood glucose levels in rat fed with cow milk, almond milk and oat milk

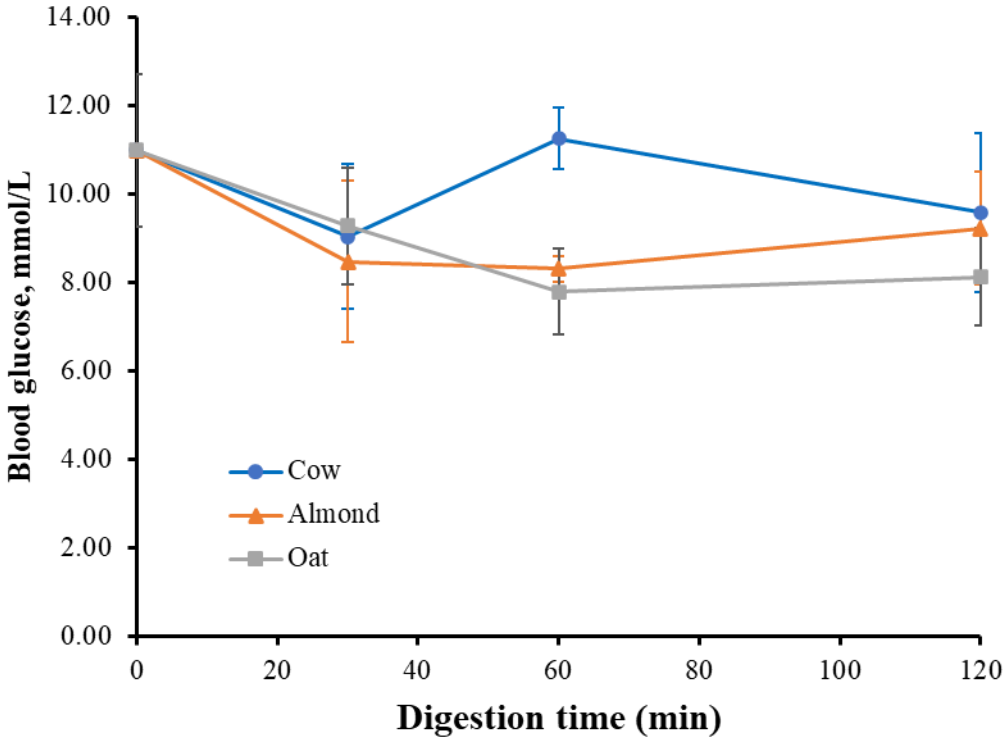

Figure A7 *Changes over time in the blood sugar (mean ± standard error of the mean) of rat fed with cow milk, almond milk and oat milk.*

Note. The blood glucose levels were measure with a Accutrend Plus monitor using whole blood. Blood sample was collected via cardiac puncture after the rat was fully anesthetised and the rats were euthanised after blood sampling. One drop of fresh whole blood was applied to the reagent area of a test strip for blood glucose measurement. A two-way ANOVA was performed to analyze the effect of digestion time and milk type on the level of serum triglycerides. The results showed that no significant effect on the serum triglyceride of either the digestion time or the milk types or their interaction.

Appendix 10: DRC 16 forms

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