Ollscoil na hÉireann, Corcaigh

**National University of Ireland, Cork**



# The impact of protein polymorphism, bovine feeding strategy and consumption temperature on the *in vitro* digestion behaviour of selected dairy products

Thesis presented by

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for the degree of

**Doctor of Philosophy**

**University College Cork**

**School of Food and Nutritional Science**

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*28/03/*2025

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# Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property. All the work described herein is the original work of the author, with the following exceptions:

Chapter 4: Stephane Quellec, Maja Musse and Guylaine Colwette and Tiphane Lucas contributed to Magnetic Resonance Imaging.

Signed

Conor Fitzpatrick

Student Number: 121101506

Date: 28/03/2025

# Abstract

**The impact of protein polymorphism, bovine feeding strategy and consumption temperature on the *in vitro* digestion behaviour of selected dairy products**

This work was carried out as part of a wider multidisciplinary research project within the Vistamilk Science Foundation Ireland (SFI) research centre entitled 'Targeted Project 8 - Digestive characteristics of dairy products. The overall aim of Target project 8 was to use *in vitro, ex vivo* and animal models to further the understanding of the digestion of dairy products. This research has fulfilled the goal of improving our knowledge of both *in vitro* digestion and dairy product digestion and has the potential to be of interest and benefit to consumers, industry and future researchers in the field of digestion.

The effect of the genetic profile of milk proteins on dairy product functionality is increasingly being studied due to implications for dairy processing and human health. The work presented in Chapter 2 of this thesis showed a significant effect of β-lactoglobulin genetic polymorphism on both the rheological properties, and subsequent digestion of skim milk yogurt. Milk that contained exclusively β-lactoglobulin B (BB) was found to form a stronger gel with lower levels of syneresis compared to milk with both β-lactoglobulin A and β-lactoglobulin B (AB) and exclusively β-lactoglobulin A (AA). This led to differences in digestion, where AA, with its looser gel compared to BB, showed faster hydrolysis (20% higher proteolysis at 60 min), while BB, with its dense curd was more resistant to gastric digestion than AA.

While protein polymorphisms affected the digestion of milk proteins in yogurt, bovine feeding strategy affected the digestion of lipids in milk. It is well known that cows fed a pasture-based diet (GRS) produce milk with distinct fatty acid profile to cows fed indoors on a total mixed ration diet (TMR), however, the release of these fatty acids during digestion had not been studied. This research showed that GRS milk released higher levels of unsaturated fatty acids (UFAs) during digestion compared to TMR milk. TMR milk released higher levels of saturated fatty acids during digestion with the lipid release profile yielding a higher Artherogenicity index during digestion.

The temperature at which milk is consumed also affects its digestion. By using MRI as a non-invasive technique, this work showed that hot milk coagulates faster in the gastric phase of *in vitro* digestion compared to cold milk. Cold milk (4°C) delayed coagulation by over 5 min compared to hot milk (60°C) and formed a lipid-rich cream layer at the top of the digesta, which was absent during gastric digestion of hot milk.

Finally, this research addressed a gap in *in vitro* digestion studies by developing a predictive model of pepsin activity accounting for the effect of both pH (1–6) and temperature (4–60°C) variations. This model was developed for both human pepsin and its porcine counterpart (commonly used as a substitute *in vitro*) based on measurements of each enzyme's activity at 37 pH/temperature combinations. It was then integrated into an excel spreadsheet to allow users of *in vitro* digestion methods to better understand their protein hydrolysis results. This tool also helps to translate *in vitro* digestion results using porcine pepsin to the *in vivo* situation with human pepsin.

The protein polymorphism findings (Chapter 2) show the importance of minor changes in amino acid sequence of proteins on both the functionality and digestion of dairy products, while the bovine feeding strategy findings (Chapter 3) reinforce the

benefits of high vs low pasture allowance in bovine feeding systems for human health. The temperature-related findings (Chapter 4) present a discussion on what temperature milk should be consumed at to potentially alter the kinetics of nutrients reaching the bloodstream. Finally (Chapter 5), this work has provided a tool for future *in vitro* digestion research that can be used to predict porcine and human pepsin activity during gastric digestion. This thesis therefore presents several novel research findings that will be of interest to researchers working both in dairy product production and dairy product digestion.

# List of publications and awards

**Peer-reviewed publications:**

***Fitzpatrick, C. J.,*** Musse, M., Feng, J., Collewet, G., Lucas, T., Timlin, M., Challois, S., Quellec, S., Dupont, D., Brodkorb, A., Freitas, D., & Le Feunteun, S. (2024). Monitoring the effect of consumption temperature of full-fat milk on *in vitro* gastric digestion using Magnetic Resonance Imaging. *Food Hydrocolloids, 109864. DOI: https://doi.org/10.1016/j.foodhyd.2024.109864* .

***Fitzpatrick, C. J.,*** Freitas, D., O'Mahony J.A., O'Callaghan T.F., Brodkorb, A. (2024). Variations in bovine milk proteins and processing conditions and their effect on protein digestibility in humans: a review of *in vivo* and *in vitro* studies. *Foods, Special Issue Modifications and Interactions of Milk Proteins in Different Processes and Products, Volume 12, Issue 22, Publisher MDPI https://doi.org/10.3390/foods13223683* .

**Manuscripts submitted for publication**

Fitzpatrick, C. J., Freitas, D., Comi, I., Vegarud, G. E., Røseth, A. G. Hayes, E., O'Callaghan, T. F, O'Mahony, J.A., Brodkorb, A. (2025). The combined effect of pH and temperature variations on human and porcine pepsin activity: A tool for protein digestion research. *Scientific Reports*, submitted Feb 2025.

## Manuscripts in preparation

***Fitzpatrick, C. J.,*** Freitas, D., Hayes, E., Daniloski, D., O'Mahony J.A., O'Callaghan T.F., & Brodkorb, A. (2024). Effect of β-lactoglobulin Protein Polymorphism on structure and digestion of yogurt.

***Fitzpatrick, C. J.,*** Freitas, D., Timlin, M., Druin G., O'Mahony J.A., O'Callaghan T.F., & Brodkorb, A. (2024). The effect of low and high pasture allowance on the *in vitro* gastric and intestinal digestion of bovine milk.

## Collaborations

Daniloski, D., Page, R. M., Lamichhane, P., ***Fitzpatrick, C. J.,*** Vasiljevic, T., Brodkorb, A., Timlin, M., Murphy, J. P., O'Callaghan, T. F., & McCarthy, N. A. (2024). Cheddar cheese production, structure and *in vitro* semi-dynamic gastric digestion: The role of β-casein phenotype. *Food Research International, 196, 115008.* *https://doi.org/10.1016/j.foodres.2024.115008.*

Egger, L., Sousa, R., Recio, I., Abbühl, L., Brügger, C., Santos-Hernández, M., Macierzanka, A., Szumała, P., Rama, A., Mulet-Cabero, A.-I., Perez-Moral, N., Brodkorb, A., ***Fitzpatrick, C, J.,*** Rieder, A., Levi, C.S., Gonçales, C., Rodriguez-Amado, I., Nobre, C., Canton, A.P., Arranz, E., Bot, F., O'Mahony, J.A., Grundy, M.M.-L., Pacheco-Cruz, I., Sepúlveda-González, A., Comi, I., Abrankó, L., Tormási, J., Gonzales, F., Ruas-Madiedo, P., Ruiz, L., Faria, M.-Â., Sobral, M.M.C., Jiménez-Munoz, L.M., Corredig, M., Jimenez, L., Molly, M., Rosa-Sibakov, N., Ménard, O., Orlien, V., Gousetti, O., Lykkepetersen, I., Ferranti, P., Olias, R., Andrade, C.D., Assunção, R., López-Nicolás, R., Simsek, S., Karakaya, S., El, S.N., Rezzi, S., Canarelli, S., Petit**,** V., Tuncil, Y.E., Hotrum, N., Broersen, K., Heimo, D., Dubois, S.,

Portmann, R.; Standardization of *in vitro* digestibility and DIAAS method based on the static INFOGEST protocol.

**Posters**

***Fitzpatrick C.J.,*** O'Callaghan T.F., Freitas, D., O'Mahony J.A., Brodkorb A. The reformulation of milk with high melting point lipids and its effect on *in vitro* gastrointestinal digestion. 7th International Conference on Food Digestion (ICFD2022), 3rd- 5th May 2022, Cork, Ireland.

***Fitzpatrick, C. J.,*** Musse, M., Feng, J., Collewet, G., Lucas, T., Timlin, M., Challois, S., Quellec, S., Dupont, D., Brodkorb, A., Freitas, D., & Le Feunteun, S. Monitoring the effect of consumption temperature of full-fat milk on *in vitro* gastric digestion using Magnetic Resonance Imaging. 8th International Conference on Food Digestion (ICFD2024), 9th – 11th April 2024, Cork, Ireland.

***Fitzpatrick, C. J.,*** Musse, M., Feng, J., Collewet, G., Lucas, T., Timlin, M., Challois, S., Quellec, S., Dupont, D., Brodkorb, A., Freitas, D., & Le Feunteun, S. Monitoring the effect of consumption temperature of full-fat milk on *in vitro* gastric digestion using Magnetic Resonance Imaging. The 13th NIZO Dairy Conference: Innovations in Milk Proteins 17th -20th October 2023, Papendal, The Netherlands.

***Fitzpatrick C.J.,*** O'Callaghan T.F., Freitas, D., O'Mahony J.A., Brodkorb A. Pepsin activity prediction model. 6th – 7th December 2022, UCC/IFSTI 50th Food Science and Technology Conference.

***Fitzpatrick, C. J.,*** Musse, M., Feng, J., Collewet, G., Lucas, T., Timlin, M., Challois, S., Quellec, S., Dupont, D., Brodkorb, A., Freitas, D., & Le Feunteun, S. Monitoring the effect of consumption temperature of full-fat milk on *in vitro* gastric digestion

using Magnetic Resonance Imaging. 15th – 18th October 2024, IDF World Dairy Summit, Paris, France.

**Oral Presentations**

***Fitzpatrick, C. J.,*** Musse, M., Feng, J., Collewet, G., Lucas, T., Timlin, M., Challois, S., Quellec, S., Dupont, D., Brodkorb, A., Freitas, D., & Le Feunteun, S. Monitoring the effect of consumption temperature of full-fat milk on *in vitro* gastric digestion using Magnetic Resonance Imaging. 51st Annual Food Science and Technology Conference Food Systems in a Changing World the Science, Challenges and Opportunities December 6-7th, Teagasc Food Research Centre, Ashtown, Dublin.

***Fitzpatrick C.J.,*** O'Callaghan T.F., Freitas, D., O'Mahony J.A., Brodkorb A. The effect of high *vs.* low pasture allowance on digestion of milk lipids. 15th November 2023, VistaMilk industry day, Cork, Ireland.

## Awards

UCC College of Food Science and Nutrition Post-Graduate Paper of the Year 2024 for the paper: ***Fitzpatrick, C. J.,*** Musse, M., Feng, J., Collewet, G., Lucas, T., Timlin, M., Challois, S., Quellec, S., Dupont, D., Brodkorb, A., Freitas, D., & Le Feunteun, S. (2024). Monitoring the effect of consumption temperature of full-fat milk on *in vitro* gastric digestion using Magnetic Resonance Imaging. Food Hydrocolloids, 109864. DOI: https://doi.org/10.1016/j.foodhyd.2024.109864. Refer to Appendix 1 for certificate.

## Funding Awards

***Fitzpatrick, C. J.*** Recipient of the Ulysses Travel Grant (€2,500) 2024 to study the effect of milk consumption temperature on the *in vitro* digestion of milk using magnetic resonance imaging (Chapter 4).

***Fitzpatrick, C. J.*** Winner of the Irish branch of International Dairy Federation (IDF) travel award to present at the IDF World Dairy Summit 2024.

## Other awards

***Fitzpatrick, C. J. Team member of MilkoSense, winning project in the*** Catalysing Connections programme, which received €5,000 in funding 'MilkoSense,' a novel concept for dairy processing aimed at reducing energy costs and improving milk protein functionality.

***Fitzpatrick, C. J.*** Vistamilk Education and Public Engagement Champion 2024.

***Fitzpatrick, C. J.*** UCC EmployAgility Award (Entrepreneurship).

# Abbreviations

ANOVA: Analysis of Variance

AUC: Area Under the Curve

AB: Heterozygous β-lactoglobulin Variant

BB: Homozygous β-lactoglobulin Variant

CLA: Conjugated Linoleic Acid

FFAs: Free Fatty Acid

FR-TSE: Fast Recovery Turbo Spin Echo

GE0: Undigested milk

GRE: Gradient Recovery Echo

G': Elastic Modulus

G'': Viscous Modulus

GRS: Grass-fed

HCl: Hydrochloric Acid

HPLC: High Performance Liquid Chromatography

PCA: Principal Component Analysis

PLS-DA: Partial Least Square Discriminant Analysis

PUFAs: Polyunsaturated Fatty Acid

SEC: Size Exclusion Chromatography

SFAs: Saturated Fatty Acid

SNP: Single Nucleotide Polymorphism

TMR: Total Mixed Ration

MRI: Magnetic Resonance Imaging

Unsaturated Fatty Acids: UFAs

U/mL: Units per Millilitre

IU: International Units

WP: Whey Protein

# Acknowledgments

I would like to express my heartfelt gratitude to all those who supported me throughout my journey as a Walsh Scholar at Teagasc Moorepark. To my family, who have been my foundation, thank you for your unwavering belief in me. To my father, John, your support and encouragement have been my guiding light. To my sister, Michelle, thank you for your continued check-ins and encouragement from Canada.

To André Brodkorb, my supervisor, mentor, and friend—thank you for your technical expertise, life advice, and unwavering support. A very special thanks to Daniela Freitas, whose support, guidance and friendship has been invaluable throughout this PhD journey. I am truly grateful for your kindness, advice, and encouragement.

To my colleagues and friends who enriched my time at Moorepark, including Elena Hayes, Cathal Dold, Sarah Gilsenan, Mark Timlin, Liam Kelly, Hannah Masterson, Leo Nunn, and those who joined later, Vinicius Pedrosa and Bianca Hosen—your camaraderie made the challenges bearable. Special thanks to colleagues in Lab 24: Talita Comunian, Gaetan Drouin, Shannon Gwala, Nefeli Lampologue, Siyi Feng, Sara Perez, Sara Pacheco, Laura Gomez, Charlotte Oliver, Maria Gloria Mora, Farhad Garavand, Gulden Goksen, and Neha Sharma.

I extend my sincere gratitude to my academic supervisors, Tom O'Callaghan and Seamus O'Mahony, for their wisdom and guidance. To my friends, especially Sean, Jack, Niall, Gareth and Katie, thank you for providing a much-needed escape and laughter. To my sports teams, Bruff R.F.C and Knockainey G.A.A, your support provided the perfect balance of focus and escapism.

A special thank you to three incredible women, Tracy, Breda, and Jean—your support has been the backbone of my success. To Eva, for your love, fun and spirit, and being a constant source of support. I am grateful to all my cousins, uncles, aunts, grandparents and extended family for their unquestioning love. Lastly, to Maxi, whom I considered more a friend than a pet, thank you for lighting up every day you had on this earth.

This work was made possible by the Teagasc Agriculture and Food Development Authority Walsh Fellowship scheme under project MDBY 0633 and VistaMilk SFI Research Centre grant number 16/ RC/3835.

# Graphical abstract

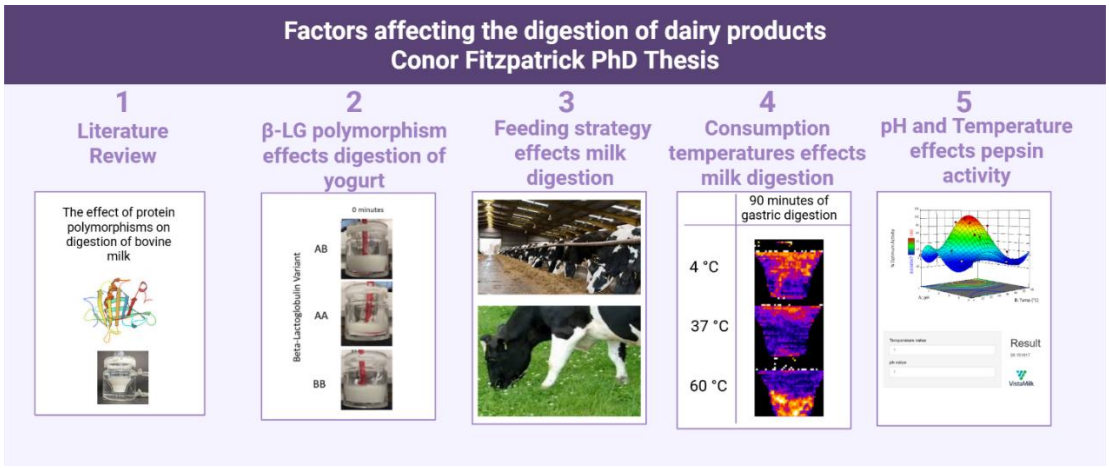


Figure 1 Graphical abstract showing the experimental Chapters of this thesis

# Chapter 1 (part 1):

# Variations in bovine milk proteins and processing conditions and their effect on protein digestibility in humans: A review of *in vivo* and *in vitro* studies

Conor J. Fitzpatrick, Daniela Freitas, Tom F. O'Callaghan, James A. O'Mahony, André Brodkorb

This chapter was published as;

Fitzpatrick, C.J., Freitas, D., O'Callaghan, T.F., O'Mahony, J.A., Brodkorb, A. *Foods* 2024, *13*, 3683. Variations in Bovine Milk Proteins and Processing Conditions and Their Effect on Protein Digestibility in Humans: A Review of *In vivo* and *In vitro* Studies.

**CRediT authorship contribution:** Conor J. Fitzpatrick: Writing – original draft, Writing – review & editing, Conceptualisation, Visualisation. Daniela Freitas: Conceptualisation, Writing – review & editing, Supervision. Tom F. O'Callaghan: Writing – review & editing, Supervision. James A. O'Mahony: Writing – review & editing, Supervision. André Brodkorb: Conceptualisation, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

## 1.1 Abstract

Bovine milk proteins account for 10% of the global protein supply, which justifies the importance of thoroughly understanding their digestive processes. Extensive research on digestion is being conducted both *in vivo* and *in vitro*. However, interpretations and comparisons across different studies require a thorough understanding of the methodologies used. Both the rate and extent of milk protein digestion can be affected by several intrinsic and extrinsic factors with potential implications for digestive behaviour and physiological responses. Among intrinsic factors, the impact of genetic variants in native milk proteins has emerged as a growing research area. To these, further complexity is added by the processing conditions frequently applied to milk prior to consumption. The main aim of this work is to provide an overview of the current knowledge on the impact of variations in milk protein profiles (particularly whey protein:casein ratio and protein polymorphisms), the treatments applied during processing (pasteurisation, homogenisation) and consumption (temperature changes) on protein digestion. To support the interpretation of the current literature, this manuscript also presents a historical perspective into research in this field and summarizes the protocols that are most frequently used, presently, on *in vitro* digestion studies.

## 1.2 Bovine milk as a human food source

"*Milk is food. Alone and unassisted it is capable not only of sustaining life for an indefinite period, but it furnishes all the elements for the complete construction of the human frame; on it alone the infant learns to talk and walk and develops all the tissues of the system. Adults have lived on it alone for weeks or months, and by it convalescents from grave sicknesses have recovered vitality and strength*" (Bulkley, 1898) .

As highlighted by Bulkley, 1898, the importance of bovine milk to human nutrition has long been understood. Studies from the late 19th and early 20th century provided observations on the changes that occur to milk in the stomach. As analytical techniques have become more sophisticated, the biochemical mechanisms have been increasingly understood and described. Advances in various technologies can offer potential for greater understanding of the nutritional and biological value of milk proteins.

Bovine milk contains approximately 29–34 g/L of high quality, digestible protein, meaning that consuming 0.5 L can account for 30–40% of a person's daily protein needs (Insel, 2013, Fox, 2003, Karman and Boekel, 1986). Bovine milk provides all the essential amino acids required for human health. This is especially important in infant nutrition. While breastfeeding is recommended for infants when possible, formula products are commonly used as substitutes, typically formulated from blends of bovine milk proteins, including whey and casein-rich components, to produce an amino acid profile closer to that of human milk (Murphy, 2015). As well as providing amino acids that are needed for human health, certain milk proteins also play a role in transporting minerals needed for human health. For example, α-La is a metalloprotein, binding one $Ca^{2+}$ per mol, which facilitates the passive absorption of $Ca^{2+}$ (Hiraoka et al., 1980, Lönnerdal and Glazier, 1985).

Studying the digestion of milk proteins is important, as changes to the rates and extents of digestion can impact physiological responses such as gastric emptying dynamics, which can affect satiety and postprandial aminoacidemia (concentration of amino acids in the blood). Techniques such as Magnetic Resonance Imaging (MRI) are increasingly being used as non-invasive methods to study digestion *in vivo* (Spiller and Marciani, 2019). However, the utilisation of human subjects poses several challenges and limitations, such as ethical considerations, reproducibility, cost and low throughput. Despite these limitations, *in vivo* studies are required for measuring certain physiological outcomes such as the rate of gastric emptying and the rate of aminoacidemia, which is important due to the role of amino acids in muscle protein synthesis. For instance, consuming free amino acids in a readily absorbable state increases the concentration of plasma amino acids compared with intact milk proteins, leading to a higher release of phenylalanine, a precursor to tyrosine, which is vital for neurotransmitter formation; however, both intact and free amino acids have been shown to increase rates of muscle protein synthesis (Weijzen et al., 2022). *In vivo* studies have also shown differences in the rate of aminoacidemia in whey protein *vs.* caseins; using intrinsically labelled leucine, it has been shown that whey proteins cause transient spikes in aminoacidemia, while casein results in a prolonged release over a longer period (Boirie et al., 1997).

Due to the limitations of *in vivo* studies, *in vitro* digestion systems are commonly used. The increasing sophistication and standardisation of *in vitro* digestion systems, such as the standardised INFOGEST method, has led to an increase in reproducible *in vitro* digestion of bovine milk proteins, without the drawbacks of ethical considerations or low throughput seen in *in vivo* studies (Brodkorb et al., 2019). These *in vitro* digestion systems can range from static *in vitro* digestions, where all food, enzymes and fluids

are added to a single tube, to dynamic *in vitro* digestions that incorporate complex churning, pH dynamics, temperature control and gastric-emptying mechanics (Brodkorb et al., 2019, Ménard et al., 2015).

However, while *in vitro* digestion studies can suggest potential physiological outcomes, they cannot completely replicate complex *in vivo* conditions. For these reasons, it is important to consider the benefits and limitations of both *in vivo* and *in vitro* digestion methods when trying to understand the digestion of bovine milk proteins.

This review paper focuses on intrinsic and extrinsic factors affecting the digestion of milk proteins, as well as discussing the various methods used to simulate milk protein digestion. The intrinsic factors reviewed here include whey protein to casein ratio and milk protein polymorphisms. The extrinsic factors reviewed here include the processing of milk and milk consumption temperatures. Certainly, other factors can affect the digestion of milk proteins and have been reviewed extensively elsewhere. For instance, the effect of physiochemical modifications of milk proteins has been extensively reviewed by Dupont and Tomé (2020). Interestingly, the effect of species on milk protein digestion has been investigated; however, while this matter is touched on briefly in this review, it is extensively reviewed elsewhere (Roy et al., 2020). The effect of processing on milk protein digestion has also been reviewed; however, the present study introduces new studies to the discussion (Ahlborn et al., 2023). This review will offer several benefits, bringing together both historical and modern research on the digestion of milk proteins, and will serve as a reference point for future research in this area.

### 1.2.1 Early observations of milk protein digestion

Studies from the 19th and 20th centuries provide findings on the digestion of milk *in vivo,* which would be difficult to obtain with modern ethical standards. One of the earliest recorded cases of the study of the gastric digestion of milk was carried out on the infamous patient, Alexis St. Martin, who had an external opening into his stomach due to a gunshot wound. These experiments revealed that milk was easily digested and provided the first evidence that heating milk affects its digestion, with raw milk "chymifying" more slowly than boiled milk (Beaumont, 1838).

Further studies examined the difference in coagulation properties between raw and boiled milk (Brennemann, 1913). By feeding raw and boiled milk to a subject at different times and subsequently having that subject return the samples from the stomach through emesis, a clear picture of what occurred during the gastric digestion of milk was observed. When boiled milk was returned after 30 min, it had formed small, soft, 'custard-like' curds, which contrasted to raw milk, which had formed firm, rubbery curds. After 3 h of digestion, raw milk curds were fewer, but larger and firmer with rounded corners. Moreover, it was found that with increasing time, the curds became increasingly difficult to regurgitate, requiring water to be consumed to evacuate from the stomach. As well as this, the contents became progressively sourer and more acidic as time after consumption increased. This indicates that the pH was decreasing as digestion continued due to the secretion of hydrochloric acid from parietal cells in the gastric wall (Engevik et al., 2020).

Mortenson et al. (1935) showed similar finding in calves: boiled milk was found to break up more easily and was therefore emptied more rapidly than raw milk using a gastric fistula. As well as this, it was found that raw milk coagulated within 10 min, while boiled milk coagulated within 8–15 min.

These studies provide valuable information on the digestion of milk in humans *in vivo.* However, the author does not condone the methods used to obtain these results due to basic ethical considerations. Certainly, the authors were conflicted on whether to discuss these studies due to the use of Alexis St. Martin as a domestic slave. However, the authors concluded that by recognising the contribution of Alexis St. Martin to the field of Gastroenterology, a recognition that he never received during the experiments, that discussing these results is justified.

### 1.2.2 Modern explanation of milk protein digestion

Early studies on the digestion of milk proteins provide valuable observations that were later explained using advanced analytical techniques. We now know that the digestion of milk proteins in humans begins in the stomach, where it is exposed to low pH and hydrolytic enzymes such as pepsin. In milk, caseins are organised into micelles, which are complex structures formed from several types of casein proteins ($\alpha_{s1}$- and $_{S2}$-, β-casein (β-casein) and κ-casein (κ-casein), as well as salts, mainly calcium phosphate, which is present as colloidal calcium phosphate, and water (de Kruif et al., 2012). Casein micelles stay in solution due to κ-casein proteins protruding from the surface, providing a negative charge and steric repulsion between micelles. However, during digestion, these κ-casein protrusions are cleaved by the action of pepsin, and, if pepsin is not present, the acidic conditions of the stomach will eventually cause the proteins to reach their isoelectric point, thereby eliminating the steric repulsion between micellar surfaces, resulting in coagulation (Sheng et al., 2021). This is the biochemical explanation of the observations of curd formation in the gastric phase provided in early studies discussed above (Brennemann, 1913, WOLMAN, 1946). The characteristics of the coagulum can be influenced by several factors and can lead to differences in rates of protein hydrolysis as shown in an *in vitro* digestion study by Mulet-Cabero et

al. (2019). As observed in early studies, a firmer, semi-solid 'coagulum' matrix, as seen in raw milk, makes it difficult for pepsin to reach the proteins entrapped within the matrix, while a more porous matrix, as seen in heat-treated milk, gives pepsin easier access, potentially modulating the rate of both protein hydrolysis and gastric emptying (Brennemann, 1913).

The presence of casein micelles in milk is an important factor in gastric coagulation. In skim milk powder with intact casein micelles, coagulation occurs within 10 min of gastric digestion. However, when casein micelles are disrupted by the removal of calcium, which may be enacted to increase the solubility of casein, coagulation does not occur until after 40 min of gastric digestion (Wang et al., 2018, Kommineni et al., 2022). Reduced calcium milk protein concentrate can be produced by injecting $CO_2$ followed by ultrafiltration (Marella et al., 2015). Further, when the amount of micellar calcium phosphate is increased, the rate of digestion of coagulum has been shown to decrease due to stronger coagulum formation (Huppertz and Lambers, 2020). This may be of interest if attempting to modulate the gastric emptying rate, and thereby the rate of protein absorption into the bloodstream.

In contrast to casein, whey protein (WP) does not coagulate in the gastric phase (Wang et al., 2018). Native WPs resist gastric digestion due to their globular structure. β-lactoglobulin for instance is largely broken down only when it moves from the acidic gastric phase to the neutral duodenal phase (Egger et al., 2016). An *in vitro* study using the INFOGEST method has demonstrated that large quantities of intact β-lactoglobulin were observed following gastric digestion and also showed a rapid disappearance of β-lactoglobulin at the beginning of the intestinal phase, therefore showing that β- lactoglobulin is highly susceptible to intestinal digestion (Sousa et al., 2020). The resistance of native β-lactoglobulin to gastric digestion is further supported

in work by Dupont et al. (2010a) where it was demonstrated to be highly resistant to adult and infant digestion *in vitro*; however, the lack of protective phosphatidylcholine in the infant stomach makes β-lactoglobulin slightly more susceptible to acidic and enzymatic degradation (Dupont et al., 2010a). Phosphatidylcholine is an abundant gastric surfactant. Proteins such as α-LA have been shown to be embedded within phosphatidylcholine during gastric digestion, which protects it from digestion (Moreno et al., 2005). In the small intestine, milk proteins are further hydrolysed by the action of pancreatic enzymes, which include trypsin, chymotrypsin, and carboxypeptidases (Dupont and Tomé, 2020). Other factors can increase the digestion of proteins, for instance, bile salts have been shown to denature proteins, increasing the rate of hydrolysis (Gass et al., 2007). This is particularly interesting in the case of β-lactoglobulin due to its resistance to digestion, with bile salts being shown to significantly increase the hydrolysis of this protein.

Much work in the area of digestion and human nutrition has been carried out on the digestion of β-lactoglobulin, largely due to its implications in milk allergy and its potentially hypotensive effects. In terms of allergenicity, β-lactoglobulin is a major milk protein responsible for milk allergy (Złotkowska et al., 2021). However, β-lactoglobulin from different species does not react in the same way to bovine β-lactoglobulin (Kapila et al., 2013). The major differences in bovine *vs.* caprine β-lactoglobulin are that caprine β-lactoglobulin precipitates at pH 6.7 to 7.1 over 80 °C while bovine β-lactoglobulin does not, and that bovine β-lactoglobulin has a lower dissociation constant than caprine β-lactoglobulin, meaning that bovine β-lactoglobulin remains in dimer format at pH 2.5, while caprine β-lactoglobulin dissociates to monomers (Crowther et al., 2018). This difference may allow greater digestion of caprine β-lactoglobulin compared with bovine; however, more research

is required. Through genetic modification, when the free cysteine residue of bovine β-lactoglobulin, located in the calyx or β-barrel region of the protein at position 121, was substituted with a serine residue, it was found that the amount of this protein that remains intact after gastric hydrolysis decreases from approximately 80 to 35%. Jayat et al. (2004) suggested that this increase in hydrolysis is due to increasing accessibility of the enzymes to the binding site due to an increased mobility of the protein.

### 1.2.3 *In vitro* digestion systems: considerations for the study of milk protein digestion

The goal of *in vitro* digestion system is to mimic the *in vivo* human digestive system. Several systems have been developed to attempt this, with examples of these methods summarised in Table 1. Each of these systems has its specific advantages and limitations which may have implications on the results obtained and conclusions that can be drawn from each study. An understanding of these aspects is essential to ensure the correct study design and outcome interpretation.

Table 1 Examples of *in vitro* digestion methods that can be used to simulate milk protein digestion in humans.

| Type | Examples | Characteristics for Milk Protein Digestion |
|---|---|---|
| Static | INFOGEST Static model (Brodkorb et al., 2019) | - Simplified digestion model with fixed enzyme ratios and pH conditions. |
| | | - Does not account for gradual changes in gastric emptying, enzyme secretion or pH. |
| | | - Suitable for comparing protein digestion across different milk formulations. |
| Semi-Dynamic | INFOGEST Semi-dynamic model (Mulet-Cabero et al., 2020b) | - Simulates dynamic aspects of gastric digestion (e.g., flow of secretions, gradual acidification): milk proteins are exposed to varying pH and enzyme concentrations over time. |
| | | - Simulates gradual changes in gastric emptying, allowing for more realistic protein hydrolysis profiles. |
| | | - Balances simplicity and physiological relevance for digestion studies. |
| Dynamic | DIDGI Model (Ménard et al., 2015) | - In addition to dynamic conditions mimicked by semi-dynamic systems, dynamic systems allow replication of other dynamic aspects such as continuous gastric emptying. DIDGI system also incorporates a dynamic intestinal phase, mimicking intestinal pH, peristalsis, and transit times. |
| | | - Release of FAA closer to *in vivo* situation than INFOGEST static protocol (Egger et al., 2019). |
| | | - Provides more physiologically relevant data but requires advanced equipment and is time-consuming. |

Prior to the implementation of a standardised *in vitro* digestion system, several authors had published work, each with different protocols, making comparisons between studies difficult or, in some cases, impossible (Hur et al., 2011). In 2014, Minekus et al. (2014) proposed a standardised INFOGEST static *in vitro* digestion protocol, which was further optimised in 2019 (INFOGEST 2.0) (Brodkorb et al., 2019). The term static is used here as the dynamic aspects of digestion (e.g., flow of secretions,

progressive gastric acidification and emptying) are not replicated. Instead, at the start of each digestive phase, food/chyme, enzymes and simulated digestive fluids are all added together, and the pH is adjusted to a fixed value. Typically, three pH adjustments are performed: once at the start of the oral phase (pH 7), once at the start of the gastric phase (pH 3) and again at the start of the intestinal phase (pH 7). As well as this, the duration of both the gastric and intestinal phases is set at 2 h for each and does not consider the volume/amount or caloric content of the meal. Because of their simplified approach, static *in vitro* digestion protocols are considered a useful, straightforward tool that facilitates a high throughput. Moreover, for the study of protein digestion, they can be particularly useful for ranking different protein sources according to their digestibility and possibly to investigate the release of bioactive sequences in the gastrointestinal tract (Bohn et al., 2018b). For milk proteins in particular, the gastric and intestinal endpoints of INFOGEST's static digestion protocol showed a good approximation to the pig *in vivo* digestion (Sousa et al., 2023). This protocol is inherently limited in its capacity to mimic the dynamic evolution of protein hydrolysis that occurs *in vivo*; however, it has been successfully validated with dairy products for gastric and intestinal endpoints (Santos-Sánchez et al., 2024, Egger et al., 2017, Sanchón et al., 2018, Egger et al., 2019). Another important aspect to consider is that this protocol is only appropriate for simulating adult digestions. However, since its publication, several papers have examined the need for adjustments when studying different population groups according to, for example, sex (Lajterer et al., 2022), disease or age, e.g., infants (Ménard et al., 2018a) and older adults (Menard et al., 2023).

At present, an international consensus has been reached by the INFOGEST network, as part of an EU project Eat4Age, on the appropriate adaptations of a static *in vitro*

model to reflect digestion in older adults (Menard et al., 2023). Several differences to the adult protocol are recommended. More specifically, in the oral phase, the elderly protocol outlines a more specific protocol for simulating chewing. However, the food to oral fluid ratio remains the same in both protocols, as does the time, pH and enzyme concentration (1:1 ratio, 2 min, pH 7, 75 U of salivary amylase/mL). The major differences occur in the gastric and intestinal phases. In the gastric phase of the adult model, the pH is set to 3, with a pepsin and lipase concentration of 2000 and 60 U/mL of gastric contents, respectively, and the time of gastric digestion is 2 h. In the elderly protocol, the pH is set to 3.7, and reduced pepsin and lipase concentrations are used (1200 and 26 U/mL of gastric contents, respectively). As well as this, the gastric digestion time is increased to 3 h. In the intestinal phase, the pH and time remain the same as the adult; however, the pancreatin and bile salts concentrations are both decreased. A recent *in vitro* digestion study has shown that gastric digestion of proteins in dairy products is significantly hindered in elderly models compared with the adult. However, the digestion of these proteins in the intestinal phase is not affected (Lavoisier et al., 2023).

Work has also been carried out to develop an infant static *in vitro* digestion model. Menard and collaborators carried out research leading to a 'first step towards a consensus' method in 2018 (Ménard et al., 2018a). This model represents a method to simulate digestion in a full-term new-born at 28 days of life. In this model, due to an observed low retention time in the oral phase *in vivo*, no oral phase is carried out. An interesting difference in this protocol compared with both the adult and elderly models is a different ratio of food to gastric fluid (63:37 *vs.* 1:1 in the infant *vs.* adult, respectively). As well as this, the enzyme concentration is dramatically decreased, with 268 and 19 U/mL of gastric fluid for pepsin and lipase, respectively. The pH is

also increased to pH 5.3, and the gastric digestion time is reduced to 1 h from 2 h in the adult model. In the intestinal phase, a difference in digestive enzymes is again recommended, with the infant model using a pancreatin concentration of 90 U of lipase activity/mL, while the adult model recommends 100 U of trypsin activity/mL. Moreover, a reduction in bile salt concentrations from 10 to 3.1 mmol/L is recommended, as well as a decrease in pH to 6.6. When the adult and infant models were compared in terms of milk protein digestion in the gastric phase, it was found that all caseins were hydrolysed significantly more extensively and rapidly in the adult model compared with the infant model; however, these differences were eliminated in the intestinal phase, with all proteins remaining after 5 min (Ménard et al., 2018a). In terms of lipolysis, no difference was seen between models in the gastric phase; however, due to decreased quantities of surfactants and lipases in the infant compared with the adult, lower levels of lipolysis were observed in the infant *vs.* adult. Adult gastric conditions also differ to infant gastric conditions in terms of phosphatidylcholine content. Phosphatidylcholine is a lipid secreted from the gastric mucosa and is present in milk. This lipid has been shown to act as a surfactant, with a protective effect on β-Lg. In a study by Mandalari et al. (2009), the breakdown of β-Lg was compared in simulated adult *vs.* infant digestion. In the infant model, a lower concentration of phosphatidylcholine was used compared with adult digestion. No effect on gastric digestion of β-Lg was found between infant and adult models; however, after 30 min of duodenal digestion, β-Lg degraded to a lesser extent when phosphatidylcholine was present (Dupont et al., 2010a). This suggests that this lipid binds to the β-Lg, potentially blocking pepsin through steric repulsion.

The INFOGEST research network has also developed an *in vitro* semi-dynamic digestion protocol based on the static adult model (Mulet-Cabero et al., 2020b). In this

model, the oral and intestinal phases remain 'static', while the gastric phase incorporates dynamic elements, including constant stirring using an overhead stirrer, the continuous addition of simulated gastric fluid and enzymes, a gradually decreasing pH (finish at pH 2) and gastric emptying based on an emptying rate of 2 kcal/min (Mulet-Cabero et al., 2020b).

As well as *in vitro* models to replicate different gastrointestinal conditions due to age, a first attempt on a gender-specific semi-dynamic *in vitro* digestion method has been published recently (Lajterer et al., 2022). Based on *in vivo* data, Lajterer, *et al.* (Lajterer et al., 2022) altered gastrointestinal conditions dependent upon sex. For example, a lower concentration of pepsin was used for females compared with males in the gastric phase, as well as a longer intestinal phase (3 *vs.* 2 h) along with lower chymotrypsin concentrations. While only small differences in the digestion of milk proteins were observed between male and female conditions, it is important to consider these factors going forward to ensure an accurate representation of *in vivo* digestion.

To enhance the similarity between *in vitro* systems and the *in vivo* situation, especially in terms of digestion kinetics, several dynamic models have been published. These models can be split into two types, mono-compartmental and multi-compartmental. Mono-compartmental systems simulate one part of the human digestion system, while multi-compartmental systems simulate several parts. Examples of mono-compartmental systems are the Human Gastric Simulator (University of California, Davis) and the Dynamic Gastric Model (DGM) (Institute of Food Research, Norwich, UK) (Kong and Singh, 2010, Thuenemann et al., 2015). These systems are similar in that they allow the precise control of pH, temperature, gastric secretions and gastric emptying, as well as the ability to mimic both chemical and physical breakdown of food as seen *in vivo*. Several differences exist between the two systems. In the DGM,

gastric mixing is achieved through contractions that are controlled by water pressure using a piston and barrel system, while the HGS uses rollers along the outside of the artificial stomach. The DGM mimics *in vivo* digestion at the end of the gastric phase by also mimicking the 'housekeeper wave' to remove all contents from the stomach, which does not seem to occur in the HGS.

Even more complex are the multi-compartmental models, which incorporate dynamic systems for several stages of digestions. These include the DIDGI system (INRAE, France), the SIMulator of Gastrointestinal tract (SIMGI) (Food Science Research CIAL, CSIC-UAM, Madrid, Spain), Engineered Stomach and Small Intestinal (ESIN) (University of Auvergne, Clermont-Ferrand, France), TIM and Tiny TIM (TNO, the Netherlands) and SHIME (Ghent University, Belgium) (Van de Wiele et al., 2015). The DIDGI system (Figure 2) simulates digestion in the gastric and intestinal phase. All functions, such as meal flow, digestive fluid flow and enzyme flow, are controlled by a computer program (SToRM®) (INRA, Grignon, France) (Guillemin et al., 2010). The gastric and intestinal phases are linked by a Teflon membrane. Unlike ARCOL, anaerobic conditions in the small intestine are simulated by pumping in nitrogen gas. This model is limited by basic mixing function not simulating the *in vivo* situation. While these models are highly useful, they do not mimic gastric motility such as is seen in the *in vivo* situation due to peristalsis and segmentation actions of the muscles of the gastric wall which may impact the digestion of protein (Davenport, 2010).

The TIM system is a still more advanced system, incorporating the stomach, duodenum, jejunum and ileum, and mimicking *in vivo* mixing conditions using peristaltic waves through water pressure on the outside of the flexible walls (Minekus, 2015). Most significantly, the TIM-2 system has the ability to mimic intestinal absorption in the jejunum and ileum using a combination of dialysis and filtration, as

well as replicating the microbial flora using human or animal faeces. The ESIN system has the ability to more accurately mimic the condition of food when it enters the gastric phase compared with all other systems (Guerra et al., 2015). This is due to the use of a reservoir that stores food and incorporates a salivary ampoule that mixes food with saliva. These two features combined, allow for the progressive release of food particles into the gastric phase, which have similar characteristics to the *in vivo* situation. The SIMGI system, similar to the TIM system, uses water pressure to simulate peristaltic movement (Barroso et al., 2015). A distinctive feature of the SIMGI is the ability to mimic physiological conditions and disease states in the gastrointestinal system due to its automated working parameters. All of the presented methods have been extensively discussed previously in Dupont et al. (2019). Dynamic digestion models are useful for studying complex physiology that cannot be achieved with semi-dynamic or static systems. For example, TIM-1 has been used to show that β-lactoglobulin and glycomacropeptide are not only the most resistant milk proteins to gastric digestion but also that they are emptied last from the gastric to the intestinal phase (Nabil et al., 2011, Minekus et al., 1995).

Several studies have compared the previously discussed *in vitro* digestion methods to each other, and to the *in vivo* situation in terms of bovine milk protein digestion. For instance, Egger, *et al.* (Egger et al., 2017) compared the digestion of milk proteins using the *in vitro* static digestion approach published by Minekus*, et al.* (Minekus et al., 2014) with *in vivo* digestion in pigs. Considering that in both the *in vitro* digestion and the *in vivo* digestion, no intact casein and a small amount of intact β-lactoglobulin was seen in the duodenal phase, it was concluded that the *in vitro* system was successful at mimicking the *in vivo* situation.


Figure 2 The DIDGI *in vitro* digestion system, STLO, INRAE, France. This is a multi-compartmental system, consisting of both gastric and small intestinal compartments. Food is automatically emptied from one stage of digestion to the next using emptying pumps.

This was further confirmed by similar peptide patterns at the end of digestion using mass spectrometry. Following the publication of a standardised static *in vitro* digestion method, Egger, *et al.* (Egger et al., 2017) again examined the similarity between the new static *in vitro* digestion system and *in vivo* digestion in pigs, as well as including an *in vitro* dynamic digestion (DIDGI system) in the comparison (Brodkorb et al., 2019). The *in vitro* static and dynamic digestions differed to the *in vivo* gastric phase in terms of casein digestion. In both *in vitro* studies, casein disappeared during the gastric phase, while in the *in vivo* situation, gastric contents were split into a solid and liquid phase, where all casein had disappeared in the liquid phase, and intact casein remained in the solid phase. However, this study did not use the same protein concentrations in skim milk powders, with the *in vivo* study using a 33% protein concentration, compared with just the 10% used in the *in vitro* studies resulting in a weaker coagulum *in vitro*. In terms of the release of free amino acids during digestion, it was found that *in vitro* dynamic digestion more closely matched the *in vivo* situation.

This may be due to several factors, including the dynamic system incorporating mixing dynamics more closely related to the *in vivo* situation, and a pH curve more accurately matching the *in vivo* situation, which can affect the activity of digestive enzymes such as pepsin and lipase. The SIMGI system has also been validated to the *in vivo* situation when digesting WP. By using peptide profile analysis, Barbé et al. (2014) found that after the duodenal phase, peptide fragments resistant to digestion were very similar in both the *in vivo* situation (using mini-pigs) and in the SIMGI *in vitro* dynamic digestion system, indicating that this is an accurate model. The correlation between results in *in vitro* and *in vivo* milk protein digestion studies have been critically reviewed previously, concluding that both gastric and intestinal endpoints of digestion *in vitro* and *in vivo* were comparable (Bohn et al., 2018a).

### 1.2.4 Digesta analysis and characterisation techniques

During *in vitro* digestions, samples are taken at set time points during oral, gastric and intestinal phases. These samples, or digesta, can be analysed in several ways to understand the rate and extent of digestion. These techniques range from qualitative techniques to complex quantitative techniques. To study phenomena such as aggregation and coagulation, microscopy techniques can be useful. Common microscopy techniques used to analyse *in vitro* digestions include light microscopy, confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) (Wang et al., 2018). Other visualisation techniques, such as MRI, can be powerful tools to examine particle formation, disintegration and particle volume, as shown by Fitzpatrick, *et al.*(2024).

Common lab assays can also be conducted to quantify the extent of digestion. These include the ortho-phthalaldehyde (OPA) assay to measure the amount of free amines in a sample as an indicator of protein hydrolysis, the bicinchoninic acid (BCA) assay

to quantify amount of protein in a sample, or the SDS-PAGE technique to visualise the breakdown of large proteins into smaller fragments (Church et al., 1985, Smith et al., 1985, Laemmli, 1970).

More sophisticated techniques can also be used to perform more advanced separation and quantification. These include chromatography methods such as size exclusion chromatography (SEC-HPLC) (Corrigan and Brodkorb, 2020). These techniques can be useful to analyse peptide release and monitor the breakdown of specific proteins. Even more advanced is mass spectrometry (MS), which can be used in tandem as MS/MS to obtain individual peptides and amino acid sequences (Egger et al., 2019). Each of these techniques has benefits and drawbacks, with the simpler techniques offering high throughput at a low cost; however, the more advanced techniques offer more complex insights.

These techniques can also be used to analyse *in vivo* digesta samples, and can be taken from human trials, or from pig trials simulating human digestion. However, techniques such as the Digestible Indispensable Amino Acid Score (DIAAS) and the Protein Digestibility-Corrected Amino Acid Score (PDCAAS) can only be carried out *in vivo* and are used to measure protein quality based on amino acid absorption (Moughan and Lim, 2024, Schaafsma, 2000). Both industry and the scientific community are asking for *in vitro* alternatives to estimate the *in vitro* DIAAS of existing and new alternative proteins. The static INFOGEST method is currently being considered as an *in vitro* protein digestibility ISO standard method (Santos-Sánchez et al., 2024, Sousa et al., 2023).

## 1.3 Factors affecting the digestion of milk proteins

### 1.3.1 Whey protein to casein ratio

Variations in the ratio of bovine milk casein to WP content, which is approximately 80:20, can be naturally influenced by breed and somatic cell count (SSC), or artificially altered for different dairy products. Studies show that different WP: casein ratios alter amino acid appearance rates in the blood post-ingestion (Boirie et al., 1997). Casein-rich meals form a firm curd in the stomach, slowing gastric emptying compared with WP-rich meals, which form a transient curd that quickly returns to a liquid phase. This difference in curd formation affects gastric emptying rates of protein from the stomach, with proteins that form firm curds being emptied more slowly than proteins that remain in the liquid phase or form soft curds. An *in vitro* digestion study by Mulet-Cabero et al. (2020c) showed that varied casein: WP ratios (0:100, 20:80, 50:50, 80:20) form a curd after 10 min, with low-casein samples solubilising quickly and high-casein samples remaining firm. This study also suggested an enzymatic effect on coagulation, likely through κ-casein cleavage at Phe105-Met106, reducing steric repulsion and allowing micelle aggregation. Boirie, *et al.* (Boirie et al., 1997) supported these findings *in vivo*, observing a rapid, transient increase in aminoacidemia after consuming a 13C-Leucine WP meal, compared with a slow, prolonged increase after a casein meal (summarised in Table 2). While the two meals were not iso-nitrogenous, with more protein being present in the whey-based meal (336 mmoles nitrogen in WP meal vs 479 mmoles nitrogen in casein meal), it was still found that the casein meal produced a lower aminoacidemia peak than WP, where leucine was 236 ± 56% above baseline after 100 min of digestion of the WP meal, compared with just 77 ± 24% in the casein meal. Although gastric emptying rates were not determined, there is a suggestion that the coagulation of the casein-based meal led

to a slower gastric emptying. This was the causal factor of a gradual release of amino acids into the blood stream from casein compared with WP.

The different digestion profiles of casein and WP can have physiological implications, with one of the outcomes that has been investigated being the relationship with muscle protein synthesis. Research in rats has shown that the co-consumption of casein and WP, combining the rapid digestion and absorption of WP with the slower kinetics of casein, can increase muscle protein synthesis compared with either WP or casein ingestion alone (Kanda et al., 2016). In humans, WP alone has been shown to be more effective at stimulating muscle protein accretion than casein alone (Pennings et al., 2011). The difference in muscle protein synthesis between WP and casein may be due to the slower emptying of casein from the gastric phase, the difference in leucine content between the two or a combination of both. WP has a higher leucine content when compared with casein, which may be a causative factor in the previously described difference in muscle protein synthesis between WP and casein, due to leucine's activation role in muscle mass regulation pathways (specifically the mTOR pathway) (Naclerio et al., 2013, Drummond and Rasmussen, 2008).

As well as this, WP has been suggested to increase short term satiety more effectively than other protein sources such as egg albumin and soy protein, as well as other macronutrients, fat and carbohydrates (Rolls et al., 1988, Anderson et al., 2004). This has been supported by longitudinal studies, whereby the increased consumption of dairy proteins was correlated to lower weight gain (Drapeau et al., 2004).

These findings have also been replicated more recently in Ireland, where higher dairy intake was associated with positive body outcomes such as lower body mass index and

lower body fat (Feeney et al., 2017). These results are indicators of the positive health outcomes of consuming dairy products.

*Table 2 Summary of factors affecting the digestion of milk proteins*

| Factors | Description | Outcome for Protein Digestion | Type of Digestion | References |
|---|---|---|---|---|
| **Whey protein: Casein Ratio** | The ratio of whey to casein proteins in milk varies due to factors such as stage of lactation and breed. | Increasing casein content decreases the rate of protein hydrolysis and gastric emptying. | *In vivo*<br>*In vitro* | (Mulet-Cabero et al., 2020c, Boirie et al., 1997) |
| **Protein Polymorphisms** | Variants in milk protein genes, such as κ-CN A, B and E, affect protein conformation. | Polymorphisms affect glycosylation levels and casein micelle size, influencing rate of digestion of milk proteins in gastric phase (AA digested more rapidly than BB) | *In vitro* | (Sheng et al., 2021) |
| **Heat Treatment** | Denaturation of proteins due to thermal processing, affecting the structure of whey proteins, particularly β-lactoglobulin, leading to interactions with κ-CN on the casein micelle. | Heat treatment affects coagulum consistency, with increasing heat treatment associated with less firm coagulum, leading to a more rapid protein hydrolysis. | *In vitro* | (Mulet-Cabero et al., 2019) |
| **Homogenisation** | Mechanical process that breaks down fat globules, reducing fat particle size, increasing the surface area for interaction with proteins. | Homogenisation increases the rate of amino acids entering the small intestine from the stomach. | *In vivo* | (Ahlborn et al., 2024) |
| **Consumption Temperature** | Milk consumed at different temperatures affects how quickly it coagulates and digests in the stomach. | Hot milk coagulates faster in the stomach, potentially leading to different gastric emptying rates. | *In vitro* | (Fitzpatrick et al., 2024) |

### 1.3.2 Milk protein polymorphisms

Bovine milk contains six major proteins encoded by six genes. The casein proteins, $\alpha_{S1}$-casein, β-casein, $\alpha_{S2}$-casein and κ-casein are encoded by the genes CSN1S1, CSN2, CSN1S2, and CSN3 genes, respectively, all located within a 250 kb region on chromosome 6, while α-LA is encoded by the gene LAA on bovine chromosome 5, and β-lactoglobulin is encoded by the gene LGB on bovine chromosome 11. Each of these genes are highly polymorphic, with several single nucleotide polymorphisms leading to amino acid changes in the protein sequence. Milk can be homozygous or heterozygous for protein variants, and Table 3 shows the identified variants in bovine milk.

In the past, studies have focused on genetic variations in milk in relation to their effect on the production of dairy products such as cheese and yogurt, with limited study on their effect on digestion (Fitzgerald et al., 1999, Aleandri et al., 1997). However, with the move towards cellular agriculture and precision fermentation-produced dairy proteins, there has been increased interest in the effects of protein polymorphisms on digestion.

*Table 3 Milk proteins and their variants.*

| Gene | Protein | Variants | References |
|---|---|---|---|
| ***CSN1S1*** | $\alpha_{S1}$- | A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, H | (Caroli et al., 2009, Grosclaude et al., 1970) |
| ***CSN2*** | β-casein | A1, A2, A3, B, C, D, E, F, G, H1, H2, I | (Rachagani and Gupta, 2008, Caroli et al., 2009) |
| ***CSN1S2*** | $\alpha_{S2}$- | A, B, C, D | (Grosclaude et al., 1982, Caroli et al., 2009, Neelin, 1964) |
| ***CSN3*** | κ-casein | A, B, B2, C, E, F1, F2, G1, G2, H, I, J | (Neelin, 1964, Caroli et al., 2009) |
| ***LAA*** | α-LA | A, B, C | (Blumberg and Tombs, 1958, Caroli et al., 2009) |
| ***LGB*** | β-lactoglobulin | A, B, C, D, E, F, G, H, I, J, W | (Aschaffenburg and Drewry, 1955, Caroli et al., 2009) |

1.3.2.1 κ-casein

It is currently accepted that 12 variants of κ-casein exist in bovine milk, with the κ-casein variant phenotype of Irish Holstein Friesians being 1.98% BB, 53.07% AA and 44.95% AB, with the E variant also being present to some extent (Holland et al., 2006). When comparing the A and B variants, two substitutions are observed: Thr→le136 and Asp→Ala148. These substitutions replace hydrophilic residues in κ-casein A with hydrophobic residues in κ-casein B (Martin et al., 2002). One amino acid substitution

seen when comparing A *vs.* E is a Ser->Gly substitution at AA 176 (Schlieben et al., 1991).

Recently, the interest in the effects of κ-casein polymorphism on milk digestion has increased. Petrat-Melin et al. (2016) investigated the gastrointestinal digestion of isolated κ-casein variants *in vitro*. As expected with casein proteins, all studied variants (A, B, and E) of κ-casein were highly digestible in the gastric phase, with none remaining intact after gastric digestion. However, different digestion kinetics were observed. It was found that after 20 min of gastric digestion, the degree of hydrolysis of variants A and B was significantly higher than that of the E variant. Although this difference was no longer observed at the end of digestion, the authors suggested that the E variant was less susceptible to digestion due to the Ser155 to Gly155 substitution causing a conformational change to the peptide bond, or due to the increased glycosylation seen in the E variant compared with the others. Furthermore, Sheng, *et al.* (Sheng et al., 2021) carried out *in vitro* digestion of milks containing different κ-casein phenotypes, AA, BB or AB. This study found κ-casein AA to be more rapidly hydrolysed during the gastric phase than other variants, with SDS-PAGE data showing this variant almost completely hydrolysed after 5 min of gastric digestion. Similarly to the study by Petrat-Melin et al. (2015) described above, no differences were observed during the intestinal phase. However, these studies did not mimic the dynamics of gastric emptying. This study did not use purified κ-casein variants as was seen in Petrat-Melin et al. (2015); therefore, the risk of confounding factors was increased, such as the recorded heterogeneities in the phenotypes of other casein types, especially the occurrence of different β-casein variants. These confounding factors were eliminated in a subsequent study by Sheng et al. (2023), whereby purified κ-casein variants were digested using the standardised INFOGEST

static *in vitro* digestion protocol. This study was of interest as it incorporated peptide profile analysis post digestion to give an insight into the non-digested peptides and potential bioactive peptide release. The results showed that during gastric digestion, κ-casein B releases significantly less peptides when compared with both A and E, indicating that B is more resistant to gastric digestion.

The observed difference in degree of hydrolysis may be due to differences in post-translational modifications (PTMs) of κ-casein variants. PTMs occur in the Golgi apparatus located in the mammary glands. It has been shown that κ-casein can be phosphorylated at one, two or three sites; however, it is more common for κ-casein to have two phosphorylation sites, while it is less common to have either no phosphorylation or to have all three sites phosphorylated (Sheng et al., 2021, Holland et al., 2006). These PTMs are influenced by genetic variants. Despite κ-casein A having more possible O-glycosylation sites (0–6) due to the presence of an extra Thr amino acid compared with κ-casein B (0–5) in the C-terminus, κ-casein B has been shown to be more highly glycosylated than A (Poulsen et al., 2016). The complexity of κ-casein is furthered by the existence of 0–2 phosphorylations due to the presence of Ser residues. Moreover, the N-terminus can participate in intermolecular disulphide bridging via its Cys residues to $\alpha_{S2}$-casein (Rasmussen et al., 1992). Research has shown that phosphorylated peptides may be more resistant to tryptic hydrolysis than non-phosphorylated variants due to the existence of salt bridges in close proximity to cleavage sites (Bubis et al., 2020, Dickhut et al., 2014). However, the cited studies did not use individual milk proteins, nor did they use the standardised INFOGEST *in vitro* digestion method; therefore, the hypothesis that the degree of phosphorylation may affect gastrointestinal digestion requires further investigation.

Another PTM that occurs in κ-casein is glycosylation. Glycosylation occurs exclusively at Thr residues via o-linked glycosylation. Glycosylation increases the ability of casein micelles to repel each other due to increased steric charges. In terms of digestion, it has been shown that the action of the main digestive enzymes involved in protein hydrolysis, trypsin, chymotrypsin and pepsin, are negatively impacted by glycosylation (Dziuba and Darewicz, 2007). Interestingly, Boutrou et al. (2008) have shown differences in digestion between glycosylated and un-glycosylated caseinomacropeptide (CMP) regions of κ-casein A, whereby decreased numbers of peptides were liberated from glycosylated CMP compared with non-glycosylated, indicating a resistance to digestion provided by O-Glycosylation's. However, these CMPs were highly processed to be isolated, and this investigation is difficult to replicate due to the use of a freshly slaughtered pig to isolate brush border membrane vesicles. In an *in vitro* gastrointestinal digestion study, it was also shown that the areas containing glycosylation were resistant to hydrolysis in κ-casein variant A; however, no other variants were studied, and the number of glycosylation's present was not elucidated (Dupont et al., 2010b). The effect of glycosylation on casein micelle size and stability is still debated. Bijl et al. (2014) hypothesised that the lower casein micelle size seen in κ-casein AB compared with AA was due to the B variant being more highly glycosylated; however, other studies have shown contradictory results (Dalgleish, 1986, Lodes et al., 1996). When isolated κ-casein protein variants were subjected to *in vitro* digestion, it was found that the variants with higher glycosylation showed a lower degree of hydrolysis: for example, variant E was highly glycosylated and showed a lower degree of hydrolysis than other variants (Petrat-Melin et al., 2016). Sheng, *et al.* (Sheng et al., 2021) also investigated the degree of glycosylation of different κ-casein phenotypes in skim milk, linking the lower degree of

glycosylation in AA milk to smaller particle sizes when compared with milk containing either AB or BB κ-casein. In the same study, Sheng, *et al.* (Sheng et al., 2021) subjected the skim milk containing different κ-casein variants to static *in vitro* digestion, finding that AA was hydrolysed more rapidly in the gastric phase; however, no differences between variants were observed after intestinal digestion. Since the amino acid substitutions that are observed between κ-casein A and B are not potential hydrolysis sites for digestive enzymes, it can be assumed that differences in digestion are brought about by factors other than a change in the number of hydrolysis sites. Differences in hydrophobicity and net negative charge could be the causative factors in differences in digestion. This observation is further supported by the findings of Sheng, *et al.* (Sheng et al., 2023), who utilised peptide analysis to show that the least number of peptides released from any κ-casein region of any variants were in the region of glycosylation sites, indicating that glycosylation inhibits enzymatic activity. Another interesting finding from this study was that while all peptides released from the non-glycosylated part of κ-casein were the same for all variants, no peptides released from the glycosylated part of κ-casein were shared.

The size of the casein micelle may be of importance when studying the effects of κ-casein variation on digestion kinetics due to the observed effects on dairy processes such as renneting. The average diameter of casein micelles in bovine milk is approximately 200 mm (De Kruif and Holt, 2003). Smaller diameter micelles are optimal for gelation, such as in renneting (Glantz et al., 2010). In a study by Devold et al. (2000), κ-Casein AB variants were associated with smaller micelle diameters when compared with either AA or AE κ-casein variants. This was in agreement with Sheng, *et al.* (Sheng et al., 2021), who found a larger casein micelle size in skim milk containing κ-casein AA compared with AB or BB. On from this, since the

concentrations of κ-casein in a milk sample can affect the size of the micelle, it is important to examine polymorphisms in the non-coding regions of the κ-casein gene. The κ-casein to total casein ratio has been found to be of importance to micelle size, and therefore influences casein digestion, and κ-casein variants have been shown to influence the total amount of κ-casein present. Laible et al. (2016) showed that by genetically upregulating the production of κ-casein, a decrease in micelle size could be observed, indicating that a higher concentration of κ-casein results in smaller diameter casein micelles. Studies analysing the effect of micelle size on digestion are limited; however, there are some data available on milk samples from various species with different micelle sizes. For example, equine milk contains a low concentration of κ-casein when compared with bovine milk, and since an inverse relationship between κ-casein content and micelle size exists, casein micelles from equine milk are significantly larger than bovine milk, at 311 and 184 nm on average, respectively (Fox et al., 1998a, Inglingstad et al., 2010). Having noted this, it has been shown, *in vitro,* that the gastric digestion of equine casein occurs more rapidly than in bovine casein, with a 70% digestion rate after 30 min, compared with 31% in bovine milk (Inglingstad et al., 2010). Differences in the *in vitro* digestion of bovine milk and sheep milk have also been found, linked to a decreased concentration of κ-casein found in sheep milk. This may lead to the finding of increased curd firmness of curds formed during gastric digestion in sheep milk, resulting in a less extensive breakdown of proteins (Saviard et al., 2024). However, when comparing milk of different species, it is difficult to overcome the obstacle of confounding factors; therefore, no assumptions can be made until further investigation is carried out on the effect of micelle diameter on digestion in bovine milk.

The study of the effect of κ-casein variants on digestion kinetics has shown some interesting results, most interestingly, changes to the degree of hydrolysis correlated to different κ-casein variants. To our knowledge, no study has investigated the effect of these variants in a dynamic digestion system, *in vivo* or *in vitro.* Further studies should be carried out to determine differences in gastric emptying rates due to genetic variations of κ-casein using semi-dynamic or fully dynamic *in vitro* digestion systems. This work could be furthered using MRI scanners to analyse the differences caused by κ-casein variants *in vivo*. It is hypothesised that differences in rates of digestion and the degree of hydrolysis rates of nutrient absorption could be affected by κ-casein genetic variants.

1.3.2.2 β-Casein

Beta-casein (β-casein) is another casein in bovine milk that is of interest in terms of genetic polymorphisms. β-Casein accounts for 40% of the casein fraction of bovine milk and is coded by a highly polymorphic gene, CSN2, located on chromosome 6. Presently, there are 15 known variants of β-casein, with A1, A2 and B most commonly found in bovine milk (Cieślińska et al., 2007). The release of bioactive peptides from β-casein is a current topic of interest, and has been of interest since the opioid activity of a β-casomorphin (BCM) was first discovered (Brantl et al., 1981). BCMs are a group of peptides with chain lengths between 4 and 11 amino acids that all begin with Tyr, and they lie dormant within the β-casein protein in milk until released by the action of gastric pepsin during digestion. The BCM first isolated by Brantl, *et al.* (Brantl et al., 1981) was BCM-7, an opioid peptide that is seven amino acids in length (Tyr-Pro-Phe-Pro-Gly-Pro-Ile). An opioid peptide is one that has similar mechanisms of action to morphine, attaching to the same opioid receptors. BCM-7 has continued to be the most highly investigated bioactive peptide to this day due to claims that is

has both positive (attenuated aging (Guantario et al., 2020)) and negative impacts (reduction in oxidative stress) (Zhang et al., 2019) on health (Daniloski et al., 2021a).

Several studies have examined the effect of β-casein genetic polymorphisms on gastrointestinal digestion and health-related outcomes. These studies have been systematically reviewed by Brooke-Taylor et al. (2017) and more recently by Daniloski, *et al.* (Daniloski et al., 2021a), mainly focusing on the release and effect of BCM-7. As well as this, Daniloski, *et al.* (Daniloski et al., 2021b) has also reviewed *in vitro* and ex vivo studies relating to β-casein and β-casomorphin. Daniloski, *et al.* (Daniloski et al., 2021b) reviewed the potential impact of BCM-7 on human health from ex vivo studies, finding that they may have potential impacts on human health. However, as highlighted in a subsequent review, despite increases in the release of BCM-7 from A2 vs A1 β-casein, Daniloski, *et al.* (Daniloski et al., 2021a) did not find evidence of clinically relevant health outcomes associated with β-casein genetic polymorphisms.

1.3.2.3 β-lactoglobulin

The digestion of whey proteins such as β-lactoglobulin is also affected by genetic polymorphisms. The coding of β- lactoglobulin occurs on chromosome 11 by the gene LCG, with 11 variants of the protein having been discovered, with B, A and C being most common in European dairy herds (Caroli et al., 2009). Bovine milk can be heterogeneous, in that it produces two variants of β-lactoglobulin, or it can be homozygous to one. Bewley et al. (1997) determined the structural differences between variants using X-ray crystallography, showing high degrees of similarities between the variants. However, local internal readjustments have been noted due to the Gly64Asp (A to B) mutation, as well as a difference in surface stabilization due to the Gln59His mutation (B to C) (Bewley et al., 1997). Studies examining β-

lactoglobulin variants and their effects on cheese production show significant differences in rennet coagulation time and curd firmness, indicating that there may also be a difference in terms of human digestion (Molina et al., 2006, Walsh et al., 1995). Creamer et al. (2004) determined the differences in tryptic hydrolysis of β-lactoglobulin A, B and C using SDS-PAGE and HPLC. This study indicated that the A variant is more susceptible to hydrolysis than the other two variants, and that the relative changes in rates of hydrolysis of β-lactoglobulin A in response to environmental changes was significantly greater than B and C which indicates that a significant structural difference exists between β- lactoglobulin A compared with the other two variants. This study also agrees with (Bewley et al., 1997), suggesting that the Val118Ala (A to B) mutation may play an important role in digestion because it is located near Cys119, which forms a disulphide bond within the FGH motif. The Asp64Gly mutation is also of interest, as the A variant introduces an additional acidic residue in the CD loop, possibly influencing the dimer–monomer dissociation equilibrium (Bewley et al., 1997, Creamer et al., 2004).

Differences in thermal stability of β- lactoglobulin genetic variants have been observed by O'Loughlin et al. (2013b), who demonstrated that β- lactoglobulin B is more resistant to heat treatment than β-lactoglobulin A. Schmidt and Markwijk (1993) also showed that all variants of β-lactoglobulin are more rapidly hydrolysed by pepsin post-heat treatment compared with native β- lactoglobulin, with heating having a greater effect on the A variant than the B variant.

Differences also exist in terms of β- lactoglobulin digestion between species. When bovine milk was digested using *in vitro* digestion, it was found that 83% remains intact at the end of gastric digestion; however, when caprine milk is digested, just 23% of β-lactoglobulin remained intact (Almaas et al., 2006). This finding is interesting as the

structure of β- lactoglobulin is highly conserved between these species. Although there are six amino acid substitutions between caprine β- lactoglobulin and the B variant of bovine β- lactoglobulin, these substitutions do not appear to result in tertiary or quaternary structural changes (Crowther et al., 2014). Further, β- lactoglobulin was found to be digested more extensively *in vitro* in bovine *vs.* sheep milk and yogurt. This was due to the formation of a denser curd in sheep's milk, reducing the access of proteases to proteins (Saviard et al., 2024).

## 1.4 Extrinsic factors affecting milk protein digestion

### 1.4.1 Processing

To ensure the microbiological safety and to increase the shelf life of commercial milk, various heat treatments can be used. According to the International Dairy Foods Association (IDFA), the most common heat treatment consists of heating to 72 °C for 15 s/90–95 °C for 20 min (High Temperature Short Time Pasteurisation, HTST) (Int Dairy). Milk can also be heated to 63°C for 30 min (Vat Pasteurisation, Low Temperature Long Time, LTLT), or to 138 °C for 2 s (Ultra High Heat Treatment, UHT). As alternatives to heat treatment, non-thermal techniques such as microfiltration and ultra-filtration can also be used to decrease the bacterial load of bovine milk. In addition to thermal/non-thermal treatments aimed at controlling the microbiological load, commercial milk typically undergoes a mechanical treatment known as homogenisation. This process can be briefly described as passing heated milk under high pressure through a small orifice, results in reduction of the average diameter of fat globules, and an increase in their total number and surface area (Goff, 2013). This leads to a reduced tendency for the creaming of fat globules, ultimately ensuring a uniform, safe and high-quality product reaches consumers. These processes

have been shown to affect the digestion of milk proteins, which will be discussed in the following sections.

#### 1.4.1.1 Heat Treatment

The heat-induced changes that occur in milk proteins during processing can affect the digestion of both WPs and caseins. HTST and micro filtered milk (which undergoes no heat treatment) have similar effects on postprandial aminoacidemia (Lacroix et al., 2008). In contrast, UHT milk leads to a more rapid increase in postprandial serum amino acids in humans (Lacroix et al., 2008). This may be due to differences in the formation of softer coagulation during digestion in UHT and HTST milk due to decreased casein micelle stability in UHT milk (Lacroix et al., 2008). *In vitro* studies offer further mechanistic insight into the digestive processes of each of these milks. It has been shown, for example, that the association of WPs with casein micelles due to pasteurisation leads to softer coagulum formation during gastric digestion (Kethireddipalli et al., 2010). Ye et al. (2016) found a higher amount of β-lactoglobulin and α-LA in clots formed during the gastric digestion of heated milk (90 °C, 20 min) *vs.* unheated whole milk, leading to softer curds in the heated milk. The authors of this study hypothesised that softer clots in heated milk likely facilitate pepsin to hydrolyse protein within the clot structures, increasing the rate of digestion, whereas accessibility was probably hindered in the denser clots formed in unheated milk explaining the slower digestion. Similarly, in another study using a more complex, fully dynamic model, it was found that heat treatment (90 °C, 10 min) led to a decreased rate of casein hydrolysis, but increased rates of β-lactoglobulin hydrolysis in skimmed milk compared with unheated skimmed milk (Sánchez-Rivera et al., 2015). Sánchez-Rivera et al. (2015) also identified differences in peptide fragments released due to heat treatment, which may have an influence on the bioactive

properties of the milk. Increasing heat treatment can further influence digestion profiles. Higher temperatures during heat treatment can lead to increased denaturation of WP, leading to an increase in WPs bound to the casein micelle, reducing the ability of casein micelles to interact with one another (Kethireddipalli et al., 2011). Mulet-Cabero et al. (2019) observed, using *in vitro* semi-dynamic digestion, that heating milk caused the production of more fragmented particles during gastric digestion. This was more pronounced for pasteurised milk (72 °C, 15 s) than UHT milk (140 °C, 3 s).

Recently, *in vivo* studies have used non-invasive techniques such as Magnetic Resonance Imaging (MRI) to study the effects of heat treatment on milk digestion in humans, with conflicting results. Milan et al. (2024) found a greater gastric content volume throughout digestion when comparing UHT milk with pasteurised milk, indicating that UHT milk was emptied to the intestinal phase slower than pasteurised milk. This finding contradicts both *in vivo* findings and *in vitro* findings. Mayar et al. (2024) used MRI to show that no difference in gastric coagulation is associated with higher heat treatment (90% WP denaturation) compared with lower heat treatment (3% WP denaturation); however, higher heat treatment did result in faster gastric emptying times than lower heat treatment. Although Milan, *et al*. (Milan et al., 2024) observed findings that seem to contradict *in vitro* digestion studies, which suggest that increasing the heat treatment of milk results in the formation of weaker curds that are emptied more rapidly from the stomach (Mulet-Cabero et al., 2019), the results of Mayar, *et al.* (Mayar et al., 2024) agrees with these *in vitro* assumptions. When conflicting results such as these are obtained, it can be useful to return to early studies published on the digestion of milk proteins. As discussed in Section 2, after 3 h of digestion of boiled milk, patients stomachs had emptied, while raw milk remained, suggesting a slower rate of gastric emptying in raw *vs.* heat-treated milk (Brennemann,

1913). While Mayar, *et al.* (2024) did not use raw milk, they did show that lower heat treatment resulted in slower gastric emptying rates, in agreement with early studies (Mayar et al., 2024). The advancement in MRI techniques should significantly advance our understanding of factors affecting milk protein digestion *in vivo*.

Heat treatment may also result in the occurrence of Maillard reactions, which may reduce the bioavailability of certain amino acids. For example, if reducing sugars such as lactose bind to Lys residues at the trypsin cleavage site, Lys may become bio-unavailable (Finot and Magnenat, 1981).

#### 1.4.1.2 Homogenisation

The impact of milk homogenisation on digestion has long been a subject of study. Important learnings can be derived from research on infant nutrition. As early as 1915, different researchers were interested in applying homogenisation to milk, hypothesising that the "more finely divided the food, the greater its accessibility to digestive fluids, and the greater its assimilation" (Ladd, 1915). For example, it has been observed that the homogenisation of human milk significantly increased gastric lipolysis, enhanced the proteolysis of serum albumin and reduced the gastric emptying rate in preterm infants (de Oliveira et al., 2017). Furthermore, homogenisation was linked to improved absorption of human milk fat in very low-birth-weight infants (Thomaz et al., 1999). Theoretically, in adults, the digestion of bovine milk could also be expected to be impacted by homogenisation; however, studies where homogenisation and heating are investigated separately are lacking. One study comparing the digestion of homogenised pasteurised milk with raw milk found that while there were no differences in postprandial levels of plasma lipids, the concentrations of some fatty acids were significantly higher following the consumption of the homogenised and heated milk (Nuora et al., 2018). Interestingly,

in an *in vivo* study using pigs as human models, it was found that both pasteurisation and homogenisation increased the rate of milk protein hydrolysis; however, only homogenisation increased the rate of amino acids entering the small intestine (Ahlborn et al., 2023). *In vitro* studies further elucidate the impact of homogenisation on digestion. Homogenisation has been shown to affect the coagulation properties of milk during *in vitro* gastric digestion. After 10 min of digestion, both non-homogenised and homogenised whole milk and milk form a coagulum; however, the coagulum formed by homogenised milk is more porous. This porous structure may facilitate pepsin's ability to access proteins, leading to a more extensive breakdown of coagulum, as demonstrated by Tunick et al. (2016).

### 1.4.2 Consumption temperature

Bovine milk can be consumed at various temperatures, and several human studies have investigated how these temperatures impact the digestive environment. Using coffee as an example, it was shown that consuming different temperature liquids can affect the temperature of the stomach, cooling or warming depending on the temperature of the liquid (Sun et al., 1988, McArthur and Feldman, 1989). It is clear that consuming liquids hot or cold can affect the temperature of the stomach for up to 30 min (McArthur and Feldman, 1989, Sun et al., 1988). This may affect the activity of pepsin, leading to changes in digestion.

When consuming bovine milk, deviations from body temperature (37 °C) during gastric digestion may impact milk protein digestion kinetics due to changes in pepsin activity at different temperatures (Horne and Lucey, 2014). *In vivo* studies with calves showed that cold milk empties more rapidly than warm milk (Webber et al., 1980). This may be due to slower coagulation at colder temperatures, causing milk to remain liquid longer, and liquids emptying more rapidly from the stomach than solids or semi-

solids (Christian et al., 1980). This difference occurs despite a high gastric pH at the beginning of gastric digestion when temperature differences occur due to the optimum activity of pepsin on κ-casein occurring at pH~6 (Salelles et al., 2021). This allows a rapid decrease in electrostatic repulsion on the surface of casein micelles due to the release of glycomacropeptide from κ-casein despite alkaline conditions (Yang et al., 2022, Horne and Lucey, 2014, Salelles et al., 2021). Yang et al. (2023) used a human gastric simulator model to digest skim milk at 4 °C, 37 °C and 50 °C, reporting that cold milk coagulated more slowly than milk at other temperatures and produced softer curds, leading to increased protein hydrolysis at 4 °C due to better pepsin access.

Fitzpatrick, *et al.* (Fitzpatrick et al., 2024) used a modified *in vitro* semi-dynamic gastric digestion under MRI to visualise the differences in the digestion of cold (4 °C), body temperature (37 °C) and hot milk (60 °C). Both Yang, *et al.* (Yang et al., 2023) and Fitzpatrick, *et al.* (Fitzpatrick et al., 2024) attempted to replicate *in vivo* gastric temperatures post-consumption of cold or hot beverages. However, Yang, *et al.* (Yang et al., 2023) began gastric digestion at 4, 37 or 50 °C, i.e., the temperature of the beverages under study, while Fitzpatrick, *et al.* (Fitzpatrick et al., 2024) simulated the consumption of milk cooled/heated to 4, 37 or 60 °C but then adjusted digestive conditions for temperature changes observed during ingestion. As a result, in that study, gastric digestion was initiated at 22 °C for cold milk and at 44 °C for hot milk and then the respective rates of return to 37 °C were simulated based on *in vivo* observations.

Despite the reduced temperature extremes in Fitzpatrick, *et al.* (Fitzpatrick et al., 2024), similar results were observed, with cold milk showing slower coagulation than milk at 37 °C or hot milk. Differences in proteolysis were found by Yang, *et al.* (Yang et al., 2023) but not by Fitzpatrick, *et al.* (Fitzpatrick et al., 2024), likely due to greater

temperature differences and more targeted protein hydrolysis measurements, such as examining the specific hydrolysis of κ-casein, in Yang, *et al.* (Yang et al., 2023). These findings show the need to carefully monitor the temperature at which milk is consumed, or simulated to be consumed, during *in vivo* or *in vitro* digestion experiments.

## 1.5 Conclusions

Bovine milk is a highly digestible source of high-quality proteins. Several factors can influence the rate and extent of protein breakdown during digestion, potentially leading to changes in the rate of uptake of amino acids into the bloodstream which, in turn, can have physiological implications. This review focused on the impact of variations in the profiles of native milk proteins and of the most common processes to which milk is exposed prior to consumption. Certainly, extrinsic factors applied to milk such as heat treatment and homogenisation lead to differences in the digestion of milk. Moreover, more subtle changes are induced by intrinsic factors such as protein polymorphisms, which can affect the nutritional quality of bovine milk, with either potential beneficial or harmful implications on human health. These subtle changes to bovine milk could be considered to produce premium milk products aimed at certain population cohorts, such as milk with increased casein content to increase the retention time of proteins, prolonging satiety and negating peaks in aminoacidemia. This paper therefore provides both academia and industry with a base of knowledge to further the dairy industry with respect to human health and nutrition.

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## 1.7 Chapter 1 (Part 2):

## Factors affecting the digestion of dairy lipids

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## 1.8 Overview of dairy lipids

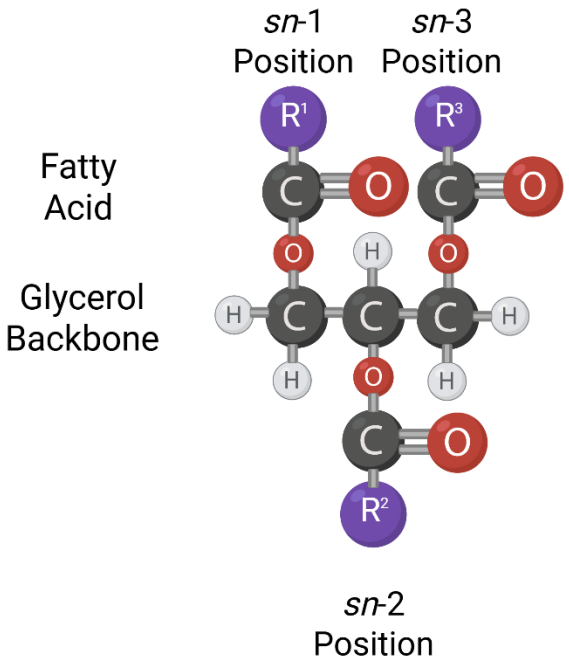


Figure 3: Schematic representation of the structure of a triacylglycerol – created using Biorender.

Bovine milk consists of 3.5-5.5% fat, depending on factors such as breed of cow, seasonality, disease and dietary intake of the cow (Jensen, 2002). Of this, over 95% exists within milk fat globules (Keenan and Dylewski, 1995). These milk fat globules are made up of triacylglycerols (TAG), retinol esters and cholesterol, enclosed within the milk fat globule membrane (MFGM), itself consisting of polar lipids, proteins and cholesterol (O'Brien and O'Connor, 2016). The MFGM, representing approximately 2% of the milk fat globule, stabilises fat droplets in the milk serum phase and protects milk fat from oxidative rancidity and off-flavours. Milk fat is primarily made up of TAGs (98.3%), with diacylglycerols (DAGs), monoacylglycerols, (MAGs), free fatty acids (FFAs), phospholipids and sterols making up a small proportion.

Despite the simple structure of TAGs, existing as a glycerol backbone esterified to 3 FAs (as seen in Figure 3), milk fat is one of the most complex fats in food due to the existence of over

400 FAs varying in carbon length, degrees of saturation, / unsaturation, double bond position, stereoisomers and origin e.g. from the diet or synthesised in the cow (Lindmark Månsson, 2008). The total weight of FAs esterified to the glycerol backbone can be used to classify TAG species into low, medium and high molecular weight.

FAs are made up of hydrocarbon chains and are synthesised through two primary pathways in the dairy cow: *de novo* synthesis and dietary uptake. The FAs can be categorised based on chain length and degree of saturation, with the categories including saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). Short- and medium-chain fatty acids (C4–C14) can be synthesised in the mammary gland, whereas longer-chain fatty acids (C16–C18) are derived from the cow's diet and mobilised adipose tissue. These FAs are esterified into TAG and distributed across different *sn*-positions on the glycerol backbone based on their chain length. For instance, long-chain fatty acids (LCFAs) such as palmitic acid (C16:0) and oleic acid (C18:1) mainly occupy the *sn*-1 position, while medium-chain fatty acids (MCFAs) are more commonly esterified at the *sn*-2 position. SFAs contain no double bonds in their hydrocarbon chains, MUFAs have one double bond, while PUFAs contain two or more double bonds. Rumen microbes can convert UFAs, such as linoleic acid (C18:2 n-6), to saturated fatty acids (SFAs) through bio hydrogenation, while desaturase enzymes in the mammary gland introduce double bonds to convert SFAs to UFAs (Santos, 2002). The most abundant SFAs in bovine milk is palmitic acid (C16:0), and other SFAs include myristic acid (C14:0) and stearic acid (C18:0) (Timlin et al., 2023c, O'Callaghan et al., 2016).

Oleic acid (C18:1) is the most abundant MUFAs in bovine milk and has been linked to positive health outcomes in humans (Kris-Etherton et al., 1999). Higher pasture allowance is associated with higher concentrations of oleic acid in milk, linking grass fed milk to positive nutritional outcomes (Timlin et al., 2023b). PUFAs, though present at lower concentrations than SFAs

and MUFAs, include essential FAs such as linoleic acid (C18:2) and alpha-linoleic acid (C18:3).

Despite being present in low concentrations in milk, milk provides 16–26% of omega-3 FAs to humans in the United States of America (Benbrook et al., 2013). The ratio and concentration of these PUFAs can vary significantly based on the cow's diet, with grass-fed systems being associated with higher levels of beneficial omega-3 FAs such as the essential FAs alpha-linoleic acid, contributing to a more favourable omega-6 to omega-3 ratio in the milk (Timlin et al., 2023b). Omega-3 FAs include alpha-linoleic acid, eicosapentaenoic acid and docosahexaenoic acid, and have reported health benefits, while increasing proportions of omega-6 FAs such as linoleic acid may be linked to inflammation.

## 1.9 Digestion of dairy lipids

Bovine milk lipids exist primarily as TAGs within fat globules, surrounded by a fat globule membrane (MFGM).

In humans, the digestion of dairy lipids begins primarily in the gastric phase. In the stomach, gastric lipase is secreted from chief cells in the stomach wall when triggered by factors such as gastrin and cholinergic stimuli. The activity of gastric lipase is dependent upon pH, temperature and substrate and has an optimum pH between 5.4 and 5.8. Since its substrate is insoluble in water, lipase works at the lipid-water interface. During gastric digestion, the pH drops below 5.5, leading to the aggregation of MFGs, and preferentially hydrolyses FAs that are esterified at the *sn-3* position of TAGs, therefore mainly releasing SCFAs and MCFAs (Mansbach, 2004). Gastric lipase has been estimated to account for 10-30% of lipid hydrolysis in milk, and up to 60% in infants (Armand, 2007, Abrahamse et al., 2012b). However, the activity of gastric lipase is gradual inhibited by the production of FFAs, blocking enzyme access to the substrate (Pafumi et al., 2002).

*Table 4 Enzymes involved in the digestion of dairy lipids in humans.*

| Enzyme | Location of action in digestive system | Reference |
|---|---|---|
| Gastric Lipase | Stomach | (Miled et al., 2000) |
| Colipase-dependent pancreatic lipase | Duodenum | (Zhu et al., 2021) |
| Pancreatic lipase-related proteins 2 (PLRP2) | Duodenum | (Zhu et al., 2021) |
| Bile-salt stimulated lipase (BSSL) | Duodenum | (Miled et al., 2000) |
| Pancreatic Phospholipase A2 | Duodenum | (Nevalainen, 1993) |

The remaining lipid digestion occurs in the small intestine due to the action of bile salts, pancreatic lipase and co-lipase (Carriere et al., 1993, Armand et al., 1999, Zhao et al., 2023). Studies have shown lipolysis to be rapid at the beginning of intestinal digestion, but slows due to the accumulation of lipolysis products at the interface, which can restrict the access of digestive enzymes (Tunick et al., 2016). However, bile salts increase the available surface area for enzymes to act by emulsification of lipids. As well as this, they participate in the formation of mixed micelles (water-soluble aggregates formed during lipid digestion with a hydrophilic exterior and a hydrophobic interior), which can increase the removal of lipolysis products such as FFAs from the surface of lipids, allowing greater enzyme access and reducing product inhibition (Sarkar et al., 2016). Because of the action of bile salts, a lag phase is rarely observed at the beginning of intestinal digestion (Tunick et al., 2016).

## 1.10 Factors affecting the digestion of dairy lipids

The digestion of lipids in milk can be affected by several factors. Some examples of these factors are discussed here.

### 1.10.1 Does stereo position affect digestion of milk lipids?

TAGs are made up of a glycerol backbone with 3 FAs attached, and FAs can be located at positions 1, 2 or 3 on this glycerol backbone, known as *sn*-1, *sn*-2, and *sn*-3, as shown in *figure 3*. Some FAs, such as C4:0 (butyric) and C6:0 (caproic) are located almost exclusively at the *sn-3* position, while FAs such as C16:0 (palmitic acid) are mostly located at either the *sn*-1 or *sn*-3 position (Månsson, 2008). The proportions of FAs located at positions on the glycerol backbone can be affected by factors such as stage of lactation. For instance, Pacheco-Pappenheim et al. (2022b) showed that the abundances of FAs such as C16:0, C18:0 and C18:1 cis9 trans 11 are present in higher amounts in early lactation *vs.* late lactation. Notably however, despite differences in abundances, the FAs were preferentially esterified at the same positions. It has also been shown that the physicochemical characteristics of dairy products, such as butter, are affected by stage of lactation. Milk from cows in late lactation has a higher level of SFAs than milk from cows in mid lactation (O'Callaghan et al., 2020). This leads to harder butter, which is less spreadable, which may influence the rate of digestion; however, studies in this area are limited. As well as this, the FAs profile of milk is affected by the diet of the cow.

In bovine milk, C16:0 and C18:0 are located at both the *sn-1/3* and *sn-2* positions in equal proportions, while human milk primarily has C16:0 located at *sn-2* position (Karupaiah and Sundram, 2007, Malacarne et al., 2002). This is of relevance in infant nutritional product formulation, as it has been shown that infant formula containing high amount of TAGs with C16:0 at *sn-2* position had higher levels of F FAs release during gastro-intestinal digestion in infants (Innis, 2011).

The position of the FAs on the TAG backbone can affect the rate of lipolysis due to lipases stereo preference for the *sn*-1 and *sn*-2 positions (Rogalska et al., 1990a) This is of interest with regards to certain FAs such as C16:0 due to its role in binding calcium. When C16:0 is esterified at the *sn*-2 position, it has been shown to increase the absorption of calcium as it remains bound to the glycerol backbone. However, when esterified at *sn*-1(3), and released from the backbone during digestion, it has been shown to decrease the bioavailability of minerals such as calcium, resulting in the loss of these minerals in the stool, and is linked to constipation in infants (Palmquist, 2006, Looijesteijn et al., 2022).

### 1.10.2 Can whey protein to casein ratio affect digestion of milk lipids?

When whey protein is consumed, it is emptied more rapidly from the stomach to the small intestine compared to casein (Mulet-Cabero et al., 2020d, Boirie et al., 1997). When a mixture of both is consumed, as in bovine whole milk, a coagulum is formed, slowing the release of nutrients from the gastric phase to the intestinal phase (Boirie et al., 1997). Increasing the ratio of casein to whey protein further slows the emptying of nutrients, both slowing the gastric digestion of lipids, and also entrapping them within a protein matrix (Mulet-Cabero et al., 2020d). Because of this, increasing the ratio of casein to whey protein decreases the rate of lipolysis (Mulet-Cabero et al., 2020d). Slowing the release of lipids into the small intestine subsequently slows the rate of lipemia (i.e., appearance of lipids in the blood stream), which may have beneficial effects on cardiovascular health. When casein was fed to healthy, overweight men as part of a lipid containing meal, it was found to reduce the amount of TAGs present in the blood, compared to when either whey protein or α-LA enriched whey protein were fed alongside the meal (Mariotti et al., 2015). This may be due to the formation of a coagulum when casein is fed, slowing the release of lipids from the gastric to intestinal phase.

### 1.10.3 How does processing affect the digestion of milk lipids?

The digestion of milk lipids can also be affected by processing such as homogenisation. During homogenisation, MFGs are reduced in size from ~1–8 μm (raw milk) to 0.3–0.8 μm and re-emulsified with MFGM fragments and adsorbed proteins, mainly caseins and whey proteins, forming smaller lipid droplets with different interfacial composition (Thiebaud et al., 2003), resulting in an increase in surface area available for lipase activity. During gastric digestion, this decrease in MFG size leads to phase separation that is not seen in non-homogenised milk, where homogenised milk creams, while non-homogenised milk sediments (Mulet-Cabero et al., 2019). As well as this, heat treatment can affect the rate of gastric emptying of lipids to the small intestine, with ultra-high temperature paired with homogenisation leading to the formation of a soft coagulum and subsequently more rapid gastric emptying compared to raw milk *in vitro* (Mulet-Cabero et al., 2019). Tunick et al. (2016) also found differences in the digestion of milk lipids during *in vitro* digestion due to processing, with homogenisation significantly increasing the rate of F FAs release when compared to raw milk. This may be due to the increased surface area of lipid droplets created during homogenisation, allowing digestive enzymes (e.g., lipases) to access and break down TAG more efficiently, which may accelerate the release of FAs. Similar results have been shown in (Gallier et al., 2012) and (Ye et al., 2017). As well as differences in the rate of lipolysis, Mulet-Cabero et al. (2019) showed, using an *in vitro* approach, that while lipids in homogenised milk cream to the top during gastric digestion, lipids in native milk sediment to the bottom; this could lead to differences in the rate of nutrient emptying from the stomach to the small intestine, and subsequently differences in uptake into the blood stream.

### 1.10.4Role of the dairy matrix in lipid digestion

Milk can be transformed into various matrices, such as semi-solid forms (e.g., yogurt) and solid forms (e.g., cheese), each affecting lipid digestion differently. One factor is the

disintegration pattern of these matrices during digestion. For example, full-fat Cheddar cheese has a less dense protein matrix than reduced-fat Cheddar, causing it to disintegrate more rapidly during *in vitro* digestion. This leads to a greater release of lipids in full-fat Cheddar compared to its reduced-fat counterpart (Fang et al., 2016). As well as this, the dairy matrix affects the movement of nutrients during gastric digestion *in vivo*. Mackie et al. (2013) demonstrated that semi-solid dairy matrices provide a greater sense of fullness compared to liquid matrices. Using MRI, the authors observed that lipids in semi-solid matrices settle at the bottom of the stomach, contrasting with the creaming behaviour of lipids in liquid matrices. This can be explained by casein micelles forming a dense coagulum in semi-solid matrices entrapping lipids (Mulet-Cabero et al., 2019). This delays lipase access to lipids. The observed sedimentation likely explains the higher satiety reported after consuming semi-solid dairy products.

### 1.10.5 Does age play a role in milk lipid digestion?

Infants digest lipids differently to adults due to both different enzyme activities and developmental factors in the digestive system. Compared to adults, infants have been shown to have lower levels of pancreatic TAG lipase (PTL) and bile salts. Because of this, they must rely on enzymes such as PTL-related protein 2 (PLRP2) to hydrolyses TAGs at *sn*-1 and 3 positions; and bile salt stimulated lipase (BSSL) to aid lipid absorption (Andersson et al., 2011). As well as this, lingual lipase can have a compensatory role for this reduction in pancreatic enzymes and bile salts in infants. As the infant develops, the role of lingual lipase in total lipid digestion declines as the infant's pancreatic function and bile salt secretion increases (Hamosh, 1979).

Lipids are essential for infant growth and neurodevelopment, therefore efficient digestion from birth is critical. Infants exhibit high gastric lipase activity (15.3–79 U/mL tributyrin in fasted infants *vs.* 15.6–64 U/mL in adults), which compensates for low pancreatic lipase levels and ensures sufficient lipid hydrolysis during early life stages (Fredrikzon and Hernell, 1977,

Armand et al., 1995, Poquet and Wooster, 2016). Interestingly, the pH of infants' stomach after meal consumption does not become as acidic as in adults due to decreased proton pump activity. This may be detrimental to pepsin activity with an optimum pH of 2; however, it may be of benefit to gastric lipase activity due to its optimum pH between 3 and 5 (Sams et al., 2016).

The amount of unabsorbed lipids, as measured by the amount of lipid in stools, is high in both pre-term infants (10-30%) and full term infants (10%). (Abrahamse et al., 2012a). However, when the low bile salt concentration and PTL secretion are accounted for, alongside the large ingestion of lipids by infants (2.5-3.5 g/kg BW/d), infant digestion is surprisingly efficient. (Abrahamse et al., 2012b).

### 1.10.6 Effect of fatty acid composition on digestion of milk lipids

Grass fed cows produced milk with decreased proportions of C16:0, leading to a lower C16:0 to C18:1, which improves the texture and spreadability of butter and other dairy products compared to TMR (O'Callaghan et al., 2016). This is highlighted by a recent Canadian controversy known as 'Buttergate'; the increased feeding of palm oil supplementation, intended to increase milk production efficiency, led to an increase in palmitic acid content, increasing the hardness of butter (Marangoni et al., 2022). In contrast to this, the increased PUFAs content in grass fed milk provides processing advantages due to the lower melting point of UFAs, improving the spreadability of butter and other dairy products (Timlin et al., 2023a). These observed textural differences may lead to differences in digestion, however, to the best of our knowledge, no studies have been carried out on this.

## 1.11Conclusion

The digestion of milk lipids is affected by several factors. The stereo-specific position of FAs on the glycerol backbone affects their digestion and absorption, with implications for both nutritional value and potential health outcomes. The ratio of whey protein to casein in milk,

types and severity of technological processing, and the dairy matrix, all may impact the rate of lipid digestion and subsequent lipidaemia. As well as this, age-related differences in digestive enzymes and gastrointestinal conditions lead to variations in lipid digestion between infants and adults, showing the need for age-specific nutritional considerations. More studies are needed to explore the long-term health implications of different milk lipid profiles and their digestion patterns.

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# Statement of research objectives

The primary objective of this research was to investigate the intrinsic and extrinsic factors affecting the *in vitro* digestion of dairy products, focusing on proteins and lipids and to create a tool to enhance the accuracy and precision of future *in vitro* digestions. The objectives of the studies carried out as part of this thesis include:

1) The movement towards precision-based dairying promotes a greater understanding of all aspects of the dairy industry, aiming to use all available possibilities to improve the industry. One factor that has the potential to be exploited to improve the productivity of the industry is to breed cows to exclusively produce certain protein polymorphisms. Previous research has outlined the potential benefits of this, such as the breeding of cows to produce milk with exclusively A2 β-casein instead of A1 β-casein. For this reason, this thesis explored the impact of genetic polymorphisms of β-lactoglobulin on the production and digestion of yogurt.
2) Ireland and New Zealand have climates that allow for cows to be fed outdoors on a pasture-based diet, in contrast to the majority of the world, who feed dairy cows indoors on a TMR diet. Several publications have shown the differences in lipid profile between milk from grass fed vs TMR diets. However, the digestion of these milks has not been studied. Therefore, this research aimed to assess how feeding strategies (grass-fed *vs.* total mixed ration) influence the release of lipids during *in vitro* digestion.
3) The activity of gastric enzymes is affected by both pH and temperature; however, the effect of meal consumption temperature on digestion is poorly studied. This research aims to investigate how milk consumption temperature affects its digestion dynamics using *in vitro* digestion under MRI. MRI enables non-invasive, real-time visualisation of these structural changes, offering insights into the effects of warm *vs.* cold milk on

nutrient bioavailability, gastric emptying, and digestive efficiency. This will improve our understanding of how temperature influences digestive physiology.

4) This research seeks to develop a predictive model for pepsin activity during gastric digestion under various physiological and dietary conditions. The activity of pepsin, an important enzyme in protein digestion, is influenced by factors such as pH and temperature. By modelling these variables, the study aims to predict enzymatic activity during gastric digestion. This predictive tool will help future researchers to understand protein digestion during *in vitro* gastric digestion.

# Chapter 2

# Impact of β-lactoglobulin genetic polymorphism on the gastric digestion of yogurt using semi-dynamic *in vitro* digestion

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**CRediT authorship contribution:** Conor J. Fitzpatrick: Writing – review & editing, Writing – original draft, Visualisation, Methodology, Investigation, Formal analysis, Data curation, Conceptualisation. Elena Hayes: Methodology, Investigation. Liam M Kelly: Resources Davor Daniloski: Writing – review & editing, Methodology. Daniela Freitas: Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualisation. Tom F O'Callaghan: Supervision, Writing – review & editing. James A. O'Mahony: Supervision, Writing – review & editing. André Brodkorb: Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualisation.

## 2.1 Abstract

This study aimed to determine the impact of the genetic variants of bovine β-lactoglobulin on the (micro) structural properties and gastric digestion dynamics of yogurts made from skim milk powder containing AA, AB and BB β-lactoglobulin variants. The viscoelastic properties of these yogurts were analysed using oscillatory rheology. Following this, the gastric digestion behaviour of each yogurt was examined using the INFOGEST semi-dynamic *in vitro* gastric digestion system. The characterisation of digestion properties was carried out using Size Exclusion High Performance Liquid Chromatography, Fourier-Transform Infrared Spectroscopy, bicinchoninic acid assay, and o-phthalaldehyde assay to evaluate extent of protein hydrolysis. Confocal microscopy was used to study the microstructure of yogurt and digesta. Our results showed that AA yogurt exhibited a less viscous and looser gel, which contributed to a more rapid protein hydrolysis during gastric digestion, compared to BB yogurt, which formed a dense protein network and tight curd, resulting in a yogurt that was more resistant to gastric digestion. This study has shown differences in yogurt rheological properties and subsequent protein breakdown during gastric digestion due to different β-lactoglobulin phenotypes present in skim milk.

## 2.2 Graphical abstract

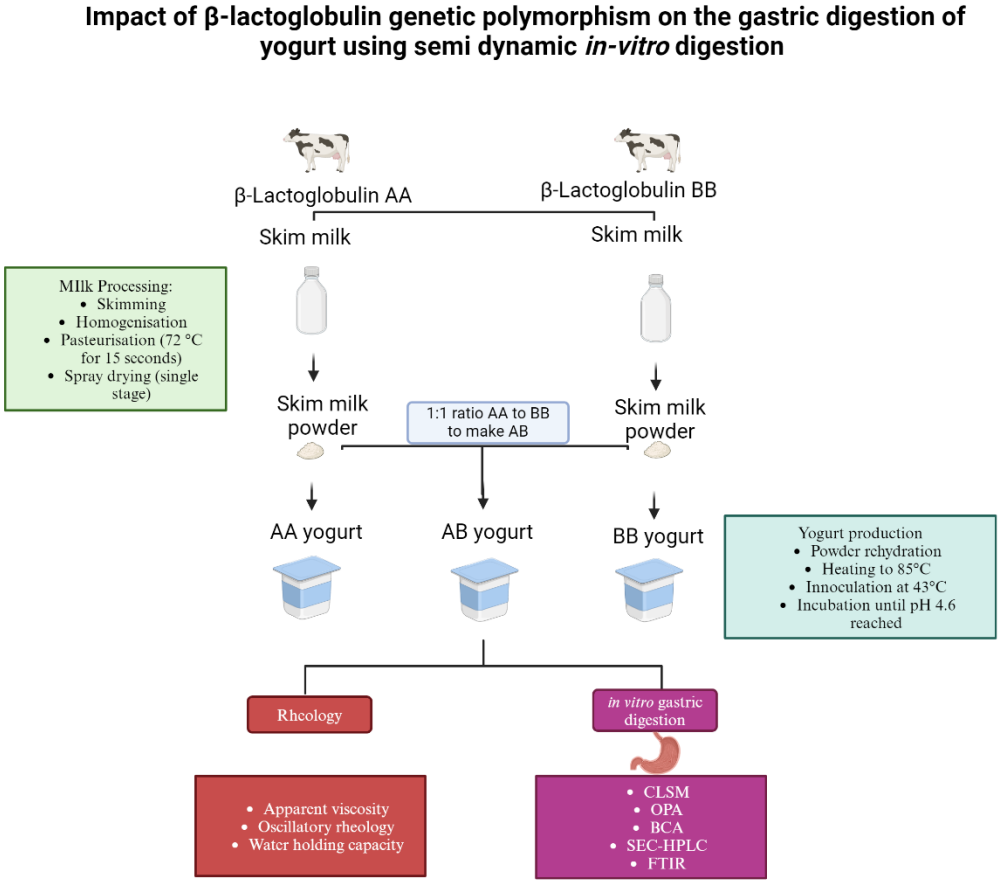


Figure 4 Schematic overview of experimental design to examine the effect of beta lactoglobulin genetic polymorphism on yogurt production characteristics and *subsequent in vitro* digestion.

## 2.3 Introduction

The functional properties of bovine milk and its dairy products are significantly affected by the quality and composition of the protein fraction. Two major protein groups are found in bovine milk; caseins, consisting of $\alpha_s$-, β- and κ-caseins, predominantly present in the form of casein micelles and whey proteins, including α-lactalbumin, β-lactoglobulin and other minor proteins, found in the milk serum (Fox and Mulvihill, 1982). These two categories of dairy proteins are crucial for the technological properties of milk as they affect gel formation in the production of cultured dairy products, with yogurt being the most popular (Lucey, 2004).

During acid gelation of milk, as the pH drops from ~6.70 to 4.60, the κ-casein layer on the casein micelles collapses, leading to a reduction in electrostatic repulsion between the casein molecules (Fox and Mulvihill, 1982). However, prior to the fermentation process essential for yogurt production, the milk is normally heat-treated at 80 - 90 °C for 10 min in order to improve the viscoelastic properties of the product (Lucey, 2004). Heat treatment causes the whey proteins, but particularly β-lactoglobulin, to denature and associate with κ-casein on the surface of the casein micelles (Lucey and Singh, 1997).This protein cross-linking via intermolecular disulphide bonds and hydrophobic interactions of caseins and whey proteins leads to formation of protein aggregates and subsequently the formation of a gel network (Lucey et al., 2022). The possibility of improving yogurt properties, such as viscosity, texture, curd firmness, water holding capacity and gelation time by selection for certain milk protein genes at primary production, has long been a subject of interest (Hallén et al., 2009, Ketto et al., 2018, Ng-Kwai-Hang et al., 1986). Phenotypes of κ and β-casein have been shown to have significant effect on production of firmer acid and yogurt gels (Daniloski et al., 2022a, Hallén et al., 2009). A similar effect may be seen as a result of the β-lactoglobulin phenotype due to correlations between its content in the starting milk with the acid gelation properties (Heck et al., 2009, Ng-Kwai-Hang et al., 1986).

ß-lactoglobulin accounts for approximately 50-55% of the nitrogen in whey proteins and contributes ~10-12% of the total nitrogen in bovine milk proteins (Farrell et al., 2004b, Fox et al., 1998b). The full amino acid sequence of β-lactoglobulin was first elucidated by Braunitzer et al. (1972) stating that this protein contains 162 amino acid residues and has a molecular weight of 18.3 kDa. This protein contains two intramolecular disulphide bonds, the first between the positions 6 - 160 in its polypeptide chain and the second, between amino acids 106 and 119. As well as this, a free cysteine exists at position 121 of its structure (O'Mahony and Fox, 2014). To date, 11 different genetic variants of bovine β-lactoglobulin have been found in the literature (Farrell et al., 2004a), nevertheless, the genetic variability within the β-

lactoglobulin gene gives rise to distinct phenotypes, denoted as AA, AB, and BB, which have been associated with different effects on dairy product characteristics (Deeth and Bansal, 2019). Although nearly identical proteins, two amino acid substitutions exist between β-lactoglobulin A and B, in position 64 and 118 within their polypeptide chains, with aspartic$^{64}$ to glycine$^{64}$ and valine$^{118}$ to alanine$^{118}$ for β-lactoglobulin A and β-lactoglobulin B, respectively (Olguin-Arredondo and Vallejo-Córdoba, 1999). Several studies examined the rheological properties of acid gels made from milk containing different β-lactoglobulin phenotypes, finding a consensus that β-lactoglobulin B is related to a positive effect on the acid gels firmness, when compared to both acid gels with β-lactoglobulin A (Allmere et al., 1998, Hallén et al., 2009). In contrast, very recently, Şahin Semerci et al. (2024) showed that the stirred yogurt made from milk with β-lactoglobulin AA showed lower syneresis values and higher values for firmness, apparent viscosity, and consistency compared to the AB yogurt.

Firmer yogurts have been related to slower rates of gastric emptying during *in vivo* gastric digestion(Le Feunteun et al., 2014). This has also been repeated in pigs with two iso-caloric yogurts with differing viscosity, showing that high viscosity yogurt was emptied more slowly from the gastric phase (Dupont et al., 2018). Since genetic variation in β-lactoglobulin affects the physical properties of yogurt and the yogurt matrix, differences may exist in the rate and extent of protein breakdown during gastric digestion, which may affect the rate of release of proteins into the small intestine for further breakdown and absorption.

Genetic selection of cows has been a factor in the increased milk yields observed since the 1960s (Brito et al., 2021). However, this genetic selection may have unintended consequences, such as altering the genetic profile of milk proteins in the national herd, which may subsequently affect milk processability (Čítek et al., 2020). For example, κ-Casein AA genotype has been associated with increased milk yields, while BB has a higher protein percentage, however, κ-Casein B is associated with better yogurt fermentation properties (Čítek et al., 2020, Poulsen et al., 2013). Therefore, if cows were genetically selected for milk yield, yogurt production may be adversely affected. In relation to the present study, several studies have shown effects of β-lactoglobulin genetic polymorphisms on milk production traits (Bangar et al., 2022, Brito et al., 2021, Čítek et al., 2020, KF et al., 1987, Lundén et al., 1997).

Therefore, the present study aims to investigate the relationship between β-lactoglobulin genetic polymorphisms and the gastric digestion kinetics of yogurt, focusing on the hydrolysis of protein fractions and the alteration of protein structure during gastric digestion. This study will investigate yogurt made from skim milk powder homozygous for β-Lactoglobulin A (referred to in this study as AA), β-Lactoglobulin B (referred to in this study as BB) or

heterozygous -50% A, 50% B (referred to in this study as AB).By examining how genetic variation influences digestion and processability traits, this study acts as a due diligence investigation highlighting the potential unintended consequences of selective breeding for enhanced milk production traits on the functional properties of dairy products.

## 2.4 Materials and methods

### 2.4.1 Materials

Yogurt starter culture (YoFlex Express 1.0: *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus*) was obtained from CHR Hansen (CHR Hansen, Hørsholm, Denmark). All chemicals used were analytical grade. Porcine pepsin (P-6887, lot no. Pepsin - SLBV 3776) was obtained from Sigma Aldrich (Wicklow, Ireland). Pierce BCA Protein Assay kit was purchased from Thermo Fisher Scientific (Cork, Ireland). All other chemicals were obtained from Sigma Aldrich (Wicklow, Ireland).

### 2.4.2 Skim milk powder production

Milk was obtained from Irish Holstein Friesian cows from Curtin's Farm (Teagasc, Moorepark, Fermoy, Co. Cork, Ireland). Genotypic analysis identified 15 cows that were homozygous for β-lactoglobulin A and 14 cows were homozygous for β-lactoglobulin B. Milk from these cows was collected into two separate 5,000 L stainless steel tanks, one for each β-Lactoglobulin polymorphism (A or B).These were analysed using Reversed Phase HPLC to confirm the β-lactoglobulin polymorphisms present (McCarthy et al., 2017). Milk was processed using the facilities at Moorepark Technology Limited (Teagasc, Moorepark, Fermoy, Co. Cork). Milk was skimmed using a Skimmer Pro Centrifuge Disk Separator (GEA, Naas, Ireland) to a fat content of 0.06 and 0.04 % for the A and B polymorphism batches, respectively. The skim milk was then immediately pasteurised by heating to 72 °C for 15 seconds using an APV Fisher stainless steel pasteuriser (SPX Flow Technology Denmark A/S, Soeborg, Denmark). Prior to spray drying, 50 kg of skim milk from each batch was evaporated to a solids content of 40 % using a Scheffers single-effect recirculating evaporator (Scheffers, Schiedam, The Netherlands). The concentrated skim samples were then dried using a using a single-stage spray dryer (Anhydro F1 Lab Dryer; Copenhagen, Denmark) with an inlet temperature of 185 °C and an outlet temperature of 85 °C. Milk from homozygous A and B cows was mixed in the ratio of 1:1 to create AB milk.

### 2.4.3 Yogurt production

Skimmed milk powder was rehydrated to 15 % total solids and 5 ± 0.3 % (w/w) protein in Milli-Q water (Millipore Corp. Bedford, MA, USA). Protein content was checked using mid-infrared spectrometry (MilkoScan FT6000, Foss Analytical A/S). Skim milk was mixed using a magnetic stirrer and heated to 50 °C for 30 min on a hot plate. Milk was left overnight in the fridge at 4 °C so that powder was fully dissolved. Prior to inoculation, milk was heated to 85°C for 10 min and then cooled to 43°C in an icebox and subsequently 0.03 g of thermophilic starter culture (YC-380*: Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus*, CHR Hansen, Hørsholm, Denmark, 500U · 2500 L−1) was weighed out and added in the rehydrated milk samples. Following this, four individual yogurts for each group (45 g each), were fermented in beakers at 43 °C until pH 4.60 was reached. The pH measurement (CyberScan pH meter 510, Eutech Instruments, Singapore) was carried out periodically on a 'trial' yogurt for each condition, preventing distortion of the gel in yogurts used in further analysis. When the required pH was reached, the beakers were removed from the water bath and were rapidly cooled in an icebox to stop the starter culture activity. Beakers were placed at refrigerated temperature overnight for further analysis (Daniloski et al., 2022).

### 2.4.4 Rheological properties of yogurts

2.4.4.1 Measurement of viscoelastic properties of yogurt

Dynamic oscillatory rheology was conducted using an AR G2 Rheometer (TA Instruments, Crawley, UK) with a 60 mm diameter aluminium parallel plate geometry and a 1,000 μm plate gap. Approximately 5 mL of yogurt was loaded onto the lower plate, equilibrated at 20 °C for 2 min, and tested without pre-shear or normal force control. Amplitude sweeps were performed to identify the linear viscoelastic region (LVR) by progressively increasing strain and monitoring the storage modulus (G') and loss modulus (G"). A strain of 0.5%, within the LVR, was used for frequency sweeps.

Frequency sweeps (0.628–628 rad/s) were conducted at 20 °C with 100 logarithmically spaced measurement points, maintaining a constant strain of 0.5%. The viscoelastic properties, including storage modulus (G'), loss modulus (G"), and loss tangent (tan δ = G"/G'), were recorded. All measurements were performed in triplicate following Baldursdottir et al. (2010).

2.4.4.2 Apparent viscosity

Rheological measurements were performed using a controlled stress rheometer (AR G2 Rheometer, TA Instruments, Crawley, UK) equipped with an aluminium plate geometry. 5 mL of yogurt was loaded onto the rheometer plate and allowed to equilibrate at 20°C for 2 min before beginning a shear rate sweep from 0.01 to 300 $s^{-1}$, with 10 points collected per decade. A total of 50 sample points were recorded. The temperature was maintained at 25°C throughout the experiment. The test was set with a sample period of 10 seconds per point, a percentage tolerance of 5%, and a maximum point acquisition time of 1 min. The experiment required three consecutive data points to fall within the tolerance range to proceed to the next shear rate. Prior to measurement, the sample was subjected to a constant rate shear of 20 $s^{-1}$ for 30 seconds to ensure homogeneous sample distribution and to minimize sample history effects.

Dynamic oscillatory rheological measurements were performed using a 60 mm diameter aluminium parallel plate geometry. The gap between the plates was set to 1000 µm, and approximately 5 mL of yogurt sample was loaded onto the lower plate for testing. Prior to measurements, the sample temperature was set to 20°C, and the system was allowed to equilibrate for 2 min. Amplitude sweeps were conducted before dynamic testing to determine the linear viscoelastic region (LVR). A strain value of 0.5%, comfortably within the LVR, was selected for all frequency sweep experiments. Frequency sweeps were performed from 626 to 0.628 Rad/s, in logarithmic mode, with 100 measurement points distributed across this range. The following rheological parameters were recorded: storage modulus, G' (elastic or solid-like behaviour, G'), loss modulus, G'' (viscous or liquid-like behaviour) and loss tangent (tan δ = G''/G'), indicating the ratio of viscous to elastic behaviour. All measurements were performed in triplicate.

2.4.4.3 Yogurt water retention

The water holding capacity (WHC) of yogurts was measured based on the method described by Daniloski et al. (2024b). Briefly, 5 g batches of yogurts were made (in duplicate) in 50 mL falcon tubes using the method described in section 2.3. Tubes were then centrifuged at 10,000 x g for 15 min at 4 °C using a Heraeus Multifuge X1R (Thermo Scientific) centrifuge. The supernatant was then decanted and weighed. The WHC was expressed as a percentage of the mass of the pellet relative to the total mass of the sample.

### 2.4.5 *In vitro* semi-dynamic gastric digestion

Yogurts were stored in fridge at 4 °C for a maximum of 48 hours before semi-dynamic *in vitro* digestion. Yogurts were digested using *in vitro* semi-dynamic digestion as described in Mulet-

Cabero et al. (2020b). The yogurt contained 5.55% protein, equating to 2.5 grams of protein per 45 grams of yogurt, as determined using the Dumas method with a nitrogen-to-protein conversion factor of 6.38.

Oral phase: 45 mL of yogurt was added to oral phase. This was carried out in the gastric digestion vessel. In the yogurt, 5.4 mL of eSSF, 33.8 µL $CaCl_2$, and 1.32 mL water was added. Following this, 5 mL of the mixture was removed for analysis. This 5 mL was added to a 50 mL falcon tube where 1 mL was immediately taken for confocal analysis; 1 mL was immediately taken for FTIR analysis.

Gastric phase: The initial volume of gastric phase was 43 mL. To begin gastric phase of digestion, the basal phase was added to the digestion vessel as follows, eSGF (0.13M HCL) 3.5 mL, $CaCl_2$ 2.2 µL, water 7.3 mL, pepsin 0.22 mL. Prior to digestion, porcine pepsin was assayed according to Minekus et al. (2014) with an activity of 3000 U/mg. Following this, eSGF and pepsin were continuously added at 1.73 mL/min and 0.197 mL/min respectively. The $t_{1/2}$ for gastric digestion was 11 min. Four gastric emptying steps were carried out, one every 5.5 min. Gastric emptying points were named as GE 1-4 in the text, corresponding to 5.5, 11, 16.5 and 22 min of gastric digestion.

### 2.4.6 Gastric emptying samples

20 mL of gastric digesta was added to a 50 mL falcon tube at each emptying point (every 5.5 min for 22 min). Samples for confocal and FTIR analysis were removed, and analysis was performed immediately. The remaining gastric emptying sample was heated at 90 °C for 10 min to denature enzyme and stop digestion. Samples were snap frozen using liquid nitrogen. Samples were then stored in freezer at - 20°C. When required for OPA and BCA analysis, samples were thawed in fridge at 4 °C.

### 2.4.7 Sample analysis

#### 2.4.7.1 Protein molecular mass distribution

Size exclusion chromatography - high performance liquid chromatography (SEC-HPLC) was used to estimate the molecular mass of proteins in the digesta (O'Loughlin et al., 2013a). SEC-HPLC was carried out as described by Freitas et al. (2022a). Briefly, Polyether sulfone (PES) membrane with 0.45 µm pore size (Captiva Premium Syringe Filters reference 5190-5095, Agilent Technologies, UK) were used to filter sample supernatants. Briefly, a TSK $G2000_{SWXL}$ column (600 × 7.5 mm; Tosoh Bioscience GmbH, Germany) with a guard column

was used, in connection with a Waters 2695 HPLC with UV/Visible detector controlled by the software EMPOWER®. An injection volume of 10 µL was eluted at 0.5 mL/min with buffer (30% v/v Acetonitrile, 0.1% v/v TFA, prepared in HPLC grade water) and monitored over 60 min at 210 nm. Estimations were made using a calibration curve based on the retention times of the following standards bovine serum albumin, carbonic anhydrase, β-lactoglobulin, aprotinin, bacitracin, histidine-leucine, phenylalanine and glycine, with respective molecular weights of 67,000, 29,000, 18,400, 6,500, 1,400, 268, 165 and 75 Da.

2.4.7.2 Confocal Laser Scanning Microscopy

Confocal Laser Scanning Microscopy (CLSM) (Leica TCAS SP5, Leica Microsystems CMS GmbH, Wetzlar, Germany) was used to examine the microstructure of the three yogurts. CLSM was carried out on yogurt prior to digestion, in the oral phase of digestion, and at each gastric emptying point. CLSM was carried out in parallel to *in vitro* gastric digestion. Aliquots of 500 µl were removed from gastric emptying samples and added to an Eppendorf containing 50 µl 0.1 % a green dye (w/v, Fast Green, FCF; $C_{37}H_{34}N_2Na_2O_{10}S_3$, Cayman Chemical, Ann Arbor, MI, USA). A dropper was used to transfer sample from Eppendorf to glass plate. Samples were covered with a cover slip and mounted on the CLSM. Images were taken using a 63 x 1.4 oil immersion objective. Fast green dye was excited at 633 nm using a HE/Ne laser, with the corresponding emission filter set at 680 - 720 nm (Gómez-Mascaraque and Pinho, 2021).

2.4.7.3 O-phthalaldehyde Assay

Proteolysis was analysed by quantifying the free amine groups using the O-phthalaldehyde assay (OPA assay) method (Nielsen et al., 2001). Briefly, samples were centrifuged at 10,000 x g for 30 min using an Eppendorf Centrifuge 5417R (Mason Technology Ltd.). Supernatant was then removed from the pellet. A standard curve was prepared using L-Leucine in the range of 10 - 0.2 mM. Samples were diluted in PBS so that they were in the linear range of standard curve. 200 µl of OPA reagent was added to 10 µL sample in a 96 well plate that was mixed using plate shaker for 1 min. The plate was covered with aluminium foil and incubated at 24 °C for 15 min. Absorbance was read using plate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany) at 340 nm.

2.4.7.4 Bicinchoninic Acid Assay

Protein released into the supernatant was analysed using the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit according to the manufacturer's protocol. Samples were centrifuged at 3,000 x g for 10 min using an Eppendorf Centrifuge 5417R (Mason Technology Ltd.). The soluble fraction was removed and diluted 10:1 in PBS buffer so that they would be in the linear range of the standard curve. A standard curve was prepared using bovine serum albumin in the range of – 0 – 2,000 µg/mL. Following this, Samples and working reagent were added to 96 well plate and incubated at 37 °C for 30 min. Plates were then cooled to room temperature and subsequently read at 562 nm on a plate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany). The standard curve was used to calculate the protein content of each sample. Dilution factors during assay and digestion were considered during calculations.

2.4.7.5 Fourier Transformed Infrared Spectroscopy

Fourier Transformed Infrared Spectroscopy (FTIR) of yogurt digests were analysed using an FTIR spectrometer (Bruker Optics GmbH, INVENIO 100453, Ettlingen, Germany) with BioATR attachment controlled by a Haake K20/DC30 external water bath (Thermo Haake, Karlsruhe, Germany) at 37 °C following the method of Daniloski et al. (2024a). Liquid nitrogen was used to cool the MCT detector, and the sample compartment was purged to remove moisture and carbon dioxide. Bruker OPUS 5.5 software was used to obtain the spectra in absorbance mode. All samples were corrected to account for peaks caused by atmospheric interferences such as moisture and carbon dioxide. Water subtraction was carried out to remove the water band, which overlaps the protein region. Vector normalization was carried out on all samples between 900 and 1,800 $cm^{-1}$ to remove baseline effects. Both band and peak assignment was performed following previously published methods of Grewal et al. (2018) and Daniloski et al. (2022b).

### 2.4.8 Data and statistical analysis

Three independent batches of yogurts were produced for each β-lactoglobulin group (AA, AB, and BB) to be used in *in vitro* digestions. Three further independent batches of yogurts were produced for rheological measurements. Spectral data analysis was carried out using MATLAB (MATLAB Release 2020b, The MathWorks, Inc., Natick, Massachusetts, USA). Two-way ANOVA and PCA plots were produced using Unscrambler software (Version 10.2, CAMO AS, Trondenheim, Norway). FTIR spectra graphs were produced using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Means for continuous variables (diverse β-lactoglobulin) were compared

using Tukey's Studentised Range post-hoc (HDS) test and one-way analysis of variance (ANOVA), thus establishing effects of the main factor (β-lactoglobulins phenotype). Statistical significance was set at 95 % level.

## 2.5 Results

### 2.5.1 Milk powder composition

Milk powders were standardised based on total nitrogen content. The composition of the skim milk powders is given in the Supplementary Table 1 S. An increased whey content was observed in A (5.95 %) polymorphism compared to B polymorphism (4.97 %). As well as this, the calcium content in BB was significantly higher than in AA and AB (1445.58 mg /100 mL and 1495.28 mg /100 mL respectively, p = 0.02), while iron contents were higher in AA and AB than in the sample with BB (0.27 mg/100 mL and 0.52 mg/100 mL, respectively).

### 2.5.2 Physicochemical properties of skim milk yogurts

2.5.2.1 Viscoelastic properties

Figure 5 shows a frequency sweep of skim yogurts. The storage modulus (*G'*), which is a measure of the elastic behaviour of the yogurt was higher for all yogurts than their corresponding viscous modulus (*G''*), which is a measure of the viscous behaviour of the yogurt. This indicates that the yogurts had elastic properties. However, a large difference was observed between yogurts due to the β-lactoglobulin polymorphism present, with BB yogurt showing the highest *G'* and *G''* values, followed by AB and AA respectively ($p < 0.05$, Figure 5). A clear difference in crossover times among the three yogurts could be seen, with the

transition, which occurred earlier for AA, followed by AB, with no crossover occurring in BB yogurt. This indicates that BB yogurt had a firmer gel, followed by AB and lastly AA sample.

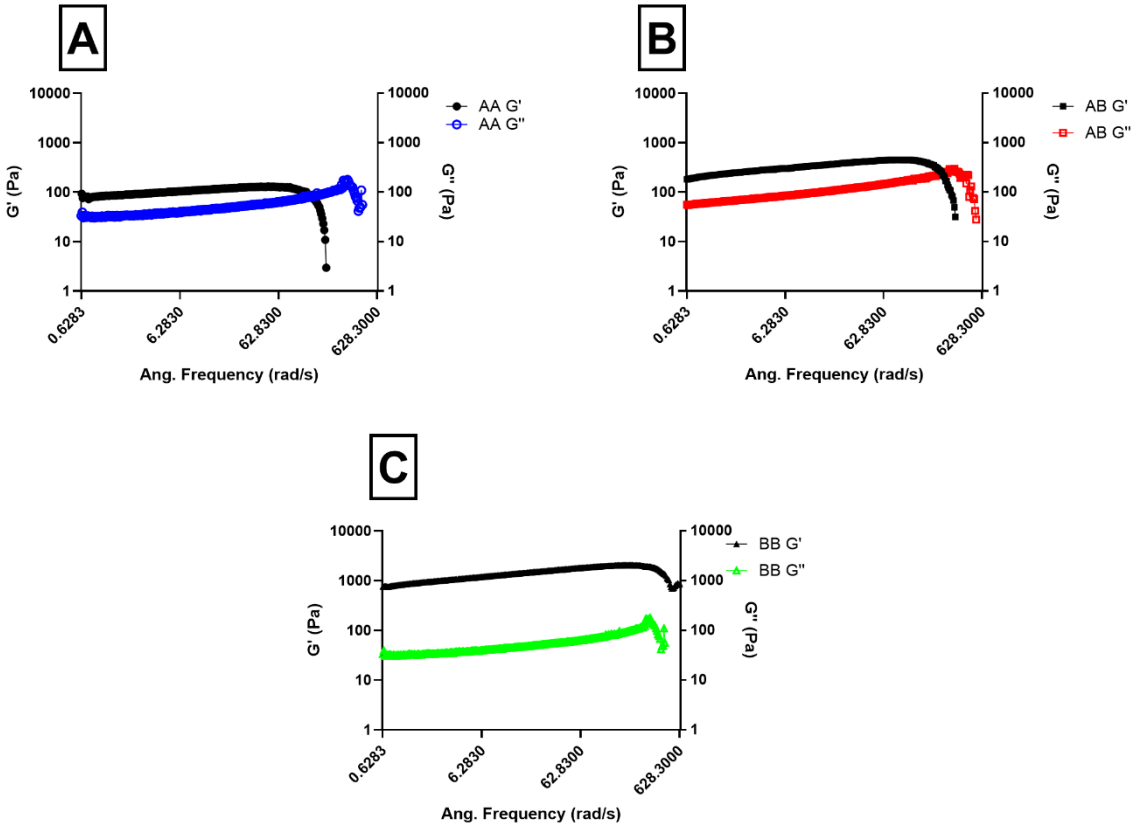


Figure 5 A, B and C: The effect of β-lactoglobulin variants on elastic modulus (G') and loss modulus (G'') on skim yogurts. Graph is representative of yogurts viscoelastic behaviour. Figure 2 (A) shows the viscoelastic behaviour of AA yogurt, (B) shows viscoelastic behaviour of AB yogurt and (C) shows the viscoelastic behaviour of BB yogurt.

*2.5.2.2* Apparent viscosity

The apparent viscosity ($\eta$) of the yogurt samples decreased with increasing shear rate, which indicates non-Newtonian behaviour (Figure 6 A). Initially, the $\eta$ of the yogurts differed greatly. The highest $\eta$ was assigned to BB (10.04 Pa. s), followed by AB (2.55 Pa. s), and AA (0.06 Pa. s). Accordingly, AA displayed lower $\eta$ across the shear rate ramp, compared to the other yogurts made from milk samples containing β-lactoglobulin B in their structure. The flow curve of AB (Figure 6 A) was between the curve for AA and BB milk. Overall, BB was the most viscous, followed by AB and finally AA yogurts (Figure 6 A).

2.5.2.3 Water holding capacity

The ability of the yogurts to retain water was affected by the β-lactoglobulin phenotype ($p < 0.05$) as shown in Figure 6 B. After maintaining the samples at low temperature overnight, the

WHC AA yogurt significantly decreased. Water holding capacity of AB and BB yogurts was approximately 60 %, indicating greater structural stability and water retention ($p < 0.05$). Moreover, a relationship between WHC and the final $G'$ of the yogurts can be observed, wherein increased final $G'$ and $\eta$ positively correlated with increased WHC of the yogurts prepared from milk samples containing the homozygous β-lactoglobulin BB, when compared to those yogurts containing the β-lactoglobulin A ($p < 0.05$). Using confocal images, this can be observed as a dense protein network in undigested BB yogurt compared to a looser network in undigested AA yogurt (Figure 7 B).

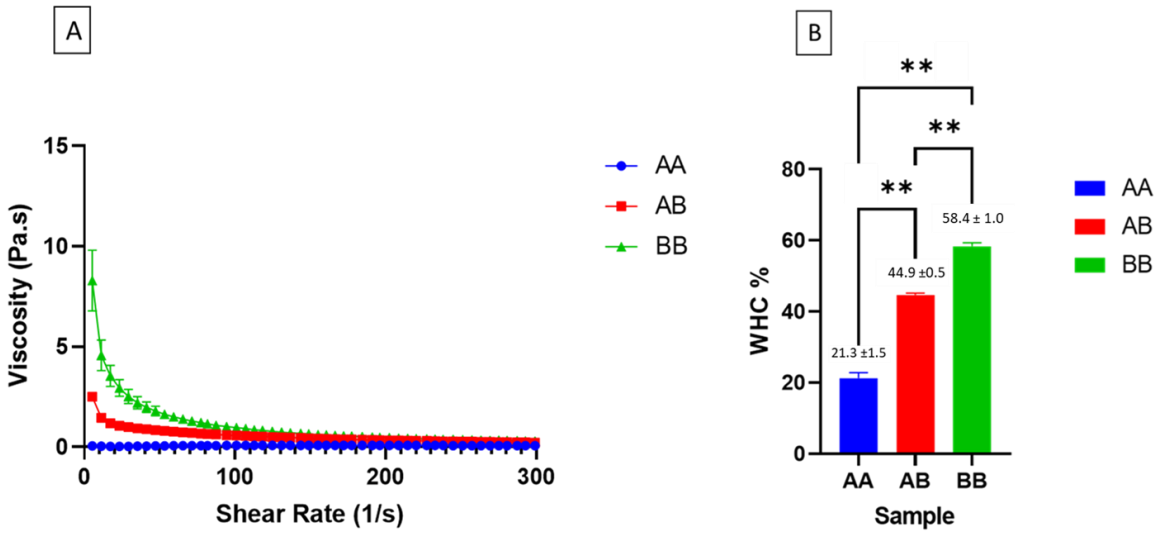


Figure 6 A) Apparent viscosity as a function of shear rate for AA (blue circle), AB (red square), and BB (green triangle) yogurts. Values are the means of data from triplicate analyses. Data presented in supplementary table 2. B) Water Holding Capacity of AA (blue circle), AB (red square), and BB (green triangle) yogurts. * Indicates significant difference ($p < 0.05$), ** indicates significant difference ($p < 0.01$).

### 2.5.3 Semi-dynamic *in vitro* digestion

Gastric digestion had a $t_{1/2}$ of 11.5 min, calculated based on a gastric emptying rate of 2kCal / min. Although similar pH curves were observed during the gastric digestion for all yogurts ($p > 0.05$), the pH of the homozygous AA yogurt was not altered for the first 5 min of gastric digestion; then all three pH curves decreased gradually, reaching a pH of around 2 after 22 min (Figure 8 A). The starting pH for all yogurts was approximately 4.60 and continuously decreased to pH 2.00 by the end of the gastric phase of digestion. This was achieved by the gradual automated addition of acidic simulated gastric fluid at a constant flow rate (1.75 mL/min). Additionally, no significant difference in buffering capacity was found between

samples, with all samples reaching pH 2 ± 0.12 by the end of the gastric digestion. This is an important factor due to the sensitivity of pepsin to pH (Pletschke et al., 1995b).

### 2.5.4 Visualisation and conformational changes of the gastric digesta

The visual observations during semi-dynamic digestion showed that AA yogurt had a looser protein network than AB and BB, with BB appearing to have the firmest structure of the three, similarly to the viscoelastic properties and the highest *G'* of this yogurt type ($p < 0.05$). By the end of gastric digestion (21 min), AA appeared to have lost its white colour compared to the other two yogurts. This could be due to an accelerated emptying of caseins from this yogurt compared to AB and BB. To confirm the depicted visual differences in the yogurt digests, CLSM was used to study the microstructure of yogurts (Figure 7 B). During the oral phase of digestion (0 min), no proteases were present; therefore, no breakdown of protein had occurred compared to the undigested sample. Despite this, AA appeared to have a less dense protein network with larger pores compared to both AB and BB (also observed in sections 3.2.1. and 3.2.2.). After 16.5 min of gastric digestion, BB retains its dense network, while both AA and AB appear to have broken up. By the end of gastric digestion, all samples had undergone protein hydrolysis as a looser network could be observed for all yogurts.

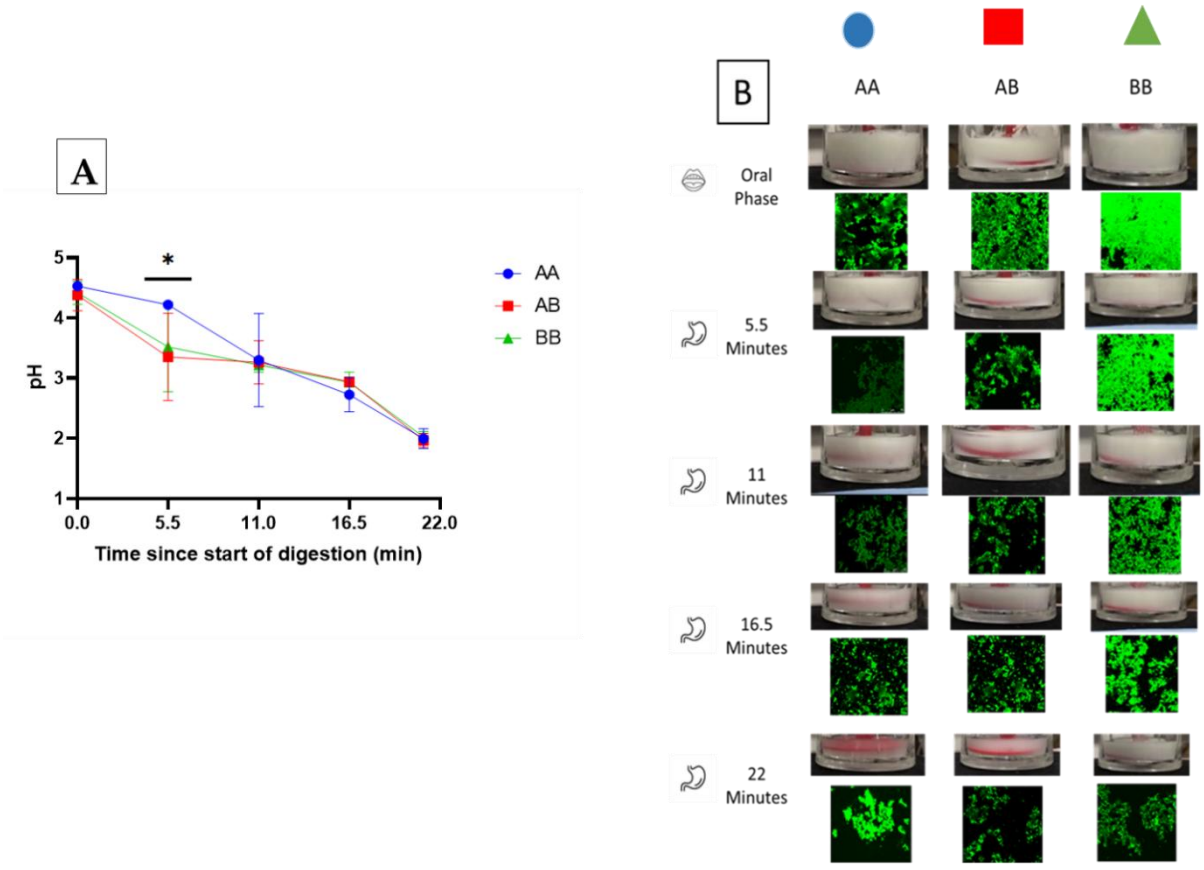

Figure 7 A) pH curve during semi-dynamic gastric digestion. * Indicates significant difference between AA and AB ($p < 0.05$) (B) Visualisation and structural properties of gastric digesta observed with CLSM. Scale bars represent 25 μm. Note: observations with the photographs, but also proven by the viscoelastic properties, suggested that initial yogurt had a less condensed gel structure in AA yogurt vs AB or BB at the start of digestion.

### 2.5.5 Release of protein into supernatant

The BCA assay was used to measure the cumulative release of protein into the supernatant (Figure 8 A) while the OPA assay was used to measure the cumulative release of free amines into the supernatant (Figure 8 B). The supernatant refers to the liquid portion of the digesta that remains after centrifugation. The supernatant contains soluble components of the digesta, such as small peptides and free amino acids that have been released during digestion. A gradual increase in protein released into the supernatant was observed for all yogurts, however AB sample released a signfiicantly higher quantity of protein into the supernatat than BB sample after 16.5 and 22 min ($p < 0.05$, Figure 8A). Similarily, a gradual increase in free amines released into the supernatant was observed for all yogurts, while AB showed a significantly greater release of free amines into the supernatant than BB sample after 22 min of gastric digestion *($p < 0.05$,* Figure 8 B). Figure 8 C shows the release of free amines divided by the release of protein into the supernatant, with no significant differences being found. It can be seen that free amine release is in proportion to the protein released, resulting in a steady value throughout digestion.

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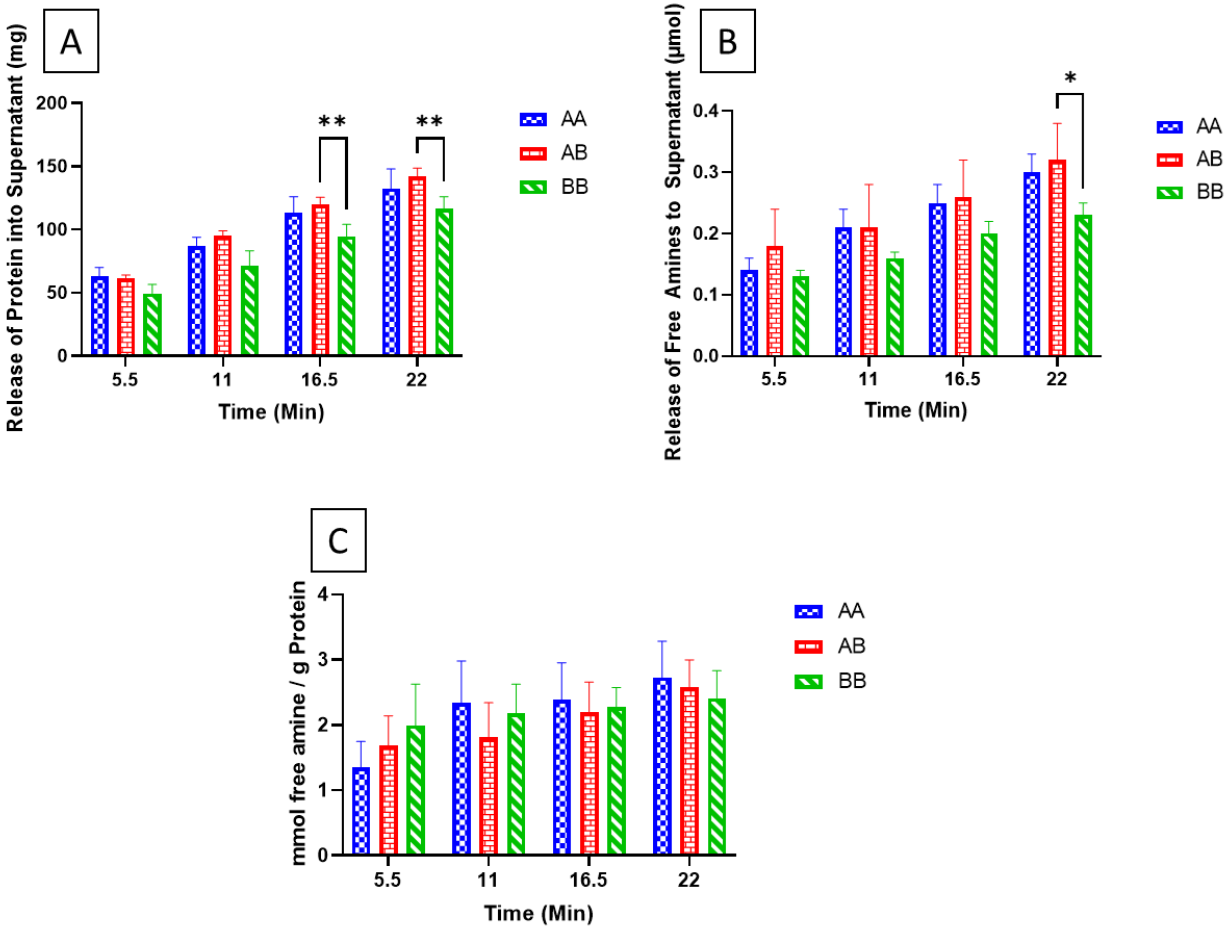


### 2.5.6 Size exclusion chromatography

SEC-HPLC was used to estimate the molecular weight distributions of proteins in samples Figure 8 Concentration of protein and free amines released into supernatant as measured by OPA (Free amines) and BCA (Protein) assays of AA (blue circle), AB (red square), and BB (green triangle) yogurts. All data is cumulative release into supernatant. (A) Release of protein into the supernatant (B) release of free amines into the supernatant (C) cumulative free amines released into supernatant divided by cumulative protein released into supernatant. * Indicates significant difference ($p < 0.05$), ** indicates significant difference ($p < 0.01$).

taken during the *in vitro* gastric digestion at all four GE points (Figure 9).

In AA yogurt, two distinct peaks appear at 21 to 11 kDa in GE1. At GE2, these shift from 19 to 16 kDa, with the 13 kDa peak disappearing. A stronger peak at 16 kDa is seen at GE4 compared to GE3, possibly due to sampling issues during digestion. For AB yogurt, two peaks are also observed at 21 to 11 kDa in GE1 but are less defined and disappear as digestion

progresses. GE4 shows the highest absorbance, followed by GE2 and GE3. A distinct 5 kDa peak is observed at GE3 for AA but not at other stages. For both yogurts, a well-defined peak at 2 kDa persists across all GE points, with the highest absorbance at GE1.

For BB, a progressive breakdown can be observed from between GE1, GE2, GE3 and GE4. Similarly, to both AA and AB yogurts, a well-defined peak can be observed at 5 kDa. This peak can also be observed in GE4, with a slight peak shift to the right and a decrease in intensity.

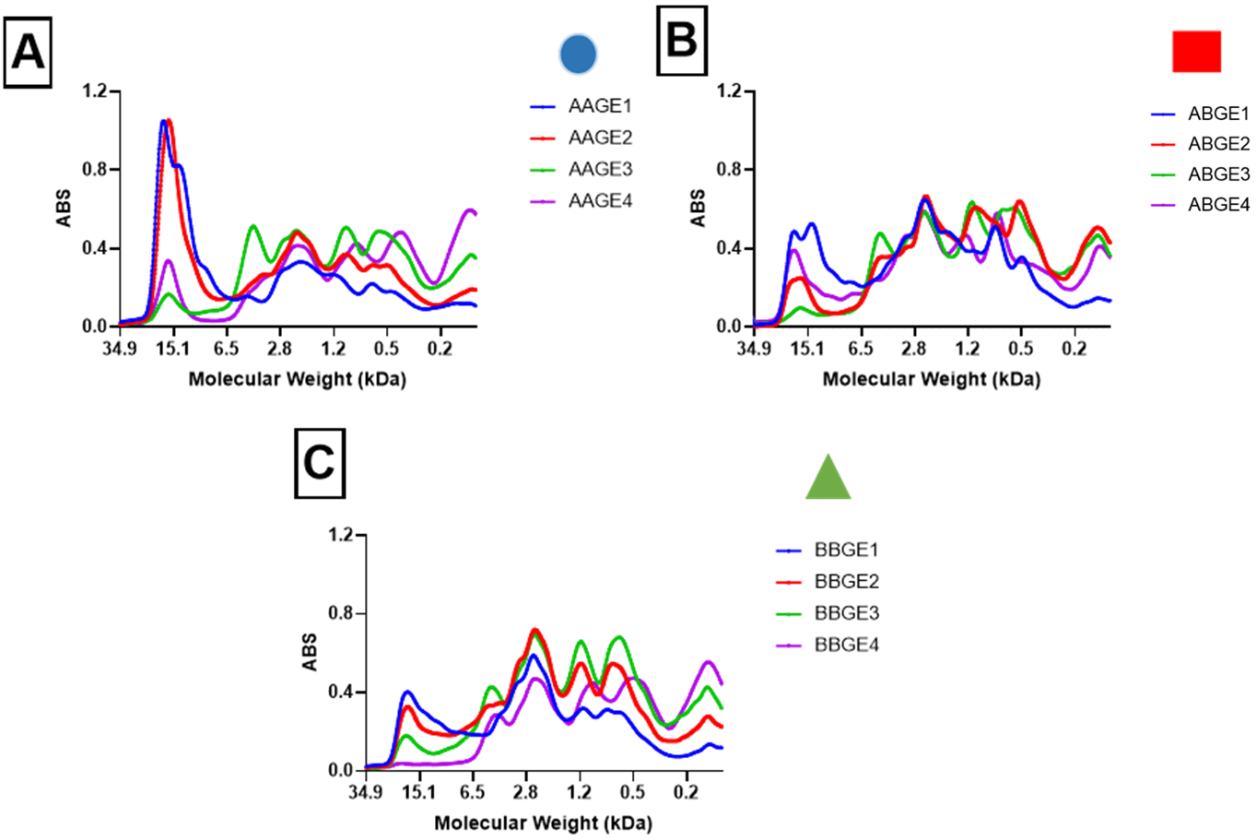


Figure 9 SEC-HPLC graphs depicting the molecular mass distributions of proteins during *in vitro* semi-dynamic digestion of AA (blue circle), AB (red square), and BB (green triangle) yogurts

### 2.5.7 Protein conformational features of digesta

The main areas of differentiation between yogurts containing different β-lactoglobulin polymorphisms (AA, AB, BB) at each stage of digestion can be found in the FTIR spectra between the ranges of 1,500 and 1,751cm$^{-1}$ *(*Figure 10 A*)*. PCA plots clearly separated yogurts containing different β-lactoglobulin polymorphisms from each other (Figure 10 B*)*. During the

GE 1 and GE 2 (between 5.5 and 11.5 mins), AA is grouped separately to AB and BB samples along PC 1, while the variation between AB and BB samples is explained by PC 2. According to the loading plot, the main variance related to PC 1 can be attributed to wavenumbers between 1,580 – 1,550 $cm^{-1}$ and 1,660 – 1,640 $cm^{-1}$. Regions between 1,580 – 1,570 $cm^{-1}$ have been associated with differences in the amount of β-turns, while regions between 1,560-1,550 $cm^{-1}$ have been attributed to differences in the amount of α-helices (Grewal et al., 2017). In the *amide I* region, loadings in the region of 1660 - 1645 $cm^{-1}$ have been attributed to differences in random coil structures. This may suggest a difference in 'the participation of caseins in the formation of aggregates' (Grewal et al., 2018). Since a peak shift is observed, it is likely that a conformational change exists between yogurts with AA *vs* AB and BB. PC2 can be explained by attributed to the 1,585 – 1,550 $cm^{-1}$ region. This region has been associated with β-turns and α-helices.

Similarly to the first two emptying points, the final two stages of gastric digestion (GE3 and GE4) of AA is grouped separately to AB and BB samples along PC 1, while the variation between AB and BB samples is explained by PC 2. As in the first 2 stages of gastric emptying, the both the region between 1,655 to 1,645 $cm^{-1}$ and 1550 to 1530 $cm^{-1}$ were important, which may be related to random coil structures and β-sheets respectively (Grewal et al., 2017).

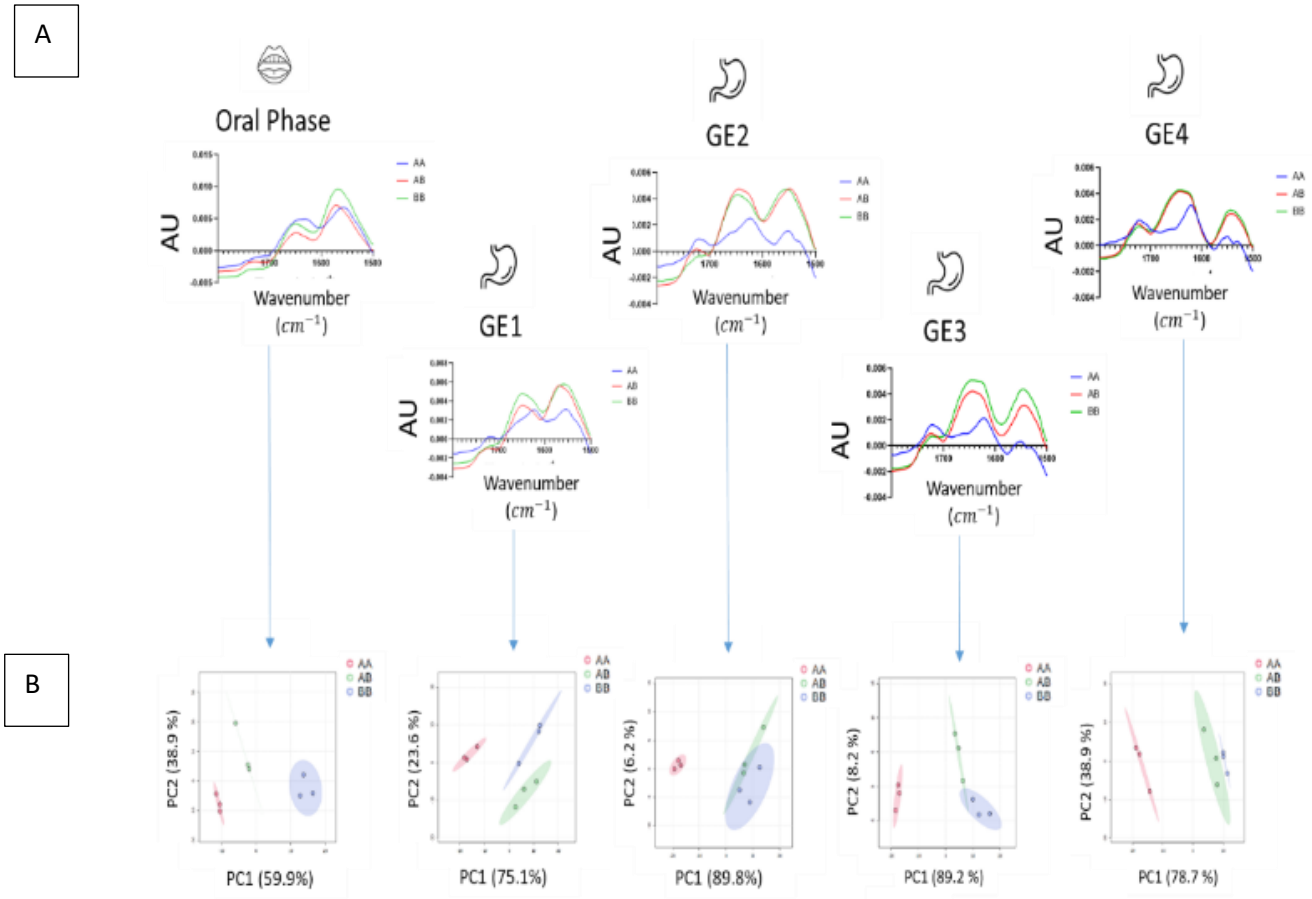


Figure 10 FTIR analysis of digests after 0, 5, 10, 15 and 20 min of gastric digestion. PCA plots indicate a clear separation between AA, AB and BB yogurt digesta at all stages of digestion.

## 2.6 Discussion

This study examines the impact of β-lactoglobulin polymorphisms (AA, AB, BB) on the rheological and microstructural properties of yogurt, and its subsequent impact on gastric digestion behaviour. With the move towards a dairy herd that is genetically selected for certain traits such as increased yield and increase fat and protein %, it is important to consider the potential outcomes this may have on milk processing.

Oscillatory rheology, viscosity analysis, WHC and CLSM analysis of the yogurt provided information about the differences among AA, AB, and BB yogurts in terms of techno-functional properties and microstructure. AA was related to a less viscous gel with weaker viscoelastic properties, lower WHC, a visibly looser protein network structure compared to BB (Figures 6 - 8, $p < 0.05$). This could be attributed to the differing conformational properties of the β-lactoglobulin polymorphisms, influencing protein interactions and network formation (Pace and Alexander, 1971, Qin et al., 1999).

Before milk fermentation, the samples were heat-treated, therefore the free $Cys^{121}$ in β-lactoglobulin variants was exposed and played an important aggregation role in the formation of yogurt (Donato and Guyomarc'h, 2009). The decreased stability of β-lactoglobulin B compared to A may result in β-lactoglobulin B exposing its free Cys more readily than β-lactoglobulin A, thereby leading to faster aggregation and more favourable yogurt production characteristics (Manderson et al., 1998). The fine gels formed due to whey protein coating of casein micelles are less likely to undergo syneresis (Vasbinder et al., 2004). In the present study, AA expelled significantly higher volumes of whey than BB indicating that a gel had not been sufficiently formed in the prior compared to the latter sample.

As well as this, the increased calcium content associated with β-lactoglobulin B may lead to the formation of stronger gels due to the role of $Ca^{2+}$ in enhancing the association between caseins and β-lactoglobulin (Mohammad-Beigi et al., 2023). It is interesting to note that firmer yogurts appear to have been produced from BB compared AA milks, despite higher whey protein contents in the reconstituted AA skim milk powder. Based on previous studies, it would be assumed that an increase in whey protein content would lead to a firmer yogurt (Wilkinson et al., 2000). During the production of the skim milk powders that were used to produce yogurt, protein content was standardised to total nitrogen content. Because of this, contents of the skim milk powders used to produce the yogurts varied in terms of whey protein content. This finding is in agreement with other studies that show an increase in whey protein content associated with the β-lactoglobulin A *vs* B (Ng-Kwai-Hang et al., 1986, Graml et al., 1989). This has also been observed in other studies (McLean et al., 1984).

Moreover, β-lactoglobulin A has a lower isoelectric point due to the presence of one extra negative charge compared to the β-lactoglobulin B (Yamamoto and Ishihara, 1999). The valine to alanine substitution at position 118 is thought to lead to the creation of a cavity at strand H, which may be the reason for β-lactoglobulin A showing more stability than the β-lactoglobulin B (Alexander and Pace 1990). This may explain why several studies have found a significant difference between β-lactoglobulin A and β-lactoglobulin B in the amount of native β-lactoglobulin remaining in milk after heating, with β-lactoglobulin B showing a significantly greater loss than β-lactoglobulin A (Allmere et al., 1998, Hillier and Lyster, 1979, Allmere et al., 1997). Other differences also exist between polymorphisms, β-lactoglobulin B has been shown to be five times more soluble than β-lactoglobulin A in distilled water (Treece et al., 1964). Recently, Anema (2024) explained that only small variations in calcium ion binding were observed between the heat-treated β-lactoglobulin A and B genetic variants. It was found that the A variant could potentially bind 13.5 calcium ions per monomer and B could bind 13

calcium ions per monomer (Anema, 2024), which might result in the different outcomes presented in the current study in both yogurt processing and gastric digestion. Nevertheless, it must be noted that the milk is a system comprised of numerous micro and macro components, which could affect these behaviours.

*In vivo*, yogurt proteins exhibit a longer gastric retention time when compared to liquid milk (Gaudichon et al., 1995, Le Feunteun et al., 2014). This suggests that the gel matrix has an impact on gastric emptying. The results of the present study indicate that during *in vitro* digestion, AA yogurt demonstrated distinct emptying behaviour compared to the other polymorphisms. During digestion, differences in protein matrix could be observed between digesta of BB yogurts *vs* AA and AB, with BB retaining its structure and both AA and AB being broken down faster. Visual observations at the final gastric emptying point confirmed this, showing AA losing its colour, indicating increased casein breakdown. In contrast, AB and BB retained their white appearance, suggesting a slower casein emptying rate and therefore a slower digestion rate. This is in agreement with an *in vivo* study on pigs, which showed that more viscous yogurts were emptied slower than less viscous yogurts from the gastric phase (Mpassi et al., 2001).

Interestingly, quantification of protein release into the supernatant using the BCA assay did not show significant difference between AA and BB samples at any stage of digestion. However, AB digesta released significantly higher protein into the supernatant compared to BB digesta after 16.5 and 22 min of gastric digestion (GE 3 and GE 4). This does not agree with *in vivo* findings, where low viscosity yogurts demonstrated a higher percentage of gastric retention after 120 min in mini pigs, therefore we would expect a difference between AA and BB samples (Ménard et al., 2018b). Differences between Ménard et al. (2018b) and the present study exist, such as their focus on drinking yogurt compared to the present study, which examined set-style yogurt.

Using the OPA assay to determine the release of free amines into the supernatant, results showed that after 22 min of gastric digestion, AB released a significantly higher amount of free amine groups compared to BB. This finding indicated that AB digesta was broken down more easily when compared to BB sample. As well as this, despite not finding a significant difference, both AA and AB digests tended to release a higher number of free amines than BB digesta at every stage of gastric digestion. Ménard et al. (2018b) showed low viscosity yogurts ($0.32 \pm 0.04$ Pa. s) to be more resistant to gastric proteolysis compared to high viscosity yogurt ($2.2 \pm 0.11$ Pa. s) in a dynamic *in vitro* system. Our results contrast with these findings, where the highest viscosity yogurt showed the lowest release of free amines during gastric digestion.

Using FTIR, an increase in absorbance was observed in the region of 1580 - 1550 $cm^{-1}$ that may be related to β-turns motifs, which are normally present due to κ-casein/β-lactoglobulin interactions. The increased absorbance in this region could explain the denser microstructure in AB and BB *vs* AA observed in the oral phase using CLSM. The formation of gel structure in yogurt making is dependent upon this κ-casein/β-lactoglobulin interactions. When milk is heated, commonly to 85 °C for 30 min, β-lactoglobulin is denatured, allowing complex formation between β-lactoglobulin and κ-CN (Anema, 2020). FTIR allowed the analysis of structural changes to proteins during digestion. The observed differences in the *amide II* and *amide I* regions of the FTIR spectra between the yogurt polymorphisms suggest alterations in secondary structure elements such as β-turns, α-helices and β-sheets. These structural changes likely contribute to the differential digestion patterns observed among the yogurt polymorphisms shown using both SEC and the OPA assay.

However, the yogurts vary in terms of viscosity, with AA showing the least viscous gel, followed by AB and finally BB. Increasing viscosity has been linked to reduced disintegration in the gastric phase *in vitro* (Rioux and Turgeon, 2012). Viscosity was also found to influence the feeling of satiation in several *in vivo* studies (Morell et al., 2015). For example, it has been shown that when fed *ad libitum*, healthy adults consumed a higher quantity of liquid milk-based products when compared to semi-solid milk-based products (Zijlstra et al., 2008). This may be due to a longer retention of nutrients in the stomach related to increased viscosity (Zijlstra et al., 2008). Although no differences were observed in the present study in terms of protein release, visual observations indicate a more rapid loss of white colour in AA yogurt compared to AB or BB samples, indicating a more rapid loss of casein proteins.

## 2.7 Conclusion

This chapter studied the differences in yogurt production due to β-lactoglobulin polymorphism and the subsequent impact on its *in vitro* gastric digestion. Firstly, the results of this study confirm that β-lactoglobulin B is associated with the formation of a firmer gel when compared to skim milk powders that carried β-lactoglobulin A, which forms weaker gels with lower viscoelasticity and greater syneresis. This was confirmed using CLSM, showing clear differences between AA, AB and BB yogurts. Secondly, this study shows that this difference led to distinct gastric digestion characteristics, with BB yogurt appearing to be more resistant to digestion compared to AA yogurt. Moreover, it is possible to clearly separate AA from BB digests at all stages of digestion using Principal Component Analysis of FTIR spectra. Future

studies could investigate the mechanisms causing these differences and their implications for human health and nutrition, such as examining differences in bioactive peptide release. As well as this, it would be of interest to adjust the ratios of β-lactoglobulin A to B, to find an optimum yogurt, with favourable rheological characteristics paired with ease of digestion. The findings of this study may be of interest to dairy producers, due to β-lactoglobulin A being more frequently observed in Holstein-Friesian cows compared to β-lactoglobulin B, which may lead to unfavourable yogurt production as indicated in this study. This study shows the importance of understanding the potential implications of genetic selection in the bovine dairy herds for both production qualities and digestion.

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# Chapter 3

# Grass to gut: impact of low and high pasture allowance on *in vitro* semi-dynamic gastrointestinal digestion of bovine milk lipids

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**CRediT authorship contribution:** Conor J. Fitzpatrick: Writing – review & editing, Writing – original draft, Visualisation, Methodology, Investigation, Formal analysis, Data curation, Conceptualisation. Daniela Freitas: Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualisation. Gaetan Drouin: Methodology, Investigation. Mark Timlin, Deirdre Hennessy, Michael O'Donovan: Resources. Tom F O'Callaghan: Supervision, Writing – review & editing. James A. O'Mahony: Supervision, Writing – review & editing. André Brodkorb: Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualisation.

## 3.1 Abstract

Feeding cows a pasture-based diet (GRS) produces milk with a different lipid profile compared to cows fed total mixed ration indoors (TMR). While this is well understood, research on the effects of these differences on milk digestion in humans remains scarce. This study investigated the impact of GRS *vs.* TMR bovine feeding systems on the *in vitro* semi-dynamic gastrointestinal digestion of milk, focusing on microstructural changes, protein hydrolysis, fatty acid (FAs) release, and health-related lipid indices. Using Confocal Laser Scanning Microscopy (CLSM), gastric coagulation entrapping lipid globules within a protein matrix was observed in both systems, with residual lipid droplets persisting post-intestinal digestion. Protein hydrolysis, analysed via free amine group release, showed no significant differences between GRS and TMR milk during gastric ($p$ = 0.65) or intestinal phases ($p$ = 0.95). Undigested TMR milk exhibited higher medium-chain FAs ($p$ = 0.005) and saturated FAs ($p$ = 0.004), while GRS milk contained increased long-chain FAs ($p$ < 0.001) and C18:2 cis-9, trans-11 CLA ($p$ < 0.001). Lipolysis was greater in GRS milk at the end of gastric digestion, with 36.8% of free fatty acids (FFAs) released *vs.* 20.4% for TMR. Health indices revealed GRS milk's higher unsaturation index ($p$ < 0.001) and lower Thrombogenicity index, with TMR showing elevated intestinal values ($p$ < 0.05). While GRS and TMR cow milks show comparable protein digestion, GRS milk releases more beneficial long-chain fatty acids including conjugated linoleic acid (CLA), with increased rate of lipolysis and more favourable health-related lipid indices, suggesting pasture-based diets may improve milk's nutritional quality despite similar *in vitro* gastrointestinal behaviour.

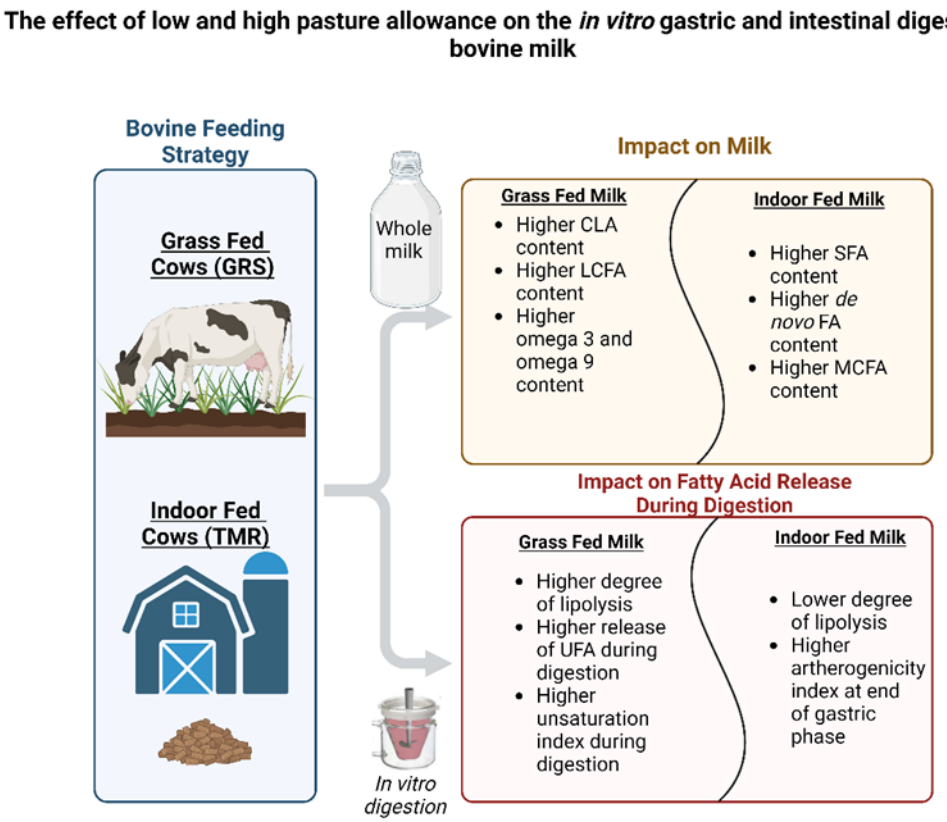


Figure 11 Graphical abstract - effect of low vs high pasture allowance on *in vitro* digestion of milk lipids

## 3.2 Introduction

Bovine milks composition varies based on factors such as genetics, stage of lactation, and feeding strategy. Its nutritional profile is influenced by feeding systems, with GRS diets increasing UFAs like CLA and omega-3 fatty acids compared to TMR diets (Timlin et al., 2023b). In temperate regions like Ireland and New Zealand, cows are primarily fed a pasture-based diet for up to 10 months of the year due to the abundance and low cost of grass. In contrast, cows in other parts of the world are typically housed indoors and fed TMR consisting mainly of silage (grass, maize, or corn) supplemented with high-energy feeds and carbohydrates (Timlin et al., 2023). While TMR feeding can increase milk yield, it also results in notable differences in milk's lipid profile, which may impact human health (O'Callaghan et al., 2016).

Milk (FAs originate from both dietary uptake and *de novo* synthesis (Osorio et al., 2016). Short- and medium-chain fatty acids (C4:0 to C14:0) are synthesized *de novo* using acetate and 3-OH-butyrate produced via ruminal fermentation (Bauman and Griinari, 2003). Approximately half of C16:0 is produced through *de novo* synthesis, while the other half is derived from the diet. Longer-chain FAs (C12 – C22) are taken up from the blood and used for triacylglycerol (TAG) synthesis in the smooth endoplasmic reticulum (Guo et al., 2024, Santos, 2002).

UFA, are associated with positive health benefits, therefore increasing UFAs in milk by increasing UFAs in cows diets may be beneficial to human health (Kim et al., 2016). However, when diets are supplemented with UFA, a large proportion (60–90%) is usually hydrogenated in the rumen into (SFAs) (Grummer, 1991). Therefore, even though it may be relatively easy to increase the proportions of UFAs in the diet, it may be difficult to increase the flow of SFAs and ultimately their ratio in the milk. However, it has been shown that increased UFAs in milk can be accomplished through GRS diets (Kelly et al., 1998, O'Callaghan et al., 2016).

FAs are essential to human health, having various roles throughout the body from energy storage to cellular signalling. Some FAs, such as omega-3 FAs and cis-9, trans-11 CLA, have been shown to exert health benefiting effects such as antitumor effects in rats (Nagao and Yanagita, 2005, Ip et al., 1994). Due to the previously mentioned differences in FAs composition, GRS fed milk has received significant attention, being seen as 'healthier' than TMR milk (O'Callaghan et al., 2016). One example is GRS milk having a lower Thrombogenicity index (Timlin et al., 2023a). This index indicates the potential of fatty acids in a given food to promote the formation of blood cloths (thrombi) in blood vessels, a risk

factor for coronary heart disease, however digestion-mediated FAs release remains unstudied. This is a critical gap given lipases' stereospecific hydrolysis of TAG positions (Rogalska et al., 1993).

Milk lipids primarily exist as (TAGs, which undergo hydrolysis and lipolysis by enzymes, such as gastric lipase and pancreatic lipase during gastric and intestinal digestion, respectively. These lipases exhibit stereo-specificities for the *sn*-1 and *sn*-3 positions of TAGs, influencing the release of specific fatty acids (Rogalska et al., 1990b). It has been shown that the feeding system can affect the position of fatty acids on TAGs (Yener et al., 2021, Pacheco-Pappenheim et al., 2022a). This raises the question, does a difference in fatty acid composition of milk equate to a difference in the released FAs during digestion? While it has been shown that pasture-based diets increase the proportion of 'healthy' fatty acids, these studies have been carried out on undigested milk, and studies are limited on the release of FAs from TAGs during digestion of milk from GRS *vs.* TMR milk.

Bovine feeding strategy may affect the digestion of milk in humans in several ways. Timlin et al. (2023a) has shown that casein protein is present in higher concentrations in TMR milk vs GRS milk. Higher concentrations of casein leads to a firmer curd formation in the gastric phase which may entrap and reduce lipolysis compared to a lower casein concentration. Interestingly however, Magan et al. (2019) has shown that pasture based diets are associated with a firmer gel strength, which may lead to slower rates of digestion.

Standardised *in vitro* digestion models, such as the INFOGEST semi-dynamic model are now commonly used to simulate digestion in humans (Mulet-Cabero et al., 2020b). These models recreate conditions of the human gastrointestinal tract, particularly dynamic aspects of gastric digestion such as continuous enzyme flow, gradual acidification as well as the simulation of gastric empting. This offers the possibility to obtain kinetic data on nutrient digestion, enabling comprehensive and detailed studies of the digestion process. This is a cost-effective method of simulating digestion without the need of live subjects, which also requires ethical considerations.

This study therefore aims to investigate the effect of high *vs.* low pasture allowance by using *in vitro* digestion methods coupled with analysis of FFAs release, protein hydrolysis and microstructural changes. It was hypothesised that GRS milk's lipid composition and fatty acid stereo positioning on the glycerol backbone would alter FAs release, acting as a precursor study for future human intervention trials.

## 3.3 Materials and methods

### 3.3.1 Ethical approval

This work is a follow on study from work carried out by Timlin et al. (2023a), where the differences in bovine milk due to feeding strategy were elucidated. Consent for experimental procedure involving cows at Teagasc, Animal and Grassland Research Centre was authorised by the Health Products Regulatory Authority and approved by the Teagasc Animal Ethics Committee. The licence number received by the Health Products Regulatory Authority for this project is AE19132-P110.

### 3.3.2 Materials

Whole milk was collected from thirty six cows divided between 2 groups within the Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co.Cork as described by Timlin et al. (2023a). Full feed composition for GRS and TMR cows is described in Fitzpatrick (2023). Briefly, the diet of GRS cows consisted of primarily perennial ryegrass (*Lolium perenne L.) (95% of total dry matter),* with concentrates only fed during milking (5% of total dry matter). TMR cows were fed maize silage, concentrates and grass silage at a ratio of 40:40:20 determined on a dry matter basis. Although previous studies by Timlin et al. (2023a) and Fitzpatrick (2023) included a third group fed Partial Mixed ration (PMR), this study focused exclusively on the GRS and TMR groups. Whole milk was collected in separate bulk tanks during evening milking during mid-lactation (June, July, August). Whole milk powder (WMP) was produced as described by Connolly et al. (2024).

In addition to common analytical grade reagents used in the digestion experiments and sample analysis, rabbit gastric extract (RGE) from Lipolytech (RGE 15, Zone Luminy Biotech Enterprises, France), porcine pancreatin (P75455, Sigma-Alrich, Ireland) and bovine bile (B3883, Sigma-Aldrich, Ireland) were also used in the digestion experiments. The activity of pepsin and lipase in the RGE used was 300 U/mg and 10.2 U/mg, respectively. The activity of trypsin in the pancreatin was 5.7 U/mg and bile salts contained 1.19 mmol of bile salts/g bile. All enzyme activity assays were carried out as previously described in Brodkorb et al. (2019). A commercially available kit, Pierce™ BCA (Bicinchoninic Acid) Protein Assay Kit (catalogue number 23225, Thermo Scientific, Ireland), was also used in sample analysis protocols. Heptane, EDTA and internal standard trinonodecanoin (C19:0) (part number: t165) were purchased from Fisher Scientific (Ireland). Reagents trichloroacetic acid, sodium hydroxide, hydrochloric acid, sodium methoxide, and acetic acid were purchased from Sigma-Aldrich.

### 3.3.3 Re-solubilisation of milk powder

Total Nitrogen, crude protein and non-casein nitrogen of WMP were determined using the Kjeldahl method with nitrogen conversion factor of 6.38. Fat content was determined using the Rose Gottlieb method (IDF, 1996).WMP contained 26.7% protein, 28.2% fat and 38.5% carbohydrates. Full gross composition of standardised milks used for the production of WMP is outlined in Timlin et al. (2023c).

The evening before use, WMP was reconstituted in Milli-Q water at 15% (w/v) at room temperature for 5 min before heating to 45°C for 30 min under magnetic stirring. Milk was then stored overnight (max 12 h) at 4°C to ensure complete rehydration. Rehydrated full-fat milk contained 4.0% protein and 3.8 – 3.9 % fat,

### 3.3.4 Digestion of milk using *vitro* semi-dynamic digestion

#### 3.3.4.1 Preparation of digestive fluids

Simulated salivary fluid (SSF 1.25 × concentrated, pH 7.0, no α-amylase), simulated gastric fluid (eSGF 0.1 M HCl 1.25 × concentrated), and simulated intestinal fluid (eSIF, 1.25 1.25× concentrated) were prepared according to Brodkorb et al. (2019). Prior to use, these fluids were pre-warmed to 37°C. A gastric enzyme solution was prepared separately by adding RGE to water at activity levels of 4,000 U/mL eSGF of pepsin and 120 U/mL eSGF of lipase. Pancreatin was dissolved in SIF to achieve 1800 units. Bile was dissolved to achieve 20 mmol/L in SIF.

#### 3.3.4.2 Oral and gastric digestion

After rehydration of WMP, *in vitro* semi-dynamic digestion was carried out according to the standardised INFOGEST protocol (Mulet-Cabero et al., 2020a). Prior to the gastric phase, 20 g of rehydrated milk was incubated with 2.38 mL simulated salivary fluid and mixed on a rotating wheel at 37°C for 2 min. This oral phase was then added to a heated gastric digestion vessel which contained acidic basal gastric fluid pre-warmed to 37°C (1.478 mL eSGF at 0.1 M HCl, 0.9 µL 0.3 M $CaCl_2$ $(H2O)_2$, 0.24 mL RGE solution). Simulated gastric fluid and RGE solution were then continuously added at a rate of 0.19 and 0.028 mL/min respectively during the gastric phase, which lasted 77 min. Gastric fluid was added using a pH stat dosing device (800 Dosino, Metrohm, Switzerland) and the enzyme solution was added using a syringe pump (New Era Pump Systems Inc., NY, USA). The flow rates of gastric fluid and enzyme solution were defined to reach a pH of 2.0 ± 0.3 and pepsin concentration of 2,000 U/mL by the end of the gastric phase. The flow rate of gastric fluid and enzymes were calculated according to

Mulet-Cabero et al. (2020a). The amount of HCl required to reach pH 2 by the end of the gastric phase was determined in preliminary tests.

The duration of the gastric phase was the same as the estimated time required for complete gastric emptying. Gastric emptying was determined to achieve a constant emptying rate (2 kcal /min) based on the total energy content of a milk serving that would be consumed *in vivo* (200 mL of milk, providing 154 kcal) as previously recommended (Mulet-Cabero et al., 2020b). Gastric mixing was carried out using a 3D printed stirrer attached to an overhead rotating device (Mulet-Cabero et al., 2020b). Gastric emptying was performed in 4 steps, by collecting 10 mL of digesta from the bottom of the gastric vessel once every 19 ± 1 min, (GE1 (Gastric Emptying 1 – 19 mins, GE2 – 38 mins, GE3 - 57 mins, GE4 – 76 mins). As is common with *in vitro* digestion of milk, by the end of the gastric phase, not all sample could be collected using a pipette, all remaining particles (regardless of their size) were collected using a spatula and included in the final gastric emptying point. From each 10 mL aliquot of gastric digesta, 4.5 mL were immediately snap frozen in liquid nitrogen and stored at -20°C for later use in intestinal phase; 250 µL aliquots were immediately analysed using confocal laser scanning microscopy; 500 µL aliquots were added to 6 mL hexane: isopropanol solution for lipid extraction; 1.5 mL was heated to 85°C for 10 min to stop enzyme activity, snap frozen in liquid nitrogen and stored at -20°C for later protein analysis; remaining digesta was snap-frozen and stored at -20°C in case additional/repeated analyses were required.

3.3.4.3 Intestinal digestion

The 4.5 mL samples taken at each gastric emptying point (GE1, GE2, GE3 and GE4) were thawed and subjected to separate *in vitro* intestinal digestions labelled I1 (Intestinal Point 1), I2, I3 and I4, respectively (Mulet-Cabero et al., 2020b, Brodkorb et al., 2019). Pancreatin activity was assayed and diluted in simulated intestinal fluid (SIF) based on trypsin activity. SIF, pancreatin and bile were added to digesta. pH was immediately adjusted to 7 using 1M NaOH. Water was added to bring the final volume to 9 mL. Intestinal phase was carried out in an incubator at 37°C and mixing was achieved using a rotating wheel set to 10 rpm. After 2 hours of intestinal digestion, 250 µL was immediately sampled for confocal laser microscopy analysis. The remaining of the digesta was homogenised using an Ultra turrax mixer and similarly to what was done with gastric digesta, aliquots were taken and were either added to hexane: isopropanol solution for lipid extraction or heated to 85°C for 10 min to stop enzymatic activity and snap frozen until required for further analysis.

### 3.3.5 Sample analysis

#### 3.3.5.1 Microstructure

Confocal Laser Scanning Microscopy (CLSM) (Leica TCAS SP5) (Leica Microsystems CMS GmbH, Wetzlar, Germany) was used to examine the microstructure prior to digestion, and in parallel to *in vitro* digestion to monitor the changes during the oral phase, and at each gastric emptying point and intestinal digestion. The method used has been previously described in detail (Gómez-Mascaraque and Pinho, 2021). Briefly, sample aliquots of 500 µl were added to an Eppendorf containing 25 µl of a solution of a 0.1% Fast Green FCF (aq.) (Cas no. 23553-45-9, Sigma Aldrich) and 50 µl of 0.02% Nile Red (in 1,2-propanediol) (Cas no. 7385-67-3, Sigma Aldrich). A dropper was used to transfer sample from Eppendorf to glass plate. Samples were covered with a cover slip and mounted on the CLSM. Images were taken using a 63× oil immersion objective with a numerical aperture of 1.4. Fast Green and Nile Red dyes were excited at 633 nm and 488 mm using a HE/Ne and Argon laser respectively.

#### 3.3.5.2 Protein hydrolysis in digesta samples

Proteolysis was analysed by quantifying the free amine groups using the OPA (o-Phthalaldehyde) assay (Nielsen et al., 2001). Briefly, samples were centrifuged at 10,000 × g for 30 min. Supernatant was then removed from the pellet. A standard curve was prepared using L-Leucine in the range of 10-0.2 mM. Samples were diluted in PBS so that they were in the linear range of standard curve. 200 µl of OPA reagent was added to 10 µL sample in a 96 well plate and mixed using plate shaker for 1 min. The plate was incubated at room temperature, protected from light, for 15 min. Absorbance was read at 340nm in a Molecular Devices Specta Max® ABS Plus absorbance plate reader (Molecular Devices, Wokingham, Berkshire, United Kingdom).

#### 3.3.5.3 Total fatty acid analysis

Total fatty acid (TFAs) profiles of the milk samples were determined in comparison with the FFAs released during digestion. Total fatty acids have been measured as fatty acid methyl esters (FAME) by GC-FID after direct transmethylation. Approximately 500 µL of sample was added to 200 µL of internal standard. Basic transmethylation has been performed at 90°C for 30 min in presence of 0.5% (w/v) methanolic sodium methylate (3 mL). After cooling to room temperature (~21°C), acidic transmethylation was performed in presence of methanolic boron trifluoride 14% (1 mL) at 90°C for 15 min. After cooling, the reaction was stopped with 0.9% NaCl (3 mL) and the produced FAMEs were extracted with hexane (2 mL) by agitation on a rotatory wheel at 30 rpm for 1 hour. After centrifugation at 600 g for 10 min at room

temperature, the supernatant was injected into the GC. FAME quantification was performed on a Varian CP-3800 GC system (Agilent Technologies, Santa Clara, California, USA), equipped with an automatic sampler with a split/splitless injector. The separation was achieved using a Select FAME CP7420 fused silica capillary column 100 m x 0.25 mm (Agilent Technologies). Helium was used as a carrier gas at a constant flow of 1.2 mL/min. Injector and detector (10 Hz) temperatures were set at 250°C and 280°C, respectively. The oven temperature was programmed at 80°C for 8 min, then raised to 200°C at 8.5°C/min, was held for 21 min at this temperature, gradually increase to 250°C at 10°C/min, and held for 20 min. Fatty acids were identified according to the retention time of commercial standard mix (Supelco 37 FAME components) and homemade FAME individual standards prepared from pure FFAs). Response factors have been calculated according to the standard dilution curve. All the identified FAs > 0.1% of the total FAs were taken into consideration. Quantification of fatty acids (g FAs / 100 g FAs) was performed using Galaxie Chromatography Data System 1.10.

3.3.5.4 Free Fatty Acid Analysis

FFAs were quantified as FAME by GC-FID after fractionation by solid phase extraction (SPE) of the total lipid extract as described previously with modifications (Kilcawley & Mannion, 2021). Total lipids were extracted twice from digesta samples (100 µL) with hexane/isopropanol (3/2, v/v) after acidification with 3 N HCl. C19:0 TAG was added as an internal control of the SPE fractionation. To fractionate FFA, an amino propyl SPE column (500 mg sorbent, 3 mL) was preconditioned with 10 mL hexane using a semi-automatic vacuum manifold (12-position) with adapter caps (Agilent Technologies) at constant vacuum of 2 inches Hg. Then all the lipid extract was added to the cartridge, the sample tubes were washed with hexane and loaded to increase recovery. Neutral lipids were eluted with 10 mL of hexane/diethyl ether (80/20, v/v). FFAs were eluted with 5 mL of diethyl ether containing 2% formic acid. FFAs fractions were evaporated under nitrogen at 35°C then derivatised as FAME with BF3 at 90°C for 1 min, as described previously to increase FFAs purity as FAME (Kochko, 2007). FAME were recovered as described for total fatty acid analysis.

FAME quantification was performed on an Agilent 7890B GC system equipped with an automatic sampler with a split/splitless injector and an FID (Flame Ionization Detector). The separation was achieved using a bonded silica capillary column (BPX 70, 60 m×0.25 mm; SGE, Melbourne, Australia) containing a polar stationary phase of 70% cyanopropyl polysilphenylene-siloxane (0.25-µm film thickness). Helium was used as carrier gas (average velocity 36 cm/s). The column temperature program started at 150°C, gradually increased to

250°C at a rate of 4°C/min and was held at 250°C for 10 min. Injector and detector temperatures were set at 280°C and 370°C, respectively, with the detector operating at a frequency of 50Hz. FAs were identified according to the retention times of a commercial standard mix (Supelco 37 FAME components) and homemade FAME individual standards prepared from pure FFAs. All the identified FAs > 0.1% of the total FAs were taken into consideration. Response factors have been calculated according to the dilution curve of the Supelco 37 FAME components standard.

Weight proportions of identified total FAs were calculated to describe the milk samples. The percentage of lipolysis in digesta samples was calculated as follows: [FFAs $_{sample}$] / [total FAs $_{undigested\ milk}$] x 100, considering FFAs from meal as negligible (<0.05% of total lipids).

### 3.3.6 Nutritional indices and Fatty Acid ratios

FAs ratios and nutritional indices were calculated for both TMR and GRS raw milk, as well as digesta at each stage of digestion. Specifically, Artherogenicity (AI) and Thrombogenicity indices (TI), as described by Ulbricht and Southgate (1991), and included in several similar studies characterizing the composition of bovine milk from cows with different diets such as Timlin et al. (2023b), Timlin et al. (2024), O'Callaghan et al. (2016), Timlin et al. (2023a) have been calculated as follows:

$$\text{Thrombogenicity index} = \frac{\text{C14:0} + \text{C16:0} + \text{C18:0}}{0.5 \times \Sigma\,\text{MUFAs} + 0.5 \times \Sigma\,\text{n6 PUFAs} + 3 \times \Sigma\,\text{n3 PUFAs} + \left(\frac{n-3}{n-6}\right)}$$

Where C14:0 is myristic acid, C16:0 is palmitic acid, C18:0 is stearic acid, ∑ MUFAs is the sum of Monounsaturated Fatty Acids, ∑ n6 PUFAs is the sum of n-6 (omega-6) Polyunsaturated Fatty Acids and Σ n3 PUFAs is the sum of n-3 (omega-3) Polyunsaturated Fatty Acids.

$$\text{Artherogenicity index} = \frac{\text{C12:0} + 4 \times \text{C14:0} + \text{C16:0}}{\Sigma\,\text{MUFAs} + \Sigma\,\text{PUFAs}}$$

Where C12:0 is lauric acid, C14:0 is myristic acid, C16:0 is palmitic acid, ∑ MUFAs is the sum of Monounsaturated Fatty Acids and ∑ PUFAs is the sum of Polyunsaturated Fatty Acids.

These indices indicate the ratios between FAs with cardio-protective effects to FAs that promote risk factors for adverse cardiovascular events. Calculations are frequently carried out using proportions of fatty acids in milk (g /100 g FAs) to allow for standardized comparisons

between foods with different FAs amounts. In addition to calculations based on relative amounts, these indices have also been calculated based on absolute FAs amounts in both milk and digesta samples as this is important for a comprehensive understanding of potential health implications.

The Unsaturation index (UI) (a measure of the degree of unsaturation in fatty acids) was also calculated for each milk sample before digestion and at each digestion sampling point (Logue et al., 2000, Timlin et al., 2023a). This calculation was carried out as follows:

$$\text{Unsaturation index} = 1 \times \%\,\text{monoenics}) + (2 \times \%\,\text{dienoics}) + (3 \times \%\,\text{trienoics})$$

### 3.3.7 Calculations and statistical analysis

A total of 81 samples were collected during *in vitro* digestions. The cumulated protein release and free amines (indicative of the degree of hydrolysis) at each time point of the digestion experiments have been calculated. Results are presented as mean ± standard deviation of three replicates unless otherwise stated.

Data was checked for outliers using the ROUT method, homogeneity of variance was checked using the F-test and normality was checked using Q-Q plots. Statistical significance between experimental conditions was assessed at each time point using 2-way ANOVA. Fisher's Least Significant Difference (LSD) test was employed for post-hoc pairwise comparisons. Rout analysis, and 2-way ANOVAs were performed using GraphPad Prism software (Prism for Windows, Version 9.0.1) and a threshold for significance of $p = 0.05$ (95% confidence interval). Q-Q plots were created using SPSS. Hierarchical clustering heat maps were created using Metaboanalyst 5.0. Data was normalised using the Auto Scaling function prior to analysis (Mean centred and divided by the standard deviation of each variable) (Timlin et al., 2023b, Pang et al., 2021).

## 3.4 Results

Whole milk samples from cows fed a pasture (GRS) *vs.* total mixed ration diet (TMR) were digested using *in vitro* semi-dynamic digestion following the INFOGEST protocol Mulet-Cabero et al. (2020a). During *in vitro* gastric digestion, emptying was performed to simulate the movement of chyme from the stomach into the small intestine. Intestinal digestions were performed on gastric digesta emptied at four time points of gastric digestion (once every 19 ± 1 min). Gastric digesta samples were collected for analysis immediately before each emptying point and intestinal samples were collected at the end of each intestinal point. The cumulated protein release (g/total g of protein), free amines and relative proportion of FFAs (g released FAs / 100 g released FAs) were compared at each sampling point. The % release of FFAs was compared by dividing the cumulative FFAs release by the total FAs in the respective milk sample. The microstructure of digestion samples was also examined.

### 3.4.1 Microstructural examinations of milk and digesta samples

Confocal Laser Scanning Microscopy (CLSM) was used to examine GRS and TMR milk samples prior to digestion and to monitor respective morphological changes throughout digestion. A representative selection of the images obtained is presented in Figure 12, where proteins are shown in red and lipids in green. In undigested samples (Figure 12 A), proteins remained in solution, making them difficult to detect. In both GRS and (TMR) milk, lipids were observed primarily as uniform globules approximately 1–2 μm in size. By the end of gastric digestion, protein coagulation was clearly visible, and the gastric coagulum was not fully broken down. Large protein particles, roughly 50 μm in length, were present in the liquid phase of both GRS and TMR digesta (Figure 12 B). To further examine the structure of the coagulum, particles were sliced and mounted on a cover slip (Figure 12 C). In these particles, lipids were entrapped within a protein matrix, indicated by the location of green colours within red protein matrix in both GRS and TMR milk. Following intestinal digestion, large coagulum was no longer visible, however, some lipid droplets remained intact, seen here as green circles (Figure 12 D).

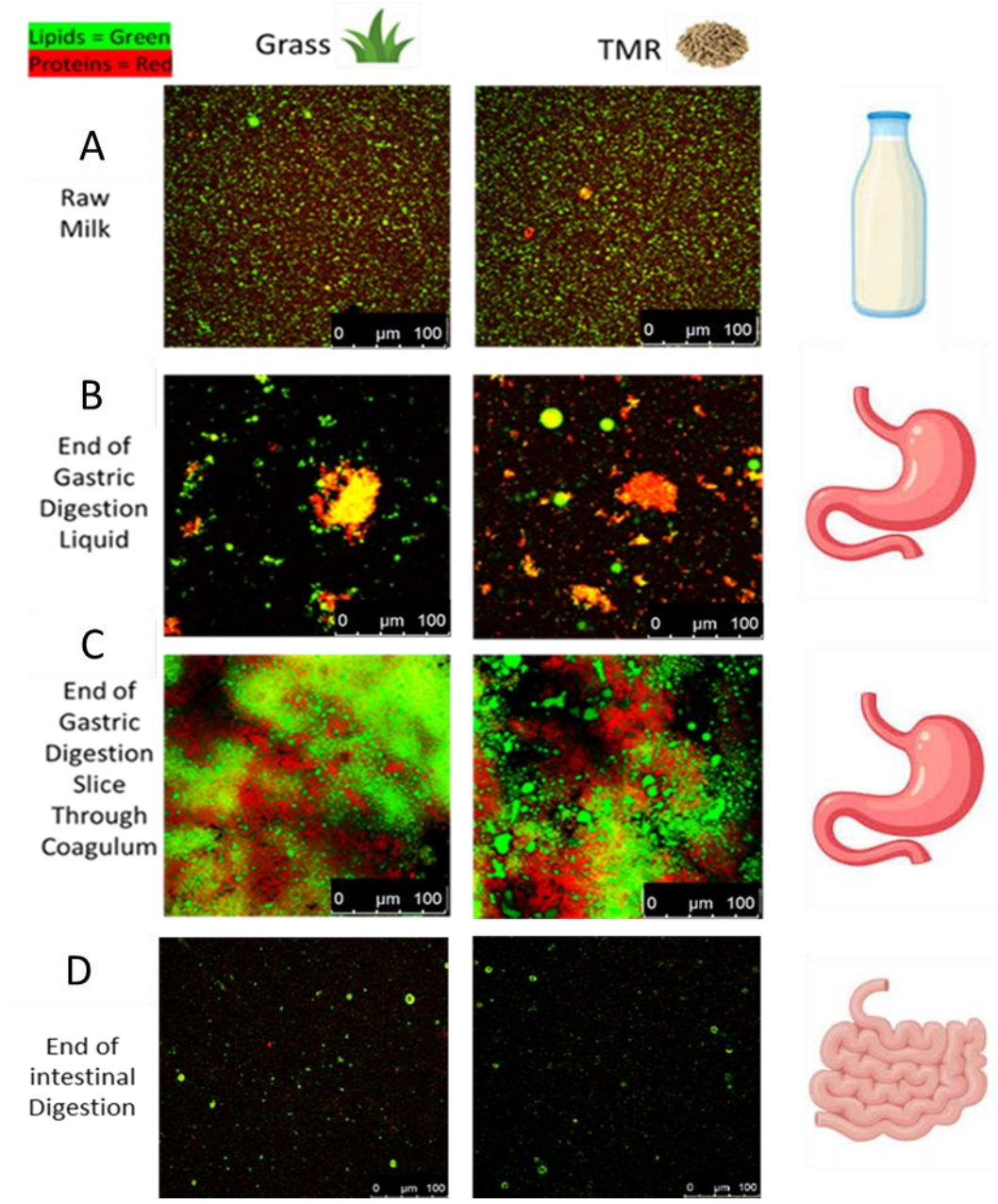


Figure 12 (A-D) Confocal Laser Scanning Microscopy (CLSM) of pasture-fed (GRS) and total mixed ration (TMR) milk before (A) and during semi-dynamic gastric digestion (B-D). At the end of gastric digestion, images were taken of a sample of liquid digesta (End of Gastric Digestion Liquid) (B) and of a slice through coagulum collected at the end of digestion (End of gastric digestion slice through coagulum) (C). Images were taken of samples at end of intestinal digestion (D). Red and green represent proteins and lipids, stained using Fast Green and Nile Red respectively.

### 3.4.2 Protein hydrolysis during gastrointestinal digestion

As described above, during the preparation of the WMP used in the present study, the samples were standardised to 3.8-3.9% fat at a ratio of 0.95 protein: fat. As protein is one of the main components of milk, protein hydrolysis was also examined as part of the current study. Figure 13 depicts the release of free amine groups ($NH_2$, expressed in mmol of leucine equivalents) during *in vitro* gastric and intestinal digestion. No significant differences were observed between GRS and TMR at any stage of the digestion process. At the final gastric digestion sampling point, GRS milk had 0.12 ± 0.03 mmol $NH_2$, while TMR milk had 0.16 ± 0.09 mmol $NH_2$, with a p-value of 0.65, indicating no statistically significant difference between the two (Figure 13 A). During intestinal digestion, TMR released higher amounts of $NH_2$ compared to GRS, however, no statistical significance was observed. At the final stage of intestinal digestion (I4), GRS had released 0.66 ± 0.30 mmol $NH_2$, while TMR had released 0.80 ± 0.38 mmol $NH_2$, ($p$ = 0.95).

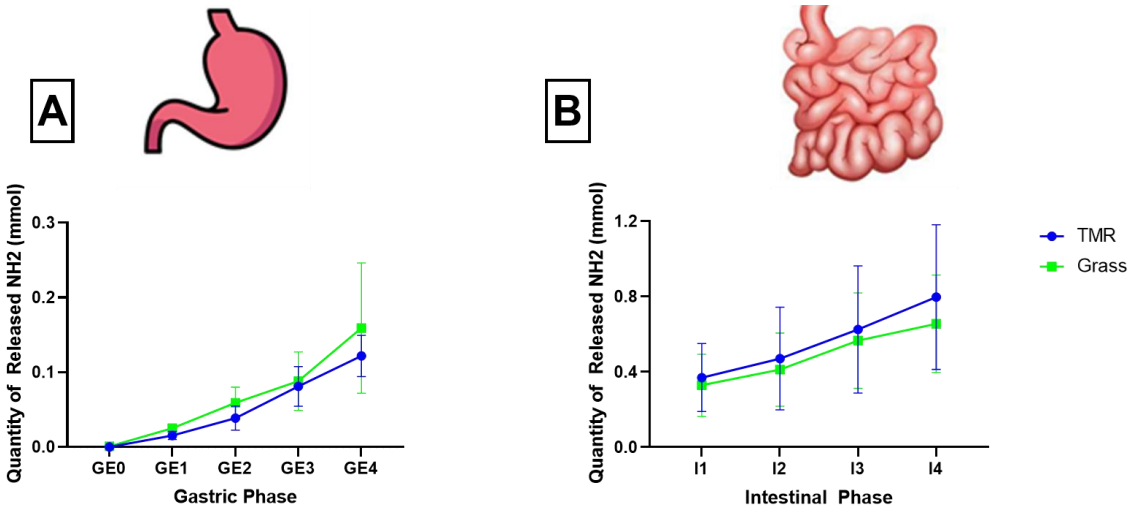


Figure 13: Cumulated release of free primary amines into the digesta supernatant during semi-dynamic gastrointestinal digestion of pasture-fed (GRS) (■) and total mixed ration (TMR) (●) milk as determined using the OPA (o-phthalaldehyde) assay. Figure 13 A presents results from gastric digestion; Figure 13 B presents results from intestinal digestion. Results are expressed as mmol of leucine equivalents. Each data point represents mean ± SD (n=3.). Significance was set at $p < 0.05$.

### 3.4.3 Fatty acid profile of undigested milk

The analysis of fatty acids in undigested whole milk showed significant differences between milk from cows fed a Total Mixed Ration (TMR) and those on a pasture-based (GRS) feeding system (Figure 14). Medium-chain fatty acids (MCFA, C12-C17) were significantly higher in TMR milk compared to GRS milk *(p* = 0.005), while long-chain fatty acids (LCFA, C18-C24) were significantly lower in TMR milk (*p < 0.001*). SFAs were significantly higher in TMR milk (*p* = 0.004), while MUFAs were significantly lower (*p* = 0.003). The *de novo* fatty acids were also significantly higher in TMR milk (*p* = 0.005). Total omega 9 fatty acids were significantly lower in TMR milk compared to GRS milk (*p* = 0.033). No significant differences were observed in omega 3 fatty acid proportions, however, GRS had higher proportions than TMR (0.44± 0.05 g/100g FAs *vs.* 0.75± 0.00 g/100g FA, for TMR *vs.* GRS respectively).

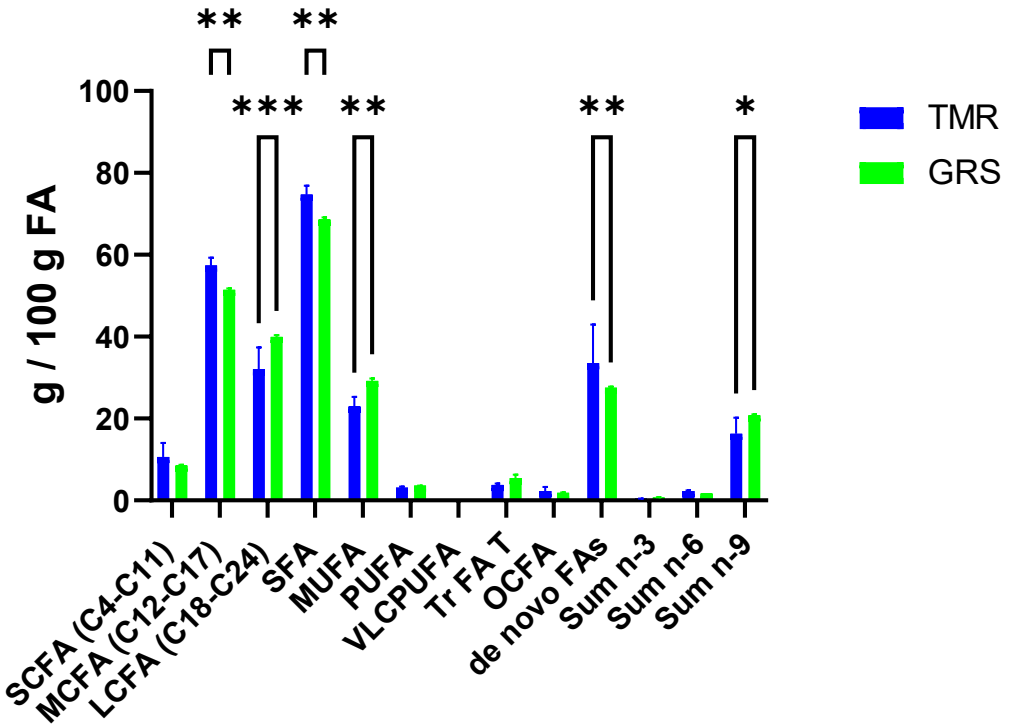


Figure 14. Comparison of fatty acid profiles in milk from cows fed Total Mixed Ration (TMR) vs. grass-based (GRS) feeding systems. (A) Fatty acid indices showing the percentage

composition of different fatty acid groups. SCFA: short-chain fatty acids; MCFA: medium-chain fatty acids; LCFA: long-chain fatty acids; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; VLCPUFA: very long-chain polyunsaturated fatty acids; Tr FAs T: trans fatty acids; OCFA: odd-chain fatty acids; *de novo* FAs: *de novo* synthesized fatty acids. Data are presented as means ± SE (n=3). Asterisks indicate significant differences between TMR and GRS: *p < 0.05, **p < 0.01, ***p < 0.001.

### 3.4.4 Lipid hydrolysis during gastrointestinal digestion

Figure 15 presents an overview of the cumulative lipolysis levels throughout gastric digestion and at the end of each intestinal digestion phase. In Figure 15A, cumulative FFAs release is expressed as a percentage of the total amount of fatty acids in milk before digestion, this is indicative of the efficiency or extent of lipolysis for each milk. In Figure 15B, these results are presented in absolute terms, providing an indication of each milk's specific pattern of lipid release. TMR milk tended to exhibit lower lipolysis rates than GRS milk throughout digestion (Figure 15 A). During the gastric phase, the rate of lipolysis for TMR milk increased from 5.74% ± 1.5 at the first gastric emptying point (GE 1) to around 20% by the end of the gastric phase (20.39% ± 3.32 at GE4), resulting in the release of 111.08 ± 78.39 mg of FFAs. Lipolysis increased during intestinal digestion with lipolysis rates ranging between 19.17% ± 2.31 (*i.e.* 102.04 ± 70.34 mg) by the end of the first intestinal step (I1), and more than 50% after the last intestinal step (53.07% ± 7.57 or 298.55 ± mg at I4). In contrast, lipolysis rates for GRS milk started at 17.18% ± 8.10 at the first gastric emptying point (GE 1), increasing to 36.81% ± 13.78 by the end of the gastric phase (GE 4), equating to a total release of 231.49 ± mg of FFAs. During intestinal digestion, FFAs release ranged between 47.55% ± 18.85 (284.04 ± mg) by the end of the first intestinal step (I1), increasing to more than 55% by the end of intestinal digestions (56.71% ± 10.95 or 356.82 ± mg, at I4). Despite GRS showing a trend towards higher total FFAs release at all stages of digestion, the differences were only statistically significant at the first and second intestinal phases in terms of total % lipolysis ($p < 0.05$).

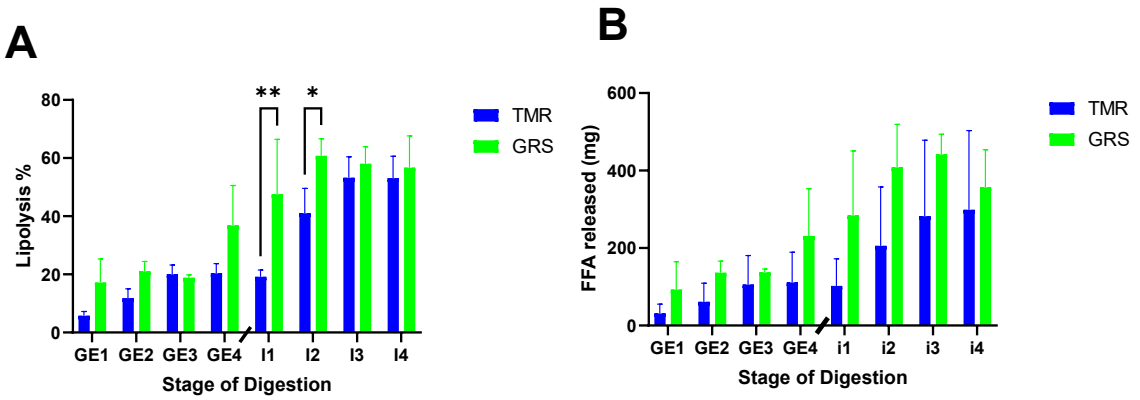


Figure 15 Cumulative release of free fatty acids during semi-dynamic gastrointestinal digestions of whole milk from cows fed a pasture-based (Grs; green bars) or total mixed ration (TMR; blue bars). Relative (% of total fatty acids in milk) and absolute (mg) amounts of fatty acids released are presented in panels (A) and (B), respectively. Each data point represents the mean of three replicates ± SD. The asterisk (*) symbol denotes statistically significant differences between grass and total mixed ration milks with the number of asterisks indicating the level of significance: for $p \leq 0.05$, ** for $p \leq 0.01$.

### 3.4.5 Release of individual fatty acids

A hierarchical clustering heat map (Figure 16) was used to visualise and compare fatty acid (FAs) release patterns for both GRS and TMR milks during gastric and intestinal digestion. This analysis showed clustering patterns and correlations between specific fatty acids and the two feeding systems.

In the gastric phase, short-chain fatty acids (SCFAs) showed a positive correlation with both GRS and TMR milks, indicating their relatively higher release compared to medium-chain fatty acids (MCFAs) and long-chain fatty acids (LCFAs). However, C18:2 cis-9, trans-11 CLA has a positive correlation with TMR milk in the gastric phase than GRS milk, despite being present in lower proportions. By the end of the gastric phase, MCFAs and LCFAs showed the largest differences between TMR and GRS milks.

The intestinal digestion phase showed different correlation pattern, with medium and long-chain fatty acids demonstrating stronger positive correlations overall. The feeding strategy (GRS *vs.* TMR) appeared to influence the correlation strengths of specific FAs: C16:0 (palmitic acid) showed a stronger positive association with TMR milk, while C18:2 cis 9 trans

11 CLA, C16:1 n-9 (palmitoleic Acid), C18:1 tr (trans vaccenic acid), and C16:1 n7 tr (trans palmitoleic acid) exhibited stronger positive correlations with GRS milk.

The release dynamics of four specific fatty acids during digestion are illustrated in Figure 17 (A-D). For C11:0 (undecanoic acid), minimal differences were observed between TMR and GRS milk throughout digestion. During the gastric phase, no significant differences were found until GE4, where TMR milk showed slightly higher C11:0 content compared to GRS milk (0.19 *vs.* 0.14, $p$=0.043). No differences were observed during the intestinal phase, indicating that C11:0 release is largely unaffected by diet.

For C16:0 (palmitic acid), GRS milk released significantly more C16:0 at GE1 ($p$=0.026) during early gastric digestion, but no further differences were observed in subsequent gastric phases. During intestinal digestion, TMR milk released higher amounts of C16:0 at mid-intestinal stages (I2: $p$=0.003; I3: $p$=0.004), suggesting delayed lipolysis in TMR systems compared to GRS milk.

The release of C18:2 cis-9, trans-11 CLA showed consistent and significant differences throughout digestion, with GRS milk releasing higher levels at all stages. In undigested milk (GE0), GRS milk contained significantly more CLA than TMR milk ($p$<0.001), and this trend continued throughout the gastric phase (GE1-GE4) and intestinal phase (I1-I4), with peak differences observed at I2 and I3 ($p$<0.001).

For linoleic acid (C18:2 n-6), no significant differences were observed during the gastric phase (GE0-GE4). However, during intestinal digestion, TMR milk released more C18:2 n-6 in the early to mid-intestinal phases (I1-I3; $p$<0.001 to $p$=0.009). Interestingly, at the final intestinal

stage (I4), this trend reversed, with GRS milk releasing significantly more linoleic acid than TMR milk ($p$=0.021).

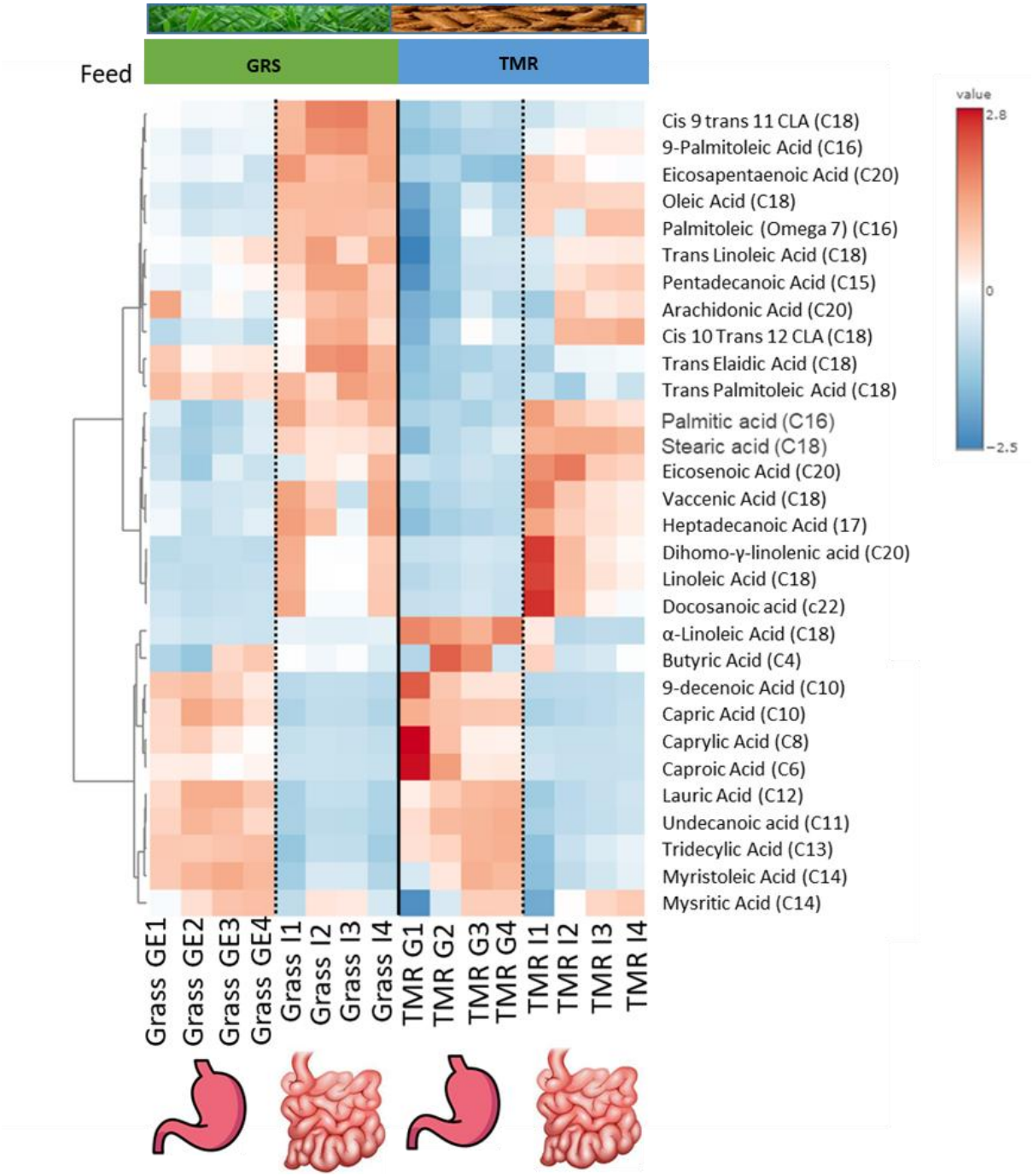


Figure 16 Hierarchical clustering heat map, depicting areas of interest in gastric and intestinal phase GRS vs TMR. Calculated based on average of 3 replicates at each gastric emptying point of grams FAs released per 100 grams of fatty acids released.

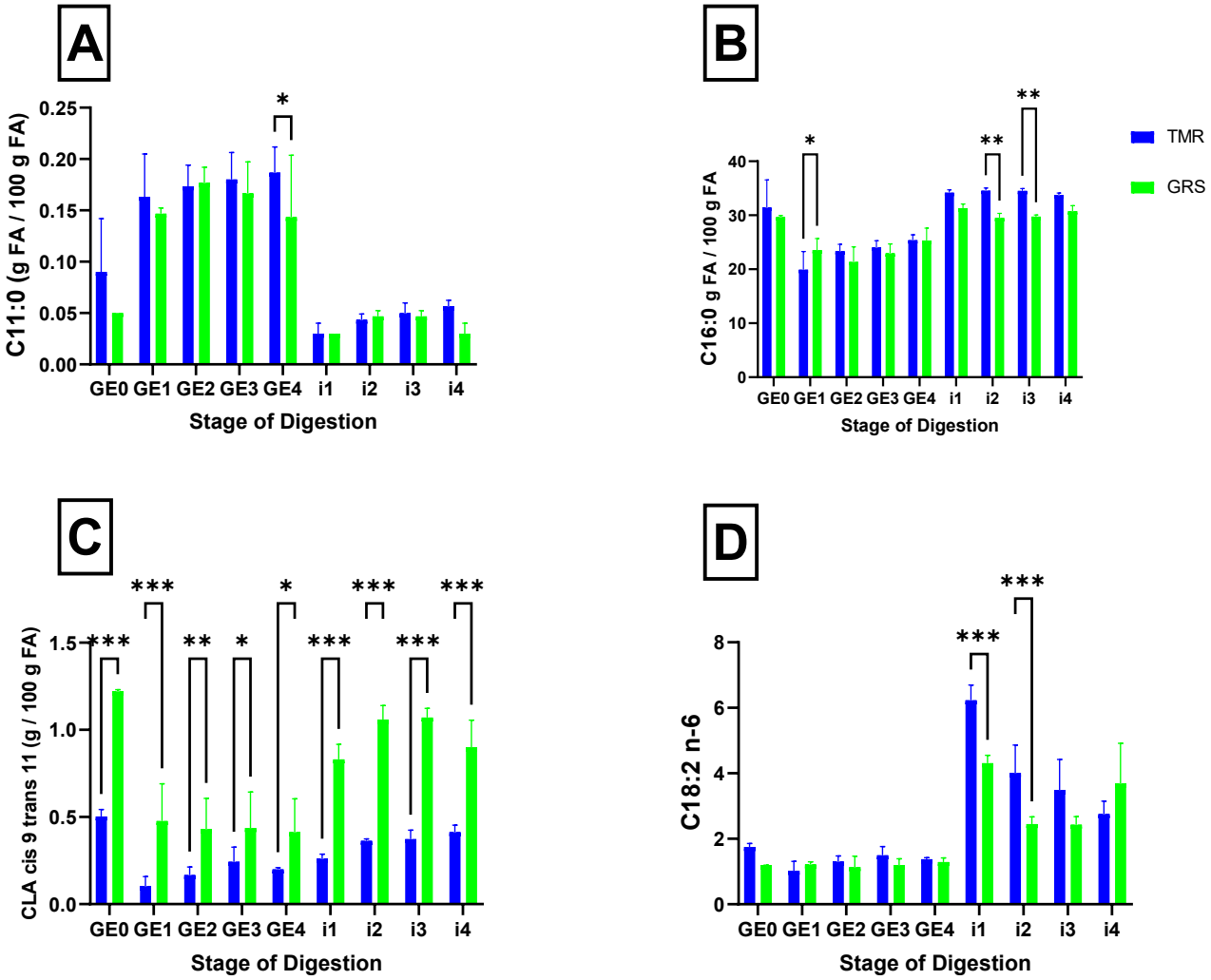


Figure 17 shows the release (g / 100 g FA) of (A) Undecanoic acid (C11:0), (B) Palmitic acid (C16:0), (C) Conjugated Linoleic Acid (CLA), and (D) Linoleic acid (C18:2 n-6) from milk from cows fed either a Total Mixed Ration (TMR, blue or Grass-based diet (GRS, green line) during simulated gastric and intestinal digestion. Digestion stages are represented as GE0 (undigested milk), GE1-GE4 (gastric digestion phases), and I1-I4 (intestinal digestion phases). Data points represent mean values (n=3), and error bars indicate standard error of the mean. Asterisks denote significant differences between TMR and GRS at each digestion stage (*p<0.05, **p<0.01, ***p<0.001).

### 3.4.6 Release of fatty acids grouped according to degree of saturation

To better understand the lipolysis profiles of each milk, FFAs in digesta samples, grouped according to the degree of saturation have also been examined. These results are presented and is presented in Figure 18. On the left side (panels A, C, and E), cumulative FFAs release is expressed as a percentage of the total amount of fatty acids in milk before digestion. On the right side of the Figure 18 (panels B, D, and F), these results are presented in absolute terms. When analysing the proportion of FAs released for each category (Figure 18 A, C and E), the trends observed, reflected the total lipolysis patterns observed (Figure 15A) in that GRS milk tended to have higher lipolysis rates at most time points of gastric and intestinal digestion. These differences were statistically significant for certain intestinal points for MUFAs (I2, $p < 0.05$) and SFAs (I2 to I4, $p < 0.05$). Interestingly, when these results were expressed in absolute terms (mg FFAs released), statistical significance was only found for MUFAs (I2 and I3 ($p < 0.05$).

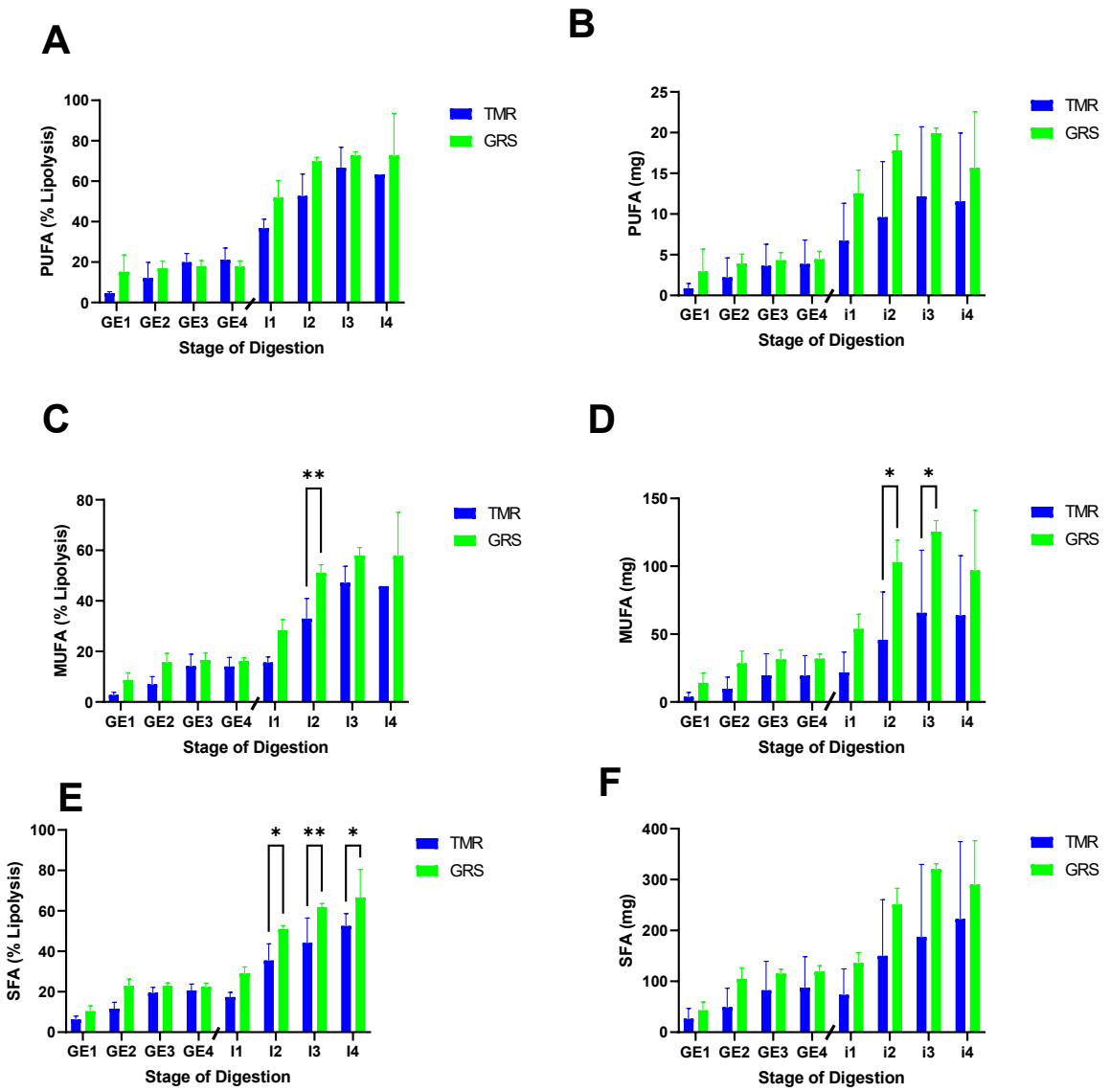


Figure 18 Figure 18 Cumulative release of FFAs grouped according to saturation degree during semi-dynamic gastrointestinal digestions of whole milk from cows fed a pasture-based (GRS; green bars) or total mixed ration (TMR; blue bars) diet. Graphs on the left (panels A, C and E) present the results obtained in digesta samples as a percentage (%) of the total FAs in milk samples. Graphs on the right (B, D, F and H) present the same results in absolute amounts (total mg released in digesta samples). (A) Lipolysis of PUFAs %, (B) Release of PUFAs in mg, (C) Lipolysis of MUFAs %, (D) Release of MUFAs in mg, (E) Lipolysis of SFAs % and (F) Release of SFAs in mg. Each data point represents the mean of three replicates ± SD. Significance was set at $p < 0.05$. $p$ values < 0.05 (*), < 0.01 (**), < 0.001 (***).

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### 3.4.7 Release of Fatty acids grouped according to carbon chain length, omega number and ratios

The FAs released during digestion were divided into 9 categories based on carbon chain length, and degree of saturation (g / 100 g FAs in milk samples prior to digestion). This information is, presented for each gastric emptying point (left side of the figure 19 panels A, B, D, and E) and intestinal digestion step (right side of the figure, panels F, G, H, and I) in figure 19.

Feeding strategy significantly influenced the proportion of fatty acids released in terms of nutritional indices, ratios and FAs categories but this effect was dependent on the stage of digestion. After 20 min of gastric digestion (GE1), TMR milk showed significantly higher release of short-chain FAs (SCFA; +21%, $p < 0.001$), saturated FAs (SFA; +9.3%, $p = 0.001$), and *de novo* synthesized FAs (+17%, $p < 0.001$), whereas grass-fed milk showed significantly higher release of medium-chain FAs (MCFA; +9.3%, $p = 0.001$), long-chain FAs (LCFA; +13%, $p < 0.001$), and MUFAs(+10%, $p < 0.001$). (Figure 19 A). While there were no significant differences GE2 – GE3 (Figure 19 B, C). At GE4, TMR milk again released significantly higher SCFAs (+4.58%, $p = 0.003$) and *de novo* FAs (+4.35%, $p = 0.004$), while grass-fed milk showed higher LCFAs (+4.13%, $p = 0.006$) and MUFAs (+3.02%, $p = 0.04$) (Figure 19 D)

During the intestinal phase, GRS released more MUFAs (+2.5%, $p = 0.017$) and *de novo* FAs (+2.2%, $p = 0.034$). The differences observed were greatest at I2 to I4: at I2, TMR milk released significantly more MCFAs (+3%, $p = 0.008$), whereas GRS milk released more MUFAs (+4.3%, $p < 0.001$) and *de novo* FAs (+2.6%, $p = 0.022$). By I3, TMR milk showed higher MCFAs release (+4.24%, $p < 0.001$), while GRS milk released significantly greater LCFAs (+3.43%, $p < 0.001$), MUFAs (+4.11%, $p < 0.001$), and trans FAs (+2.16%, $p = 0.018$). This differentiation peaked at I4, with TMR milk showing significantly greater release of SCFAs (+3.4%, $p < 0.001$), MCFAs (+6.3%, $p < 0.001$), SFAs (+5.1%, $p < 0.001$), and *de novo* FAs (+5.7%, $p < 0.001$); conversely, GRS milk exhibited significantly higher LCFAs (+8.3%, $p < 0.001$), MUFAs (+4%, $p < 0.001$), polyunsaturated FAs (PUFA; +2%, $p < 0.001$), and trans FAs (+1.5%, $p = 0.009$).

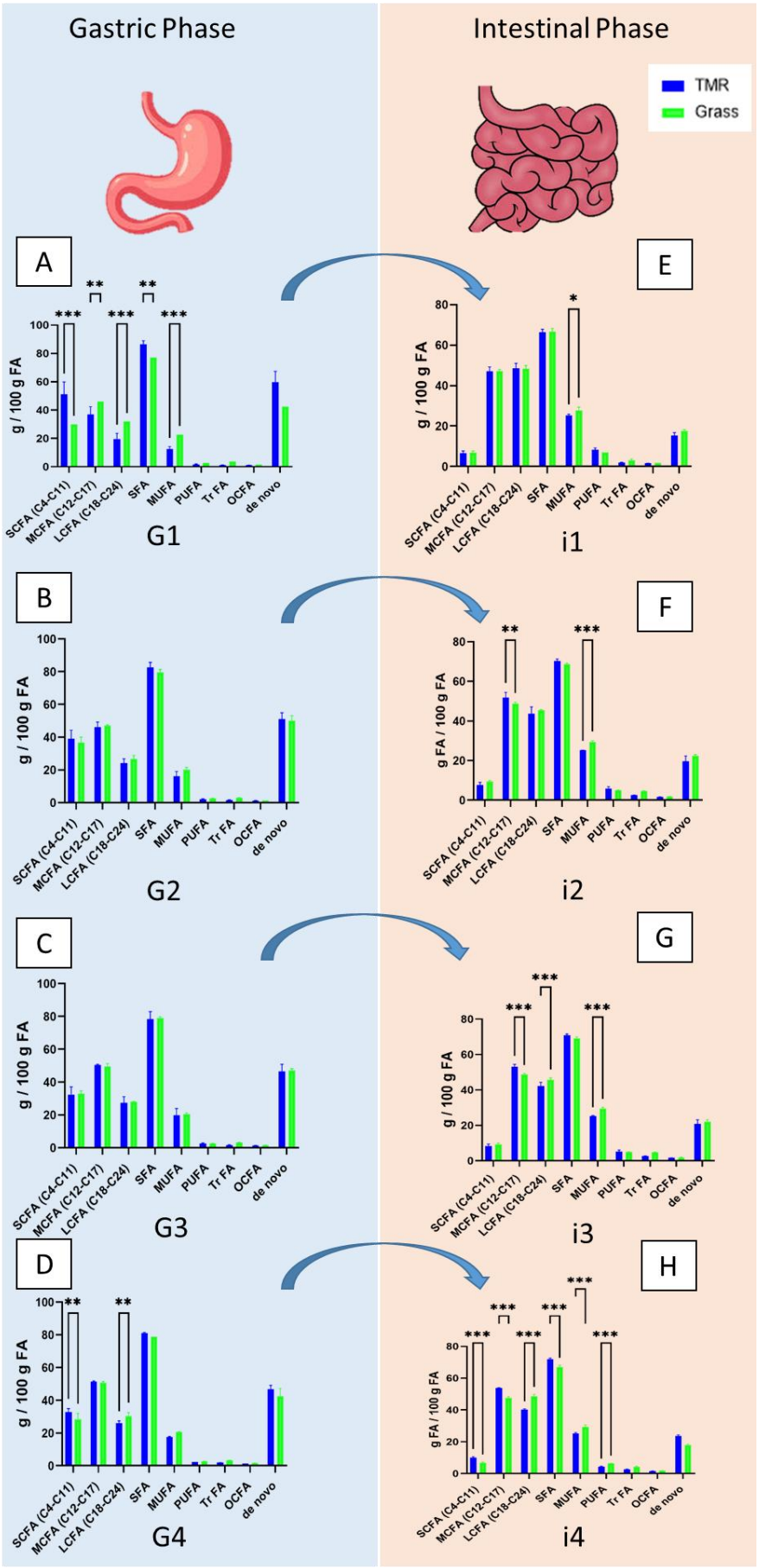
Gastric Phase
Intestinal Phase
TMR
Grass
A
B
C
D
E
F
G
H
G1
G2
G3
G4
i1
i2
i3
i4
g / 100 g FA
g FA / 100 g FA
SCFA (C4-C11)
MCFA (C12-C17)
LCFA (C18-C24)
SFA
MUFA
PUFA
Tr FA
OCFA
de novo

Figure 19 Proportion of FAs at each gastric emptying point and respective intestinal digestion step (g FAs / 100 g FAs). FAs are divided into 9 categories based on carbon chain length, omega number. Each data point represents mean ± SD (3 rpt.). Each graph represents one sampling point during digestion. Graphs A-D represent 4 stages of gastric digestion. Graphs E-H represent 4 stages of intestinal digestion. Significance was set at $p < 0.05$. p values < 0.05 (*), < 0.01 (**), < 0.001 (***). SCFA: Short-Chain Fatty Acids, MCFA: Medium-Chain Fatty Acids, LCFA: Long-Chain Fatty Acids, SFA: Saturated Fatty Acids, MUFA: Monounsaturated Fatty Acids, PUFA: Polyunsaturated Fatty Acids, tr FA: Trans Fatty Acids, OCFA: Odd-Chain Fatty Acids, de novo: De novo synthesized fatty acids.

### 3.4.8 Calculation of health-relevant indices based on FAs release

To understand the potential health related differences between consuming milk from cows fed a pasture-based vs TMR diet, three health related indices were calculated based on undigested (Figure 10A) and at each stage of digestion (Figure 20 B-C). In undigested milk, significant differences were observed between Total Mixed Ration (TMR) and Grass (GRS) feeding systems for the unsaturation index. GRS-fed cows exhibited a significantly higher unsaturation index compared to TMR-fed cows (33.0 *vs.* 30.0, $p < 0.001$). However, no statistically significant differences were found for the Thrombogenicity index (4.0 *vs.* 2.9, $p = 0.072$) and Artherogenicity index (3.8 *vs.* 2.7, $p = 0.072$) in undigested milk between the two feeding systems.

Throughout the digestion stages, the unsaturation index demonstrated significant variations between TMR and GRS feeding systems (Figure 20B). GRS-fed cows showed significantly higher unsaturation indices at stages GE2 (32.0 *vs.* 20.0, $p < 0.001$), GE3 (30.0 *vs.* 19.0, $p < 0.001$), I1 (37.0 *vs.* 22.0, $p < 0.001$), and I2 (31.0 *vs.* 24.0, $p = 0.025$). No significant differences were observed at stages GE1, GE4, I3, and I4 ($p > 0.05$). For the Artherogenicity index, significant differences between TMR and GRS feeding systems were observed at two digestion stages (Figure 20C). TMR-fed cows exhibited significantly higher Artherogenicity indices at stages GE1 (8.3 *vs.* 5.5, $p = 0.0069$) and GE4 (8.7 *vs.* 5.9, $p = 0.0025$). No significant

differences were found at stages GE2, GE3, I1, I2, I3, and I4 ($p > 0.05$). The Thrombogenicity index also exhibited significant differences between the feeding systems across several digestion stages (Figure 20 D). GRS-fed cows had significantly higher Thrombogenicity indices at stages GE1 (3.1 *vs.* 1.7, $p = 0.001$), GE3 (3.3 *vs.* 2.5, $p = 0.035$), and GE4 (3.6 *vs.* 2.5, $p = 0.006$). Conversely, TMR-fed cows showed significantly higher Thrombogenicity indices at stages I2 (3.6 *vs.* 2.8, $p = 0.030$), I3 (3.7 *vs.* 2.8, $p = 0.023$), and I4 (3.7 *vs.* 2.7, $p = 0.020$). No significant differences were found at stages GE2 and I1 ($p > 0.05$).

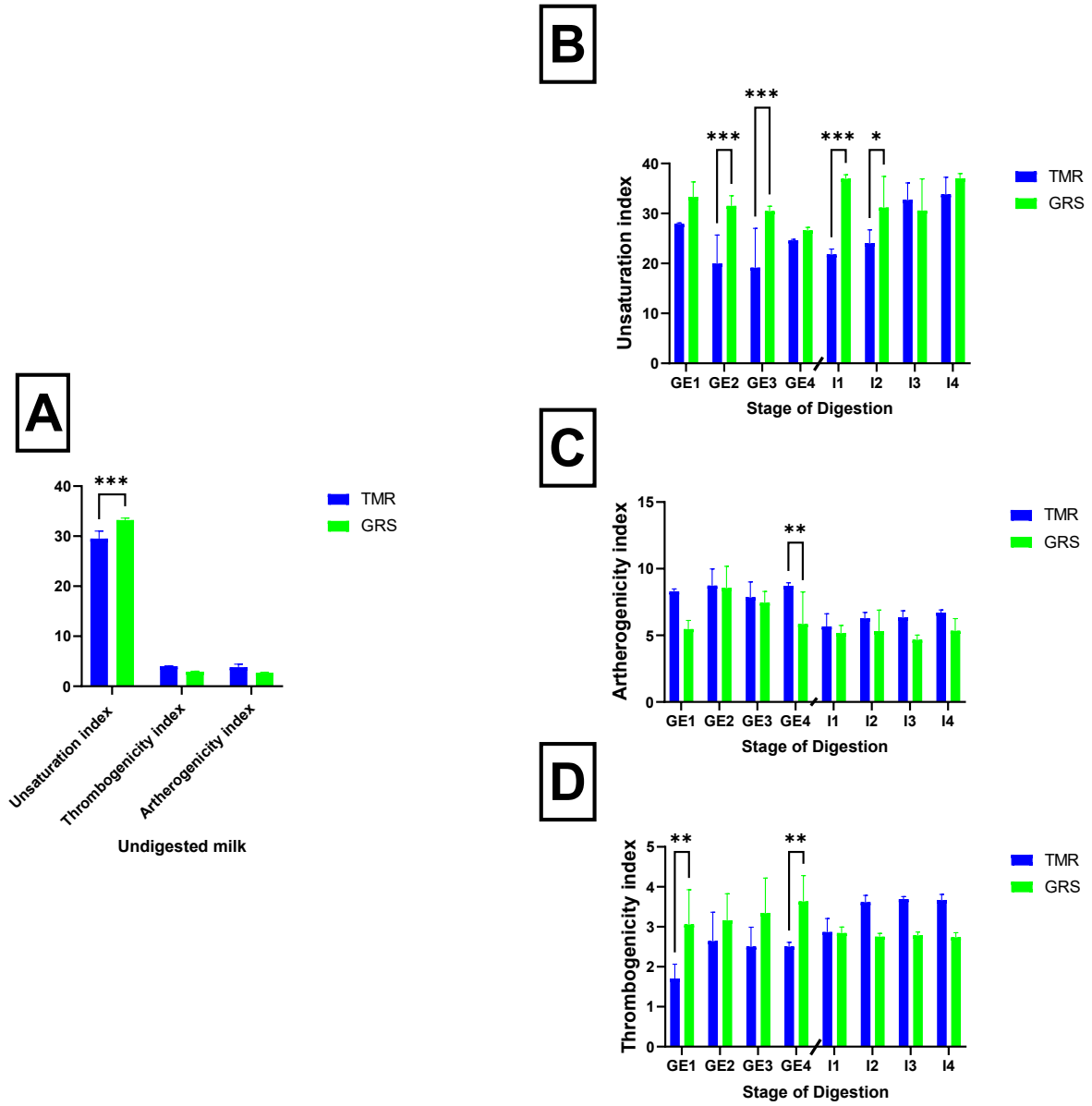

Figure 20 Health-related indices derived from fatty acids (FAs) released during the in vitro digestion of pasture-fed (GRS; green bars) and total mixed ration milk (TMR; blue bars). (19 A) Unsaturation index calculated as (1×% monoenoic)+(2×% dienoics)+(3×% trienoics), Artherogenicity Index calculated as (4×C12:0+C14:0+C16:0)/(MUFA+PUFAs), and Thrombogenicity index calculated as (C14:0 + C16:0 + C18:0) / (0.5 * MUFAs + 0.5 * PUFAs n-6 + 3* PUFAs n-3 + (n-3 / n-6)) in undigested whole milk. (19 B-D) Unsaturation index , Artherogenicity index and Thrombogenicity index at each stage of digestion. Significance was set at $p < 0.05$. p values < 0.05 (*), < 0.01 (**), < 0.001 (***).

## 3.5 Discussion

Several studies have examined the impact of bovine feeding strategy on milk composition. This is however, the first study to our knowledge to investigate the effect of feeding strategy on milk digestion in humans using *in vitro* digestion methods.

The milk coagulation patterns during gastric digestion observed via CLSM are in agreement with previous reports of casein micelle aggregation during *in vitro* semi-dynamic digestion, forming a protein-lipid matrix that slows gastric emptying of nutrients (Mulet-Cabero et al., 2019). As expected, no significant differences in protein hydrolysis between GRS and TMR milks was observed (Figure 13). Since the protein content of milks was standardised, any potential difference in protein hydrolysis between GRS and TMR milk may have been masked. Differences in protein hydrolysis could have been observed in non-standardised, non-homogenised whole milk although this difference is still expected to be minor.

However, some interesting previously unknown differences, were observed during *in vitro* digestion. The present study has shown that GRS shows more extensive release of FFAs (lipolysis) when compared to TMR at all stages of digestion. During gastric digestion, lipolysis reached 36.81 ± 13.78% and 20.39 ± 3.32% for GRS and TMR milks, by the end of this digestive step. Further increases were observed during intestinal digestion with final lipolysis levels (i.e. at the end of I4) reaching 56.71 ± 10.95% and 53.07 ± 7.57% for GRS *vs.* TMR milk (Figure 15). This is consistent with previous *in vitro* digestion studies that also show incomplete lipolysis during gastrointestinal digestion of dairy lipids (Garcia et al., 2014). The extent of lipolysis of milk is affected by processing, size of lipid globule and chemical composition (Gallier and Singh, 2020). Both milks in this study have been processed in the same way. Additionally, the milk fat globule size of these milks has been studied by Timlin et

al. (2023a), finding no difference in the milk fat globule size due to diet. This implies that the observed differences in lipolysis are due other factors. One explanation for this could be due to the higher proportion of long chain unsaturated fatty acids in GRS vs TMR milk, potentially causing a more fluid lipid core compared to a crystalline lipid core in TMR milk due to higher saturated and medium chain fatty acid content leading to lower enzyme access in TMR vs GRS. Another factor could be the whey protein to casein ratio. Timlin has shown that TMR has a greater casein to whey protein ratio compared to GRS milk. This could lead to a firmer coagulum formation during digestion, entrapping lipids within the protein matrix to a greater extent than in GRS. This could be the explanation for lower degree of lipolysis in TMR vs GRS milk.

The differences in FAs profiles of undigested GRS and TMR milks, particularly the higher C18:2 cis-9, trans-11 CLA content in GRS and omega-3 levels (Figure 17) is in agreement with previously published research in this area (Timlin et al., 2023b, O'Callaghan et al., 2016). Previous work with the same samples has shown that milk from cows fed a TMR diet had significantly higher proportions of saturated fatty acids (SFAs) compared to pasture-fed (GRS) (Timlin et al., 2023a). This finding is in agreement with previous studies by O'Callaghan et al. (2016) and Baltušnikienė et al. (2008), who also demonstrated a trend towards higher proportions of SFAs in pasture fed cows. These studies also showed increased levels of MUFAs and PUFAs in GRS milk, consistent with the current study. The higher unsaturated fatty acid content in GRS milk may be associated with the greater UFAs content in grass compared to maize silage (Chilliard et al., 2001).

The release of C18:2 cis-9, trans-11 CLA remains high from GRS milk during intestinal digestion This may be important, as CLA's has several beneficial properties such as anti-inflammatory, anticancer and immunomodulatory effects (Benjamin and Spener, 2009). Another benefit associated with an increased C18:2 cis-9, trans-11 CLA content is anti-obesity effects. However, many of the human intervention studies investigating C18:2 cis-9, trans-11 CLA supplementation have used synthetic rather than natural sources of C18:2 cis-9, trans-11 CLA (Mądry et al., 2020). Therefore, future studies should focus on human intervention studies using naturally occurring C18:2 cis-9, trans-11 CLA sources such as grass-fed milk.

C18:2n-6 has been shown to be released in significantly higher proportions from TMR milk during intestinal digestion. This might be due diet affecting the structural specificity of fatty acids. In TMR milk, α-linoleic acid is likely positioned in *sn*-2 position, where lipases have

specificity for. TMR's TAG structure seems to favour releasing linoleic acid earlier in digestion, while grass-fed milk releases it later. Previous research has shown that cows' diet influences TAG composition and stereospecific FAs positioning. For instance, feeding hydrogenated fatty acids, such as seen in TMR milk, leads to preferentially esterifies medium-chain saturated FAs (e.g., C16:0) at *sn-2* position (Pacheco-Pappenheim et al., 2022c).

Similarly, the release of palmitic acid (C16:0) during digestion is of particular interest. In human milk, palmitic acid is typically esterified at the *sn-2* position of TAG, while it is found at the *sn*-1,3 positions in most infant formulas (Bongers et al., 2007). This positional difference has been shown to reduce lipolysis in formulas (Yuan et al., 2020). In this study, a greater release of C16:0 from TMR vs GRS milk during intestinal digestion (Figure 17 B) may be due to similar reasons. Future studies should investigate FAs positional analysis on TAGs to better understand how feeding strategies influence lipolysis kinetics (Yuan et al., 2020). Furthermore, palmitic acid has been implicated in inflammatory processes and hypothalamic dysfunction, potentially impacting insulin sensitivity and metabolic regulation, highlighting the need to further explore the health implications of TMR vs GRS milk in human diets (Milanski et al., 2009, Hernández-Cáceres et al., 2019).

To understand FAs release patterns, FAs have been grouped into different categories according to degree of saturation (Figure 18). A significantly higher degree of lipolysis of MUFAs in GRS vs TMR at I2 was observed, which led to a significantly higher release (in mg) of these FAs at both I2 and I3. SFAs also displayed a significantly higher degree of lipolysis in GRS vs TMR at I2, I3 and I4. However, although this trend was also observed for the quantitative release of SFAs, no significant differences were observed. This trend is contradictory to what is expected to be observed since raw TMR milk contains significantly higher proportions of SFAs (Figure 14), which has also been observed in previous studies (Timlin et al., 2023a). This may be due to differences in stereo position of fatty acids, but more research is required to validate this. Heat map observations revealed that short-chain fatty acids (SCFAs) were preferentially released during gastric digestion from both GRS and TMR milk. Interestingly, it has been shown that SCFAs are preferentially esterified at the *sn*-1(3) position while MCFAs are preferentially esterified at *sn*-2 position (Yener et al., 2021). Moreover, both rabbit gastric lipase (as used in the present study), and human gastric lipase, show stereo preference for the *sn*-1(3) position (Rogalska et al., 1990a). This stereo specificity may explain the preferential release of short chain fatty acids during gastric digestion, which was observed for both GRS and TMR milks.

During gastric digestion GRS releases higher LCFAs, MUFAs, PUFAs and Tr FAs, with the difference between GRS and TMR milk being more significant during intestinal phases. This is likely due to the increased levels UFAs from pasture and increased levels of ruminal bio hydrogenation (Chilliard et al., 2007). In contrast, TMR milk showed increased release of SCFAs, MCFAs, SFAs, and *de novo* synthesised FAs, which is in agreement with the higher proportion of these FAs in associated with indoor fed cows (O'Callaghan et al., 2016). Since MCFAs and LCFAs are more easily absorbed in the intestinal phase compared to LCFAs, the early release of SCFAs and MCFAs from TMR milk could provide rapid nutrition to the consumer. MUFAs and PUFAs are released in greater proportions from GRS vs TMR milk, with the largest differences being observed at the end of intestinal phase (i4), which may prolong the postprandial release of these cardio protective fatty acids into the blood stream (Calder, 2015).The differences in FAs release between GRS and TMR milk have important nutritional implications. TMR milk exhibited higher AI and TI, which are associated with a higher risk of cardiovascular disease (Ulbricht and Southgate, 1991). However, these differences were not as obvious during digestion. Despite TMR displaying a higher AI index at all digestion points, a significant increase was only observed at GE4 when quantitative data was analysed, while no significant differences were observed when proportions were analysed. Interestingly, despite a significantly higher TI value in undigested TMR vs GRS milk, GRS milk had significantly higher TI values at GE1 and GE4 when proportional release of FAs was analysed. GRS had a significantly higher unsaturation index at several digestion points which may have several benefits for human nutrition. For instance, UFAs are linked to reducing LDL cholesterol which lowers the risk of heart disease (Hunter et al., 2010).

Future studies should use fully dynamic models such as the Human Gastric Simulator that could increase accuracy to the *in vivo* situation. As well as this, while FFAs quantification provides an understanding of lipid hydrolysis, absorption efficiency remains unstudied. Future studies should couple *in vitro* digestion with Caco-2 monolayers to better understand bio accessibility of released fatty acids.

## 3.6 Conclusion

This study has shown that bovine feeding systems effect the *in vitro* digestion lipids. GRS milk showed more extensive gastric lipolysis. GRS milk demonstrated increased release of long-chain fatty acids and conjugated linoleic acid with C18:2 cis-9, trans-11 CLA release continued throughout intestinal digestion. TMR milk, conversely, released higher proportions of medium-chain and saturated fatty acids during digestion, as well as increased Thrombogenicity in the intestinal phase. Results such as the more favourable unsaturation in the gastric phase of

GRS milk digestion suggest potential cardiovascular benefits. These findings therefore show the potential role of feeding systems in changing fatty acid release, with GRS milk offering greater health related benefits through factors such as increased C18:2 cis-9, trans-11 CLA levels. Future work should validate these outcomes in fully dynamic *in vitro* digestion models, model intestinal cells and finally in *in vivo* clinical trials to better understand effects on human health.

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# Chapter 4

# Monitoring the effect of consumption temperature of full-fat milk on *in vitro* gastric digestion using Magnetic Resonance Imaging (MRI)

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This chapter was published in the journal Food Hydrocolloids Volume 152, July 2024, 109864

*Awarded the University College Cork School of Food and Nutritional Sciences Postgraduate Research Publication of the Year 2024*

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## 4.1Abstract

This study employed Magnetic Resonance Imaging (MRI) to assess the impact of the consumption temperature of full-fat milk (4 °C, 37 °C, and 60 °C) on its behaviour during *in vitro* gastric digestion. Using the INFOGEST semi-dynamic protocol, and replicating human gastric temperature profiles for cold, warm and hot beverages, it was observed that consuming milk at 4 °C delayed protein coagulation compared to 37 °C and 60 °C by more than 5 min. 3D-MRI lipid quantitative analyses showed that fat-rich particles tended to float to the top of the digesta in a process similar to creaming with the 4°C and 37°C milks, a phenomenon that was not observed with the 60°C milk. The quantities of released proteins and free primary amines in the digesta supernatant, indicative of pepsin activity, showed no significant variation with milk temperature. These findings highlight the influence of consumption temperature on the structural reorganization of whole milk during gastric digestion and prompts further inquiry into the potential implications of milk temperature on nutrient delivery into the small intestine.

## 4.2 Introduction

During gastric digestion, bovine milk separates into liquid and solid phases because casein micelles coagulate, while the other milk proteins, termed whey proteins, remain soluble. Physiologically, the coagulation of caseins in the stomach is thought to be for better control of the release of proteins from the gastric to the intestinal phase, governing their further breakdown and absorption (Soop et al., 2012). Indeed, gastric digestion plays an important role in the rate of appearance of nutrients in the bloodstream by modulating the emptying of nutrients into the small intestine. Among the factors that may influence the gastric digestion of bovine milk, the kinetics of gastric acidification is important to consider. The pH increases rapidly following the consumption of a neutral pH meal, such as milk, and then decreases gradually throughout digestion down to pH ~ 2 due to the secretion of acidic gastric fluid (Clark et al., 1993). As during yogurt making, this decrease in pH contributes to the coagulation of casein micelles. It is also essential to enable proteolytic and lipolytic activity in the gastric compartment. Pepsin is the only proteolytic enzyme present during the gastric phase. Although pepsin is generally considered to have an activity optimum at approximately pH 2 (Piper & Fenton, 1965), it can hydrolyze caseins over a broad range of pH (Salelles, Floury, & Le Feunteun, 2021) and induce their coagulation in a similar way to rennet. For instance, *in vitro* studies with porcine pepsin have shown that this enzyme can hydrolyse 73% of κ-casein at pH 6.3 in 10 min at an activity level of only 2.75 U of pepsin/mL (Yang et al., 2022). This clearly indicates that, for milk, gastric proteolysis may begin straight after the consumption of a meal, even if the pH is close to neutral levels. Therefore, during the gastric digestion of milk in humans, both pH-driven and enzymatic-induced coagulation of casein micelles are at work.

Another factor that may influence the gastric coagulation of caseins, often overlooked, is the effect of the milk consumption temperature. *In vivo* studies have shown that the consumption of a hot or cold beverage can move gastric temperature away from 37°C for up to 30 min (Sun, Houghton, Read, Grundy, & Johnson, 1988; McArthur & Feldman, 1989). Moreover, it has also shown that the consumption of liquid foods at different temperatures can affect physiological responses, such as gastric emptying time. For example, it has been observed that beverages consumed at 60°C tend to exhibit faster gastric emptying times compared to cold liquid meals or beverages (Fujihira, Takahashi, Shimamura, & Hayashi, 2022; Mishima et al., 2009). Studies on the effect of consumption temperature of bovine milk on digestion are limited, however. One recent study investigated the effect of the ingestion temperature of milk on *in vitro* digestion by examining differences in κ-casein hydrolysis when skimmed milk is consumed at different temperatures using *in vitro* semi-dynamic digestion (Yang et al., 2023).

This study showed that the initial rate of hydrolysis of κ-casein by pepsin was higher with a 50 °C milk compared to 37 °C and 4 °C milks. However, at the end of digestion, the overall hydrolysis of κ-casein was lowest in the 50 °C milk. This study also suggested that milk consumed at 4 °C may be digested more easily due to the formation of looser and softer curds when compared to milk consumed at 37 °C or 50 °C. Several studies have also investigated the effect of temperature on the rennet-induced milk coagulation. This is of interest as rennet, a mixture of chymosin and pepsin, functions in a similar way to pepsin alone, cleaving κ-caseins, reducing casein micelle repulsion and allowing coagulation to occur. It has been shown that the incubation temperature of milk with rennet affects both coagulation time and curd firmness, with faster coagulation times and harder curds being formed at higher temperatures, which may indicate a similar occurrence in the gastric phase of milk digestion (Nájera, De Renobales, & Barron, 2003).

As well as protein digestion, lipid digestion is also initiated in the gastric phase. Most lipids in bovine milk exist within the milk fat globule as triacylglycerides (TAGs), with phospholipids and cholesterol mostly present on the surface of the milk fat globule membrane. The breakdown of these lipids begins upon the action of gastric lipase, with 10-30% of lipid hydrolysis occurring in this phase (Hamosh, Bitman, Wood, Hamosh, & Mehta, 1985). Gastric lipase has a stereo-specificity for the *sn*-3 position of TAGs and, despite having an optimal activity at pH 4-5.4, it is active over a wide range of pH (2-7) (Sams, Paume, Giallo, & Carrière, 2016). Milk fat has been shown to cream during the gastric phase of digestion. This is thought to occur due to enzymatic hydrolysis of proteins located on the surface of the milk fat globular membrane, leading to a destabilisation of the milk fat globules (Ye, Cui, & Singh, 2011). Therefore, apart from the pH, variations in any other conditions susceptible to influencing pepsin activity in the early stages of gastric digestion, such as the meal temperature, have the potential to influence this digestive process.

To better understand the effect of the consumption temperature of milk on its gastric behaviour and digestion under physiologically relevant conditions, *in vitro* digestion is a useful tool that can be combined with many analyses such as microscopy, chromatography, and rheology. While these are all powerful techniques, they are also invasive, making it impossible to study digestive processes without some level of disruption to the mechanism under study. Conversely, MRI is a dynamic and non-invasive imaging technique, which has been used to monitor the digestion of foods and beverages, including milk, both *in vitro* and *in vivo*. A variety of MRI imaging protocols has been recently proposed to monitor gastric digestion *in vitro*, providing insights into structural and chemical transformations of foods. For example,

Deng, Seimys, Mars, Janssen, & Smeets (2022) used MRI relaxation parameters to predict the release of protein into the supernatant during semi-dynamic digestion of protein gels, while Chemical Exchange Saturation Transfer (CEST) and Magnetization Transfer (MT) was used to study the milk protein breakdown with MRI (Mayar, Smeets, van Duynhoven, & Terenzi, 2023). Musse et al. (2023) also showed that MRI can be used to monitor the disintegration of solid food particles using ultra-short echo time (UTE) during semi-dynamic *in vitro* digestions of a cheese and bread meal and quantitatively assess the hydrolysis and creaming of lipids using gradient multi-echo (GRE) for separation of water and lipid signals.

In continuation of those studies, the objective of this article was to evaluate by MRI the effect of consumption temperature (4 °C, 37 °C and 60 °C) of full-fat milk on its behaviour during semi-dynamic *in vitro* gastric digestion. The term 'consumption temperature' will be used throughout this study for the sake of clarity and simplicity, even though it is based on an *in vitro* approach. The milk consumption temperatures we investigated were selected to reflect the likely temperatures at which a human will consume milk. 4 °C was chosen to mimic the consumption of milk directly out of the fridge, 60 °C milk was chosen to mimic the consumption of a hot cup of milk, and 37 °C milk was chosen to allow an experiment where no gastric temperature change occurs. Furthermore, full fat milk was chosen over skim milk upon the hypothesis that milk fat could behave differently depending on the consumption temperature, and to take full advantage of the capabilities of MRI. MRI experiments were designed to assess the formation of the milk curd, the disintegration kinetics of the casein particles upon pepsin action, and to quantify their creaming kinetics. All MRI-related findings are compared to chemical analysis results and discussed in relation to the effect of temperature on digestive enzyme activities and milk coagulum properties.

## 4.3 Materials and methods

### 4.3.1 Materials

Whole Milk Powder (WMP) was obtained from Moorepark Technology Limited (MTL), Teagasc Food Research Centre, Moorepark Fermoy, Co. Cork, Ireland. This WMP was produced from cows fed a grass-based diet during mid-lactation in Fermoy, Co. Cork, Ireland. Briefly, bulk tank milk was standardised to 0.95 true protein:fat ratio. The standardised milk was pasteurised and homogenised and subsequently evaporated to 45% solids using single stage falling film evaporator at 65°C (Anhydro F1 Lab, Copenhagen, Denmark). The milk was then spray dried using a single stage drier with nozzle atomizer, air inlet temperature 180°C, air outlet temperature 85°C (Anhydro Spray Dryer, SPX Flow Technology Denmark A/S,

Soeborg, Denmark). In addition to common analytical grade reagents used in the digestion experiments and sample analysis, rabbit gastric extract (RGE) from Lipolytech (RGE 15, Zone Luminy Biotech Enterprises, France) was also used in the digestion experiments. The activity of pepsin and lipase in the RGE used was 300 U/mg and 10.2 U/mg, respectively, as measured from the assays described in Brodkorb et al. (2019).

### 4.3.2 Experimental design

In this study, we investigated four experimental conditions simulating the digestion of full-fat milk consumed at 37 °C in the absence of enzymes (no-enzyme control condition), and the digestion of milk consumed at 37 °C (temperature control condition), at 4 °C, and at 60 °C in the presence of enzymes.

### 4.3.3 Milk preparation

WMP was analysed as per Timlin et al. (2023). WMP contained 26.7% protein, 28.2% fat and 38.5% carbohydrates. The evening before use, WMP was reconstituted in osmosis water at 15% (w/v) at room temperature for 5 min before heating to 45 °C for 30 min under magnetic stirring. Milk was then stored overnight (max 12 hours) at 4 °C to ensure complete rehydration. Rehydrated full-fat milk contained 4.0% protein, 4.2% lipid, and 5.8% carbohydrates.

### 4.3.4 Semi-dynamic gastric *in vitro* digestion

The gastric phase of the semi-dynamic INFOGEST protocol (Mulet-Cabero, Egger, et al., 2020) was used with some modifications to enable the reproduction of gastric temperature curves observed *in vivo* as further described below (Sun et al., 1988; McArthur & Feldman, 1989). All digestions were carried out in triplicate using RGE as the source of lipase and pepsin.

#### 4.3.4.1 Preparation of digestive fluids

Simulated salivary fluid (SSF 1.25x concentrated, pH 7.0, no amylase) and simulated gastric fluid (SGF 1.25× concentrated, pH 1.5) were prepared according to Brodkorb et al. (2019). RGE was added as a separate solution, prepared by adding RGE to water at activity levels of 80,000 U of pepsin and 2,400 U of lipase /mL.

#### 4.3.4.2 Digestion protocol

To allow optimum temperature control, reconstituted full-fat milk (20 mL) was set to the required temperature (see section 2.4.3.) and added onto the basal secretions already contained in the gastric digestion jacketed vessel (ref. 6.1418.150, Metrohm, Ireland): 1.458 mL of SSF,

10.2 µL of $CaCl_2(H_20)_2$, 1.645 mL of SGF, 0.109 mL of RGE (or water for the no enzyme control experiments) and 0.790 mL of water. This gastric digestion vessel, which was connected to a cryo water bath to enable the reproduction of temperature curves observed in human studies (see section 2.4.3.), was then placed in the centre of the MRI scanner. Due to the constraints of conducting the experiments in an MRI scanner, the addition and stirring of digestive fluids throughout the gastric phase were performed manually. More specifically, SGF and enzyme should have been added at a rate of 0.2808 mL/min and 0.0148 mL/min, respectively, according to Mulet-Cabero, Egger, et al. (2020). In this experiment, SGF was added using a stepwise approach with 1.404 mL every 5 min (0.281 mL/min × 5 min) for the first 20 min and 2.809 mL every 10 min, thereafter, decreasing the pH from 6.0 ± 0.5 to pH 2.0 ± 0.5. The RGE solution (or water for the no enzyme control experiments) was added at 0.0740 mL every 5 min for the first 20 min and 0.148 mL every 10 min thereafter. Gastric contents were mixed manually right after the additions of digestive fluids. To standardise stirring, the same person carried out stirring with the plastic pipette used to add the solution using 5 rotations each time. In accordance with the recommendation of Mulet-Cabero, Egger, et al. (2020), the duration of the gastric phase was calculated as twice the half-gastric emptying time, as estimated based on the caloric content of the meal to be digested *in vivo* and the assumption that the gastric content will be emptied at a mean rate of 2 kcal/min from ingestion to the half-gastric emptying time. This is intended to digest the milk samples in the same conditions to which they would be exposed when consumed as part of a meal *in vivo*. Our present study mimics the *in vivo* digestion of a 200 mL cup of whole milk containing 0.77 kcal/mL, leading to a total calorie content of 15.4 kcal for our meal. This corresponds to a half-gastric emptying time of 38.5 min, that was rounded up to 40 min to allow gastric emptying every 10 min for simplification. We also added 10 min at the end of our digestion to allow for a fat measurement sequence to be run on the MRI, bringing our total time of digestion to 90 min. Gastric emptying was carried out from the bottom of the vessel every 10 min up to 90 min using a 10 mL plastic pipette with a cut tip (inserted into digesta from above) to allow for particles < 3 mm to be collected with the fluid (total of 4.5 mL each time). These samples were then stabilised by heating in a thermal block set to 100 °C for 10 min to reach a T °C > 80 °C for 5 min (measured inside the Eppendorf), before cooling on ice. The pH of the emptied samples was measured approximately 10 min later to monitor the kinetics of gastric acidification and stored at -20 °C until required for analysis.

#### 4.3.4.3 Temperature control

The temperature within the gastric digestion vessel was controlled using a circulating cryobath. To mimic the increase/decrease of gastric temperature seen in the *in vivo* situation, including the pre-heating or cooling in the mouth and oesophageal tube, different temperature kinetics were designed. When 350-400 mL of coffee or orange juice are consumed at 4 °C, the gastric temperature reaches about 22 °C right after ingestion and returns to body temperature (37 °C) within 20-30 min (Sun et al., 1988; McArthur & Feldman, 1989). Likewise, the consumption of hot versions of these liquid foods lead to an initial gastric temperature of approximately 44 °C, returning to body temperature 20-30 min after consumption (Sun et al., 1988; McArthur & Feldman, 1989). This is why milk was added to the gastric digestion vessel at 22 °C to replicate the consumption of milk at 4 °C and at 44 °C to replicate the consumption of milk at 60 °C. To ensure these temperature kinetics, different cryobath settings were developed for each consumption temperature. For the 4 °C consumption temperature, the cryobath was initially set to 22 °C, and ramped to reach 37 °C over 30 min inside the vessel. For the 60 °C consumption temperature, the vessel was initially heated to 44 °C and subsequently cooled to reach 37 °C over 30 min. In the control experiment, in which the consumption of milk at physiological temperature (37 °C) was simulated, gastric digestion was performed at a constant temperature. Temperature inside the vessel was continuously monitored using a pre-calibrated optical fibre connected to a data logger (UMI8, FISO Technologies Inc., Canada).

### 4.3.5 MRI acquisition

MRI experiments were carried out on a 1.5T MRI device (Magneton, Avanto, Siemens, Germany) with a 4-element head RF receiver coil. The following protocols for the acquisition of tri-dimensional (3D) images were used:

A fast recovery turbo spin echo (FR-TSE) sequence for morphological images with effective echo time equal to echo spacing 9.8 ms, Turbo Factor 6, TR 150 ms, FOV 86 mm x 86 mm x 43 mm, matrix size 128 × 128 × 64, bandwidth 200 Hz/ pixel, 1 scan. Acquisition time was 3 min 51 s and image resolution was 0.7 mm × 0. 7 mm × 0.7 mm.

A gradient multi-echo (GRE) sequence to separate water and lipids signals as described in Hernando, Kellman, Haldar, & Liang (2010). Parameters were set at first TE 1.48 ms, echo spacing 2.81 ms, 6 echoes per echo train, TR 75 ms, flip angle 15°, FOV 112 mm × 128 mm × 64 mm, matrix size 112 × 128 × 64, bandwidth 888 Hz/ pixel, 1 scan. Acquisition time was 8 min 59 s and image resolution was 1 mm × 1 mm × 1 mm.

FR-TSE sequence was carried out every 5 min between t = 0 and 40 min, and between t = 50 and 75 min. A final FR-TSE sequence was carried out at t = 85 min. A GRE sequence was carried out between t = 40 and 50 min and between t = 75 and 85 min. To carry out a GRE sequence, the water flow to heat the vessel was halted to prevent artefacts from appearing in the images.

### 4.3.6 Estimation of the particle volume, number, and size from MRI

The volume of digesta was semi-manually analysed in FR-TSE images using Avizo 3D Pro software (FEI Company, version 2021.2). Thresholding was carried out to segment digesta from the digestion vessel and external environment. The threshold value was selected by visual inspection. This value was adjusted for each 3D image, as the contrast between the digesta voxels and the background was not constant. The fill function was used to fill any gaps in the digesta that were not automatically detected. In some cases, such as when the optic fibre was visible in the images, manual editing was carried out. The selected voxels were then used to compute a total digesta volume. Within digesta volumes, particles were characterized using Label Analysis Avizo function. This function was applied on binary images, where particles were manually thresholded. The algorithm first identified particles by applying a label to connected voxels (3D neighbours segmented voxels), so as each particle was assigned to a unique label. Then the total particle volume and the number of individual particles were quantified. To estimate the average particle size, the total particle volume was divided by the number of particles. This was carried out for all 3 repetitions at each condition.

### 4.3.7 Lipid quantification

Separation of water and lipid signals was carried out from the 3D GRE images using the "water-fat separation" method detailed in Picaud, Collewet, & Idier (2016). This method is based on the difference in signal frequency between the hydrogen protons of water molecules and those of lipid molecules. It enables the disentanglement of the water signal from that of lipids in each voxel. The method requires the composition of the lipids since the frequency of the lipid protons signal depends on their position on the triglyceride chain as described in Berglund et al. (2012). For the composition of triglycerides, we used one composition of dairy fat data provided in https://www.agroscope.admin.ch/agroscope/fr/home/themes/denrees-alimentaires/alimentation-sante/lait-produits-laitiers/graisse-du-lait.html, and which is reproduced in Supplementary Material S1. Using this data, we calculated the average number of protons at each position on the triglyceride chain and established one average frequency spectrum of the lipids described by nine different frequencies.

For each pixel of the 3D images, the signal of the lipids was quantified and then converted to grams by using a dedicated experiment on the rehydrated full-fat milk alone. 2D vertical lipid maps were finally obtained by averaging the lipid maps over one of the horizontal direction regarding the vessel. The reduction of information from 3D to 2D was necessary to improve the signal-to-noise ratio of the result and to facilitate interpretation. However, 3D acquisitions were relevant since they made it possible to measure the lipids in the whole vessel.

### 4.3.8 Analysis of digestion samples

All samples were centrifuged (3000 g, 5 min, 4 °C) and the supernatant was further filtered through 0.8 µm syringe filters (Acrodisc® 25 mm w/0.8 µm Supor® STRL, Pall Corporation) to remove particles and dairy fat. In the no-enzyme control experiments, the samples collected at t = 10 and 20 min of digestion were still liquid. As those liquid samples were useless to monitor the release of proteins/peptides from the particles, they were not analysed.

#### 4.3.8.1 Released proteins

The concentration of soluble proteins, released peptides and amino acids in the supernatant, further referred to as released proteins, was measured using 4th derivative UV absorbance using the method proposed by (Lüthi-Peng & Puhan, 1999) that is insensitive to the whey proteins to caseins ratio. This was performed using a 6M guanidine-HCl and 0.1M Na acetate buffer at pH 5.0, quartz cuvettes, and a UV spectrophotometer (UVmc2, Safas Monaco, Monaco, France). Results were converted into masses using a calibration curve established with skim milk as a standard (0–1.5 g/L of proteins) and the volume of the digesta at the time at which the samples were was collected. The mass of released proteins that was withdrawn during preceding gastric emptying steps was then added to the data obtained at time t to compute the masses (mg) of released proteins into the digesta supernatant.

#### 4.3.8.2 Free primary amines ($NH_2$)

The release of free primary amino groups ($NH_2$) in the supernatant, which is indicative of both the mass and the length of released peptides, was measured using a previously described o-phthalaldehyde (OPA) spectrophotometric assay in microplates (Lorieau et al., 2018) that was adapted from the protocol of (Church, Swaisgood, Porter, & Catignani, 1983). Results were corrected for digestive secretions (blanks with no food) and converted into free primary $NH_2$ quantities (mmol) using a calibration curve created with L-methionine (0–2 mM) and the volume of the digesta at the time at which the sample were collected. The $NH_2$ quantity that had been withdrawn during preceding gastric emptying steps was then added to the data

obtained at time t to compute the quantity of free primary $NH_2$ (mmol) in the digesta supernatant.

### 4.3.9 Statistical Analysis

Results are presented as mean ± standard deviation of three replicates unless otherwise stated. Statistical significance between experimental conditions was assessed at each time point using a two-way analysis of variance (ANOVA) and post hoc pairwise comparisons using the Tukey's HSD test. This analysis was performed with GraphPad Prism software (Prism for Windows, Version 9.0.1) and a threshold for significance of $p = 0.05$.

## 4.4 Results

### 4.4.1 Gastric temperature

The temperature curves obtained in digesta under MRI experiments are presented in Figure 21. As described previously, these curves were carefully designed to match as closely as possible to *in vivo* data available for liquid foods in the literature. Those temperature profiles are similar to the ones used by Yang et al. (2023) who studied the effect of consumption temperature of skim milk on its *in vitro* gastric digestion, but with further consideration of the temperature change that occurs in liquid foods before reaching the human stomach (Sun et al., 1988; McArthur & Feldman, 1989). This is why, for instance, the gastric digestion of the cold (4 °C) milk began at 22 °C in our experiments and not 4 °C as in Yang et al. (2023). In the same way, in the experiment replicating the consumption of hot (60 °C) milk, gastric digestion was initiated at 44 °C. During gastric digestion of the 4 °C and 60 °C milk samples, the standard digestion temperature (37 °C) was reached, on average, after 30 min. Note that the drops in temperature curves beyond 50 min were due to switching off water circulation to allow images for lipid quantification to be acquired.

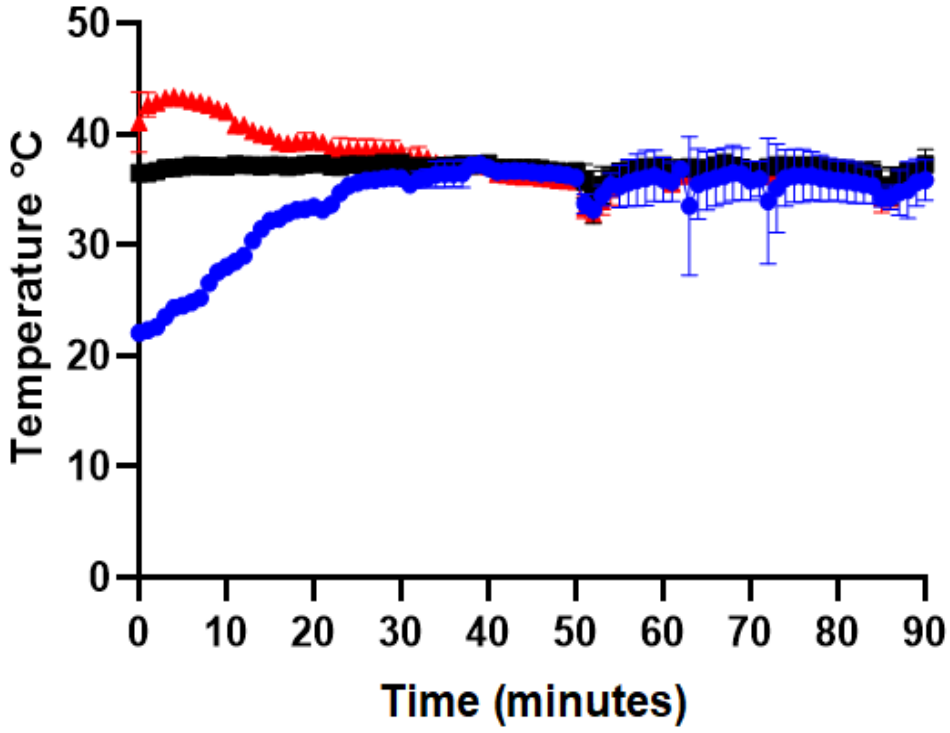


Figure 21 Temperature during gastric digestion for the consumption of milk: at 4 °C (●), at 37 °C (■), and at 60 °C (▲). Data are means ± SD over three replicates.

### 4.4.2 Gastric pH

The mean pH profile of each experimental condition is shown in Figure 22. The pH increased rapidly from 2 (basal secretions) up to approximately 6 upon the addition of the milk. Following this, the pH decreased continuously throughout digestion reaching values around 4 halfway through the digestion and pH 2 by the end of the experiments, as recommended in the semi-dynamic INFOGEST protocol (Mulet-Cabero, Egger, et al., 2020). All curves followed a similar trend except for the no enzyme control, for which the pH tended to decrease faster below pH 4. This is due to the lack of enzymatic hydrolysis of proteins in this experiment. When peptide bonds are hydrolysed at acidic pH by pepsin, some protons are consumed by the amino groups ($–NH_2$) at the *N-terminus* of the peptides produced. This pepsin-related consumption of protons, which adds to the initial buffering capacity of the food, does not exist in the absence of enzymes and explains why the pH decreases faster. This is a well-known phenomenon that can even be used to monitor pepsin activity with a pH-stat device (Mat, Cattenoz, Souchon, Michon, & Le Feunteun, 2018).

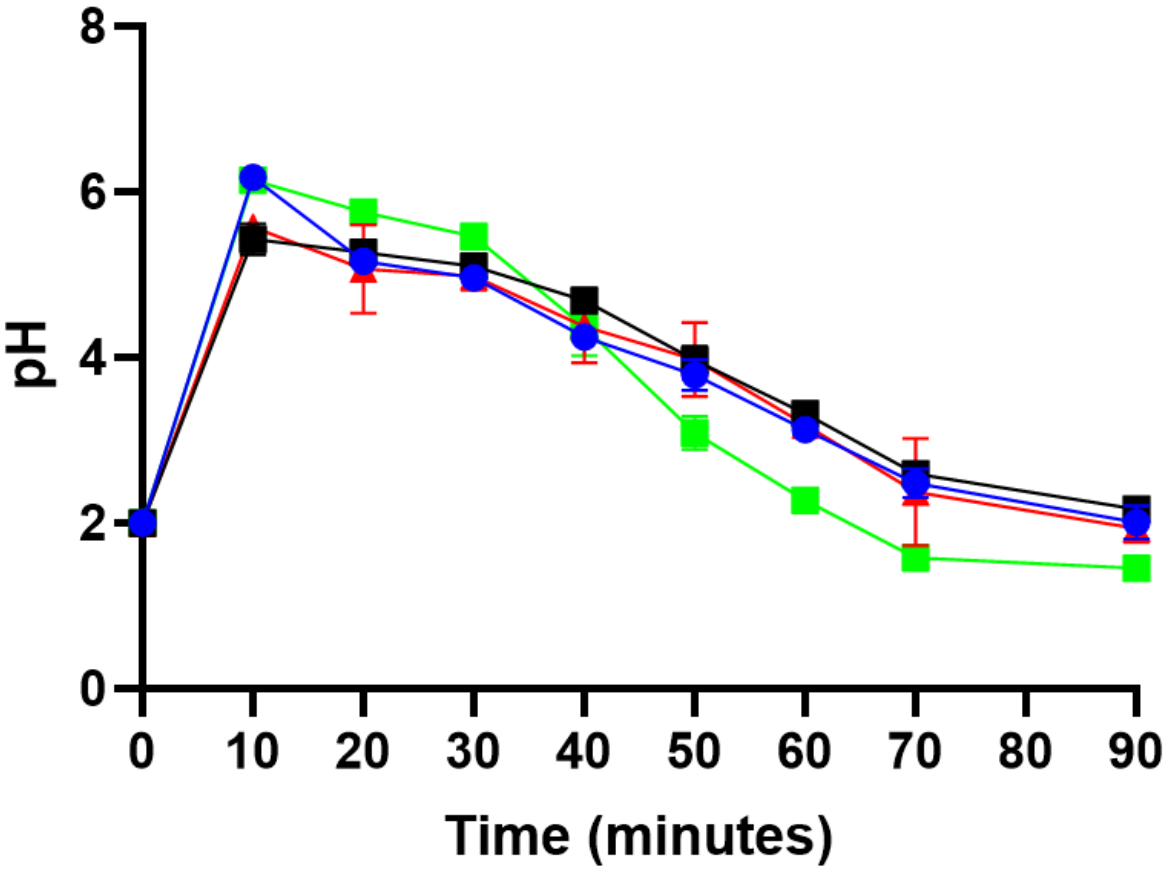


Figure 22 pH during gastric digestion for the consumption of milk: at 4°C (●), at 37 °C in the absence (■) and in the presence of gastric enzymes (■ ), and at 60°C (▲). Data are means ± SD over 3 replicates.

### 4.4.3 MRI monitoring of particle formation and disintegration

Figure 23 presents the middle slice of FR-TSE MRI 3D images acquired during the first 32.5 min of digestion and at the end of the experiments. In these images, the contrast is mainly due to differences in T2 relaxation time, which may be interpreted as a probe of the biopolymer structure and concentration (Mariette, 2009). Because no significant change in protein structure and water content occur during gel formation, a similar MRI dark grey signal was observed before and after milk coagulation. It was, therefore, not possible to distinguish liquid milk from a macroscopic gel spanning over the entire vessel in these images (Figure 23). Consequently, gel particles, which appear dark within a bright surrounding solution, were only visible in the next MRI image, *i.e.* after the gel was broken down into pieces by the manual mixing step between MRI scans: at t = 32.5 min with the no-enzymes control at 37 °C, from t = 12.5 min with the milk at 4 °C, and at t = 7.5 min with the milks at 37 °C and 60 °C. This

was true for all 3 replicates for each condition (Supplementary Material S2). Knowing that those times correspond to the mean time of the FR-TSE MRI 3D acquisitions, and that these lasted for 5 min, it may be concluded that milk coagulation took place within 25-30 min in the absence of enzymes, within 5-10 min for the milk at 4 °C, and within 0-5 min for the milks at 37 °C and 60 °C. These ranges of coagulation times were supported by our visual observations made when fresh gastric secretions were added to the milk between successive MRI scans. They were later confirmed by trying to study the effect of the initial gastric temperature (22 °C, 37 °C and 44 °C) on the onset of milk coagulation induced by the basal secretions (no further SGF addition) using rheological measurements. A coagulation time of 27.7 ± 4.2 min (triplicates) at a constant temperature of 22 °C was found, while coagulation was so quick at 37 °C and 44 °C (≤ 30s) that it was not possible to obtain fair and repeatable rheological measurements (Data not shown).

The FR-TSE images shown in Figure 23 were further processed to characterize the particles observed throughout gastric digestion in terms of total volume, number, and mean volume per particle. These results are presented in Figure 24, in which the data from the control experiments with and without enzymes at 37 °C are presented on the left-hand side (Figure 24A, 24C and 24E), and the data obtained at the three studied consumption temperatures in the presence of enzymes are presented on the right-hand side (Figure 24B, 24D, 24F).

At 37 °C in the presence of enzymes, the particle total volume (Figure 24A) and the mean volume per particle (Figure 24E) were maximal right after the gel formation, that is at t = 2.5 min. Just after the breaking down of the gel into particles upon stirring, at t = 7.5 min, a reduction of the total particle volume by half in only ~ 5 min was observed (Figure 24A). Because the pH was still unfavourable for pepsin action at that time (Figure 22), and because gastric emptying was not undertaken between t = 2.5 and 7.5 min, this result can only be explained by an intense syneresis of the particles, *i.e.* particle shrinking with whey expulsion. Later, the decrease in particle total volume was more gradual. Conversely, in the absence of enzymes, the mean and total particle volumes remained negligible until the onset of coagulation (between 25 and 30 min) and was maximal around 35 min, remaining stable until the end of the experiments (Figure 24A and 24E). Although caution must be taken with these results of semi-automatized analyses, large numbers of very small particles (Figure 24C) seemed to form in the no pepsin control before coagulation was initiated, peaking at 22.5 min (pH ~5, Figure 22 and Figure 24C and 24E), a phenomenon that could be attributed to the local formation of particles when adding the drops of HCl. When coming closer to the isoelectric point of caseins, a sharp decrease in particle number and an increase in the total and mean

particle volumes occurred, indicating that larger particles were being formed. During the second half of gastric digestion, when pH conditions were conducive to casein aggregation, but also most favourable to pepsin action, the total and mean particle volumes remained higher in the no enzyme control than in the digestions containing pepsin. The opposite was observed with the number of particles, which was higher for the enzyme experiment confirming that pepsin was the main causative factor in the breakdown of particles.

Turning to the effect of milk temperature consumption (Figure 24B, 24D and 24F), some significant differences in particle total volume (Figure 24B), number of particles (Figure 24D) as well as mean volume per particle (Figure 24F) were observed for the milk consumed at 4 °C compared to either 37 °C or 60 °C for $t \leq 12.5$ min ($p < 0.05$) because of its delayed coagulation. Of note is that at t = 7.5 min, the particle total volumes were 10.9 ± 5.5 $cm^3$ at 37 °C and 10.1 ± 6.3 $cm^3$ at 60 °C, corresponding to 41.3% and 40.4% of total digesta volume, respectively. These values further show that the gel particles underwent a significant syneresis after the breaking down of these gels, and that this phenomenon was of similar magnitude for both the warm and hot milks. Interestingly, after the breaking down of the gel into particles with the milk at 4 °C, from t = 12.5 min (Figure 24B), initial differences vanished and the total particle volume became very similar for all the studied temperatures at all time points (Figure 24B), with no statistical differences in terms of mean volume per particle (Figure 24F). Despite different milk coagulation kinetics and properties in the early stages of gastric digestion, the overall behaviours of the particles were thus comparable later on, consistent with pepsin action being the main factor in the breakdown of particles, even though the number of particles in the 37 °C experiments tended to be slightly higher than for the 4 °C and 60 °C milks from t = 57.5 min.

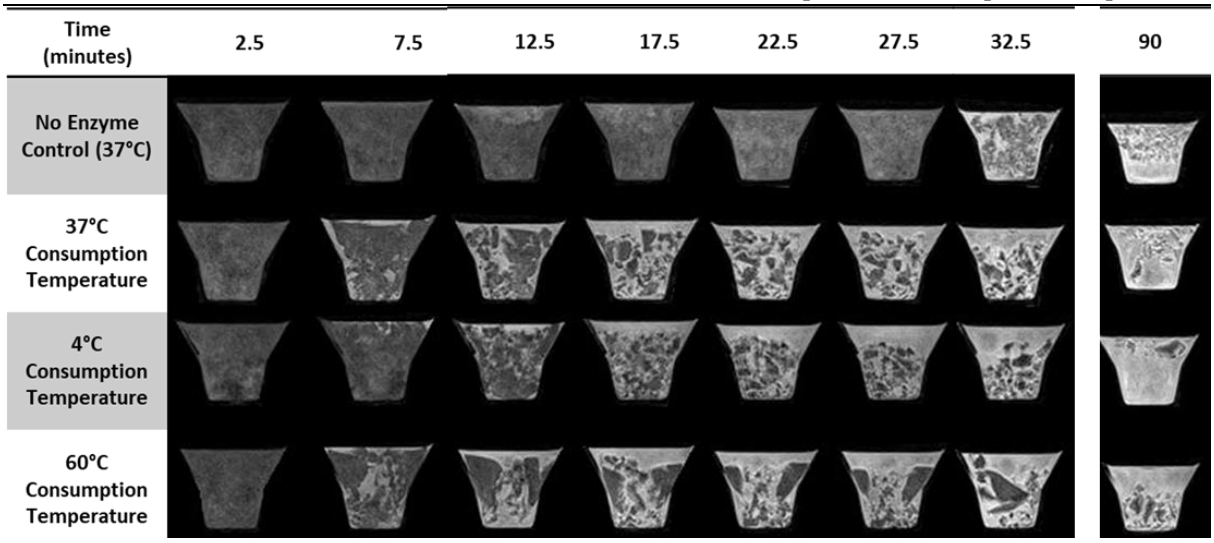


Figure 23 Examples of MRI images (from 1 replicate over the 3) acquired with the Fast Recovery Turbo Spin Echo (FR-TSE) sequence, illustrating the monitoring of gel formation and subsequent disintegration of dairy gel particles. Prior to onset of coagulation, the digesta, which is liquid, appears dark. After onset of coagulation, liquid digesta appears bright, and coagulated particles appear dark. Each time point involved the acquisition of 3D data sets comprising 64 images, capturing the entirety of the digestion vessel. The presented images showcase the middle slice from each acquisition, specifically focusing on those obtained during the first 32.5 min of digestion and at the conclusion of the experiments.

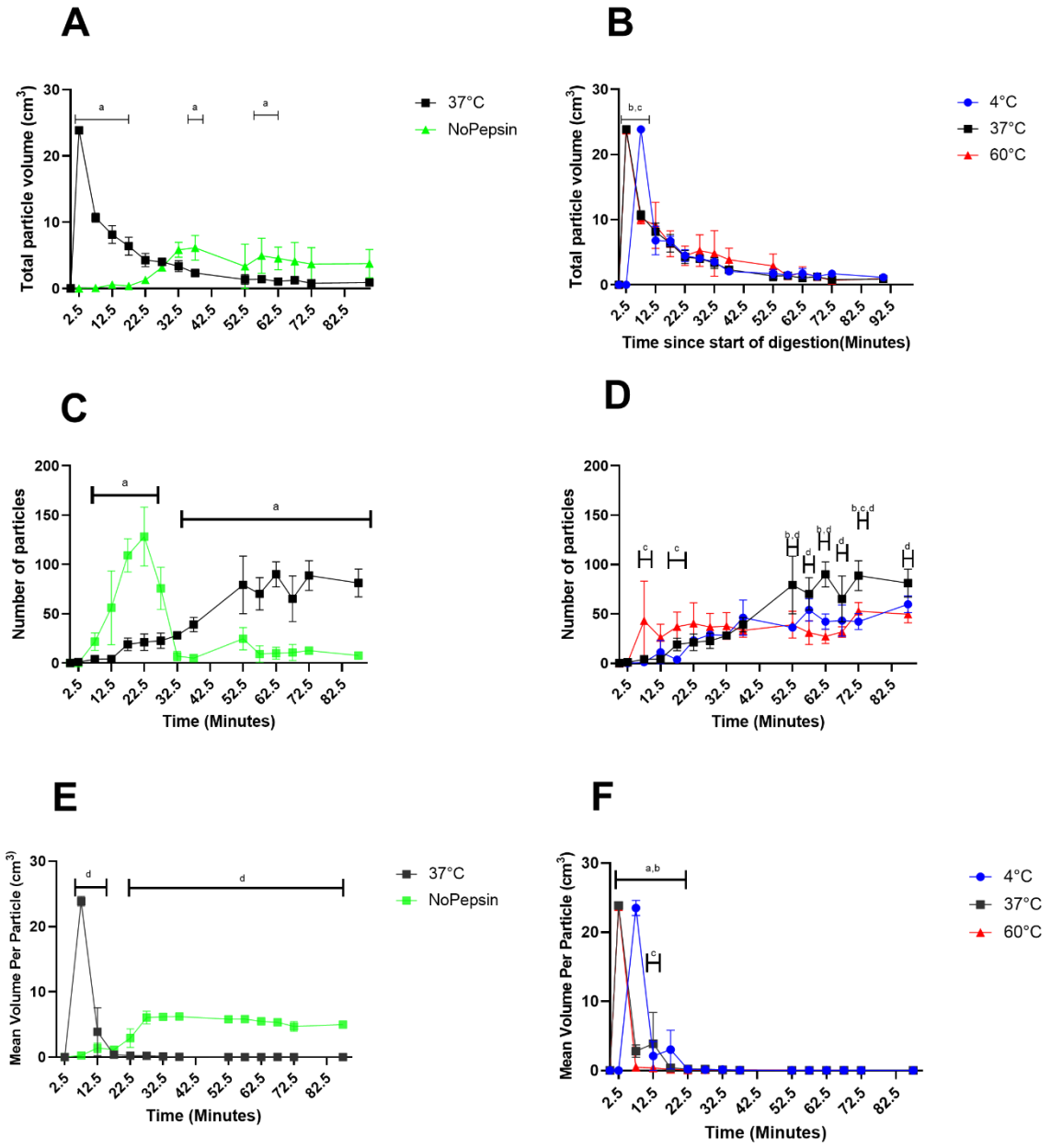


Figure 24 Particle analysis of MRI images. The left-hand-side subplots (A, C, E) compare the results obtained at 37 °C in the presence (□) and absence of enzymes (□). The right-hand-side subplots (B, D, F), compare the results obtained in the presence of enzymes at 4 °C (●), 37 °C (□) and 60 °C ( ▲ ). Data are means ± SD over 3 replicates. Statistical differences ($p<0.05$) are indicated by letters (A: 37°C with enzymes vs 37 °C without enzymes: B: 4 °C vs 37 °C, C: 4 °C vs 60 °C, D: 37 °C vs 60 °C.)

### 4.4.4 Fat quantification by MRI

Figure 25A shows the spatial distribution of lipids at the mid-point (t = 40 min) and at the end (t = 80 min) of gastric *in vitro* digestions in the presence of enzymes Figure 25B shows the estimated lipid mass as a function of the height in the vessel. At t = 40 min, when milk is

consumed at 4 °C, lipids were found to be spread between the bottom and top of the digestion vessel, while when consumed at 37 °C and 60 °C, lipids remained at the bottom of the vessel with no lipid creaming to the top. At t = 80 min (end of digestion), all the lipids had migrated to the top of the vessel for the milk consumed at 4 °C, most lipids had creamed when milk was consumed at 37 °C, and all lipids remained at the bottom when milk was consumed at 60 °C. The same observation could be made for the particles in the FR-TSE images (Figure 23 and Supplementary Material S2), in which the particles tended to float to the surface around mid-digestion with the milk at 4 °C, at the end of the digestions with the milk at 37 °C, but never with the milk at 60 °C. This indicates that the lipids observed in Figure 25 are not free lipids alone, they rather correspond to milk fat entrapped within the particles.

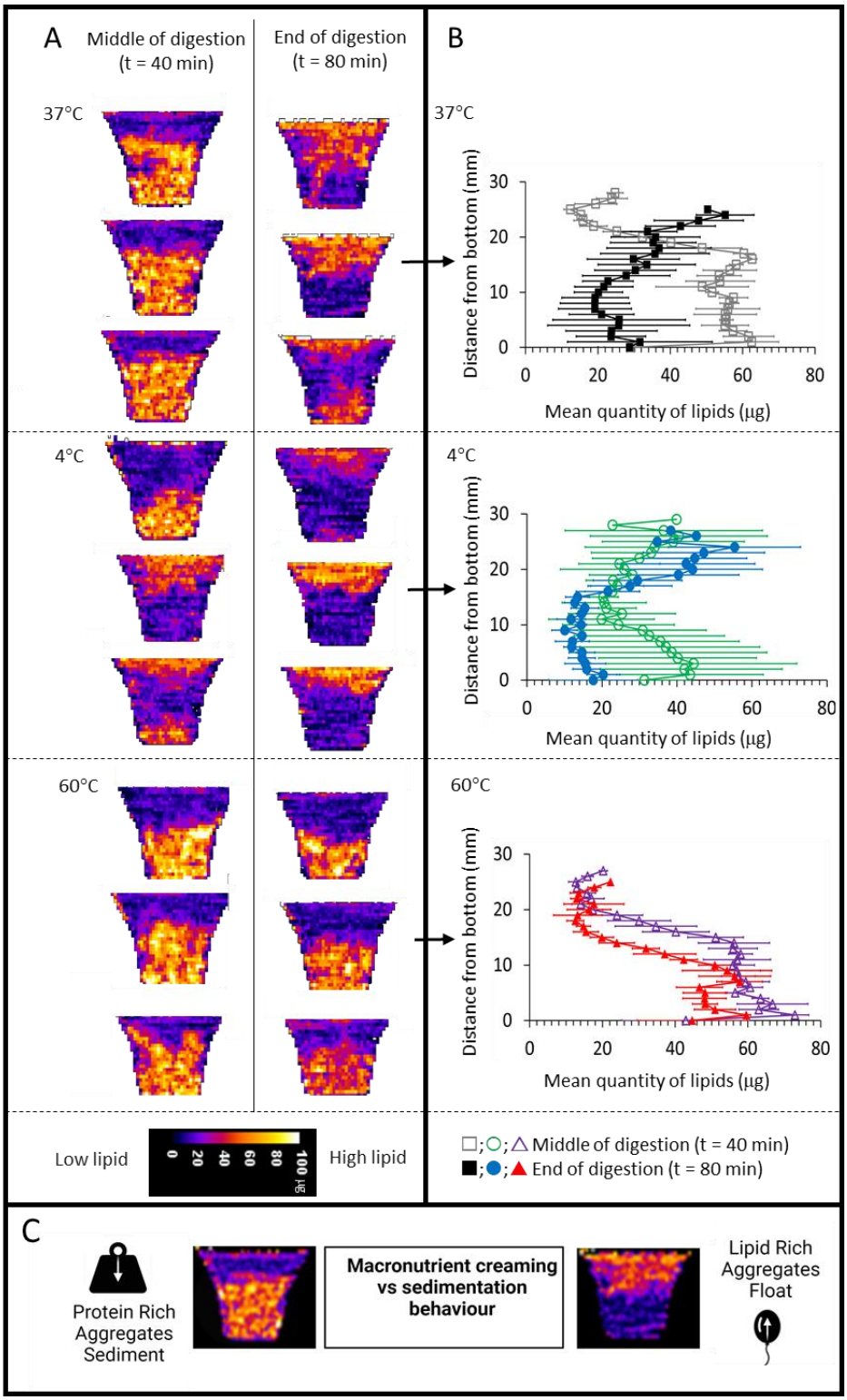


Figure 25 (A) Colour images represent lipid maps (mean value over the projection direction) at t = 40 (Left Column) and t = 80 min (Right Column) for the three replicates. Orange colour represents areas of high lipid amount. (B) Graphs represent the mean quantity of lipid signal over 3 repetitions calculated at each millimetre along the height of the digestion vessel. (C) Schematic representation of the effect of proteins vs lipids in particles in relation to creaming and sedimentation.

#### 4.4.4.5 Protein analysis in the digesta supernatant

Figures 26A and 26B depict the mass of proteins/peptides released into the supernatant, and Figures 26C and 26D show the quantity of free primary amines in the digesta supernatant. Of note is that only the samples collected after the onset of gel formation were considered in the no enzyme control experiment, that is for t ≥ 30min. As expected, both the mass of proteins and quantity of free amines released into the supernatant at 37 °C were significantly higher in the presence of enzymes than in their absence in ($p < 0.0001$, Figure 26A and 26C from t = 30-40 min). In the absence of enzymes, the estimated mass of proteins/peptides released into the supernatant plateaued at around 120 mg (Figure 26A). Considering that the amount of proteins in the milk was initially close to 800 mg, it can be estimated that about 15% of the initial milk protein content was released into the supernatant by the end of gastric digestion. This value, which is close to the expected 20% of whey proteins in the milk, is thus compatible with a release of whey proteins from the particles into the supernatant. In the presence of enzymes at 37 °C, coagulation occurred by the first emptying point, hence leading to a gradual release of proteins into the supernatant that is indicative of the breakdown of protein particles into peptides that release into the supernatant. The rise in the quantity of free amines and mass of protein in the digesta within the first 30 min, when the pH was above 5 (Figure 22), indicates that protein hydrolysis by pepsin was effective from the early stages of gastric digestion. The differences in the free amines released in the presence and absence of enzymes at 37 °C were significant from t = 30 min. The masses of protein released under both conditions were similar at this stage, but the curves started diverging from this point, and the differences became significant from t = 40 min. During the second half of digestions (after t = 40 min), the magnitude of the gap between the results obtained with and without enzymes increased rapidly, hence suggesting that pepsin activity increased significantly when the pH was brought down to 5 and below (Figure 22). According to the results of both Figure 26B and 26D, the release kinetics of soluble peptides did not depend on the milk consumption temperature, however. In the presence of enzymes, the estimated mass of proteins/peptides released into the supernatant was about 350-400 mg in all experiments (Figure 26B), indicating that about half of the initial protein milk content was emptied from the gastric digesta in the form of soluble proteins and peptides during the experiments. The other half was therefore emptied from the gastric digesta in the form of dairy particles.

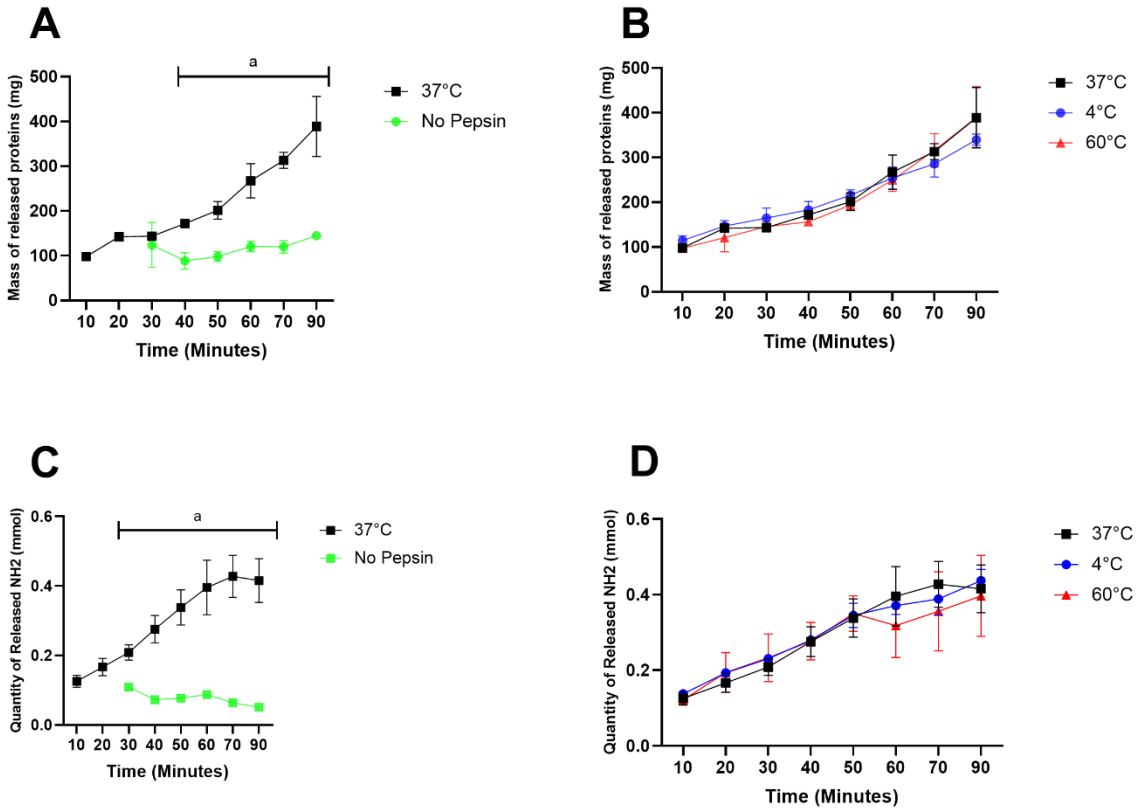


Figure 26 Release of proteins/peptides and free primary amines in the digesta supernatant. The left-hand-side subplots compare the mass of released peptides (A) and the quantity of released free NH2 (C) obtained in the presence (■) and absence of enzymes (■). The right-hand-side subplots compare the mass of released peptides (B) and the quantity of released free NH2 (D) obtained in the presence of enzymes at 4 °C (●), 37 °C (■) and 60 °C (▲). Statistical differences ($p<0.0001$) are indicated by letters (a: 37 °C with enzymes vs 37 °C without enzymes).

## 4.5 Discussion

Thanks to the use of MRI and gastric temperature profiles relevant to the digestion of cold (4 °C), warm (37 °C) and hot (60 °C) liquids in humans, the present study shows that the consumption temperature can influence the behaviour of full-fat milk during semi-dynamic gastric *in vitro* digestion. The delay before the onset of milk coagulation and the creaming properties of the fat-rich dairy particles appeared to be particularly affected by the milk temperature. However, both the kinetics of particle disintegration monitored by MRI, and the release kinetics of peptides into the supernatant remained largely unaffected.

### 4.5.1 Particle formation and disintegration

In the absence of digestive enzymes at 37°C, the formation of milk particles occurred due to the increasing acidity, when pH approached the isoelectric point of caseins. This phenomenon took place within 25-30 min, which coincided with a pH of ~ 5 (Figure 22). This result is in excellent agreement with the knowledge on acid-induced milk coagulation, showing that the onset of milk coagulation typically occurs around that pH (Lucey & Singh, 1997). Past this time point, casein particles were progressively diluted due to further additions of the acidic secretions and gastric emptying steps but no breakdown of particles (Figure 24E) nor any gradual release of peptides (Figure 26A and 26C) was seen in the absence of pepsin. These observations show that the strong acidity of the gastric environment was not sufficient by itself to disintegrate the dairy particles. In the presence of enzymes, the onset of milk coagulation took place much more rapidly for all milk consumption temperatures; that is in less than 10 min, at pH ≥ 5.5, and before the first addition of SGF on top of the basal secretions. These findings are in excellent agreement with the study of Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb (2019) on the semi-dynamic *in vitro* gastric digestion of different full-fat milks, which all coagulated in less than 10 min as well. They also confirm the role of pepsin in the formation of the milk coagulum during gastric digestion and indicate that enough pepsin was already contained in the basal secretions to reach the critical amount of κ-casein hydrolysis for coagulation to occur at all considered temperatures and despite a pH still close to 6. Although pepsin is often considered to be active at low pH only, this is not the case when it is acting on casein micelles. It has indeed long been known that pepsin can be active on caseins at pH close to neutrality, as recently reemphasised by Salelles et al. (2021) who have investigated the dependence of pepsin activity as a function of pH on casein micro particles. According to another recent study, approximately 73% and 33% of κ-caseins must be hydrolysed at pH 6.3 and 5.3 to induce coagulation, respectively, hence suggesting that about half of the κ-caseins contained in our milk could be hydrolysed within the first min of gastric digestion (Yang et al., 2022).

Our results further show an increased time to protein coagulation, by 5 to 10 min in our experimental conditions, when milk was consumed at 4 °C compared to 37 °C or 60 °C (Figure 23 and 24B). This observation was reinforced by our rheological trials in the presence of basal gastric secretions showing that milk coagulation occurred in more than 20 min at 22 °C but in less than 1 min at 37 °C and 60 °C. These data are consistent with the study of Yang et al. (2023), who also reported a delayed coagulation of skim milk with a decreasing consumption temperature. Although the literature lacks data on the effect of temperature at high pH on rabbit

pepsin acting on caseins, as used in the present study, it is well known that the activity of pepsin increases with temperature. It has for instance been reported that porcine pepsin activity on haemoglobin at pH 2 can be about twice as high at 44 °C as at 22 °C (Brier et al., 2007). The high sensitivity of pepsin to temperature therefore explains why the critical level of κ-casein hydrolysis needed to induce the formation of dairy particles was reached later with the lukewarm temperature (~22 °C) used at the start of the digestion of the 4 °C milk.

However, no significant variation could be found in terms of protein hydrolysis during gastric digestion as a function of the milk consumption temperature (Figure 26B). The same was true for the evolution of the total volume of particles estimated from the FR-TSE MRI images (Figure 24B). These findings suggest that the progressive breakdown of particles upon pepsin action was largely independent of milk consumption temperature in our experimental conditions. This contrasts somehow with the results obtained by Yang et al. (2023) on the effect of the consumption temperature of skim milk on gastric digestion. In their study, protein hydrolysis was found to be relatively faster for a 4 °C milk than for milks at 37 °C and 50 °C, a phenomenon that was attributed to the looser and softer protein network structure formed with the 4 °C milk during the early stages of digestion. They also reported that, after 240 min of digestion, the total curd weights of the warm and hot skim milks were higher than that of the 4 °C milk. The discrepancy between these findings and ours might relate to the presence of lipids in full fat milk, either in terms of interfacial dynamics and/or lipid crystals for the period below 37 °C that may have increased the firmness of the curds formed during our experiments. The experimental conditions used in both studies most certainly influenced the results as well. In the study of Yang et al. (2023), the starting gastric temperatures were initially set at 4 °C and 50 °C to investigate the digestion of cold and hot milk, with a return to 37 °C within 60 min. In the present study, our analysis of the *in vivo* literature led us to consider starting temperatures of 22 °C and 44 °C with a return to 37 °C within about 30 min as realistic physiological conditions for the same purposes. This is because the temperature of a drink changes significantly before it reaches the stomach according to the human studies we could find (Sun et al., 1988; McArthur & Feldman, 1989). Moreover, while we used a stepwise mixing procedure to allow for MRI acquisitions to be performed in the absence of fluid movements, Yang et al. (2023) used the Human Gastric Simulator (HGS), which enables a more reliable simulation of the mixing that should occur within the stomach. In comparison to our protocol parameters, the mixing conditions and the temperature profiles used in the study of Yang et al. (2023) should both contribute to the formation of more fragile dairy particles with the milk at 4 °C. All these considerations are likely to explain why they observed a small but significant increase in protein hydrolysis with cold milk, while we did not. More studies

with considerations of physiologically relevant gastric mixing, gastric temperature profile, and gastric emptying (which also seemed different in both studies) are thus needed to better evaluate if the consumption of cold milk can significantly influence the extent of protein hydrolysis at the gastric stage. In any case, the combined results of both studies suggest that such an effect should remain of a rather limited extent. Employing more advanced analytical techniques such as amino acid analysis, as in Jacobs et al. (2019), may also appear as an interesting mean to obtain more information on the breakdown of the dairy particles and to estimate the caseins to whey proteins ratio in the emptied material and/or the released peptides.

### 4.5.2 Creaming of fatty particles

Another phenomenon we observed is that the dairy particles tended to cream during gastric digestion (Figure 23 and Figure 25). To understand this phenomenon, one needs to understand why the density of these fat-rich particles can decrease during digestion. As schematically represented in Figure 25C, an analogy can be used with lipids acting as balloons, causing particles to rise to the top, and with proteins acting as anchors, causing particles to sediment to the bottom. With this analogy, the creaming of the particles can be ascribed to a progressive increase of the lipid-to-protein ratio within the particles during digestion, or in other words, the release of proteins within the supernatant was more rapid than for lipids. This could happen because of a tendency of the lipid droplets to remain attached and accumulate at the periphery of the eroding protein network, a phenomenon that has been observed using confocal microscopy (Luo, Ye, Wolber, & Singh, 2021). This could also be explained by a progressive release of proteins that were not directly involved within the protein network. This second mechanism is likely to have occurred during our experiments since some dairy proteins, notably whey proteins, can remain solubilized or suspended within the aqueous phase upon milk coagulation and thus be progressively expelled from the particles later. Interestingly, such a process should be enhanced as a function of digestion time because of the increasing dilution of the digesta by the gastric secretions. This could indeed conveniently explain why the particles also creamed before the end of the digestion experiments in the absence of digestive enzymes at 37 °C (Figure 24).

Moreover, the time at which the dairy particles creamed was also influenced by the milk consumption temperature (Figure 25). When milk was consumed at 4 °C, the lipid-rich particles creamed in the middle of digestion. The same phenomenon took place later with the milk at 37 °C, between t = 40 and 80 min, while the lipid-rich particles always remained sediment at the bottom of the vessel when milk was consumed at 60 °C. Several hypotheses could be put forward to explain this effect of consumption temperature on the kinetics of

particle creaming. For instance, the amount of β-caseins in casein micelles has been shown to be temperature-dependent, with up to 60% of β-caseins that can be removed from milk when centrifuged at 4 °C, though noting that the milk temperature was only brought down to 22 °C to simulate the behaviour of a 4 °C milk (McMahon & Oommen, 2008). It could nonetheless be assumed that there was a lower content of β-caseins within the dairy particles formed with the cold milk, or a more rapid protein impoverishment of the particles, leading to a sooner creaming than for the hot milk. A similar reasoning could be put forward based on the assumption that a looser curd was formed when milk was consumed at 4 °C when compared to 60 °C, consistent with the findings of Yang et al. (2023). Additionally, it is also possible that a firmer curd formed at high temperatures would be more resistant to pepsin action, even though we fail to see significant differences during our monitoring of the disintegration of dairy particles and the release of peptides. It is also noteworthy that milk fats are partially in their solid form below 37 °C and that crystalline milk fat is less susceptible to lipolysis (Smoczyński, 2017). It is difficult to know whether these phenomena could also contribute to the earlier creaming of dairy particles at low consumption temperature. Considering that the minimum temperature used in the gastric phase was 22°C, and that the milk fat should have been in a comparable state with the milks at 37 °C and 60 °C, any interpretation of our results based on the lipid melting point is quite unlikely. More studies would be welcome to better understand the exact mechanisms explaining how the consumption temperature of whole milk may influence the kinetics of particle creaming.

### 4.5.3 Physiological relevance

The observed differences in both the kinetics of protein coagulation and particle creaming may affect the rate of nutrient delivery to the small intestine *in vivo*. This is because the gastric behaviour of dairy products can directly influence the composition of the material that is emptied by the stomach. For instance, Mulet-Cabero, Rigby, Brodkorb, and Mackie (2017) demonstrated *in vitro* that, when digesting a liquid oil-in-water emulsion *vs.* a semi-solid dairy product made of yogurt and Gouda cheese particles, the emptying of lipids was delayed with the liquid emulsion due to lipid creaming, leading to a low amount of lipids available for intestinal absorption during the early stages of digestion. A similar phenomenon may therefore also apply when particles containing lipids tend to float during gastric digestion, as observed with our 4 °C and 37 °C milks. In Mulet-Cabero et al. (2017), the lipids contained in the semi-solid dairy product were entrapped within the food matrix and tended to remain sediment to the bottom of the digestion vessel, similar to what we observed for the milk at 60 °C. Although liquid milk was added to the digestion vessel in the present study, the very rapid formation of

dairy particles entrapping lipids at high temperatures resulted in a system very similar to the semi-solid matrix investigated in the study of Mulet-Cabero et al. (2017). Similar kinds of phenomena have also been observed *in vivo* with pigs fed with differently processed milks (Ahlborn, et al. 2023). In that study, it was shown that the fat from non-homogenised milks tended to cream and induce a much slower release of lipids to the small intestine (lipid half gastric emptying time = 93 min and 228 min) than for homogenized milks that are less prone to fat creaming (lipid half gastric emptying time = 11 and 51 min). Therefore, one potential physiologically relevant outcome of the present study is that lipids may be emptied more rapidly into the small intestine when milk is consumed hot compared to cold or body temperature. Interestingly, a human study by Mackie, Rafiee, Malcolm, Salt, and van Aken (2013) also suggests that the slower emptying of semi-solid dairy food as compared to liquid foods can be accompanied by enhanced appetite suppression. This increased appetite suppression was concluded to be due to the retention of food boluses in the antrum of the stomach activating mechanoreceptors within the gastric wall (Mackie et al., 2013). It is therefore plausible that the rheological properties of the particles formed after the gastric coagulation of milk can modulate appetite suppression, raising the potential for hot milk to promote satiety during the late stages of digestion when compared to cold milk.

When drawing physiological implications from *in vitro* data, the limitations in our semi-dynamic *in vitro* model system must be acknowledged. While efforts have been made to simulate many aspects of *in vivo* conditions, such as gastric mixing, emptying, and gastric juice production, this model does not entirely replicate the complexity of the *in vivo* situation. These differences may affect the translation of our results to the *in vivo* situation. Another difference that occurs *in vivo,* but not *in vitro* is the variation in the rate of gastric emptying due to consumption temperature. Several studies have examined the effect of consumption temperature on the rate of gastric emptying. With a carbohydrate beverage containing a known quantity of $^{13}$C-sodium acetate, Fujihira et al. (2022) showed that a higher $^{13}CO_2$ percentage tended to be excreted in the breath when a hot beverage (60 °C) was consumed compared to a cold beverage (4 °C), suggesting a more rapid rate of gastric emptying at 60°C. Using a comparable experimental design, Mishima et al. (2009) found that a liquid meal consumed at 60°C was emptied significantly more rapidly in comparison to the same meal consumed at 4°C. Sun et al. (1988) also reported that orange juice was emptied significantly faster when drunk hot vs cold. All these studies therefore suggest that a hot meal can be emptied more quickly from the stomach than the equivalent cold meal. Because the magnitude of this effect remains limited, and because we decided to adhere to the standardised international consensus for *in vitro* semi-dynamic digestion (Mulet-Cabero, Egger, et al. 2020), differences in gastric

emptying rates were not considered in the present study. Consequently, caution must be taken when extrapolating the observed effects from our semi-dynamic model to *in vivo* settings, and future *in vitro* experimentation should consider accounting for this observed difference. Since MRI is suitable for studying digestion in human studies, another major perspective of this work would be to carry out *in vivo* MRI experiments and to test in this way the physiological relevance of these results obtained *in vitro*. Our results, with the compromises that must be made, pave the way for MRI monitoring of the *in vivo* kinetics of protein coagulation and particle creaming under different conditions.

## 4.6 Conclusion

In this study, we used MRI for *in vitro* investigation of the effects of consumption temperature on the gastric behaviour and digestion kinetics of whole milk. The results obtained have highlighted two key differences when milk is consumed cold *vs.* when milk is consumed warm and hot. Firstly, we have confirmed that the coagulation of milk in the gastric phase is delayed when milk is consumed cold compared to when milk is consumed warm or hot. Secondly, we have shown in an original way that, although gastric temperature conditions only vary for the first half an hour of digestion, the creaming kinetics of particles appeared to be affected up to the end of digestion. When hot milk is consumed, dairy particles remain sediment at the bottom of the digestion vessel in contrast to when milk is consumed cold, where particles float to the top in the middle of digestion. These differences indicate that variations in terms of gastric emptying rates of lipids, and possibly satiety, may be affected by the temperature at which milk is consumed. As a result, there is a need for additional *in vivo* MRI investigations to study the impact of milk consumption temperature on gastric digestion. This also raises further questions regarding the effect of consumption temperature on other foodstuff that behaves differently to milk during digestion, such as carbohydrate rich foods.

## 4.7 References

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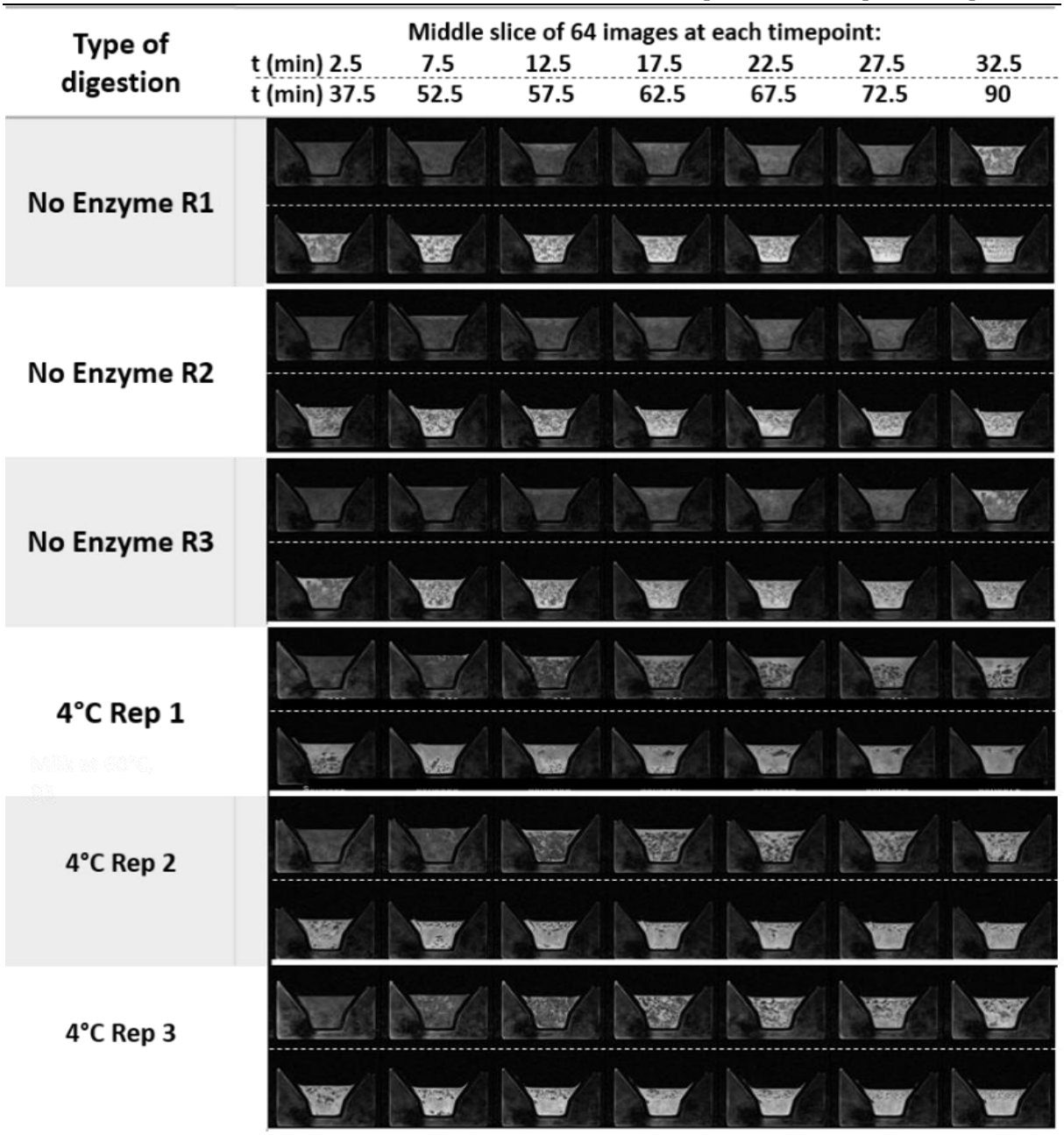
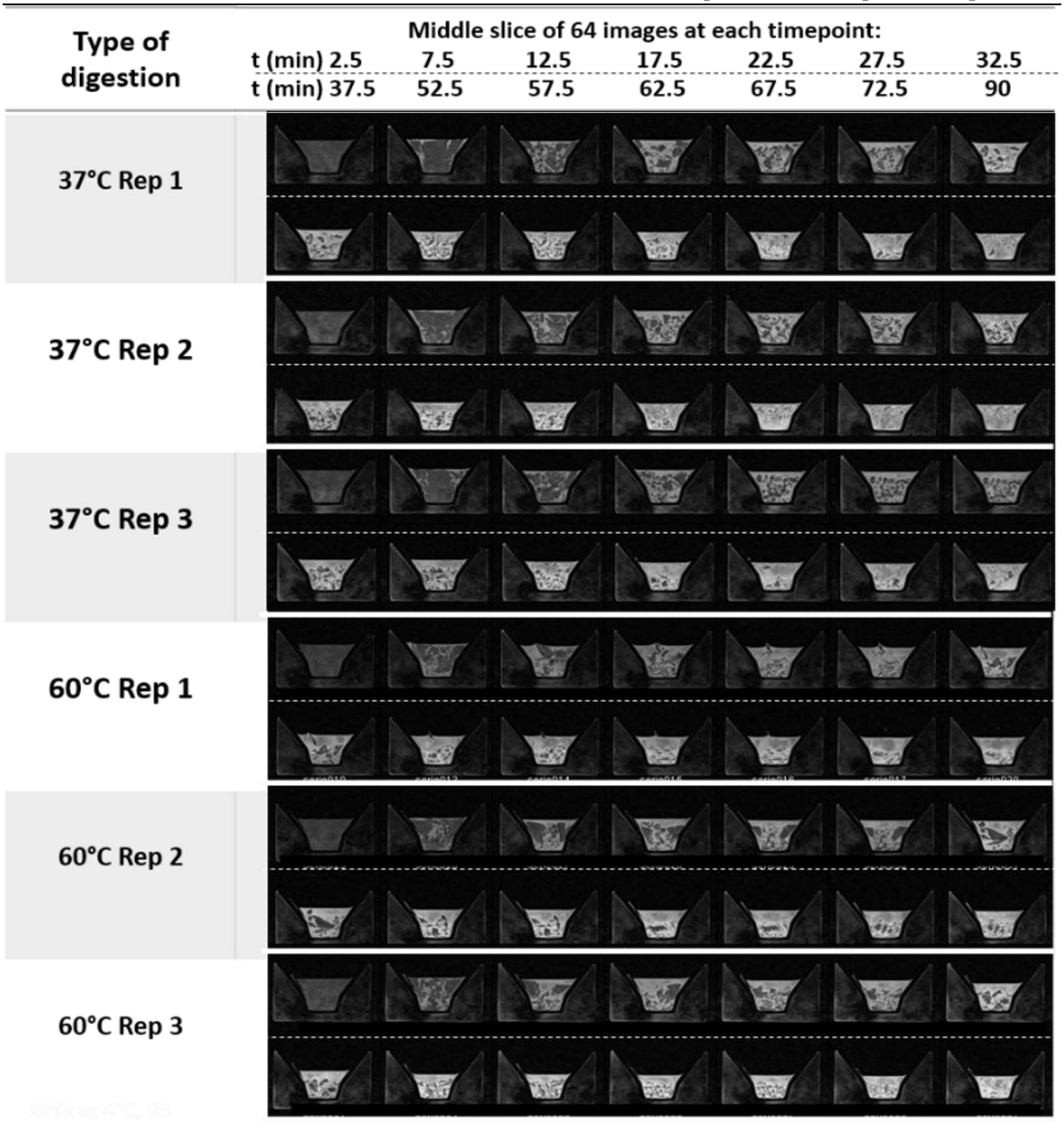
## 4.8 Supplementary material

**Supplementary figure 1.** Mean triacylglycerol composition of dairy fat as proposed in https://www.agroscope.admin.ch/agroscope/fr/home/themes/denrees-alimentaires/alimentation-sante/lait-produits-laitiers/graisse-du-lait.html

| Fatty acid | Formula | Content in g/100 g fat |
|---|---|---|
| Butyric | C4H8O2 | 3.13 |
| Hexanoic | C6H12O2 | 2.02 |
| Caprylic | C8H16O2 | 1.16 |
| Capric | C10H10O2 | 2.47 |
| Decanoic | C10H20O2 : C10:1 | 0.30 |
| Lauric | C12H24O1 | 2.95 |
| Myristic | C14H28O2 | 9.83 |
| Pentadecanoic | C15H30O2 | 1.08 |
| Palmitic | C16H32O2 | 26.11 |
| Palmitoleic | C16H30O2 | 1.25 |
| Margaric | C17H34O2 | 0.56 |
| Stearic | C18H36O2 | 8.07 |
| Oléic | C18H34O2 : C18:1 c9 | 16.47 |
| Linoleic | C18H32O2 : C18:2 c9,c12 | 1.21 |
| Alpha-linolénic | C18H30O2 : C18:3 c9,c12,c15 | 0.76 |
| Arachidonic | C20H32O2 : C20:4 (omega-6) | 0.15 |
| Eicosapentaenoic acid (EPA) | C20H32O2 : C20:5 (omega-3) | 0.08 |
| Docosapentaenoic acid (DPA) | C20H32O2 : C22:5 (omega-3) | 0.11 |
| Docosahexaenoic acid (DHA) | C22H32O2 : C22-6 (omega-3) | 0.01 |
| Various minor fatty acids | - | 22.28 |




**Supplementary figure 2**. As a complement to Figure 3 of the article, the entire kinetics for each replicate in each condition MRI images acquired with the Fast Recovery Turbo Spin Echo (FR-TSE) sequence are given below. Prior to onset of coagulation, the digesta, which is liquid, appears dark. After onset of coagulation, liquid digesta appears bright, and coagulated particles appear dark.




**Supplementary figure 3**. As a complement to Figure 3 of the article, the entire kinetics for each replicate in each condition MRI images acquired with the Fast Recovery Turbo Spin Echo (FR-TSE) sequence are given below. Prior to onset of coagulation, the digesta, which is liquid, appears dark. After onset of coagulation, liquid digesta appears bright, and coagulated particles appear dark.

# Chapter 5

# The combined effect of pH and temperature variations on the activity of human and porcine pepsin: A tool for protein digestion research

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*This chapter has been submitted for publication in Scientific Reports*

**CRediT authorship contribution:** C.J. Fitzpatrick, A. Brodkorb, and D. Freitas conceived and designed the study. C.J. Fitzpatrick and D. Freitas conducted the experimental work, with E. Hayes contributing specifically to spectroscopy-related methodology and data analysis. I. Comi, A.G. Røseth, and G.E. Vegarud were responsible for the collection of human gastric samples and performed preliminary pepsin activity assays prior to sample transfer. The initial manuscript draft was prepared by C.J. Fitzpatrick, D. Freitas, and A. Brodkorb. Project supervision was provided by D. Freitas, T.F. O'Callaghan, J.A. O'Mahony, and A. Brodkorb. All authors (C.J. Fitzpatrick, D. Freitas, I. Comi, G.E. Vegarud, A.G. Røseth, E. Hayes, T.F. O'Callaghan, J.A. O'Mahony, and A. Brodkorb) critically reviewed, edited, and approved the final manuscript.

## 5.1 Abstract

The digestion of food using *in vitro* digestion methods is becoming increasingly popular due to the introduction of standardised protocols and the validation of results with *in vivo* data. There is currently interest in understanding all aspects of *in vitro* digestion to improve reliability and reproducibility. Porcine pepsin is the major gastric enzyme used as a replacement for human pepsin during the gastric phase of *in vitro* digestion models. However, until now, limited information is available on the combined effect of the temperature and pH variations that occur during digestion on the activity of these enzymes. This study investigated the effect of pH 1-7 and temperature 4-60 °C on the activity of human and porcine pepsin, finding that human pepsin retained a broader activity range compared to porcine pepsin. Human pepsin retained 80% of its activity at pH 3 and 46% at pH 4, while porcine pepsin retained only 47 and 13%, respectively. Predictive activity models for both porcine and human pepsin were developed based on these findings. Additionally, porcine pepsin was shown to be completely inactivated when heated to 75 °C for at least 5 min, whereas heating to 65 °C for 15 min was inadequate to achieve complete inactivation.

This research enables users of standardised *in vitro* digestion models to compare results across different digestions of the same food. It also allows for the prediction of pepsin activity that would occur with human instead of porcine pepsin or vice versa, bridging findings from *in vitro* models to *in vivo* conditions. This research also informs users of the optimal inactivation conditions of porcine pepsin for sample preparation.

## 5.2 Graphical Abstract

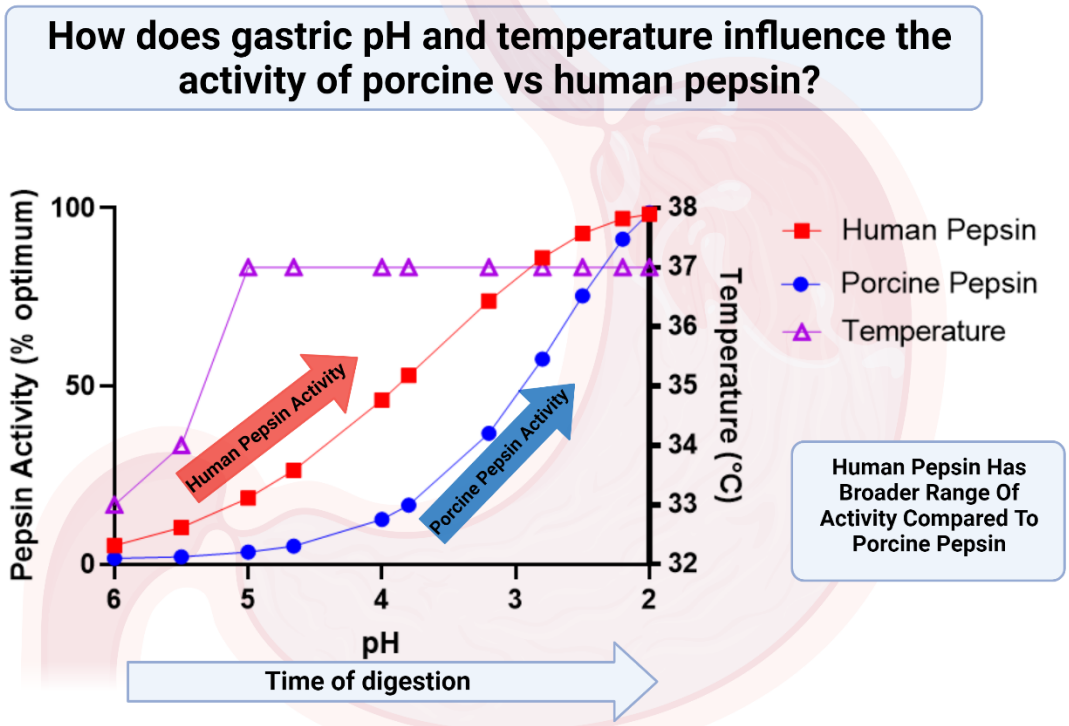


Figure 27 Graphical abstract displaying the difference between human and porcine pepsin activity during gastric digestion

## 5.3 Introduction

Pepsin (EC 3.4.23.1) is an endopeptidase that plays a critical role in digestion. This enzyme is secreted in an inactive form (pepsinogen) by gastric chief cells, and is activated on reaching the acidic environment of the stomach. As with all enzymes, its activity can be strongly affected by pH and temperature. As a result, changes in these parameters within the gastric compartment can influence the rate, pattern and extent of protein digestion.

The activity of pepsin in human gastric fluid (hereinafter referred to as human pepsin) has been shown to remain stable at pH up to 6; however while stable, very little activity remains at pH > 5.5, with optimum activity at pH 2 at 37 °C (Piper and Fenton, 1965). Gastric pH is strongly affected by the pH and buffering capacity of the meal consumed, as well as the dynamic aspects of gastric digestion (secretion of hydrochloric acid from parietal cells). Several studies have shown the large fluctuations in gastric pH, as described in Table 1.

Table 5 Summary of gastric pH observations in response to different meal types and compositions.

| Meal Description | Gastric pH Observations | Time to pH 2 | Reference |
|---|---|---|---|
| **1,000 kcal meal: hamburger, bread, hash brown potatoes, vegetables, milk (~600 g total weight).** | Fasted state pH: 1.7; increased to 4.3–5.4 (peak: 6.7) during ingestion; decreased to pH 2.0 gradually. | 107 ± 70 min | (Dressman et al., 1990) |
| **Meal: lean steak, boiled potatoes, vegetables, salad (575-675 g); dessert (200 mL), water (200 mL). Meal pH 5.9** | Gastric pH increased to 4.5 after meal ingestion; returned to pH 2.0 more gradually. | Average of 240 min | (Gardner et al., 2002) |
| **Liquid meal: 500 mL (376.5 kcal: 14 g protein, 52 g carbohydrate, 12.5 g lipid); meal pH: 7.** | Initial pH: ~7 (similar to meal pH); decreased gradually as the stomach emptied; reached pH 2 when 90% of the meal was emptied. | >150 min | (Carriere et al., 1993) |

Studies have also shown that a large variation in gastric temperature can occur due to the ingestion of a meal. When 400 mL of orange juice was consumed at 50°C, it was found that gastric temperatures increased to 43°C, while consuming the same beverage at 4°C caused gastric temperatures to reduce to 21°C (Sun et al., 1988). Gastric temperature then returned to within 1°C of body temperature within 20 min of ingestion of the warm drink and within 30 min of ingestion of the cold drink. McArthur and Feldman (1989) also investigated gastric

temperature fluctuations using 360 mL of coffee (infused into the stomach) as a test drink. They found that when coffee was consumed at 4 or 58°C, it took 16.7 ± 2.7 min and 23.8 ± 1.1 min to return to 37°C (McArthur and Feldman, 1989).

These findings demonstrate the large variations that can be observed in both gastric pH and temperature in the postprandial period and, since pepsin activity is affected by these parameters, suggest possible implications for protein hydrolysis during this digestive step(Yang et al., 2023).

Studying digestion *in vivo* is often difficult; therefore, *in vitro* digestion studies are becoming increasingly common. These *in vitro* digestion protocols use simulated digestion fluids and non-human enzymes to mimic *in vivo* digestion. Porcine pepsin is commonly used as a replacement for human pepsin as it exhibits some important similarities with the human counterpart, while being more readily available. Both human and porcine pepsin exhibit similar configurations, displaying 84% structural conservation (Fujinaga et al., 1995) and both enzymes have been shown to work with optimum activity at pH close to 2 and temperature close to 37°C (Piper and Fenton, 1965, Wang et al., 2017, Eriksen et al., 2010, Schlamowitz and Peterson, 1959). However, the effect of concurrent variations in pH and temperature of the food when ingested in each of these enzymes remains poorly understood. As a result, establishing comparisons between the proteolytic capacity of human and porcine pepsins is not straightforward.

As well as this, regardless of the type of *in vitro* digestion used, pepsin activity must be inhibited in digesta samples collected throughout the experiments and after digestion to prevent over estimation of protein breakdown during analysis. Several methods to inhibit enzyme activity exist and should be chosen based on the enzymes present and on the post-digestion analysis to be carried out (Brodkorb et al., 2019, Minekus et al., 2014). One method of pepsin inhibition that is commonly used is heat treatment at 100°C. Indeed, exposure to high temperatures can result in the loss of structural integrity of pepsin, and consequent inactivation.

However, this may have repercussions for other components of interest in the samples and possibly impact the outcomes of the study. Therefore, it is best to use the lowest heat treatment possible to minimise the impact on other food compounds, e.g. proteins in the digesta.

Consequently, the main aim of this study was to understand the combined effect of variations in environmental pH and temperature on the activity of human and porcine pepsin and to develop a user-friendly tool that allows users to predict and compare the proteolytic activity of these two enzymes based on the pH and temperature conditions during *in vitro* digestion. Furthermore, this paper aims to investigate the minimum heat treatment required to inhibit pepsin activity in *in vitro* digestion samples.

## 5.4 Materials and methods

### 5.4.1 Materials

Bovine haemoglobin (SBV9700, Sigma-Aldrich®) was purchased from Merck (Co. Wicklow, Ireland). Haemoglobin was dissolved 2% (w/v) in water. Porcine pepsin (SLBZ 7282, Sigma-Aldrich®), also purchased from Merck (Co. Wicklow, Ireland), was dissolved to 1 mg/mL in 10 mM tris buffer with 150 mM NaCl at pH 6.5. Human gastric fluid pepsin was collected which contained pepsin (Refereed to in this paper as human pepsin) from a total of 10 fasting volunteers at Lovisenberg Diaconal Hospital, Oslo. GI fluids were collected, treated and further tested as detailed described according to Asledottir et al. (2024).

### 5.4.2 Ethical approval

The aspiration of gastric fluid and its subsequent storage were conducted in accordance with ethical guidelines and approved by the Norwegian Ethics Committee. The study protocol for gastric fluid aspiration was approved under ethics license number REK 2012/2030. Additionally, the storage of human aspirates in the Biobank was approved under ethics license number REK 2012/2010. All volunteers provided written informed consent prior to participating in the study.

### 5.4.3 Pepsin activity determinations

When pepsin is assayed using the method outlined in the standardised INFOGEST protocol, it is recommended to use bovine haemoglobin as a substrate (Brodkorb et al., 2019, Anson and Mirsky, 1932). During this assay, pepsin is incubated with haemoglobin, releasing tyrosine-containing peptides soluble in trichloroacetic acid (TCA) that can be detected spectrophotometrically (at 280 nm). Unit definition is as follows "One unit will produce a $\Delta A_{280}$ of 0.001 per min at pH 2.0 and 37°C, measured as TCA-soluble products" (Brodkorb et al., 2019). In the present study, both porcine and human samples were firstly assayed in triplicate, using this method (at pH 2, 37°C) and the resulting values were taken as the respective maximum activities. A modified version of the assay was then used to analyse each sample at a range of pH and temperature conditions (pH 1-7, temp 4–60°C) as described below.

### 5.4.4 Activity of human pepsin under optimum pH and temperature

A 50 µL/mL solution of human pepsin in 10 mM HCl was prepared. Haemoglobin solution was adjusted to pH 2 and heated to 37°C. An aliquot (100 µL) of the human pepsin solution in 10 mM HCl was added to 500 µL of haemoglobin and incubated for 10 min at 37°C. Following this, 1 mL of TCA (5% w/v) was added to stop the reaction. Samples were centrifuged (6,000 × g, 4°C, 30 min) using a Thermo Scientific Heraeus Multifuge X1R (Thermo Fisher Scientific, USA) centrifuge and supernatant was removed to fresh tubes. Absorbance of supernatant was read at 280 nm.

### 5.4.5 Activity of porcine pepsin under optimum pH and temperature

The same procedure was followed, starting from a 1 mg/mL porcine pepsin solution which was diluted to 20 µg/mL in 10 mM HCl and used in place of the human pepsin.

### 5.4.6 Effect of pH and temperature on human and porcine pepsin activity

A response surface methodology (RSM) approach was used to systematically select pH and temperature combinations relevant to gastric digestion (Hooda et al., 2012). A D-optimal

design was generated using Design Expert 12 (Design-Expert® version 12.0.9.0, Stat-Ease Inc., USA), selecting 26 combinations of pH and temperatures relevant to gastric digestion to ensure a balanced and orthogonal experimental design. Additionally, 11 extra points were included to optimise the model for semi-dynamic digestions. The impact of these 11 pH and temperature combinations on porcine pepsin had been previously studied in our laboratory, and presented with results of an *in vitro* digestion study by Freitas et al. (2022b). Human and porcine samples were therefore assayed at a total of 37 different temperature and pH combinations (presented in Supplementary Tables 1 and 2, respectively). The pH and temperature of the haemoglobin solution (500 µL) was adjusted to the required conditions prior to the addition of human or porcine sample solutions (100 µL) prepared as described above. The incubation was then followed at the required temperature prior to the TCA precipitation and spectrophotometric determination steps carried out according to the recommended protocol described above (Brodkorb et al., 2019). Enzyme activity was expressed as proportion (%) of optimum activity, calculated by dividing obtained absolute activity by the activity of the same enzyme at pH 2, 37°C. When not in use, stock and sample solutions were stored on ice.

### 5.4.7 Development of predictive pepsin activity models

Using the pepsin activity data described in the previous section, two pepsin activity prediction models were developed using Design Expert for porcine and human pepsin activity. For porcine pepsin, A base 10 log transformation was used on the data due to a high maximum to minimum value ratio (114.3 to 0.54 = ratio of 212.80). Terms included in the model were the intercept, A (pH), B (Temp), AB, $A^2$, $B^2$, and $AB^2$. The F-Value of this model was 17.63, indicating a significant model, with a 0.01% chance of an F-value of this size occurring due to noise. This model had a predicted $R^2$ of 0.63 and an adjusted $R^2$ of 0.75. To improve the model, three outliers, identified by using residuals *vs.* predicted plots, were omitted from the data (Run No.6, pH 3, Temp 4°C, measured activity 20.62% and runs 26 and 27 pH 7, 37 °C, measured activities 22.62, 7.63 and 11.28%, respectively).

For human pepsin, A base 10 log transformation was used on the data due to a high maximum to minimum value ratio (100 to 0.6 = ratio of 165.4). Terms included in the model were the intercept, A (pH), B (Temp), AB, $A^2$, $B^2$, $AB^2$, $A^3$, and $B^3$. The F-Value of this model was 29.39, indicating a significant model, with a 0.01% chance of an F-value of this size occurring due to noise. This model had a predicted $R^2$ of 0.81 and an adjusted $R^2$ of 0.86. To improve the model, one outlier, identified by using residuals *vs.* predicted plots, was omitted from the data (Run No.23, pH3, 37°C – measured activity 14.3%).

The produced equations were integrated into separate Excel spreadsheets (Supplementary material Excel file), created to allow users to input specific pH and temperature values from their own *in vitro* digestions. These tools then allow to predict pepsin activity (either human or porcine) throughout digestion experiments as a percentage relative to the optimum conditions (pH 2 and 37°C).

### 5.4.8 Case Studies

To test the spreadsheets developed and explore the potential uses of this work in future protein digestion studies, three case studies have been conducted.

*Case study 1* has been defined to test the model's suitability to establish comparisons between projected levels of pepsin activity by human *vs.* porcine pepsin samples during gastric digestion *in vitro*. The conditions of an *in vitro* semi-dynamic gastric digestion of whole milk (20 mL), following standardised INFOGEST protocols, were used to capture pH and temperature data (Brodkorb et al., 2019; Mulet-Cabero et al., 2020). This data was input to the relevant pepsin activity prediction model, to predict the difference in activity between human and porcine pepsin.

*Case study* **2** has been outlined to test the model's usefulness in comparing predicted pepsin activity levels when using different *in vitro* digestion models. The conditions of a semi-dynamic *vs.* a static digestion protocol applied to whole milk were compared following the

respective standardised INFOGEST protocols (Brodkorb et al., 2019; Mulet-Cabero et al., 2020). In both scenarios, the standard digestion temperature (37°C) was considered and, in combination with the respective pH data, entered the spreadsheets developed (see Supplementary Material) to estimate pepsin activity levels (expressed as the percentage of optimal porcine pepsin activity) in each scenario based on the predictive models that were developed in the present study.

*Case study 3* has been conducted to compare estimated pepsin activity levels in adults to those in older adults during digestion of a model meal, based on parameters extracted from the literature (Table 2). The basal gastric volume was set at 33 mL, as reported by Bryson Roberts et al. (2007). The basal concentration of pepsin in the gastric fluid was 0.4 mg/mL, also referenced from Bryson Roberts et al. (2007). For the simulations, a meal volume of 50 mL containing 50 kcal was used. The gastric emptying rate was set to 2 kCal/min equalling 2 mL/min in this case (Watson et al., 2019). The gastric secretion rate was established at 1 mL/min, set at normal fasting rate of secretion for the purpose of this model (Roelofs et al., 2024). Additionally, for simulations modelling digestion in older adults (69-98 years old), a 40% reduction in pepsin output was incorporated, based on findings by Feldman et al. (1996). These parameters were taken into consideration alongside gastric pH curves, based on approximate pH curves from two *in vivo* digestion studies to estimate total pepsin activity during an *in vivo* gastric digestion (Figure 29C) (Malagelada et al., 1979, Gardner et al., 2002).

### 5.4.9 Inactivation of pepsin

5.4.9.1 Impact of temperature and time

To determine the heat treatment required to irreversibly denature porcine pepsin, the following conditions have been tested. A porcine pepsin solution in water (20 µg /mL) was preheated to 65, 75, 85 or 95°C for 5, 10 or 20 min. Following this, the pepsin was cooled to 37°C and assayed under optimum conditions (pH 2, 37°C) as described above. Three repetitions were carried out for each condition.

Table 6 Parameters selected to estimate total pepsin activity in vivo

| Parameter | Value | Units | Reference |
|---|---|---|---|
| Basal gastric volume | 33 | mL | (Roelofs et al., 2024) |
| Basal pepsin concentration | 0.4 | mg/mL | (Bryson Roberts et al., 2007) |
| Rate of pepsin increase in stomach | 46.8 | mg/hour | (Bryson Roberts et al., 2007) |
| Meal volume | 50 | mL | - |
| Meal energy | 50 | kcal | - |
| Meal emptying rate | 2 | mL/min | (Lentner, 1986, Roelofs et al., 2024) |
| Rate of gastric secretion | 1 | mL/min | |
| Pepsin output reduction in older adults | 40 | % | (Feldman et al., 1996) |

5.4.9.2 Study of conformational changes by Fourier Transform Infrared spectroscopy

FTIR analysis was used to visualise structural changes in porcine pepsin activity following heat treatment at 65, 75, 85 or 95°C for 5, 10 or 15 min. A Bruker Invenio S with a BioATR with zinc selenide crystal attachment was used (Bruker Optics GmbH, INVENIO 100453, Ettlingen, Germany). Liquid nitrogen was used to cool the MCT detector, and the sample compartment was purged to remove moisture and carbon dioxide. Bruker OPUS 5.5 software (Bruker, Germany) was used to obtain the spectra in absorbance mode. Each spectrum was measured in duplicate and consisted of 50 scans averaged. The background was measured with water and was subtracted from the spectrum. Atmospheric correction pre-treatment was applied to remove moisture and carbon dioxide. Vector normalisation was carried out in the

amide region (1,500-1,700cm$^{-1}$) to eliminate baseline effects. Spectra from native pepsin were subtracted from those of heat-treated samples (Kehoe et al., 2008).

### 5.4.10 Statistical analysis

Statistical analysis was conducted using Design Expert version 12.0 (Stat-Ease Inc., USA). The activities of human and porcine pepsin at the different temperature and pH combinations tested were fitted to a reduced cubic model, and analysis of variance (ANOVA) was used to evaluate the significance of the model and its terms. The quality of the model fit was assessed through the coefficients of determination: $R^2$, adjusted $R^2$, and predicted $R^2$. Model adequacy was further verified using diagnostic plots, including the normal plot of residuals to evaluate the normality assumption and residuals *vs.* predicted values to identify potential outliers and assess the homoscedasticity of residuals. Outliers were identified using residual *vs.* predicted plots in Design Expert version 12.0. Visualisation of data and results was performed using both Design Expert 12.0 and GraphPad Prism version 9.0 (GraphPad Software LLC, USA). Results and Discussion

## 5.5 Results

### 5.5.1 Pepsin activity at optimum pH and temperature

Porcine and human pepsin activities were determined at pH 2 and 37°C. Porcine pepsin activity was measured at 3,095.2 ± 328.7 U/mg. Human gastric fluid (HGF) pepsin activity was assessed in two laboratories—Teagasc Moorepark, Ireland, and the Norwegian University of Biosciences (NMBU), Norway—to confirm that activity was not lost during transport on dry ice. The activity measured in Ireland was 931.0 ± 110 U/mL, which closely aligned with the measurement in Norway (944 ± 1,127.90 U/mL). The values obtained in Ireland were established as the optimal (100%) pepsin activities and were used as references to calculate the percentage of pepsin activity at different pH and temperature conditions.

### 5.5.2 Production of human and porcine pepsin activity prediction models

Following the activity determinations under optimum pH and temperature conditions, each type of pepsin (human or porcine) was analysed at 37 different pH and temperature combinations (Supplementary Table 1 and 2, respectively) using a modified version of the INFOGEST pepsin assay (Anson, 1938) (described in Section 2.2). The data collected from these analyses were used to create the human and porcine pepsin activity models. The human pepsin predictive equation is displayed in equation 1. From this equation, both contour graphs and 3D surface model graphs were created, highlighting areas of differentiated pepsin activity (Figure 1 A and B). Human pepsin had high activity close to its optimum conditions at pH 2, 37°C. Its proteolytic activity then decreased as conditions moved away from this pH and temperature, as evidenced by the transition from the red to the blue area of the graph.

$$\log(Pepsin\ Activity) = -0.0702332 + (0.670378 * \mathrm{pH}) + (0.085 * \mathrm{Temp}) - (0.012 * \mathrm{pH} * \mathrm{Temp}) - (0.0765 * pH * pH) - (0.001 * Temp * Temp) + (0.000266 * pH * Temp * Temp)$$

Equation 1: Human Pepsin activity prediction equation. Users can input pH and temperature, giving the result as a percentage of optimum activity

The porcine pepsin predictive equation is displayed in equation 2. From this equation, both contour graphs and 3D surface model graphs were created (Figure 27 C and 27D*).* Porcine pepsin exhibited high activity close to its optimum conditions at pH 2, 37°C. However, its activity quickly decreased as conditions moved away from this point, as indicated by the green and blue colours.

$$log(Pepsin\ Activity) =$$

$$= -0.104906 + 1.50841 * pH + 0.036848 * Temp + -0.0208757 * pH * Temp + -0.492622 * pH^2 + 0.000705041 * Temp^2 + 0.000450138 * pH * Temp^2 + 0.041292 * pH^3 + -0.000025538 * Temp^3$$

Equation 2: Porcine Pepsin activity prediction equation. Users can input pH and temperature, giving the result as a percentage of optimum activity

### 5.5.3 Comparison between human pepsin and porcine pepsin

When comparing the activity profiles of porcine and human pepsin (Figure 27), differences in their behaviour across pH and temperature ranges were evident. Human pepsin demonstrated broader regions of high activity, indicated by red areas on the graphs (Fig 27 (A) and 27 (B)), signifying that it retained greater enzymatic activity over a wider range of temperatures and pH levels compared to porcine pepsin (Figure 27 (C) and 27 (D)). At pH 2 and 37°C, human pepsin displayed peak activity. Even as the pH was increased to 3, human pepsin retained over 90% of its activity at 37°C, with activity still at approximately 38% at pH 4 under the same temperature conditions. In contrast, porcine pepsin exhibited a more rapid decline in activity as pH increased. At pH 2 and 37°C, porcine pepsin displayed optimum activity, similar to human pepsin. However, at pH 3 and 37°C, the activity decreased to ~57%, decreasing further to below 9% at pH 4. This sharp reduction was more pronounced compared to human pepsin, which still retained considerable activity at higher pH levels. This demonstrated the more restricted range of activity of porcine pepsin, which rapidly lost activity as pH increased beyond 2.50, particularly at physiological temperature (37°C).

Other factors may also explain the broader range of activity observed in human pepsin compared to porcine pepsin. For instance, human pepsin only underwent centrifugation to

separate from mucin; as such, it must be considered that it may contain iso-enzymes of pepsin and enzymes other than pepsin, such as gastricsin (Tang et al., 1967, Eriksen et al., 2010). This may partly explain the broader activity range identified in our study, as gastricsin (Pepsinogen II) has been shown to have an optimum pH at 2.9, and also shows specificity towards the substrate used in the current study (haemoglobin) (Becker and Rapp, 1979). In contrast, the porcine pepsin sample was a commercial enzyme preparation which had been subjected to several purification processing steps.

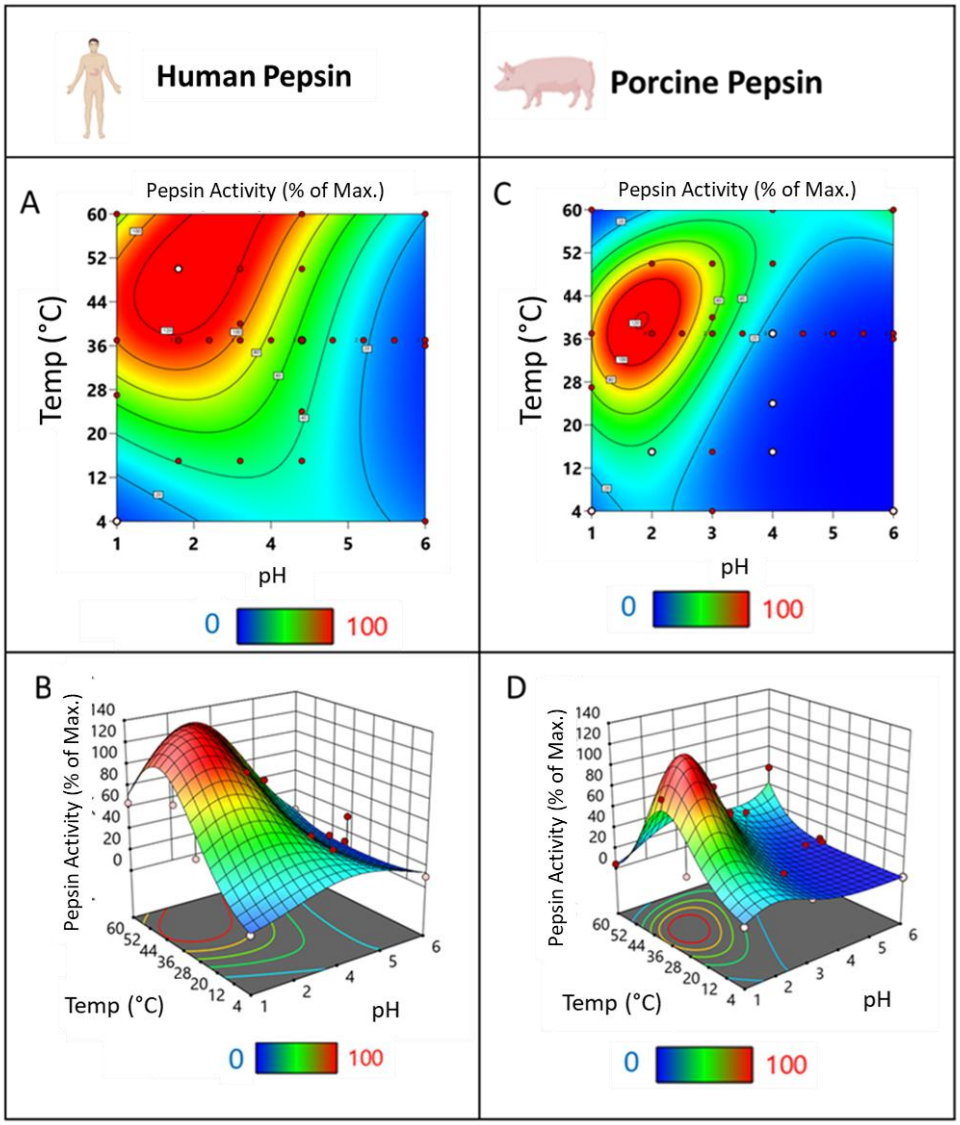


Figure 28 Figure 28 Effect of pH and temperature on pepsin activity. Contour graphs (A and C) and 3D surface models (B and D) of pepsin activity in human pepsin (A and B) and porcine pepsin (C and D) across a range of pH and temperature (pH 1-7, temp 4°C – 60°C). Blue areas represent low activity while red areas represent high activity. Pepsin activity is expressed as a percentage of the activity measured under optimum conditions (pH 2 and 37°C) using haemoglobin as substrate, i.e., 931.0 ± 110 U/mL and 3,095.2 ± 328.7 U/mg for human and porcine pepsin, respectively. One unit is defined as the amount of enzyme that produces a ΔA280 of 0.001 per min at pH 2.0 and 37°C, measured as TCA-soluble products. Panels B and D show potential activity over 100% due to pepsin activity at certain condition being higher than at pH 2, 37°C.

Nevertheless, these findings can have implications for *in vitro* digestion studies as our work suggests that the proteolytic capacity of porcine pepsin under dynamic gastric pH and temperature conditions *in vitro* may differ from what would be expected from human pepsin.

### 5.5.4 Case studies

The prediction models created as part of this study for porcine and human pepsin were designed to allow users to quantify pepsin activity throughout *in vitro* gastric model digestion and to compare human *vs.* porcine pepsin directly. Users can input pH and temperature data from their digestion studies into the Excel-based spreadsheets. These tools calculate pepsin activity at each data point relative to optimal conditions (pH 2, 37°C), enabling visualisation of activity profiles and area under the curve (AUC) analysis for comparative assessments.

*Case study 1*: To provide an example of the use of this model, gastric digestion parameters (temperature, time, pH) were estimated based on an *in vitro* semi-dynamic gastric digestion of whole milk (Mulet-Cabero et al., 2020a). Temperature was assumed to be 37°C for all points in each digestion. This data was then input to both the porcine pepsin model and the human pepsin model; Using both the porcine and human pepsin prediction models, similar curves for human and porcine pepsin during *in vitro* semi-dynamic digestion were observed (Figure 28, (A)), with pepsin activity being low at the start of digestion and slowly increasing as pH decreases during digestion. However, human pepsin showed a broader range of activity, as illustrated by a greater Area Under the Curve (AUC) value *vs.* porcine pepsin (Figure 28 (B).

*Case study 2:* To test the application of these models for comparisons of the levels of pepsin activity in different *in vitro* digestion models, estimated porcine pepsin activity was compared in static *vs.* semi-dynamic digestion (Figure 28 (C)). Static *in vitro* digestion, as expected, showed the same activity throughout digestion due to the maintenance of a constant pH (pH 3), while pepsin activity increased throughout the semi-dynamic digestion. AUC calculations

(Figure 28 (D)) were again performed, showing that despite static digestion never reaching the optimum pH of pepsin (pH 2), it had a higher AUC value compared to semi-dynamic digestion over a 240 min gastric digestion. The main reason for this is that in semi-dynamic digestion, users should add HCl to "reach pH 2 at the end of the gastric digestion" (Mulet-Cabero et al., 2020a). Despite these differences, studies have found a lower release of free amino acids from milk proteins when comparing the static INFOGEST gastric digestion to a dynamic model (Egger et al., 2019). This could be due to several confounding factors, such as improved mixing in the dynamic system compared to the static system allowing improved pepsin access to substrate, or a different pH curve in the dynamic model, leading to increased pepsin activity. However, the different substrates in each of these studies are likely one of the main reasons for these differences. Our prediction model would suggest that a higher degree of hydrolysis should be seen when using the static model. In the present experiment, it was shown, using haemoglobin as a substrate that the activity of porcine pepsin was negligible at pH > 4 and increased at pH 3, to an optimum activity at around pH 2. These findings agree with those of Crévieu-Gabriel et al. (1999) and Pletschke et al. (1995a), where haemoglobin was used as a substrate for pepsin activity. In the presence of a different substrate, such as the milk proteins it is possible that pepsin is active at a higher pH (Egger et al., 2019). Indeed, changes to the pH range at which porcine pepsin is active with different substrates have been shown elsewhere (Salelles et al., 2021). Porcine pepsin activity, when measured by the rate of hydrolysis of casein micelle micro aggregates, has been shown to be almost constant in the pH range 1-5 (Salelles et al., 2021). Interestingly, this is consistent with findings from a previous *in vitro* semi-dynamic digestion study, where rapid milk coagulation was observed at the start of the gastric phase (pH ≈ 6) (Fitzpatrick et al., 2024). These findings indicated that pepsin was active at the beginning of digestion, when pH was above the isoelectric point of casein, showing that, although pepsin activity is negligible at high pH when the substance is haemoglobin, activity levels were sufficient to cause coagulation when the substrate was casein.

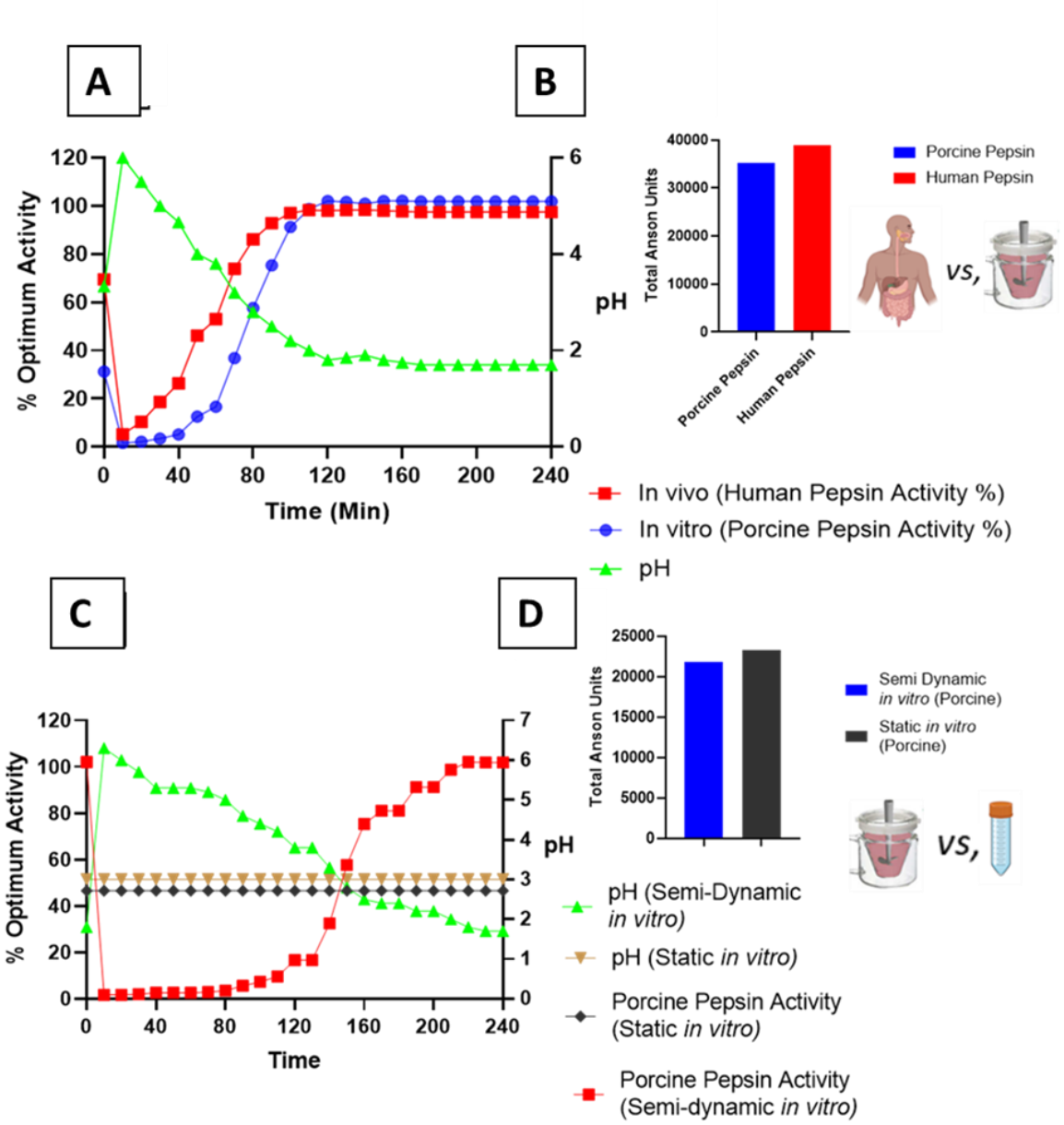


Figure 29 Comparison of different digestion methodologies in terms of pepsin activity. (A) Using the same pH curve (Green Triangles), predicted human pepsin activity (Red squares) was compared to predicted porcine pepsin activity over an entire gastric digestion of milk. (B) Estimate human pepsin activity was compared to estimated porcine pepsin activity using an area under the curve analysis. (C)) The predicted porcine pepsin activity was compared in a semi-dynamic in vitro digestion (Red squares) to a static in vitro digestion (Black Diamonds). Semi-dynamic pH (green triangles) decreased gradually over digestion, while static pH remained constant throughout (Brown upside down triangles).

The capacity of human and porcine pepsin to hydrolyse specific substrates may also be different. As noted by Eriksen et al. (2010), pepsin isoforms may interact differently with whey protein compared to haemoglobin. Other studies have also shown that altering the substrate can influence pepsin activity and stability, with Schlamowitz and Peterson (1959) finding that the denaturation of substrates prior to interaction with hog pepsin can increase the pH range that pepsin is active.

*Case study 3:* The pepsin activity prediction model developed in this study can be applied to *in vivo* data to estimate pepsin activity levels during gastric digestion under different conditions. In the present study, adult *vs.* elderly digestive conditions were compared. Several physiological parameters must be considered in this type of analysis, including basal gastric volume, postprandial gastric fluid volume, and pepsin concentration (Table 2). For this simulation, the ingestion of a 50 mL, 50 kcal liquid meal has been considered. By considering gastric fluid secretion rate (0.9 mL/min), rate of pepsin increase (46.8 mg/h) and gastric emptying rate (2 mL/min), the total amount of pepsin in the stomach is calculated at any given time. Pepsin activity is estimated by multiplying the total amount of pepsin by the optimal activity value (2,000 U/mg), and the actual activity is predicted using conditions specific to stomach pH and temperature, based on pH data obtained from Gardner et al. (2002) and Malagelada et al. (1979). As well as this, the model allows for the simulation of reduced pepsin activity in older adults by implementing a 40% reduction in pepsin output (Feldman et al., 1996).

Figure 28 (A) shows the predicted change in pepsin activity during digestion of a meal considered here for both adults and older adults *in vivo*. Total pepsin units (main y-axis) are initially low due to the low amount of pepsin present in the stomach, paired with unfavourable conditions for pepsin activity (pH 6) in adults (blue curve). As digestion progressed, an increase in pepsin activity units was observed as more pepsin was secreted into the stomach.

At the same time, pH decreased towards pH 2 due to hydrochloric acid secretion, further increasing pepsin activity. After approximately 100 min of digestion, pepsin activity units began to decline as the favourable gastric conditions no longer offset the loss of pepsin due to gastric emptying. A similar trend is seen for older adults (red curve), but with a 40% reduction in pepsin output, resulting in a peak pepsin activity of just over 20,000 units compared to 42,000 units in adults. On the secondary y-axis, pepsin concentration in gastric fluid (mg/mL) is shown for both adults (blue) and older adults (red). Pepsin secretion rate exceeds the loss of pepsin through gastric emptying, leading to a continuous increase in pepsin concentration throughout the digestion period. Over the entire digestion, the area under the curve values for adult total pepsin units is almost twice the value observed for older adults ($3.6\times10^{\wedge9}$ *vs.* $2.1\times10^{\wedge9}$) for adult *vs.* older adult, respectively) *(*Figure 30 (B)).

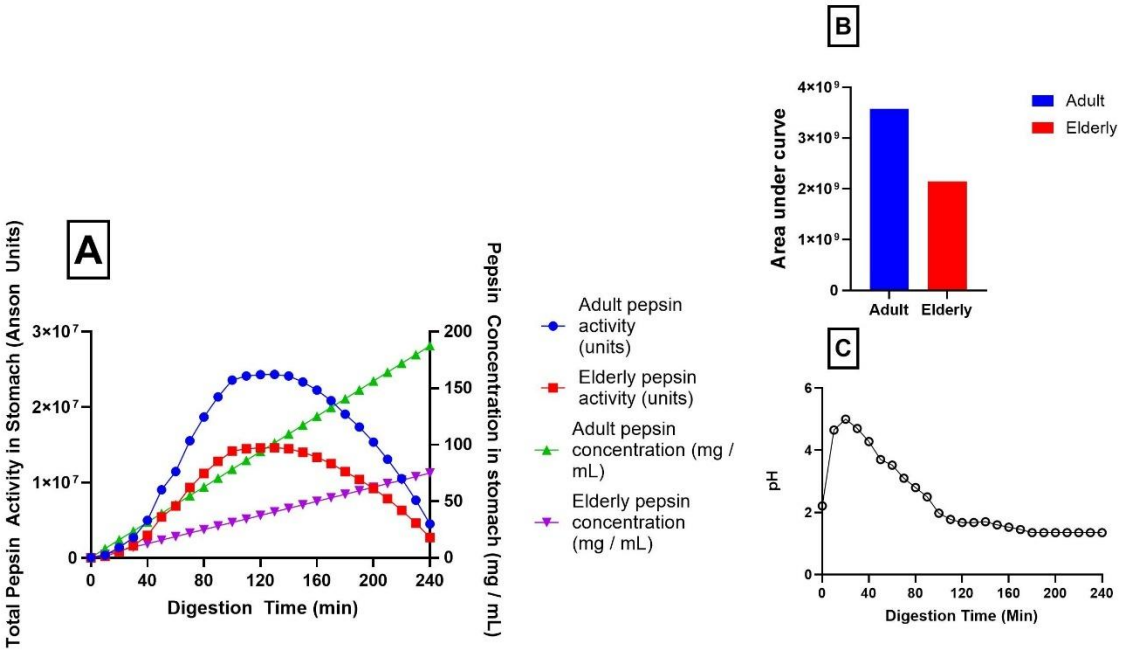


Figure 30 Simulation of total pepsin activity (units) in stomach after the consumption of 50 mL, 50 kCal liquid meal, accounting for basal pepsin concentration, pepsin secretion rate, gastric emptying of pepsin, and pepsin activity based on pepsin activity prediction model. (A) Pepsin concentration continues to increase throughout digestion due to continuous pepsin secretion. Pepsin activity increases to approximately 90 min and then begins to decrease due to decreasing total volume of pepsin in stomach due to gastric emptying. (B - Inset) Area under the curve calculation for adult vs. older adult digestion. (C) pH curve used for this simulation.

### 5.5.5 Heat deactivation of pepsin

Heating is a common method of enzyme deactivation used to stabilise samples collected during *in vitro* model digestions. However, if the intensity of heat treatment is too high, it may negatively impact on other proteins of interest in the samples, potentially influencing the outcomes of subsequent analyses. Therefore, best practice is to apply the minimum heat treatment necessary to irreversibly denature pepsin, reducing the risk of undesirable effects on other labile components.

In the present study, porcine pepsin was heated to between 65 to 95°C for set time periods (5, 10 or 15 min), and activity was subsequently measured at 37°C. The measured activity was compared to the previously determined 'optimum' activity and expressed as a percentage of that value, as shown in Figure 31 A. Heating for 15 min at 65°C was insufficient to fully deactivate pepsin; after 5 min of heating at 65°C, pepsin retained 95.35 ± 0.75% of its activity. Extending the heating duration to 10 or 15 min led to a significant reduction in activity, but 72.37 ± 0.26% and 45.21 ± 0.43% of activity was still retained, respectively. A significant difference ($p < 0.01$) was found at 65°C between 5 and 10 min ($p < 0.01$), 5 and 15 min ($p < 0.01$), and 10 and 15 min ($p < 0.01$). Heating at higher temperatures led to faster and complete inactivation of pepsin, with 5 min at 75°C being sufficient to achieve irreversible inactivation. All heat treatments tested at 75°C, and above, were effective in irreversibly inactivating pepsin and no significant difference was found between heating times. When comparing temperatures, significant differences were found between 65°C and all other temperatures.

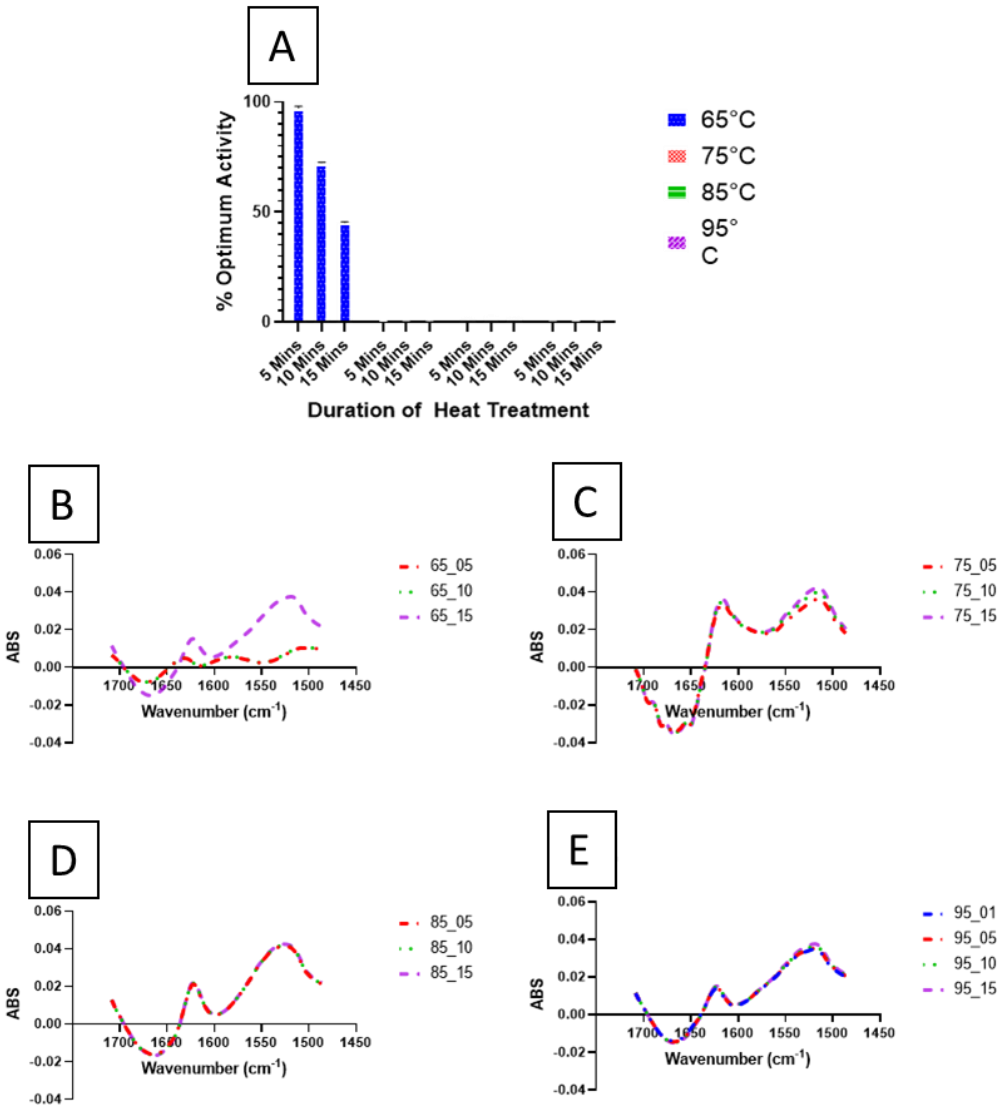


Figure 31 Thermal inactivation of porcine pepsin and corresponding FTIR spectral analysis. (A) Pepsin activity was measured at 37°C (pH 2, using haemoglobin as substrate) following heat treatment at 65°C, 75°C, 85°C, and 90°C for 5, 10, and 15 min. Activity was preserved after heating at 65°C across all time points, while any exposure above 75°C for 5 min resulted in irreversible inactivation (hence, no bars are visible at T > 75°C). (B) FTIR spectra of pepsin under various thermal conditions with native pepsin spectra subtracted: (B) 65°C, (C) 75°C, (D) 85°C, and (E) 95°C. The native pepsin spectrum is provided in supplementary materials (Sup. Figure 4)

To further investigate the structural changes induced by the different heat treatments and the reversibility of pepsin denaturation after heat treatment, FTIR analysis was carried out on porcine pepsin samples subjected to the same heat treatments (heating to 65–95°C, over 5–15

min). The results are presented in Figure 30, showing FTIR spectra for samples heated at 65°C (B), 75°C (C), 85°C (D), and 95°C (E). The amide I and amide II regions, (1,700-1,600 $cm^{-1}$ and 1,570-1,500 $cm^{-1}$, respectively) were analysed to examine changes in pepsin structure upon heating. The region 1,630-1,640 $cm^{-1}$ is associated with β-sheet structural changes in proteins (Markoska et al., 2019). A peak shift from 1,636 to 1,626 $cm^{-1}$ was observed after 15 min of heating at 65°C (Figure 30 (B), and at all-time points, in all measured temperatures greater than 65°C (Figure 30 (C-E), indicating a tighter packing of β-sheets, possibly due to aggregation, which occurs when protein is denatured. When temperature was increased from 65 to 75°C and higher, an increase in absorbance in this amide I region was seen, which could be related to increased intermolecular β-sheet structures, and an increase in protein unfolding (Ren et al., 2022).

The *amide II* region is mainly associated with N-H bending and C-N stretching vibrations of molecules. Changes in absorbance in this region can be related to changes in the α-helical structure of proteins, or conformational changes in proteins. There was an increase in absorbance in the *amide II* region with heating at 65°C for 15 min (compared to the same temperature and shorter periods), likely resulting from unfolding of the secondary structures. These absorbance levels were comparable to those observed for all heating periods at temperatures of 75, 85 and 95°C. At these temperatures, pepsin was rapidly denatured with no activity detected (at 37°C), regardless of the duration (Figure 30 A). Interestingly, nearly overlapping spectra were observed for samples heated 5 and 15 min at each of these higher temperatures. These findings, when considered with the fact that pepsin activity is not recovered after heating to 75°C and above, suggests that the conformational changes to pepsin are irreversible after heating to this temperature. Conversely, the activity of pepsin after heating at 65°C, even after 15 min, is partly retained (upon cooling to 37°C). This finding can be explained by the FTIR data, where no peak shift can be seen at the amide 1 region when pepsin is heated to 65°C, compared to clear changes in this region at all other tested temperatures.

## 5.6 Conclusion

This study demonstrates how human and porcine pepsin differ in activity across varying pH and temperature levels, showing the potential impact on proteolysis when substituting porcine pepsin for human pepsin in *in vitro* digestion protocols. Predictive models for pepsin activity were developed, providing tools to predict activity levels under different digestion scenarios and for various population groups. A limitation of the models is their reliance on haemoglobin as a substrate, which may not fully reflect real food matrices. Future work should explore alternative substrates, such as caseins or plant proteins, to improve the applicability of these models. This study also confirms that a 5 min heat treatment at 75°C is sufficient to irreversibly denature pepsin after digestion. These results contribute to a better understanding of *in vitro* gastric protein digestion and presents a tool for improving future digestion research.

## 5.7 References

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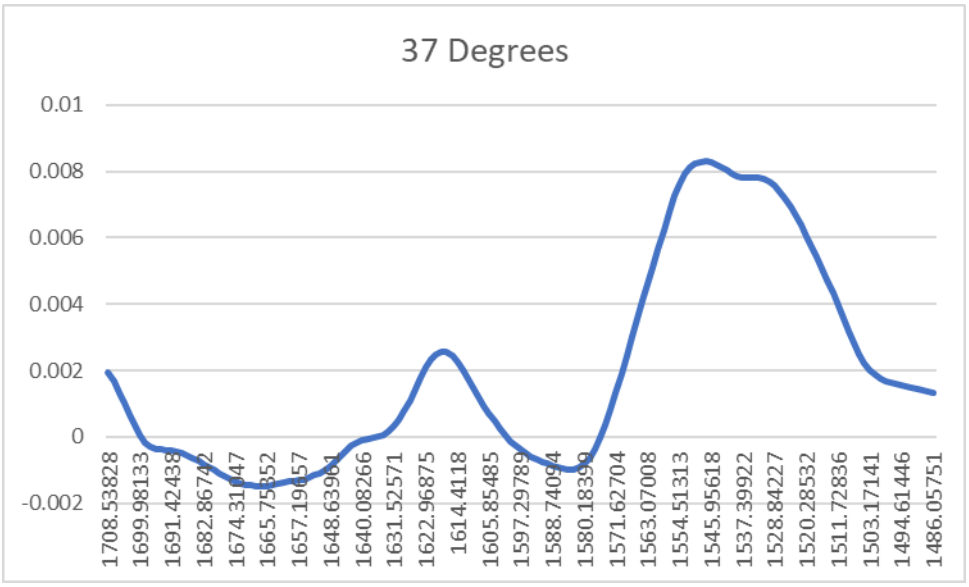
**Supplementary Material**

*Supplementary Table 1: Human Gastric Fluid Pepsin Activity.*

| Run | A:ph | B:Temp (°C) | Pepsin Activity (% of maximum) |
|---|---|---|---|
| **1** | 1 | 27 | 45.54 |
| **2** | 4 | 60 | 92.91 |
| **3** | 6 | 60 | 20.73 |
| **4** | 3 | 40 | 88.51 |
| **5** | 1 | 60 | 64.45 |
| **6** | 3 | 4 | ~~20.62~~ |
| **7** | 6 | 36 | 35.45 |
| **8** | 4 | 24 | 44.25 |
| **9** | 6 | 4 | 9.67 |
| **10** | *1* | *4* | *5.59* |
| **11** | 2 | 15 | 31.04 |
| **12** | 2 | 37 | 103.76 |
| **13** | *2* | *50* | *96.99* |
| **14** | 3 | 15 | 43.07 |
| **15** | 3 | 37 | 98.39 |
| **16** | 3 | 50 | 103.54 |
| **17** | 4 | 15 | 40.60 |
| **18** | 4 | 37 | 38.02 |
| **19** | 4 | 50 | 42.75 |
| **20** | 5 | 37 | 6.77 |
| **21** | 1 | 37 | 83.24 |
| **22** | 2 | 37 | 113.64 |
| **23** | 3 | 37 | 92.80 |
| **24** | *4* | *37* | *38.24* |
| **25** | 6 | 37 | 5.91 |
| **26** | 7 | 37 | ~~7.63~~ |
| **27** | 7 | 37 | ~~11.28~~ |
| **28** | 6.5 | 37 | 0.54 |
| **29** | 6 | 37 | 9.88 |
| **30** | 5.5 | 37 | 19.87 |
| **31** | 5 | 37 | 10.10 |
| **32** | 4.5 | 37 | 15.25 |
| **33** | 4 | 37 | 37.49 |
| **34** | 3.5 | 37 | 87.76 |
| **35** | 3 | 37 | 93.77 |
| **36** | 2.5 | 37 | 93.56 |
| **37** | 2 | 37 | 114.29 |

Supplementary Table 2: Porcine pepsin activity

| *Run* | *A:ph* | *B:Temp °C* | *Pepsin Activity (% of maximum)* |
|---|---|---|---|
| *1* | 1 | 27 | 28.50 |
| *2* | 4 | 60 | 15.03 |
| *3* | 6 | 60 | 58.68 |
| *4* | 3 | 40 | 58.59 |
| *5* | 1 | 60 | 4.21 |
| *6* | 3 | 4 | 12.33 |
| *7* | 6 | 36 | 1.94 |
| *8* | 4 | 24 | 4.06 |
| *9* | 6 | 4 | 0.60 |
| *10* | 1 | 4 | 9.96 |
| *11* | 2 | 15 | 26.67 |
| *12* | 2 | 37 | 99.00 |
| *13* | 2 | 50 | 70.00 |
| *14* | 3 | 15 | 24.05 |
| *15* | 3 | 37 | 59.84 |
| *16* | 3 | 50 | 80.71 |
| *17* | 4 | 15 | 2.26 |
| *18* | 4 | 37 | 8.91 |
| *19* | 4 | 50 | 12.52 |
| *20* | 5 | 37 | 3.52 |
| *21* | 1 | 37 | 89.27 |
| *22* | 2 | 37 | 99.59 |
| *23* | 3 | 37 | 14.33 |
| *24* | 4 | 37 | 4.30 |
| *25* | 6 | 37 | 4.84 |
| *26* | 7 | 37 | 5.82 |
| *27* | 7 | 37 | 0.76 |
| *28* | 6.5 | 37 | 0.99 |
| *29* | 6 | 37 | 2.00 |
| *30* | 5.5 | 37 | 3.00 |
| *31* | 5 | 37 | 3.50 |
| *32* | 4.5 | 37 | 6.00 |
| *33* | 4 | 37 | 8.90 |
| *34* | 3.5 | 37 | 55.00 |
| *35* | 3 | 37 | 57.00 |
| *36* | 2.5 | 37 | 88.00 |
| *37* | 2 | 37 | 100.00 |




Supplementary Figure 4 Native Pepsin spectra (37 °C)

# Chapter 6

# General discussion and suggestions for future work

## 6.1 Summary of key findings

This work has contributed to the scientific knowledge in both *in vitro* digestion and dairy chemistry and has the potential to be of interest and benefit to consumers, industry and future researchers in the field of digestion. The key findings of this work are summarised as:

1) The genetic heterogeneity of milk proteins influences the production of yogurt with subsequent implications for protein digestion. This has been demonstrated here in terms of β-lactoglobulin polymorphisms, where the B variant is associated with improved yogurt production characteristics. Moreover, this study has shown that the yogurt produced from skim milk containing the B variant is more resistant to gastric protein hydrolysis compared to the A variant. Considering the results of this study along with the constant evolution of dairy herds linked to the Economic Breeding Index (EBI) and genetic merit, the subsequent effects of genetic differences and processability of milk warrants consideration prior to a move towards or away from a certain polymorphism.
2) Pasture-fed cows have previously been shown to produce milk with a more nutritionally beneficial fatty acid profile compared to indoor feeding systems. This study showed that these nutritional differences persisted throughout *in vitro* digestion.
3) Hot milk coagulates more rapidly during *in vitro* semi-dynamic gastric digestion when compared to cold milk. It was also found that in cold milk, a cream layer is formed at the top of the gastric phase, which is not observed in hot milk. These differences open questions about the possible implications for

gastric emptying rate to the small intestine, however; this needs to be validated *in vivo*.

4) Several studies have examined the effect of either pH or temperature on pepsin activity. This thesis combined both factors and produced a model that can be used to predict human pepsin activity at any pH / temperature combination relevant to gastric conditions. An equivalent predictive model has also been developed for porcine pepsin, which is used in *in vitro* digestions. This has been integrated into a freely accessible tool, enabling comparisons between human and porcine pepsins and further supporting our understanding of the results of *in vitro* and *in vivo* digestion studies.

## 6.2 General discussion

This work was carried out as part of a wider multidisciplinary research project within the Vistamilk SFI research centre entitled 'Targeted Project 8 - Digestive characteristics of dairy products'. The overall aim of Target Project 8 was to use *in vitro, ex vivo* and animal models to further the understanding of the digestion of dairy products. While other researchers focused on *ex vivo* and *in vivo* studies, this thesis focused on *in vitro* digestion. The initial aim of the project was to be a *'Study exploring the impact on digestion of changes to the protein and lipid profile of bovine milk and dairy products'.* This research not only achieved this goal, by improving our understanding of how genetic profiles of bovine milk may impact yogurt digestion and how bovine feeding strategies influence milk lipid digestion but also extended beyond its initial scope. For instance, this PhD thesis produced valuable findings on the effects of milk consumption temperature and produced a novel equation to predict pepsin activity during gastric digestion. These additional outcomes benefit future research

methodologies and the understanding of dairy product digestion, surpassing the project's initial objectives.

It is important to understand all the implications associated with altering the genetic profile of the national herd in Ireland. One such protein that could be bred for is β-lactoglobulin. In this study, the yogurt production characteristics and subsequent digestion differences between β-lactoglobulin A and B were investigated. The β-lactoglobulin B variant produced yogurt with a firmer gel and subsequently increased hydrolysis resistance compared to the A variant, likely due to stronger casein-β-lactoglobulin interactions forming denser curds (Figure 5 and 6). This reduced proteolysis by ~20% compared to the A variant during gastric digestion. This suggests that BB yogurt will remain in the stomach longer than AA yogurt. The specific effects on gastric emptying times and possible implications for hunger/satiety perceptions could be addressed in dedicated clinical studies in the future. Conversely, if *in vitro* findings translate into the *in vivo* situation, the faster breakdown associated with β-lactoglobulin A may increase the rate of nutrients passing from the gastric to intestinal phase. This may be beneficial in certain populations, specifically older adults, who have been shown to have delayed amino acid absorption (Milan et al., 2015).

Using the Economic Breeding Index (EBI), it is possible to breed for certain traits such as longevity, fertility and more recently, the protein profile of milk. However, breeding for certain traits should be done with care. For instance, if β-lactoglobulin A is associated with higher milk production, we must also consider the implications of changes in the technological properties of the milk (e.g., acid gelation performance in yogurt production). If the national herd moves towards a certain genetic polymorphism, dairy processing characteristics, such as yogurts time to gel formation,

cheese ripening and digestive behaviour should be monitored closely to identify any differences that are occurring.

This thesis also aimed to advance the understanding of high versus low pasture allowance on milk lipid digestion and fatty acid release. It was shown that GRS milk exhibited higher rates of gastric lipolysis (36.8% GRS *vs.* 20.4% for TMR) (Figure 15), likely driven by its (UFAs)-rich lipid core (e.g., CLA, omega-3). CLSM revealed smaller, dispersed lipid droplets in GRS coagulum (Figure 12), likely due to UFAs compared to TMR. GRS milk sustained CLA release during intestinal phases (Figure 17), which, when linked to murine studies showing anti-inflammatory and anti-carcinogenic effects, suggested human health benefits (Ip et al., 1994).

Despite the potential nutritional benefits that GRS milk has over TMR milk, the pasture-based system must remain profitable for the farmer when compared to TMR based systems. Several factors come into play when considering this. For instance, while it may be possible to achieve a higher price for the 'Grass-fed' image, cows fed on an indoor based system produce approximately 25% more milk solids that grass fed milk (2024). As well as this, indoor feeding systems allows farmers to accurately plan the feeding needs of the herd, while the pasture-based system is susceptible to seasonal grass shortages due to the climate. However, the pasture-based system currently is a comparatively lower input feeding system compared to TMR, at approximately €94 / tonne dry matter *vs.* €355 / tonne dry matter on a TMR system. Therefore, while TMR increases total solids produced from milk, it increases feed costs (O'Donavan, 2024). It should also be considered that in a time of labour shortages on Irish farms, high input TMR systems may increase gross margin on farms but also requires intensive management.

As well as looking at intrinsic factors that affect the digestion of milk and dairy products, this thesis also studied how extrinsic factors can affect the digestion of milk. During gastric digestion, hot milk (60°C) lead to protein and lipid sedimentation and a potential increase in rate of gastric emptying of nutrients compared to cold milk (4°C) (Figure 25). It was also shown that cold milk formed a cream layer which wasn't observed in hot milk (Figure 25). This raises questions on how milk temperature may impact gastric emptying profiles. One hypothesis is that cold milk could potentially lead to prolonged gastric retention which, in turn, could influence satiety through distension-induced vagal signalling (Marciani et al., 2009). However, further research would be needed to investigate this. Conversely, hot milk's rapid gastric transit may benefit clinical nutrition requiring rapid nutrient delivery (e.g., post-surgical recovery, sports nutrition etc.). The differences observed here were hypothesised to be due to differences in pepsin activity due to different gastric temperature post consumption of hot vs cold milk, however, a full quantification of pepsin activity at specific time points was not possible until a later study in this thesis provided a method to do this.

The use of MRI in this study allowed a powerful, non-invasive tool to visualise the minor differences in the digestion of hot vs cold milk that would not have been observed using biochemical methods. For instance, in preliminary studies, while it was possible to visually observe differences in protein coagulation between milks of different temperatures, it was not possible to find differences using techniques such as the OPA assay for the breakdown of proteins. Using MRI allowed the quantification of the visual observations made in the preliminary trials.

To better understand how temperature variations within the gastric environment might influence proteolysis, a pepsin activity prediction model was created. It is well known that pepsin activity is dependent upon both pH and temperature, however, no model

incorporating both has previously been developed. The predictive model produced as part of this thesis, based on the quantification of pepsin activity at 37 pH and temperature combinations across pH 1–6 and between 4–60°C for both human and porcine pepsin. It was found that human pepsin showed a broader activity ranged compared to porcine pepsin. This may be important for future versions of the INFOGEST standardised digestion protocol to take into consideration. According to the results shown here, it could be recommended to increase the amount of porcine pepsin used in *in vitro* digestions. This tool can also be used to understand differences in gastric proteolytic capacity at different stages of life. For instance, during gastric digestion in infants, pH remains elevated compared to in adults. If a digestion of a specific food was carried out using an adult model, the pepsin prediction model could be used to predict the results of an infant model by altering the pH values to mimic infant digestion. As well as this, the model could be used to predict protein breakdown in clinical conditions such as dyspepsia. Another benefit of this model is that the result output is expressed as a percentage. This means that any user can use this model regardless of the activity level of their own human or porcine pepsin sample. For instance, if user X has porcine pepsin sample with an activity 2,000 U / ml, and user Y has porcine pepsin with 3000 U / mL, both can use the model. If the output of the model was 50% activity, user X would have 1,000 U / mL, while user Y would have 1,500 U / mL. This part of the research work addressed the lack of understanding of pepsin activity under varying gastric pH/temperature conditions and provides a route to establish comparisons of *in vitro* results to the *in vivo* situation.

As well as the main outputs of this thesis, collaborative efforts carried out during this PhD, not shown in this thesis, explored the digestion of several dairy products. For example, studying A1 *vs.* A2 milk in Cheddar cheese revealed differences in digestion

profiles (Daniloski et al., 2024). A novel method was also developed by the author of this thesis to simulate the oral digestion of solid dairy products like cheese, which enhances our capacity to model complex foods (Daniloski et al., 2024). Additionally, as described above, research on the impact of β-lactoglobulin polymorphisms in infant formula digestion was carried out too, furthering research using infant models of instead of adult models during *in vitro* semi-dynamic digestion (Unpublished work). Finally, towards the goal of contributing to further development of *in vitro* digestion protocols, contributions were made towards the 'standardization of *in vitro* digestibility and DIAAS method based on the static INFOGEST protocol' by taking part an international ring trial

## 6.3 Future considerations

### 6.3.1 Investigation of other bovine milk protein polymorphisms in relation to digestion

To advance our understanding on the impact of genetic variations on the digestion of dairy products, future research could focus on other genetic variants such as caseins, on digestion of dairy products. Previously studied variants include β casein A1 vs A2 which found that β casein A2 releases a lower amount of β casomorphin 7 due to the amino acid substitution of proline at position 67 reducing pepsin hydrolysis at this site compared to A1 milk (Daniloski et al., 2021b). κ-Casein B has also been associated with increased curd firmness in chymosin hydrolysis compared with other variants which could lead to longer retention times in the gastric phase of digestion if the same results were observed with pepsin instead of chymosin (Guggisberg et al., 2024). Genetic differences such as these could have effects on digestion, especially in complex dairy matrices like cheese. Examining these variants across different dairy

products could reveal advantages for certain genotypes and allow the development of dairy products that optimise digestion and nutrient bioavailability. Since κ-casein cleavage is responsible for processes like rennet coagulation and gastric coagulation, and since it has been shown that κ-casein B produces firmer curds in cheese making, genetic variants of this protein should be studied in relation to digestion. As well as this, differences in κ-casein genetic variants may lead to non-coagulating milk which has implications for milk digestion (Nilsson et al., 2020). This may have implications for rate of appearance of amino acids in the blood stream or may lead to a decrease in total milk digestibility.

### 6.3.2 Altering the ratio of β- lactoglobulin A to β-lactoglobulin B to find the optimum ratio for yogurt production while maintaining digestibility

This work has shown that skim milk containing exclusively β-lactoglobulin B is more favourable for yogurt production compared to skim milk containing exclusively β-lactoglobulin A. However, it has also shown that β-lactoglobulin B yogurt is more difficult to break down during gastric digestion. It would therefore be of interest to explore different ratios of these two variants to find the optimum for both yogurt production and digestion ability.

Future *in vivo* studies should investigate milk protein polymorphisms effect on yogurt gastrointestinal tolerability through human trials. This could be done through monitoring postprandial symptom profiles, such as Visual Analogue Scale (VAS) scores for bloating and gastric discomfort, where VAS is a psychometric tool that measures subjective symptoms by asking participants to rate their intensity on a continuous scale, typically ranging from 'no symptom' to 'unbearable' (Bengtsson et al., 2011). A hypothesis that could be used to form these human clinical trials would

be ‘β-lactoglobulin BB yogurt is more difficult to digest than AA yogurt leading to longer gastric retention, greater gastric discomfort and an altered pepsin release pattern as measured by ELISA testing’.

### 6.3.3 Comparison of dairy products produced from indoor vs grass fed milk

The digestion profiles of dairy products such as cheese, butter, and yogurt should be studied to assess how feeding strategies, such as GRS *vs.* TMR milk may affect digestion of lipids. For instance, the effect of bovine feeding system on butter has previously been studied, showing that pasture feeding improves several characteristics such as texture and importantly nutritional values. Therefore, it would be of interest to examine differences in digestion, however, since butter is not typically eaten as a meal, it would have to be examined along with another foodstuff such as fat free bread.

This research should also be extended to *in vivo* studies. Previous human acute studies of grass fed vs TMR cheese pushed the boundaries of how much cheese a volunteer will consume, feeding approximately 120g of cheese / day, but did not report differences between TMR vs GRS diets in terms of human health effects (O'Connor et al., 2024). More recently, Rooney et al. (2025) carried out a randomised control trial in overweight adults over 6 weeks, comparing the daily consumption of GRS vs TMR fed cheddar cheese. The results show that GRS reduced circulating SFAs, increased circulating CLA and had more favourable omega-3 to omega-6 ratios compared to TMR.

To further this, longer term (6 months +) studies would build on this, potentially identifying long-term effects of grass-fed *vs.* TMR-derived dairy products on cardiovascular, metabolic, or gut health. However, there is a no guarantee of seeing a

long-term effect, leading to the issue of expensive studies showing no results. Studies therefore should be carefully designed to maximise their potential for meaningful results. One approach, as used in Rooney et al. (2025) who investigated overweight adults, could be to include populations where dietary interventions might have more pronounced impacts, such individuals with metabolic syndrome. Since GRS milk has higher CLA concentrations and greater subsequent release during digestion, biomarkers such as IL-6, and oxidative stress markers could be examined over a 6-month trial, feeding fat- and protein- standardised GRS vs TMR milk. The GRS milk may reduce markers of inflammation and oxidative stress compared to TMR milk. This should be balanced with the need for diverse, representative study populations and any such study design decisions should not only be scientifically justified and ethically sound but also consider the broader applicability of results to the general population. Additionally, researchers should ensure appropriate safeguards for all participants, particularly those from potentially more vulnerable groups.

### 6.3.4 Expanding effect of consumption temperature to infant formula

It is recommended to feed infant formula at 37°C, however, this best practise may not always be carried out. Repeating the milk consumption temperature study with infant formula instead of bovine milk would be of interest as temperature-related digestion effects may be especially relevant for infant nutrition due to their underdeveloped digestive system.

### 6.3.5 *In vivo* MRI studies

Future research should focus on validating the *in vitro* findings of this thesis through *in vivo* analysis. MRI studies to observe and compare the digestion of lipids and

proteins in hot *vs.* cold milk would allow for direct observation of how temperature affects milk digestion dynamics within the human gastrointestinal tract *in in vivo* and enable further comparisons with *in vitro* digestion results. However, it is likely that the results obtained would not be impactful enough to justify the high cost of running such an investigation. Therefore, it would be more likely that an *in vivo* investigation be carried out on infant formula rather than bovine milk due nutritional needs of infants as well as the size and value of the infant formula commercial market. However, if this research was to be undertaken, it should be carried out with careful ethical consideration to ensure that the primary objective is to benefit infant health rather than commercial gains.

### 6.3.6 Expansion of Prediction Models

It is well known that pepsin activity is substrate dependent (Salelles et al., 2021). The pepsin activity model in this thesis exclusively use haemoglobin as a substrate due to its use in the standardised pepsin activity test recommended by the INFOGEST international research network (Brodkorb et al., 2019). Therefore, future research should aim to expand these models to a broader range of substrates such as caseins and egg white proteins as examined in Salelles et al. (2021) to promote wider applicability to other food products.

Lipase is another important digestive enzyme for which, to date, there is no method/tool to predict activity during gastric digestion. Therefore, using a similar approach to create a lipase activity model would offer a more comprehensive view of digestive enzyme dynamics, particularly for studies focusing on fat digestion. Similarly to the pepsin activity model created in this thesis, both the *in vitro* (Rabbit gastric lipase) and *in vivo* (human) enzymes should be modelled. By expanding these

prediction models, researchers could better understand simulations of digestion for both protein and lipid components.

It should also be noted here that artificial intelligence models may soon be utilised to predict enzyme activities during digestion, however, these models require 'ground-truthing' by investigation such as the pepsin activity prediction model presented in this thesis.

## 6.4 Final conclusion to thesis

This thesis has contributed to the global scientific knowledge of dairy digestion by investigating knowledge gaps identified in Vistamilk SFI's Targeted Project 8 strategic objectives. By investigating the digestion of dairy products affected by genetic variants, feeding strategies and consumption conditions, this work supports Vistamilk's goal of highlighting Ireland's position as a global dairy leader in both production and research. The findings on pasture-fed vs indoor fed systems provide scientific validation for Ireland's grass-fed marketing strategy, showing that the nutritional advantages of grass-fed milk persist throughout *in vitro* digestion with potential nutritional implications through greater release of bioactive compounds such as CLA. The novel application created in this thesis, modelling both human and porcine pepsin contributes to international standardisation efforts in digestion research as part of the INFOGEST network. As well as this, this work includes multi-disciplinary and international collaborations, contributing to Vistamilk's key performance indicators.

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## Appendix 1


Figure 32 UCC College of Food and Nutritional Sciences Post Graduate Research Publication of the Year award for the paper 'Monitoring the effect of consumption temperature of full-fat milk on in vitro gastric digestion using magnetic resonance imaging'