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Characterising the techno- and bio-functional properties of milk protein-polyphenol complexes for application in performance nutrition

Thesis presented by

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Doctor of Philosophy

in

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Declaration

By signing this document, I declare this work is my own in its entirety and has not been submitted for an academic degree to any other university or higher education institute.

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Tessa M. van de Langerijt Date: 15th of June 2023

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"Nothing in life is to be feared; it is only to be understood."

– Marie Curie

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Abstract

There is increased consumer interest in performance nutrition, focusing especially on enhancing muscle strength and recovery. Dairy-plant hybrid beverages, rich in milk proteins and polyphenols, have considerable potential in this area, because both proteins and polyphenols have been positively linked to muscle strength and recovery. However, there is limited knowledge of the effects of protein-polyphenol interactions on the techno- and bio-functional properties (e.g., physical stability of casein micelles, heat stability, bioaccessibility and bioavailability) of liquid dairy systems. In these studies, the addition of polyphenols to systems containing casein micelles increased the attractive forces within the casein micelles, due to complex formation, preventing casein micelle dissociation. When milk protein systems contained blackberry polyphenols, their heat stability increased in the pH range 6.2-6.4. In this pH range, blackberry polyphenols bound calcium, which prevented protein aggregation and increased heat stability. Co-concentration of milk proteins and polyphenols was achieved by ultrafiltration, enabled by milk protein-polyphenol complex formation, allowing the complexes to be retained. Milk fat in mixed milk protein-blackberry systems positively influenced the bioaccessibility of polyphenols but had a negative effect on their bioavailability after *in vitro* digestion. Increasing the leucine content in these systems did not impact the bioaccessibility or bioavailability of polyphenols. In contrast, proteins increased the bioaccessibility of polyphenols and reduced the hydrolysis of polyphenols during *in vitro* digestion, which resulted in an increased polyphenol bioavailability. In conclusion, protein-polyphenol complexes showed considerable promise for preventing casein micelle dissociation, improving heat stability, facilitating co-concentration by ultrafiltration and improving the bioaccessibility and bioavailability of blackberry polyphenols. This new knowledge may help support the successful development of protein-polyphenol rich beverages for performance nutrition.

List of abbreviations

2-h-β-c	2-hydroxypropyl-β-cyclodextrin
α-La	α-lactalbumin
ABTS	Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AC	Apical chamber
AM	Acidified milk
ANS	1-anilino-naphthalene-8-sulphonic acid ammonium salt
ANOVA	Analysed statistically with the analysis of variance
AH	After heating
BBJ	Blackberry juice
BC	Basolateral chamber
BCC	β-casein concentrate
BCCM	Blackberry casein concentrate milk
β-CM	β-casein micelle
β-CM	β-casein micelles
BH	Before heating
BJDB	Blackberry juice dairy beverages
β-Lg	β-lactoglobulin
BBP	Blackberry phenolics
BM	Blackberry milk
BSA	Blood serum albumin
BSCM	Blackberry sodium caseinate milk
C	Catechin
CE	Catechin equivalent
CF	Concentration factor
CCM	Casein concentrate milk
CCP	Colloidal calcium phosphate
CM	Casein micelle
CMs	Casein micelles
CNP	Casein nanoparticles
cC-3-GE	Cyanidin-3-glucoside equivalent
D[3,2]	Sauter mean diameter
D[4,3]	Volume weighted diameter

DBBs	Dairy blackberry blends
DPPH	2,2-diphenyl-1-picrylhydrazyl
ECG	Epicatechin gallate
EE	Encapsulation efficiency
EGCG	Epigallocatechin gallate
ESI	Electrospray ionization
FDBP	Freeze-dried blackberry puree
FRAP	Ferric reducing ability of plasma
FBS	Foetal bovine serum
ΔG	Gibbs free energy
ΔH	Enthalpy
HBSS	Hanks' balanced salt solution
HCT	Heat coagulation time
HFD	High fat diet
HLM	High leucine milk
HPLC-Q-ToF	High-performance liquid chromatography quadrupole-time of flight
HPM	High protein milk
Igs	Immunoglobulins
Leu	Leucine
LH-SMP	Low-heat skimmed milk powder
LM	Leucine milk
M	Milk
MCC	Milk casein concentrate
MRM	Multiple reactor monitor
MPC	Milk protein concentrate
ND	Normal diet
NWP	Normalized water permeability
Papp	Apparent permeability
PD	Particle diameter
PES	Polyethersulfone
PM	Protein milk
PP	Polyphenol
PPs	Polyphenols
PSD	Particle size distribution

R	Rejection
r-CMs	Re-assembled casein micelles
RDI	Recommended daily allowance
ΔS	Entropy
S0	Sieving coefficient
SD	Standard deviation
SCM	Sodium caseinate milk
SCN	Sodium caseinate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SM	Skimmed milk
SMUF	Simulated milk ultrafiltrate
SPE	Solid phase extraction
SSM	Semi-skimmed milk
TAC	Total anthocyanin content
TEM	Transmission electron microscopy
TFC	Total flavonoid content
TMP	Transmembrane pressure
TPC	Total phenolic content
TPP	Tea polyphenol
tR	Retention time
UF	Ultrafiltration
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry
W	Water
WM	Whole milk
WPI	Whey protein isolate

Introduction and research objectives

Introduction

Performance nutrition is a rapidly expanding industry, with a global market value of $42.9 billion USD in 2022 and an expected growth of 7.4% from 2023 to 2030 (Grand View Research, 2023). One of the main focus areas for performance nutrition is food that enhances muscle recovery, endurance and strength (Carey et al., 2023). This type of nutrition is not only important for athletes, but also for the elderly population in terms of maintaining muscle mass (Oikawa et al., 2022). Products in the performance nutrition category may include hybrid dairy-plant beverages, rich in proteins and polyphenols. Milk proteins are positively associated with enhancing skeletal muscle mass and strength in athletes (Gryson et al., 2014) and maintaining muscle mass during aging (Deutz et al., 2014). Blackberries (*Rubus fruticosus*) can be grown on a commercial scale in Ireland and are classify as a native fruit specie (Strik et al., 2008). The polyphenols in blackberries, which are rich in anthocyanins, phenolic acids, flavonols and ellagitannins (Oszmiański et al., 2015), are associated with increased recovery from exercise-induced muscle damage (Carey et al., 2021), reduced instances of cardiovascular disease and improved metabolic health (Heneghan et al., 2018). Besides the individual effects of milk proteins and polyphenols, milk proteins can also partially protect polyphenols from oxidation in the gastro-intestinal tract (Lang et al., 2021).

In the past, research on milk protein polyphenol systems mainly focused on the formation of milk protein polyphenol-complexes with different types of individual proteins or protein structures (e.g., reassembled casein micelles, β-casein micelles, β-lactoglobulin aggregates) with pure polyphenols, such as curcumin, quercetin or catechin (van de Langerijt et al., 2023). In these studies, the techno-functional properties investigated were the types of interactions between polyphenols and milk proteins, size, morphology, encapsulation efficiency and antioxidant properties of the complexes. It remains unclear how polyphenol addition to beverage systems with different milk protein ingredients and protein contents influences functional properties. It is uncertain, for example, how complex

formation influences casein micelle dissociation. Some research has been performed on the influence of individual polyphenols and coffee, tea and cocoa extracts on the heat stability of milk systems (O'Connell et al., 1998; O'Connell & Fox, 1999). To our knowledge, the influence of polyphenols originating from berries has not been investigated. Another relatively unexplored opportunity within beverage production, is exploiding complex formation to co-concentrate milk proteins and polyphenols by ultrafiltration.

In terms of bio-functional properties, research has been done on the influence of individual milk proteins on the bioaccessibility and bioavailability of specific polyphenols (van de Langerijt et al., 2023; Zagury et al., 2019). Most performance nutrition beverages will contain a variety of different milk proteins and contain other milk compounds, such as, milk fat. They might also be supplemented with the amino acid leucine, due to their link with muscle recovery (Churchward-Venne et al., 2014). However, the influence of these different ingredients on the polyphenols present in blackberry puree specifically is unclear. It is therefore important to study systems containing a variety of milk proteins and blackberry polyphenols, in formulations with different protein, fat and fortified leucine levels. This thesis focuses on the outlined knowledge gaps .

Research objectives

This thesis has the overall aim of developing a hybrid dairy-plant beverage system, rich in milk proteins and blackberry polyphenols, designed to improve post-exercise muscle recovery and strength in active individuals. This thesis is part of a wider project, called RubusElite, which focuses on the influence of blackberry polyphenols incorporated in dairy systems on the improvement of musculoskeletal health. The specific goal of this thesis was to design, develop, formulate, process, analyse and optimise a beverage system that delivers the protein and polyphenol targets required from a nutrition perspective, toa chieve the overall project objectives, as well as analyse the digestive fate of both the proteins and polyphenols, aligned with the overall aims of the Rubus Elite project.

This thesis investigates the influence of polyphenols on the physical and heat stability of high milk protein systems. The reason why physical stability needs to be investigated is because high-protein beverages are often formulated with a variety of different milk protein ingredients. However, the effects of mixing different milk protein ingredients on the stability of casein micelles and the influence of polyphenols thereon is largely unknown. The influence of polyphenols, originating from polyphenol-rich fruits, on the heat stability of milk and protein-enriched milk systems will need to be studied as most commercial milk and dairy products undergo a heat treatment to reduce microbiological load (van den Oever & Mayer, 2021). Creating beverages with the levels of milk protein and polyphenols needed to mediate the beneficial health effects can be challenging. Protein-polyphenol complex formation for co-concentration by ultrafiltration could be a processing option, which will be explored in this thesis. This thesis also investigates the influence of composition representative of performance nutrition on the bioaccessibility and bioavailability of a variety of different polyphenols. Studying these techno- and bio-functional properties is essential for the effective development of dairy-plant hybrid beverages.

The specific objectives of this thesis were to:

- Develop a comprehensive overview on the structural, binding, and functional properties of milk protein-polyphenol systems.
- Study the influence of sodium caseinate and β-casein concentrate on the stability of casein micelles and the influence of polyphenols thereon.
- Investigate the influence of blackberry polyphenols on the heat stability of different milk systems.
- Determine if ultrafiltration could potentially be used to co-concentrate milk proteins and rutin in the retentate due to milk protein-rutin complex formation.

- Investigate the influence of milk proteins and milk fat on the bioaccessibility and bioavailability of polyphenols and organic acids present in blackberry puree after *in vitro* digestion.

- Study the influence of leucine, formulated innately within milk protein or in fortified form, on the bioaccessibility and bioavailability of phenolic components in blackberry puree after *in vitro* digestion.

- Link and discuss the outcomes of the studies in this thesis and provide recommendations for future research.

A schematic overview of the thesis is provided in Figure 1.

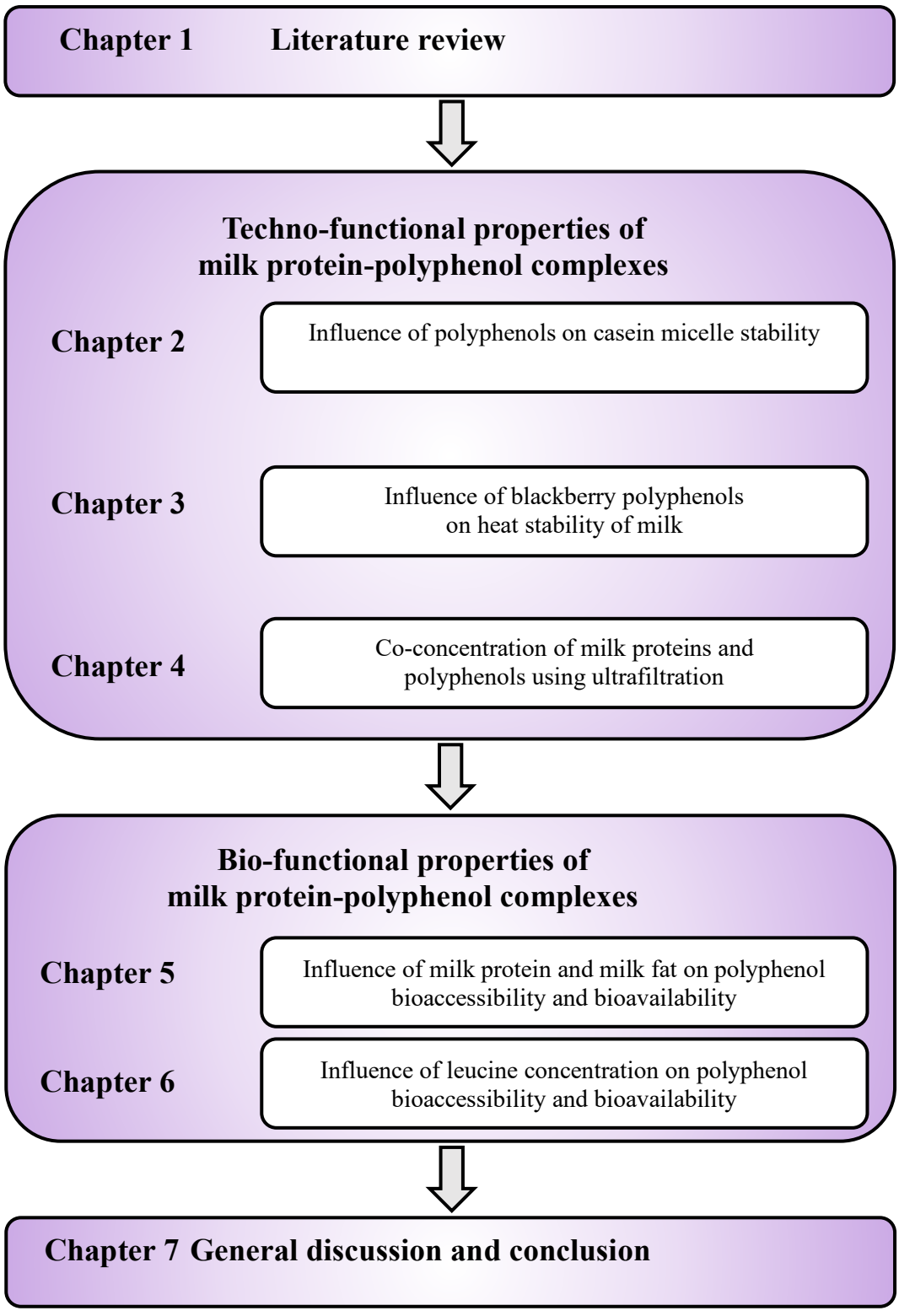

Figure 1. Schematic overview of the thesis structure and flow.

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Scientific outputs

List of publications

van de Langerijt, T. M., O'Mahony, J. A., & Crowley, S. V. (2022) The influence of sodium caseinate and β-casein concentrate on the physicochemical properties of casein micelles and the role of tea polyphenols in mediating these interactions. *LWT - Food Science and Technology*, 154. 112775

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van de Langerijt, T.M., Tzima K., O'Mahony J. A., Rai D. K., & Crowley S. V. (2023) Co-enrichment of milk and polyphenols using membrane filtration. *Journal of Food Engineering*. [submitted]

van de Langerijt, T.M., O'Callaghan Y. C., Tzima K., Carey, C.C., Lucey, A., O'Brien N. M., O'Mahony J. A., Rai D. K., & Crowley S. V. (2023) Influence of blackberry-dairy blends containing different leucine concentrations on the bioaccessibility of phenolic compounds after *in vitro* digestion and their apparent permeability in a Caco-2 cell model. *Food Bioscience.* [submitted]

List of additional publications

Amagliani, L., van de Langerijt, T. M., Morgenegg, C., Bovetto, L., & Schmitt, C. (2023). Influence of charged and non-charged co-solutes on the heat-induced aggregation of soy and pea proteins at pH 7.0. *Food Hydrocolloids*, 137, 108392

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Conference contributions

12th NIZO Dairy Conference, Online (10/21)

Poster presentation: van de Langerijt, T. M., Murphy T. R., O'Mahony, J. A., Crowley, S. V., (October 2021) *The influence of blackberry polyphenols on the heat stability of protein-enriched milk systems.*

4th Food Structure and Functionality Symposium, Online (10/21)

Poster presentation: van de Langerijt, T. M., O'Mahony, J. A., Crowley, S. V., (October 2021) *Comparison of the physicochemical and nano-structural properties of milk protein concentrate after blending with beta-casein or sodium caseinate.*

7th International Conference on Food Digestion, Ireland, Cork (05/22)

Poster presentation: van de Langerijt T. M., O'Callaghan Y. C., Tzima K., O'Brien N. M., Rai D. K., O'Mahony J. A., Crowley S. V., (May 2022) *Influence of milk constituent-polyphenol interactions on bioavailability of blackberry polyphenols, Poster presented at the 7th International Conference on Food Digestion.*

Chapter 1

Structural, binding and functional properties of milk protein-polyphenol systems: A review

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Declaration

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1.1. Abstract

Polyphenols (PP) are linked to health benefits (e.g., prevention of cancer, cardiovascular disease and obesity), which are mainly attributed to their antioxidant activity. During digestion, PP are oxidised to a significant degree reducing their bio-functionality. In recent years, the potential of various milk protein systems, including β-casein micelles, β-lactoglobulin aggregates, blood serum albumin aggregates, native casein micelles and re-assembled casein micelles, to bind and protect PP have been investigated. These studies have yet to be systematically reviewed.

The functional properties of the milk protein-PP systems depend on the type and concentration of both PP and protein, as well as the structure of the resultant complexes, with environmental and processing factors also having an influence. Milk protein systems protect PP from degradation during digestion, resulting in higher bioaccessibility and bioavailability, which improve the functional properties of PP upon consumption.

This review compares different milk protein systems in terms of physicochemical properties, PP binding performance and ability to enhance the bio-functional properties of PP. The goal is to provide a comprehensive overview on the structural, binding, and functional properties of milk protein-polyphenol systems. It is concluded that milk protein complexes function effectively as delivery systems for PP, protecting PP from oxidation during digestion.

1.2 Introduction

Polyphenols (PP) are secondary plant metabolites containing an aromatic benzenoid ring and a hydroxyl group (Belščak-Cvitanović et al., 2018). There is a great variety of PP, with estimates of approximately 8,000–10,000 different compounds; with sizes ranging from simple monomers to complex polymers (El Gharras, 2009; Eran Nagar et al., 2020). The structures of the PP most commonly studied in the literature are summarized in Figure 1.1.

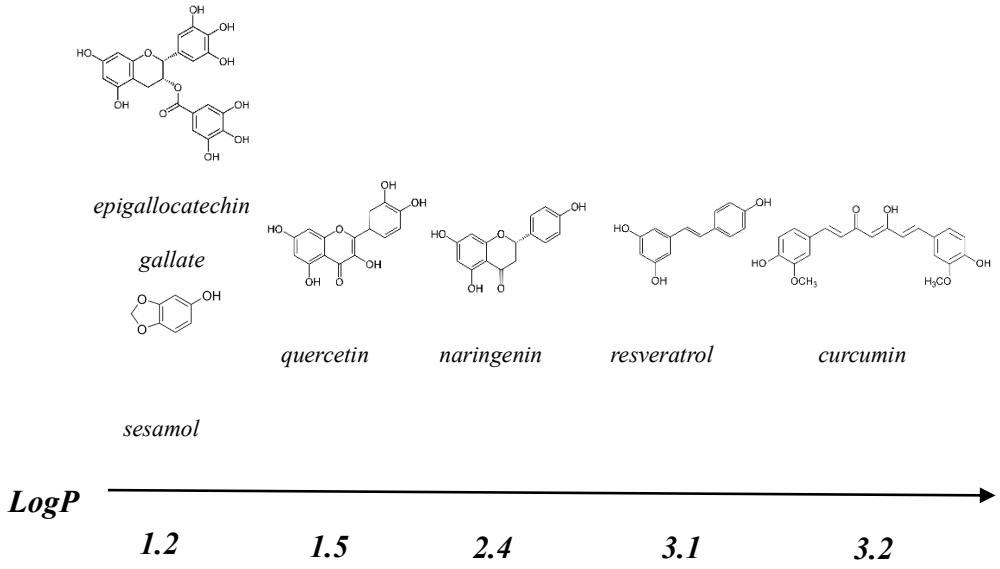

Figure 1.1: Molecular structure of different polyphenols ordered from left to right in terms of their decreasing polarity, expressed as LogP, which values were calculated by XLogP3 3.0 (PubChem release 2021.05.07).

PP have many different biological functions in plants, including pigmentation, growth and reproduction (Lattanzio et al., 2006). In recent years, consumption of PP has been linked to positive health effects, due to their antioxidant properties, which protect cells from oxidative damage, and positive effects on the gut microbiota. This could potentially reduce the risk of certain diseases, e.g., cancer, cardiovascular disease and inflammation (Rodríguez-Daza et al., 2021; Scalbert et al., 2005), making PP of considerable interest as functional ingredients in the food industry. However, many PP have low solubility in water, because of their nonpolar aromatic benzenoid rings (Z. Fang & Bhandari, 2010). They are also sensitive to oxidation during digestion, due to the slightly

alkaline conditions of the small intestine, which leads to a decrease in bioaccessibility and lower bioavailability (Parada & Aguilera, 2007; H. Zhang & Tsao, 2016). Binding of PP by milk proteins could potentially improve their water solubility and limit oxidation during digestion (Esmaili et al., 2011; Ghayour et al., 2019; Pan et al., 2013).

Bovine milk contains ~3.3% (w/w) protein, which consists of more than 100 different types of proteins, with the two main families being caseins (80%) and whey protein (20%) (DePeters & Cant, 1992). Caseins consist of four major different types, α_{s1}-, α_{s2}-, β- and κ-casein, which represent 38, 10, 35 and 12% of the caseins, respectively (Goulding et al., 2020). They are mostly present in colloidal aggregates, called casein micelles (CMs), and have a high concentration of calcium phosphate (Horne, 2011). The CMs have an average diameter of 150-180 nm and average molecular weight of 108 kDa (O'Mahony & Fox, 2013; Swaisgood, 2003). There are multiple models describing the structure of CMs, but in general it is believed that α_{s1}-, α_s2- and β-casein form loose aggregates through hydrophobic interactions, which are stabilized by interactions between colloidal calcium phosphate (CCP) and casein phosphoserine. κ-Casein forms a hairy layer at the surface of the CM, providing steric and electrostatic stabilization (Dalgleish, 2011; Holt, 1992; Horne, 1998). Water channels run through this structure, which makes it possible for small molecules, such as PP, to penetrate into the core of the CMs (Dalgleish, 2011; Yazdi & Corredig, 2012).

The main types of whey proteins are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), blood serum albumin (BSA) and immunoglobulins (Igs), which represent ~60, 20, 10 and 10% of total whey protein, respectively (Goulding et al., 2020). Whey proteins are globular proteins and have a high degree of secondary structure. Heating of these proteins above their denaturation temperature (which range between ~60-80 °C), will lead to partial unfolding. This causes the exposure of hydrophobic groups that were previously buried within the inside of the structure, which can result in aggregation (Brodkorb et al., 2016),

to an extent depending on environmental conditions including temperature, pH and ionic strength (Nicolai et al., 2011).

There is a great variety of milk protein types and structures, depending on the origin and processing history of the milk. Individual proteins or groups of protein can be separated by membrane filtration or ion-exchange chromatography, generating concentrates, isolates and fractions, such as milk protein concentrate and isolate. Other milk protein products are produced by acidification, enzyme treatments and/or salt addition, including acid casein, caseinates and rennet casein (Goulding et al., 2020). It is uncertain how structural differences between such milk protein-rich ingredients, associated with processing history, may affect binding to PP.

The main interactions between milk proteins and PP are non-covalent bonds consisting of hydrophobic and hydrophilic interactions, which include hydrogen bonds, van der Waals and electrostatic interactions (Chanphai et al., 2018; Chen et al., 2019):

- Hydrophobic interactions occur between the aromatic benzenoid rings of PP and the hydrophobic regions of proteins. These interactions are temperature-dependent and a reduction in temperature decreases the strength of the hydrophobic interaction between proteins and PP.
- A hydrogen bond is a dipole-dipole interaction, involving hydrogen and electronegative ions, e.g., Cl, F, N, O and S. The hydrogen bonds between proteins and PP are mainly between the hydroxyl groups of the PP and the polar amino acids of the proteins, and an increase in temperature will lead to a decrease in the amount of hydrogen bonds.
- Van der Waals interactions are the attractive forces between dipoles of non-charged atoms and molecules and the distance between the dipoles determines the strength of the van der Waals interactions. Temperature has a small influence on the strength of van der Waals interactions.

- Electrostatic interactions are the interactions between two charged atoms or molecules. Depending on pH, the amino acid residues of proteins can be positively charged (arginine, histidine and lysine) or negatively charged (aspartic acid and glutamic acid). These amino acids can interact with PP of an opposite charge through electrostatic interactions. In addition, salt ions can screen the charges present on the protein and PP, which will lead to decreased electrostatic interactions.

PP-protein binding is often studied by fluorescence spectroscopy (Moeiniafshari et al., 2015; Wu et al., 2011; Yazdi & Corredig, 2012), with the amino acids tryptophan, tyrosine and phenylalanine having a fluorescence emission. After an excitation the systems fluorescence emission can be measured, which is typically completed for systems with the same protein concentration and an increasing concentration of PP. When the fluorescent amino acids interact with PP, fluorescence intensity quenching follows, which results in a decrease in fluorescence emission upon an increase of the PP concentration (Figure 1.2) (Chanphai et al., 2018). This fluorescence data can be used to calculate the binding constant of the PP to the protein and the thermodynamic parameters, changes in enthalpy (ΔH) entropy (ΔS) and Gibbs free energy (ΔG), of the interaction.

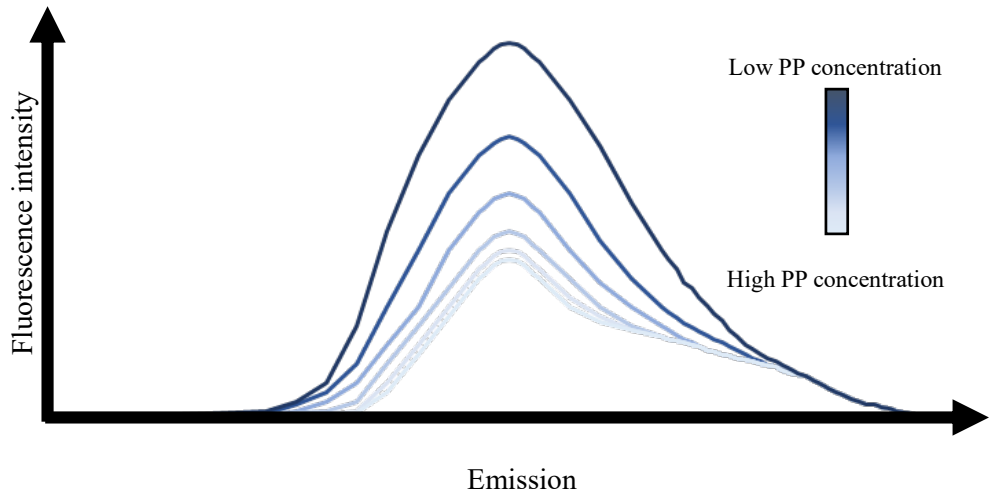

Figure 1.2: Schematic representation of a fluorescence emission spectra, in which the fluorescence emission decreases with an increasing polyphenol (PP) concentration.

As an example, in milk, the PP curcumin predominantly binds to the CMs. Milk, containing both CMs and whey proteins, had a binding constant (K_b) of 11 μM^{-1}, whereas pure CMs have a K_b of 0.22 μM^{-1}, at a temperature of 25 °C (Yazdi & Corredig, 2012). Whey proteins, the other main proteins in milk, form aggregates upon heating, which attach to the surface of the CMs and increase the degree of curcumin binding to the CMs (Yazdi & Corredig, 2012). There is a great variety of milk proteins and milk protein ingredients with potential to bind PP, with different possible encapsulation efficiencies (EE).

Milk proteins are food-grade and widely researched as delivery systems for bioactive components. They can form complexes with hydrophobic and hydrophilic regions, which enables them to bind molecules, such as PP, with different polarities. Nutrition research often focuses on whey proteins, due to its high leucine content, which is positively linked to muscle recovery (Sousa et al., 2014). However, casein-based systems can be preferred from a techno-functional point-of-view, due to properties including high heat stability and surface activity that can be important in practical applications (Dumpler et al., 2020; Elzoghby et al., 2011). During digestion, the release of caseins from the stomach to the intestine is slower than whey proteins, due to coagulation of casein in the stomach, which results in a delay in digestion (Boirie et al., 1997; Soop et al., 2012), potentially influencing the bioaccessibility and bioavailability of PP. The size of milk protein-PP complexes can have a wide range, with a minimum of 9 nm for BSA with quercetin and maximum of 100 μm for whey protein isolate aggregates with fruit juice (Diaz et al., 2020; R. Fang, Hao, et al., 2011; R. Fang, Jing, et al., 2011; Schneider et al., leng2016). The colloidal stability of the systems will depend on the size of the protein-PP complexes and systems with smaller particles will have a greater colloidal stability. It is also known that physico-chemical properties of proteins, such as size and morphology can influence digestion (S. Zhang & Vardhanabhuti, 2014), which could influence the protective effect of the protein on the PP. The main objective of this review was to provide a comprehensive overview of how the structure of different milk protein systems — which

can depend on factors including processing history — may influence the binding of PP and the properties of resultant complexes. For example, PP binding may affect structural properties of protein-PP structures, as well as the antioxidant activity and digestibility characteristics of the PP. Such knowledge can play an important role in the application of polyphenols in milk-protein-containing systems.

1.3. Casein based systems

1.3.1. Native casein micelles

The hydrophobic and hydrophilic regions of CMs can bind both hydrophobic and hydrophilic PP, such as curcumin and resveratrol (Figure 1.3A) (Yazdi et al., 2014). The most widely studied PP in CM-based systems have been curcumin, resveratrol and green tea varieties (Table 1.1), with the structure of these PP provided in Figure 1.1.

Table 1.1. Summary of the different casein-based encapsulation systems for polyphenols, including details of their particle diameter (PD) and encapsulation efficiency (EE), antioxidant activity assay and anti-tumor recorded cells. In which ABTS stands for azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and FRAP stands for ferric reducing ability of plasma. The abbreviations n.r. stands for not recorded and n.a. for not applicable.

Protein	Polyphenol	PD (nm)	EE (%)	Antioxidant activity assay	Antitumor recorded cells	Ref.
Casein micelles	curcumin	138-150	n.r.	n.r.	n.a.	(Yazdi et al., 2014)
		<200	n.r.	n.r.	cervical	(Sahu et al., 2008)
		n.r.	n.r.	n.r.	n.a.	(Nadi et al., 2015)
		176-187	n.r.	n.r.	n.a.	(Khanji et al., 2015)
		n.r.	n.r.	n.r.	n.a.	(Hudson et al., 2019)
		n.r.	97	FRAP and ABTS	n.a.	(Khanji et al., 2018)
	epigallocatechin gallate	n.r.	18-95	n.r.	n.a.	(Haratifar & Corredig, 2014)
	green tea flavonoids	-n.r.	n.r.	n.r.	n.a.	(Yuksel et al., 2010)
	resveratrol	138-150	n.r.	n.r.	n.a.	(Yazdi et al., 2014)
	tea catechins	n.r.	n.r.	n.r.	adenocarcinoma	(Guri et al., 2014)
	tea polyphenols	n.r.	n.r.	n.r.	n.a.	(van de Langerijt et al., 2022)
Reassembled casein micelles	curcumin and quercetin	73-187	93-97	n.r.	breast	(Ghayour et al., 2019)
	epigallocatechin gallate	68	85	n.r.	n.a.	(Malekhosseini et al., 2019)
	sesamol	158-166	28-35	n.r.	n.a.	(Santos Basurto et al., 2018)
	quercetin	182-334	26-97	n.r.	n.a.	(Ghatak & Iyyaswami, 2019)
Casein nano particles	curcumin	169	83	ABTS	n.a.	(Pan et al., 2013)
		104-213	70-100	n.r.	colon	(Pan et al., 2014)
	curcumin and quercetin	187	>99	n.r.	breast	(Ghayour et al., 2019)
	epigallocatechin gallate	162-246	n.r.	n.r.	n.a.	(Malekhosseini et al., 2019)
with (2-hydroxypropyl-β-cyclodextrin)	quercetin	171-251	75-83	n.r.	n.a.	(Peñalva et al., 2019)
β-casein micelles	curcumin	n.r.	n.r.	ABTS	n.a.	(Esmaili et al., 2011)
	naringenin	~30	n.r.	n.r.	n.a.	(Moeiniafshari et al., 2015)
		~20	n.r.	n.r.	n.a.	(Li et al., 2019)
		9-12 and 79-359	n.r.	n.r.	n.a.	(Li et al., 2020)
	resveratrol	n.r.	n.r.	n.r.	n.a.	(Ghorbani Gorji et al., 2015)
		6-13	59 or 69	n.r.	n.a.	(Cheng et al., 2020)

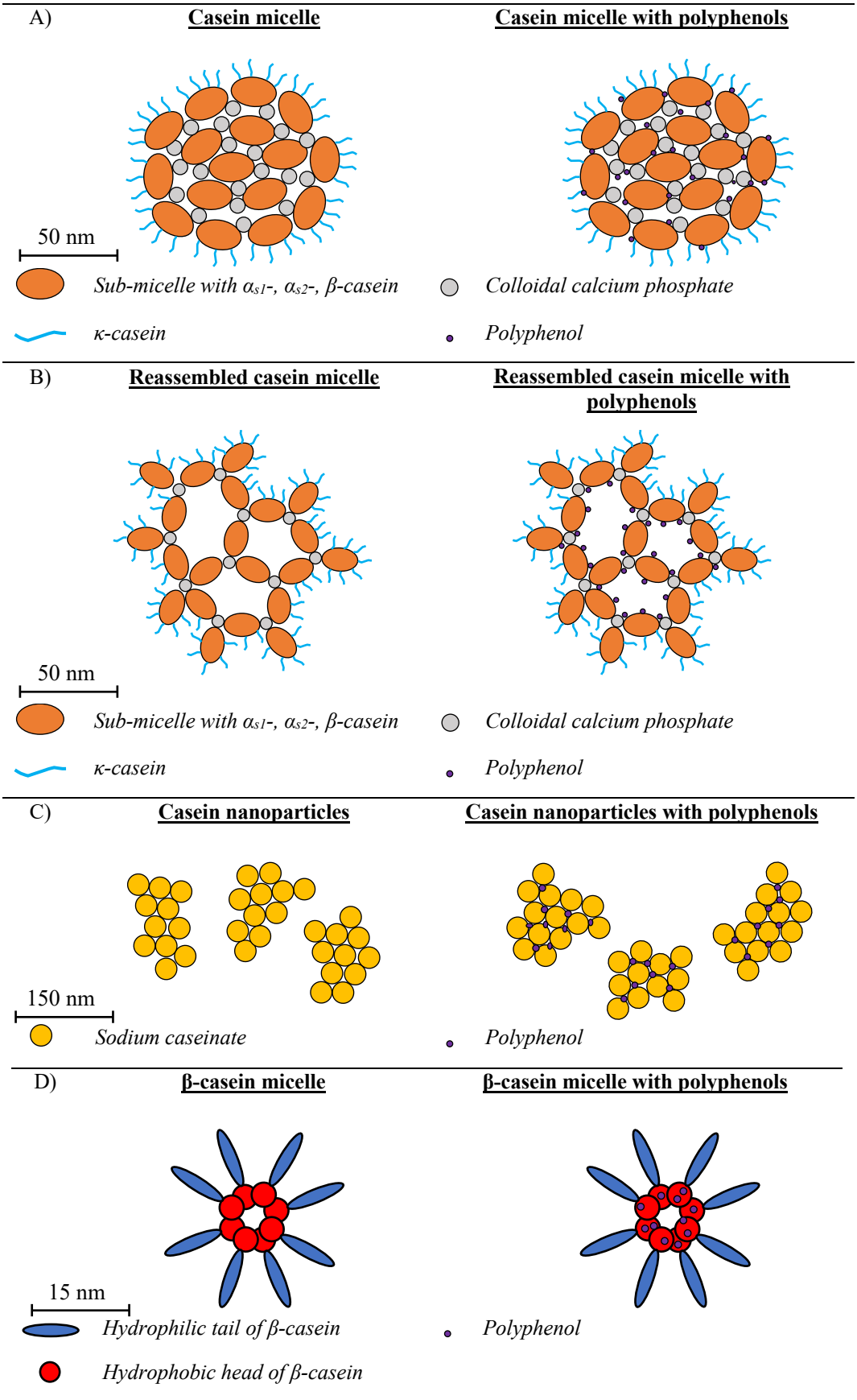

Figure 1.3: Schematic representation of casein systems with and without polyphenols. A). Casein micelles are colloidal particles, consisting of casein sub-micelles, which are stabilised by colloidal calcium phosphate,

resulting in formation of micellar complexes, which are surrounded by a hairy layer of κ-casein (Dalgleish, 2011; Griffin et al., 1988). The interaction between polyphenols, such as curcumin and green tea flavonoids, and casein micelles are mainly hydrophobic (Nadi et al., 2015; Sahu et al., 2008; Yuksel et al., 2010). More hydrophilic polyphenols, e.g., resveratrol, also interact with casein micelles through hydrogen bonds (Yazdi et al., 2014). The interactions had no influence on the size and morphology of the casein micelles (Khanji et al., 2015; Sahu et al., 2008). Hydrophobic polyphenols mainly interact with available hydrophobic patches within the core of casein micelles and more hydrophilic polyphenols with the hydrophilic areas in the casein micelles (Yazdi et al., 2014). B). Re-assembled casein micelles are artificially made micelles. They are made by adding tri-potassium citrate, calcium chloride and di-potassium hydrogen phosphate to sodium caseinate (Semo et al., 2007). Their size and morphology are similar to native casein micelles, only their surface appears to be rougher, probably due to the presence of κ-casein aggregates (Ghayour et al., 2019; Malekhosseini et al., 2019). The interaction between re-assembled casein micelles and polyphenols are similar to native casein micelles. For hydrophobic polyphenols, such as curcumin, the main interaction is hydrophobic and for more hydrophilic polyphenols, such as sesamol, the main interaction are hydrogen bonds (Ghayour et al., 2019; Santos Basurto et al., 2018). C). Casein nanoparticles are small aggregated protein structures made of sodium caseinate (Malekhosseini et al., 2019). The interaction between sodium caseinate and the polyphenol resveratrol was through hydrogen bonds and hydrophobic interactions (Acharya et al., 2013). D). β-casein has a strong amphiphilic character and will self-associate into micelles with a hydrophobic core and hydrophilic outer layer (O'Connell et al., 2003). The polyphenols curcumin, naringinin and resveratrol mainly interact with β-casein micelles through hydrophobic interactions, in the hydrophobic core of the micelle (Esmaili et al., 2011; Ghorbani Gorji et al., 2015; Moeiniafshari et al., 2015).

Physicochemical Properties of Casein Micelle-Polyphenol Complexes

Sahu et al. studied the interaction between CMs and curcumin using fluorescence spectrophotometry. When comparing free curcumin in water to curcumin interacting with CMs, the low intensity broad fluorescence peak shifted from 540 to 500 nm, after excitation at 420 nm. This indicated that the main mechanism of interaction between curcumin and CMs were hydrophobic (Sahu et al., 2008). This was confirmed by Nadi et al. (2015), who performed a thermodynamic analysis of the binding process, the positive values for both ΔH, 35.00 $kJ.mol^{-1}$, and ΔS, 0.23 $kJ.mol^{-1}$, showed that the main type of interaction was hydrophobic. With the aid of scanning electron microscopy, atomic force microscopy, dynamic light scattering and small angle X-ray scattering, it was determined

that binding of curcumin did not alter the structure of CMs (Khanji et al., 2015; Sahu et al., 2008). Other PP also interact with CMs through hydrophobic interactions. The anilinonapthalene-8-sulfonate (ANS) binding study of Yuksel et al. (2010) investigated the binding interactions between CMs and green tea flavonoids. The signal at 480 nm, after excitation at 390 nm, of samples with CM concentrations between 1 and 4% (w/w) and an ANS concentration of 135 mM was between 1073 ± 60 nm and 1295 ± 95 nm. Addition of 200 ppm phenols from green tea flavonoids decreased the signal for all CM systems to values between 393 ± 64 nm and 676 ± 18 nm. Green tea flavonoids decreased the available hydrophobic patches of CMs, demonstrating the role of hydrophobic interactions (Yuksel et al., 2010).

In the study of Yazdi & Corredig (2012), the fluorescence intensity between 300 and 440 nm of diluted skimmed milk in permeate (1:20) was almost completely quenched when the curcumin concentration was increased from 2.5 and 45 μM, after excitation at 280 nm. This demonstrated that curcumin penetrates the CMs and can interact with proteins in the inner core of the CMs. Yazdi et al. (2014) dissociated β-casein from CMs, creating CMs with a more hydrophobic core. They measured the fluorescence emission spectra of curcumin between 450 and 650 nm after excitation at 330 nm, and for resveratrol between 350 and 550 nm after excitation at 320 nm. For curcumin (hydrophobic) an increase in β-casein removal from the CM resulted in an increase of the binding constant. Therefore, the authors state that the increase in available hydrophobic patches within the core of the CMs, caused by the removal of β-casein, resulted in the higher binding affinity for β-casein-depleted CMs compared to normal CMs. This suggests that depletion of β-casein from CMs results in a loosing of the CM structure, due to loss of phosphoserine clusters, leaving the CM association solely due to CCP. The loosening in structure could cause an increase of water molecules in the core of the micelles, which could lead to a more favourable environment for the formation of hydrogen bonds between the CM and resveratrol. The authors imply this is the reason that resveratrol has a higher binding

affinity for β-casein-depleted CMs than CMs. This study demonstrated that the environment in the core of CMs has a strong influence on PP binding.

Both Khanji et al. (2015) and Sahu et al. (2008) Sahu found that the interaction between PP had no influence on the size (150–180 nm) and morphology of the CM. To understand the thermodynamics of CM-curcumin binding, Hudson et al. performed partitioning, surface plasmon resonance and thermal degradation kinetic experiments (Hudson et al., 2019). It was shown that at 25 °C and pH 6.6, one CM was able to bind 18,000 curcumin molecules, indicating that the CM:curcumin ratio is the main factor influencing the EE. The studies reviewed in this article have different terms to describe the percentage of the total PP in the system bound to the proteins, e.g., EE (Ghayour et al., 2019), loading efficiency (Chanphai et al., 2018), total PP yield of particles (Schneider et al., 2016), PP retention (Khanji et al., 2018), and in this review the term EE will be used. An increase in epigallocatechin gallate (EGCG) concentration from 0.01 mg/mg protein to 0.50 mg/mg protein led to an EE decrease from 95 to 18%, which is probably due to saturation of the CM. Haratifar & Corredig (2014) confirmed this for EGCG, because the EE of EGCG in relation to CM depended on the EGCG concentration.

The effect of green tea PP on the stability of CMs was studied by van de Langerijt et al. (2022), by measuring the turbidity, sedimentation profile and surface hydrophobicity of CM dispersions with and without tea PP. The tea PP reduced the turbidity loss of the CM dispersions over time, increased the integral transmission of the sedimentation profiles and decreased the available hydrophobic surface of the CMs. These results indicate that hydrophobic bonds between the tea PP and hydrophobic patches of the CM stabilise the CM structure.

Effect of Casein Micelles on the Antitumor Activity of Polyphenols

Sahu et al. studied the cytotoxicity of CM-curcumin complexes (Sahu et al., 2008). This was achieved by measuring the amount of viable human cervical cancer cells (HeLa cells) after 48 h of exposure to free curcumin and CM-curcumin complexes, which resulted

in an IC50 value of 14.85 and 12.69 μM, respectively. This indicates that CM-curcumin complexes have a higher cytotoxicity than free curcumin, which is probably due to higher curcumin absorbance of CM-curcumin complexes by the HeLa cells compared to free curcumin. Sahu et al. did not study the influence of digestion on the CM-curcumin complexes, which could potentially influence the cytotoxicity of both free and CM-curcumin complexes. In the study of Guri et al. (2014) two digestion models were used, a batch and dynamic digestion model, to study the influence of digestion on the cytotoxicity of EGCG in CM-EGCG complexes. After digestion, the viability of adenocarcinoma cells was tested, and it was shown that the cytotoxicity after digestion with both models was preserved by the CMs.

Influence of Spray Drying and Rehydration on the Structure and Antioxidant Activity of CM-Curcumin Complexes

In the food industry, milk proteins are mostly processed to a powder form, due to storage and transport benefits, after which they are rehydrated to be used in their final formulation. The most common technique for drying is spray drying (Schuck, 2002). Khanji et al. (2018) produced a powder containing CM-curcumin complexes and investigated the influence of spray drying and rehydration on the structure and antioxidant activity of CM-curcumin complexes. Small angle X-ray scattering measurements demonstrated that addition of curcumin did not alter the internal structure of the CMs, both before and after spray drying. The rehydration time of the powders was determined by measuring the time it takes to decrease the mean diameter (D50) to 0.7 nm, which corresponds to the D50 of native CMs, and was slightly shorter (i.e., faster rehydration) for the CM-curcumin complex powders (430 min) compared to the pure CM powders (470 min). This was likely because the main type of interaction between curcumin and CMs is hydrophobic and therefore less hydrophobic sites are available within the CMs, making the overall system more hydrophilic. The ferric reducing ability of plasma (FRAP) assay and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay indicated that the

antioxidant activity of curcumin within the CM-curcumin complexes was not affected by the spray drying. These assays also indicated that storage of the spray dried complexes for 60 d at 40 °C preserved the antioxidant activity of the complexes when rehydrated.

1.3.2. Sodium Caseinate

Sodium caseinate (SCN) is produced by first lowering the pH of skimmed milk to 4.6, which will lead to the dissociation of CCP from the CMs and result in isoelectric precipitation of casein, after which the whey is typically separated using a decanter centrifuge. A washing step removes residual lactose and minerals etc, and the caseins are resolubilised by adding sodium hydroxide and increasing the pH to 7 (Barraquio & van de Voort, 1991; O'Regan & Mulvihill, 2011). At neutral pH, SCN forms small aggregates with a diameter of approximately 20 nm, with the exact size depending on the ionic strength (HadjSadok et al., 2008). SCN can be used to create different structures, e.g., re-assembled casein micelles or casein nanoparticles.

1.3.2.1. Re-assembled Casein Micelles

Re-assembled casein micelles (r-CMs) are made by addition of tri-potassium citrate, calcium chloride and di-potassium hydrogen phosphate to SCN. The salts create artificial CCP which enables SCN to form aggregates due to calcium-bridged phosphoserine-to-phosphoserine linkages, with a similar size (between 150 and 180 nm) and morphology to CMs (Hindmarsh & Watkinson, 2017; Sáiz-Abajo et al., 2013; Semo et al., 2007; Swaisgood, 2003; Zimet et al., 2011). These r-CMs are capable of binding hydrophobic components, thereby increasing their solubility in aqueous solutions and minimising their chemical degradation. If desired, hydrophobic components can be added to the SCN before it is converted to r-CMs. Therefore, hydrophobic components do not need to diffuse into the r-CMs structure but are incorporated upon r-CMs formation. This will potentially lead to incorporation of more hydrophobic compounds in r-CMs compared to native CMs. Semo et al. (2007), was the first to demonstrate that r-CMs bind 5.5 times more vitamin D2 (a hydrophobic vitamin) compared to native CMs, showing the potential

of r-CMs as encapsulating agents. Based on their findings, several studies were published on the encapsulation of PP by r-CMs, as shown in Table 1.1. A schematic representation of r-CMs with and without PP can be found in Figure 1.3B.

Physicochemical Properties of Re-assembled Casein Micelle-Polyphenol Complexes

Within r-CMs there are both hydrophilic and hydrophobic regions, enabling r-CMs to interact with PP through hydrophilic and hydrophobic interactions (Ozdal et al., 2013). Fluorescence spectroscopy studies were performed on r-CMs loaded with curcumin and sesamol (Ghayour et al., 2019; Santos Basurto et al., 2018). The maximum emission wavelength of curcumin, after excitation at 287 nm, was at 348 nm independent of the curcumin concentration, when binding to r-CMs (Ghayour et al., 2019). In contrast, the maximum emission wavelength of sesamol increased from 338 to 355 nm, after excitation at 280 nm (Santos Basurto et al., 2018). This indicated that the main type of interaction between curcumin and quercetin with r-CMs was hydrophobic, while the main interaction with sesamol (a more polar PP) was mainly via hydrogen bonds. Thus, the polarity of PP primarily determines the type of interaction between r-CMs and PP. These findings indicate that r-CMs can potentially be used to bind both hydrophobic and hydrophilic PP.

The morphology of the r-CMs was investigated by scanning electron microscopy and transmission electron microscopy (Ghayour et al., 2019; Malekhosseini et al., 2019; Santos Basurto et al., 2018). When native CMs are visualised using electron microscopy a spherical shape with a smooth surface was observed (McMahon & Oommen, 2008). The electron micrographs of r-CMs showed a spherical shape similar to native CMs, but their surface seemed to be rougher. This might be due to the κ-casein aggregates attaching to the surface of the r-CMs (Ghayour et al., 2019; Malekhosseini et al., 2019).

The size of r-CMs is influenced by several factors, such as the salt concentration, protein and PP concentration, type of PP, degree of heating and presence of surfactants (Ghatak & Iyyaswami, 2019; Ghayour et al., 2019; Malekhosseini et al., 2019; Santos Basurto et al., 2018). In the study of Ghatak and Iyyaswami, r-CMs made with 0.5% (v/w)

SCN had a mean diameter of 182 nm, an increase in SCN resulted in an increase of the mean diameter, with the largest diameter of 296 nm at 5% SCN (Ghatak & Iyyaswami, 2019). It is suggested that small protein aggregates attach to the r-CMs, thereby increasing the particle size.

The EE of sesamol by r-CMs was researched by Santos Basurto et al. (2018), who observed that an increase in SCN concentration led to an increase in EE. The highest EE of 36% was found at a SCN concentration of 5% (w/v) and a sesamol concentration of 1 mg/ml. This increase in EE was probably due to an increase of available hydrophobic patches (caused by the increase in SCN concentration), which can interact with sesamol. Ghatak & Iyyaswami (2019) observed a different relationship between the EE of quercetin and SCN concentration. An increase of SCN concentration led to a decrease in EE and the maximum EE of ~84% was found with 0.5% (w/v) SCN. The authors attributed this to the differences in size and structure of r-CM caused by the different SCN concentrations. The size of the r-CMs was smaller with lower SCN concentrations; indeed, these data suggest that the smaller r-CMs formed at lower SCN concentrations have a higher EE. It is suggested that at higher protein concentrations the proteins favour hydrophobic interactions with each other instead of with quercetin, leading to the increase in particle size and decrease in EE of r-CMs. The difference in influence of SCN concentration on the EE of curcumin and quercetin was probably due to the difference in the conditions under which the r-CMs were formed. This may have led to differences in the structure of the r-CMs, which influenced the EE. It might also be that the two different relationships between the SCN concentration of r-CMs and the EE of sesamol and quercetin was due to the different properties of the PP. The type of PP incorporated in the r-CMs also influenced the EE of the system, similar r-CMs incorporating curcumin, EGCG and quercetin, had an EE of 97, 85 and 93%, respectively (Ghayour et al., 2019; Malekhosseini et al., 2019). Even though the EE of all these PP was high, the results indicate that the EE is dependent

on the type of PP, which could be due to the difference in polarity and binding coefficient of the PP.

The influence of quercetin concentration (12–22 μM) on the EE of r-CMs made with 0.5% (w/v) SCN was studied by (Ghatak & Iyyaswami, 2019), with a decrease in EE from 97 to 7% observed when quercetin concentration increased from 12 to 22 μM. The authors suggest that this decrease in EE was due to limitations in the amount of quercetin that could interact with the hydrophobic patches of the r-CMs.

The influence of pH on the EE of curcumin, EGCG and quercetin by r-CMs has also been studied (Ghatak & Iyyaswami, 2019; Ghayour et al., 2019; Malekhosseini et al., 2019). With pH values greater than 7, the EE of EGCG and quercetin decreased, whereas the EE of curcumin was largely unchanged. An increase in pH increases the overall negative charge of r-CMs, which results in a looser micellar structure, due to electrostatic repulsion (Malekhosseini et al., 2019), causing EGCG and quercetin dissociation and formation of anionic species, with consequent reductions in EE. However, the EE of curcumin was not influenced by the increase of pH, suggesting that influence of pH on the EE of r-CMs is dependent on the type of PP.

When the effect of storage time of the EE of r-CMs loaded with curcumin, EGCG and quercetin was studied, a period of 21 d had almost no influence on the EE of r-CNs. This indicates that the type of PP does not influence the effect of time on the EE (Ghayour et al., 2019; Malekhosseini et al., 2019).

Ghatak & Iyyaswami (2019) studied the influence of tri-potassium citrate, calcium chloride and di-potassium hydrogen phosphate on the EE of quercetin in r-CMs. Tri-potassium citrate is a calcium chelating agent and actively binds calcium. Concentrations up to 1 M increase the EE from 55 to 83%, with higher concentrations decreasing the EE. Low concentrations of citrates are known to increase the number of cross-links within r-CMs, whereas higher concentrations cause destabilisation and removal of CCP from the r-

CMs (Loewen et al., 2018). The increase in cross-linking enabled the growth of the colloidal mass of the r-CMs, which may have resulted in more extensive quercetin binding and increased the EE. In contrast, the destabilisation of r-CMs resulted in less quercetin binding and a decrease in EE. The influence of calcium chloride concentration between 0 and 1 M was studied and the highest EE of 87% was measured at 0.5 M. Higher concentrations than 0.5 M led to aggregation of r-CMs, resulting in precipitation of the r-CMs, with associated reductions in EE. Di-potassium hydrogen phosphate was used to stabilise the r-CM structure, but concentrations between 0 and 1 mM had no influence on the EE of r-CMs.

Influence of Re-assembled Casein Micelles on the Antitumor Activity of Polyphenols

The PP curcumin and quercetin are known cytotoxic molecules. The work of Ghayour et al. studied the effect of PP binding on r-CMs, the impact of digestion on the PP in r-CM-PP complexes, and the effect of r-CMs on the cytotoxicity of curcumin and quercetin, by measuring the cell viability of MCF-7 human breast cancer cells (Ghayour et al., 2019). It was shown that binding of curcumin and quercetin by r-CMs improved their cytotoxicity. Digestion of these loaded r-CMs improved the cytotoxicity even further, leading to the conclusion that r-CMs improve the delivery of curcumin and quercetin and can potentially be used as part of novel drug delivery systems.

When comparing the different studies on PP binding by r-CMs it becomes clear that once the optimal parameters are identified (e.g., SCN concentration, pH, salt concentration), the EE of r-CMs can be high for both hydrophobic and hydrophilic components. This, together with the observation that r-CMs improve the cytotoxicity of curcumin and quercetin, implies strong potential for r-CMs as PP delivery systems.

1.3.2.2. Casein Nanoparticles

Casein nano particles (CNP) are small protein structures made of SCN with an average diameter between 100 and 250 nm. In recent literature, four different methods were used to create casein nanoparticles to study their binding of PP; a representation of

their general structure can be found in Figure 1.3C. In the simplest method, SCN was dispersed simultaneously with quercetin, curcumin or EGCG in deionised water at 25 °C (Ghayour et al., 2019; Malekhosseini et al., 2019). In the method of Pan et al. (2013) SCN was dispersed in 40% (v/v) aqueous ethanol and heated for 5 min at 60 °C, after which curcumin was added. The unbound curcumin was removed by centrifugation and the CNP were spray dried. The study of Pan et al. (2014) involved preparation of pH-driven CNP by dispersing SCN in water at room temperature, adjusting the pH to 12 with NaOH, adding curcumin and adjusting pH to 7 with HCl. Peñalva et al. (2019) added 2-hydroxypropyl-β-cyclodextrin (2-h-β-c) to CNP, to prevent the metabolism of quercetin prior to absorption in the intestine. The CNP were produced by dispersing SCN, lysine and 2-h-β-c in water, after which quercetin (dissolved in ethanol) and calcium chloride were added.

Physicochemical Properties of Casein Nanoparticle-Polyphenol Complexes

The binding of resveratrol by SCN was studied by Acharya et al. (2013) by measuring fluorescence quenching. The excitation wavelength was 280 nm, after which the emission intensity between 300 and 600 nm was measured. SCN had an emission band around 350 nm, which was quenched (decrease in intensity), with an increasing resveratrol concentration. Further thermodynamic analysis of the results gives a negative value for the ΔH, $-21.2\ kJ.mol^{-1}$, a positive value for the $\Delta S°$, $37.9\ kJ.mol^{-1}$. The negative value for ΔH, indicates the presence of hydrogen bonds and positive value for ΔS indicates the presence of hydrophobic interactions (Acharya et al., 2013).

The mean diameter of CNP is typically in the range 100–250 nm, with the exact size dependent on the preparation method. Dissolving 1% (w/w) SCN in water resulted in CNP with an average diameter of 162 nm (Malekhosseini et al., 2019) or 187 nm (Ghayour et al., 2019), a heat treatment of 20 s at 74 °C increasing the average diameter to 246 nm (Malekhosseini et al., 2019). The spray dried CNP, made in aqueous ethanol, had an average diameter of 169 nm, which was similar to the size of the unheated CNP (Pan et al.,

2013). When the CNP were prepared by the method of Pan et al. (2014), the average diameter of the CNP depended on the initial curcumin concentration in the system; for example, a decrease in diameter from 213 to 104 nm, was observed when the curcumin concentration increased from 0 to 2 mg/mL. The CNP in the study of Peñalva et al. (2019) had a diameter of 138 nm without quercetin, 251 nm with quercetin and 171 nm with quercetin and 2-h-β-c.

All CNP had a high EE between 70 and effectively 100%. The EE of quercetin and curcumin by CNP, made with 0.5% (w/w) SCN and a 1:1 molar ratio of quercetin or curcumin with SCN, was determined in the study of Ghayour et al. (2019), and was higher than 99%. This was attributed to the high number of hydrophobic interactions and hydrogen bonds between the PP and SCN. The ethanol spray dried CNP made with 2% (w/w) SCN and 0.5% (w/w) PP, had an EE of 83%, and significantly higher solubility of curcumin in water, 11 ng/mL in water compared to 137 μg/mL in a colloidal dispersion with 1.25% (w/v) CNP (Pan et al., 2013). Just like the size, the EE of the pH-driven CNP prepared in the study of Pan et al. (2014) depended on the curcumin concentration. The CNP prepared with 2% (w/w) SCN decreased the EE from 100 to 70%, when the curcumin concentration increased from 0.4 to 2 mg/mL. However, the total amount of curcumin bound by the CNP increased with increasing curcumin concentration. The CNP in the study of Peñalva et al. (2019) increased in EE from 75 to 83% when 2-h-β-c was added.

Functional Properties of Casein-Polyphenol Nanoparticles

Using the ABTS assay, Pan et al. (2013) reported that binding of curcumin increased the antioxidant activity of aqueous solutions from <1.0 to 8.9 mM. Free curcumin precipitates in water, resulting in the low antioxidant activity of the sample. Binding to CNP helped to keep the curcumin solubilised, which increased the antioxidant activity of the sample.

In the study of Malekhosseini et al. (2019) a shelf-life stress test was performed on the CNP to measure the effect of CNP on EGCG degradation. This was done by heating

the CNP to 45 °C for 31 d and extracting EGCG from the CNP with a diluted ethanol solution at different time points. The amount of extracted EGCG was determined by measuring the UV absorbance of the ethanol extract at 274 nm. It was shown that CNP did not influence the degradation rate of EGCG, possibly attributable to the open structure of the CNP. Ghayour et al. (2019) performed the same experiment on curcumin and quercetin and measured the absorbance of the ethanol extract at 468 and 373 nm, respectively. Curcumin was better protected from degradation over a time span of 28 d compared to quercetin, the authors suggested that this was due to the thermal sensitivity of quercetin at 45 °C.

Influence of Casein Nanoparticles on the Bioavailability of Polyphenols

Peñalva et al. (2019) studied the *in vitro* digestive release of quercetin, and the influence of CNP (both with and without 2-h-β-c) on this release. The samples were subjected to 2 h of gastric digestion at pH 1.2 and 24 h intestinal digestion at pH 6.8. The CNP, both with and without 2-h-β-c, released 20% of the EGCG during the gastric digestion. After the first 4 h of intestinal digestion, the release rate of the CNP with and without 2-h-β-c was 60 and 80%, respectively. The authors suggested that this decrease in release rate caused by 2-h-β-c was due to formation of CNP with a more compact matrix. Peñalva et al. (2019) also performed an *in vivo* digestion study, in which the quercetin concentration present in blood plasma of rats was measured, after being fed samples with 2.5 mg/mL quercetin, in the form of free quercetin, CNP or CNP with 2-h-β-c. Consumption of free quercetin resulted in a low concentration of quercetin in the blood plasma, which rapidly decreased, resulting in a bioavailability of 4%. The concentration of quercetin in the blood plasma after consumption of quercetin bound to CNP and CNP with 2-h-β-c was significantly higher compared to consumption of free quercetin. After consumption of quercetin bound to CNP, the concentration of quercetin in the blood plasma decreased slowly for 6 h after consumption. After 24 h, only a low amount was detected, with an associated bioavailability of 12%. The quercetin concentration in the

plasma after consumption of CNP with 2-h-β-c remained high up until the last measurement 72 h after consumption, resulting in a bioavailability of 37%. This improvement in bioavailability might have been due to partial protection from gastric digestion, which resulted in more quercetin absorption in the intestine.

Effect of Casein Nanoparticles on the Antitumor Activity of Polyphenols

The *in vitro* cell proliferation assay, with colon cancer cells (HCT-116) of Pan et al. (2014) determined the effect of binding curcumin (1, 5, 10 and 20 μg/mL) by CNP on the cell viability. As expected, an increase in curcumin resulted in a decrease in cell viability. The bound curcumin also had a lower cell viability (~50%) than the free curcumin (~75%) at the highest curcumin concentration of 20 μg/mL; this was likely due to the improved solubility of curcumin by its binding to casein nanoparticles. Ghayour et al. (2019) measured the cell viability of breast cancer cells (MCF-7) when exposed to curcumin or quercetin bound to CNP. As in the previous study, an increase in PP resulted in decreased cell viability. Binding of curcumin at a concentration of 1800 μM to CNP resulted in a greater decrease of cell viability (~15%) than binding of quercetin to CNP (50%) at a concentration of 1800 μM.

1.3.3. β-Casein micelles

The protein β-casein has 209 amino acids, the unique combination of which results in a molecular weight of ~24 kDa and an isoelectric point of 4.9. Its highly negatively charged hydrophilic N-terminus and weakly positive charged hydrophobic C-terminus, give β-casein an amphiphilic character (Huppertz, 2013; O'Mahony & Fox, 2013; Pfaff & Anderer, 1988). The hydrophobic interactions between the C-terminuses of the β-casein proteins leads to self-association, resulting in formation of small micelles, called β-casein micelles (β-CMs). Their average diameter is typically in the range 20–30 nm and the β-CMs have a hydrophobic core and hydrophilic outer layer (O'Connell et al., 2003). Due to their hydrophobic core, it is suggested that they present strong potential for nano-

encapsulation of hydrophobic molecules, such as PP (Figure 1.3D) (Esmaili et al., 2011; Shapira et al., 2010).

Physicochemical properties of β-casein micelle-polyphenol complexes

Moeiniafshari et al. (2015) performed fluorescence spectroscopy studies on the interactions between β-CMs and naringenin at neutral pH, with the results used to perform a thermodynamic analysis on the binding interactions. It was shown that the binding between β-CM and naringenin is mainly caused by hydrophobic interactions, but also by van der Waals interactions and hydrogen bonds. Other fluorescence binding studies confirmed that the interaction between β-CM with curcumin and resveratrol was also predominantly hydrophobic (Esmaili et al., 2011; Ghorbani Gorji et al., 2015).

The size and morphology of the β-CM-PP complexes depended on the pH of the system. Li et al., (2019) showed that at pH 7, the amino acids tryptophan and tyrosine moved from an apolar to a more polar environment, when binding with naringenin, indicative of a change in conformation of the β-CMs. In contrast, at pH 2, this change in tryptophan and tyrosine to a more polar environment did not occur. The C-terminus loses most of is negative charge at pH 2, which reduces its hydrophobic nature, leading to the formation of larger β-CM-naringenin complexes (Li et al., 2019). At pH 7, the addition of NaCl should screen the charges present on β-CMs, leading to formation of larger β-CM-naringenin complexes, but the main peak in the size distribution remained at 28 nm. It is suggested this is caused by prevention of micelle formation, due to the screening of the negative charges of the N-terminus.

At neutral pH, temperature influences the size of the β-CMs without PP. At temperatures less than 15 °C, they have a relatively small diameter, compared to at temperatures greater than 15 °C. This is due to the reduced strength of hydrophobic interactions at lower temperatures, with most β-casein being present in the monomeric form. At higher temperatures the strength of the hydrophobic interactions increases, and the monomers will turn into micelles (de Kruif & Grinberg, 2002; O'Connell et al., 2003).

When naringenin was added to β-CMs the size of the micelles remained constant between a temperature of 5 and 55 °C (Li et al., 2019). Li et al. (2020) attempted to stabilise the β-CM-naringenin complexes with genipin. The genepin cross-linked the β-caseins within the micelles by intra-micellar covalent interactions. The cross-links improved the colloidal stability of the β-CM-naringenin complexes at pH 2.0. The genipin cross-links also decreased the release rate of naringenin from β-CM.

Esmaili et al. (2011) found that β-CMs improve the solubility of curcumin in aqueous solutions up to 2500 times. The EE of resveratrol depended on the β-casein concentration and type of resveratrol isomer. An increase in β-casein resulted in an increased EE, with the highest value reported at 200 μM β-casein, and 69% for trans-resveratrol and 59% for cis-resveratrol (Cheng et al., 2020).

Functional Properties of β-Casein Micelle-PP Complexes

Using a ABTS assay, Esmaili et al., (2011) demonstrated that the antioxidant activity of β-CM-curcumin complexes with 20 mM curcumin had a higher antioxidant activity than 120 mM curcumin dissolved in 10% ethanol; this indicated that binding of curcumin by β-CMs significantly improved the antioxidant activity of curcumin. An *in vitro* cytotoxicity study showed that β-CM-curcumin complexes have a higher cytotoxicity than free curcumin for human leukaemia cells (cell line K-562); the IC50 value for β-CM-curcumin complexes and free curcumin was 17.7 and 26.5 mM, respectively (Esmaili et al., 2011).

1.4. Whey Protein-Based Systems

Around 20% of the proteins in bovine milk consist of whey proteins, of which the main types are, β-lactoglobulin (β-Lg), α-lactalbumin (α-La), blood serum albumin (BSA) and immunoglobulins (Igs), which represent ~60, 20, 10 and 10%, respectively (Deeth & Bansal, 2018). They are globular proteins and unfold when heated at temperatures greater than their denaturation temperature. Depending on the pH, denaturation leads to formation of different aggregate shapes: spherical particles, flexible strands and semi-flexible fibrils

(Schmitt et al., 2007). There are numerous studies investigating different whey proteins as delivery systems for PP (Table 1.2, Figure 1.4).

Table 1.2. Summary of the different whey protein-based encapsulation systems for polyphenols, including details of their particle diameter (PD) and encapsulation efficiency (EE), antioxidant activity assay and antitumor recorded cells. In which ABTS stands for azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH stands for 2,2-diphenyl-1-picrylhydrazyl, FRAP stands for ferric reducing ability of plasma and ORAC stands for oxygen radical absorbance capacity. The abbreviations n.r. stand for not recorded and n.a. for not applicable.

Protein	Polyphenol	PD (nm)	EE (%)	Antioxidant activity assay	Antitumor recorded cells	Ref.
whey protein isolate	blackcurrant juice, cranberry juice and muscadine grape juice	5000, 30,000 and 100,000	28-84	n.r.	n.a.	(Schneider et al., 2016)
	blueberry juice and cranberry juice	1000-100,000	21–46	n.r.	n.a.	(Diaz et al., 2020)
with pectin	Anthocyanin rich extract	175-204	35 or 55	n.r.	n.a.	(Arroyo-Maya & McClements, 2015)
	Grape seed extract, hibiscus extract, tannic acid and chatechin	2000 - 17,000	n.r.	n.r.	n.a.	(Thongkaew et al., 2014)
β-lactoglobulin (unheated)	(+) catechin	< 458	n.r.	ABTS and ORAC	n.a.	(Oliveira et al., 2016)
	green tea extract	33	n.r.	n.r.	n.a.	(von Staszewski et al., 2014)
		33-955	n.r.	n.r.	breast, colon, glioma, kidney, lung, melanoma, ovarian and prostate	(von Staszewski et al., 2012)
		15-200	62-85	n.r.	n.a.	(Rodríguez et al., 2015)
	tea catechins	n.r.	n.r.	n.r.	n.a.	(Kanakis et al., 2011)
β-lactoglobulin (heated)	epigallocatechin gallate	<50	n.r.	n.r.	n.a.	(Shpigelman et al., 2010)
		<50	60-70	n.r.	n.a.	(Shpigelman et al., 2012)
		43	40	FRAP and ORAC	n.a.	(Zagury et al., 2019b)
		2-50	74	n.r.	n.a.	(Zagury et al., 2019a)
		31	59	n.r.	adenocarcinom, cervical, eosophageal carcinoma, gastric, lung, melanoma ovarian and prostate	(Wu et al., 2017)
Blood serum albumin	quercetin	9-12	n.r.	ABTS and DPPH	n.a.	(Fang et al., 2011b)
	quercetin	~10 nm	n.r.	ABTS and DPPH	n.a.	(Fang et al., 2011a)
	tea catechins	n.r.	45-65	n.r.	n.a.	(Chanphai & Tajmir-Riahi, 2019)

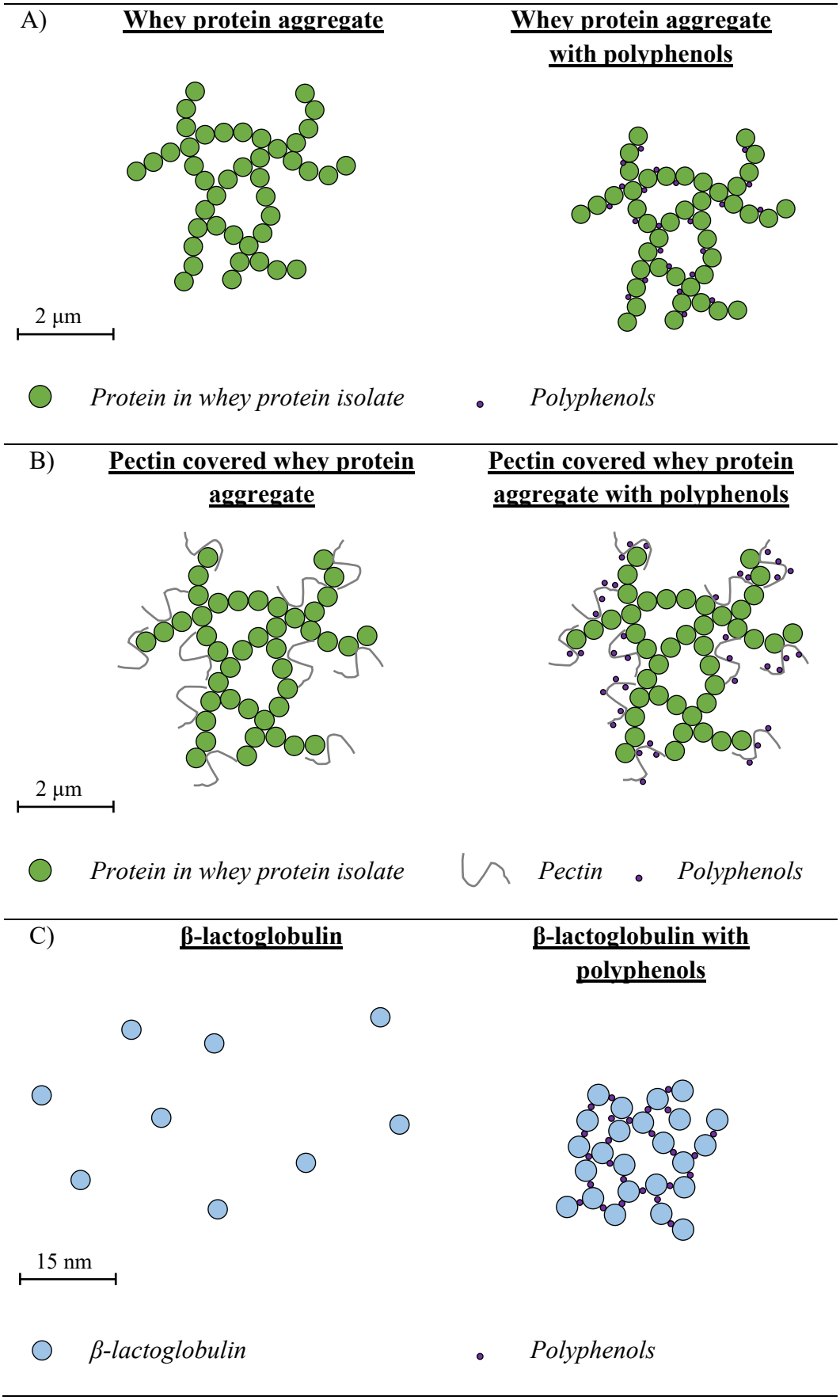

Figure 1.4: Continued

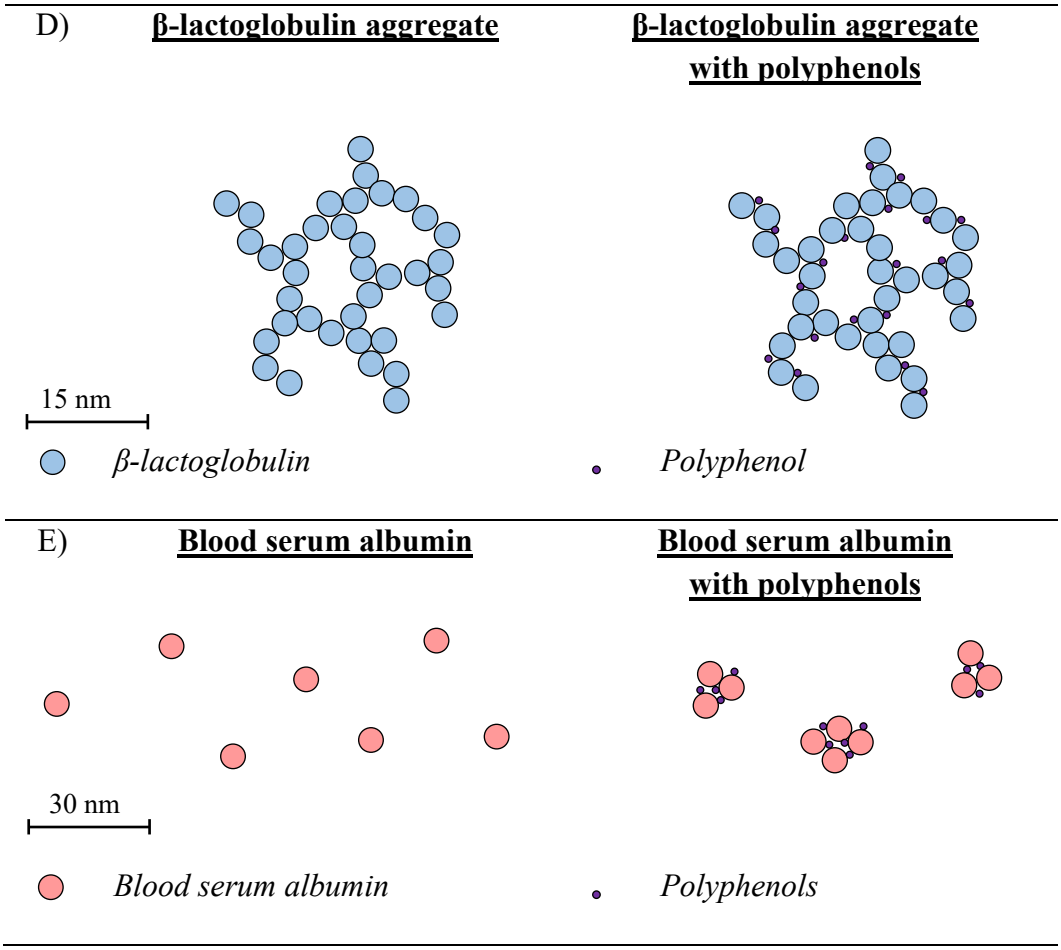

Figure 1.4: Schematic representation of whey protein systems with and without polyphenols. A). Whey protein aggregates are made by lowering the pH of the system to 4.5, close to the isoelectric point of the whey proteins, which will lead to the formation of protein aggregates, due to due to minimal protein charge–charge repulsions (Krebs et al., 2007; Schneider et al., 2016). Polyphenols interact with whey protein aggregates through hydrophobic interactions (Schneider et al., 2016). The size of the whey protein-PP aggregates depends on the pH, type of polyphenol and concentration of polyphenols added to the system (Diaz et al., 2020; Schneider et al., 2016). B). Pectin covered whey protein aggregates are made by heating whey protein isolate dispersions, which will lead to protein denaturation and aggregate formation. A solution with pectin was added to the whey protein aggregates and the pH was lowered to 3.2, giving the aggregates a positive and pectin a negative charge, resulting in the formation of pectin covered whey protein aggregates (Thongkaew et al., 2014). It is suggested that polyphenols, like anthocyanins, bind to the surface of pectin covered whey protein aggregates (Thongkaew et al., 2014). C). β-lactoglobulin is a globular protein and around neutral pH it is mainly present as a non-covalent linked dimer (Boland, 2011). Addition of catechins to β-lactoglobulin leads to complex formation, an increase in polyphenol concentration results in an increase in the size of these complexes (Rodríguez et al., 2015). The interactions between catechin and β-lactoglobulin are both hydrophobic and hydrophilic (Kanakis et al., 2011). D). Heating of β-lactoglobulin leads to denaturation, resulting in heat induced aggregates which

are able to bind more epigallocatechin gallate then unheated β-lactoglobulin. This is probably the result of more available hydrophobic amino acids, due to heat induced denaturation, which have a hydrophobic interaction with epigallocatechin gallate (Shpigelman et al., 2010). E). Blood serum albumin is a relatively large whey protein which is highly structured but flexible (Deeth & Bansal, 2018). They can bind to polyphenols such as quercetin, mainly through hydrophobic interactions and tea catechins through both hydrophobic and hydrophilic interactions (Chanphai & Tajmir-Riahi, 2019; R. Fang, Jing, et al., 2011). The size of blood serum albumin with and without quercetin is similar to each other (R. Fang, Hao, et al., 2011; R. Fang, Jing, et al., 2011).

1.4.1. Whey Protein Isolate

Whey protein isolate (WPI) is a highly pure source of whey protein, with protein content ≥90% and is prepared using ion exchange chromatography technology to selectively enrich proteins from liquid whey (Morr & Ha, 1993), with the four main whey proteins in WPI present in the same ratio as in milk (Goulding et al., 2020).

1.4.1.1. Whey Protein Isolate Aggregates

Schneider et al. (2016) used WPI aggregates to bind PP (Figure 1.4A). WPI was added to blackcurrant, cranberry and muscadine grape juice with different PP concentrations, 50, 250 or 500 mg of PP/L juice, to obtain a solution with a final protein content of 20% (w/w). Diaz et al. (2020) produced the same aggregates, but added different WPI concentrations (10, 15 and 20%, w/w, protein) to blackberry and cranberry juice with 1800 PP mg/L juice. The pH in both studies was adjusted to 4.5 and after 30 min the WPI aggregates binding PP were separated by centrifugation, after which they were redispersed in water (Diaz et al., 2020). These redispersed WPI-PP complexes were spray dried in the study of Schneider et al. (2016) and freeze-dried in the study of Diaz et al. (2020). In both studies, the size of the particles was measured before and after spray/freeze drying. The size of the WPI-PP aggregates depended on the pH, type of PP and the concentration of PP and protein, but in general, the size was in the range of 2–200 μm.

The EE of the WPI aggregates depended on the type of PP and the PP and protein concentration, for all types of PP, the EE decreased with increasing PP concentration. This

is probably because PP were able to interact with the available hydrophobic areas of WPI aggregates. Once these hydrophobic areas were occupied it was less likely that PP would bind to the whey proteins. The maximum EE of 84% (w/w) was found for cranberry juice, with a PP concentration of 50 mg/L and protein concentration of 20% (w/w) WPI.

1.4.1.2. Whey Protein Isolate-Pectin Aggregates

Arroyo-Maya & McClements (2015) and (Thongkaew et al. (2014) prepared PP delivery systems of WPI-pectin aggregates (Figure 1.4B). In the study of Thongkaew et al. (2014), a WPI and pectin dispersion was prepared by dissolving WPI (5% w/w) and apple pectin (2% w/w) in a 10 mM sodium phosphate buffer of pH 7. The WPI dispersion was heated for 40 min at 65 °C to form WPI aggregates. These WPI aggregates and the pectin dispersion were combined in a 1:1 ratio, after which the pH was adjusted to 3.2. This gave a positive charge to the WPI aggregates and a negative charge to the pectin, resulting in WPI-pectin aggregate formation due to electrostatic interactions. Different concentrations of grape seed extract, hibiscus extract, tannic acid and catechin (1, 3 and 5 mg/mL) were added to the WPI-pectin aggregates. The same WPI aggregates were also made without pectin for comparison. In the study of Arroyo-Maya & McClements (2015), WPI and sugar beetroot pectin were dissolved separately in water and thereafter combined to create a dispersion with 0.5% (w/w) WPI and 0.25% (w/w) pectin. The pH was adjusted to 5.8 and the solution heated at 90 °C for 5 min to form WPI aggregates. Afterwards, the pH was reduced to 4, creating WPI-pectin aggregates. An anthocyanin-rich extract (0.30 and 0.60 mg/mL PP) was added either before or after the heating step, to yield particles with different sizes. Thongkaew et al. (2014) reported particle sizes around 12.4 μm, depending on the PP type and concentration. This size was similar to the size of the WPI aggregates made without pectin (Diaz et al., 2020; Schneider et al., 2016). However, Arroyo-Maya & McClements (2015)reported a size between 175 and 204 nm. These WPI-pectin aggregates showed an increase in size and EE when the PP were added before heating. The EE increased with decreasing PP concentrations as well, which was also found in the WPI

aggregates without pectin (Diaz et al., 2020; Schneider et al., 2016). The maximum EE of 55% was reported for WPI-pectin aggregates to which 0.3 mg/mL anthocyanins were added before heating.

The influence of complex formation on the colour stability of anthocyanins, once ascorbic acid was added, was studied over a period of 7 d. It was shown that complex formation did not improve the colour stability of anthocyanins, which was probably because ascorbic acid was small enough in size to diffuse into the WPI-pectin aggregates. Both WPI aggregates and WPI-pectin aggregates were able to form protein-PP complexes. The PP present in one portion of blueberries (75 g) could be served in 12 g of protein-PP complexes, and the PP in one glass of cranberry juice (236 mL) in 17 g protein-PP complexes (Diaz et al., 2020). An advantage of WPI-pectin aggregates over WPI aggregates is the lower protein precipitation capacity, which resulted in a more stable system (Thongkaew et al., 2014). However, pectin WPI-pectin aggregates demonstrate greater sensitivity to pH than WPI aggregates, therefore, choosing one over the other depends on the desired system properties.

1.4.2. β-Lactoglobulin

The main whey protein in milk is β-Lg, which contains 162 amino acids and has a molecular weight of approximately 18 kDa (Boland, 2011). Its isoelectric point is ~4.6 or 5.2, depending on the genetic variant and its denaturation temperature is ~78 °C (Brodkorb et al., 2016; O'Regan et al., 2009). Around 50% of the total protein in WPI is β-Lg, and this section will focus on pure β-Lg systems.

1.4.2.1. Unheated β-Lactoglobulin

Most research on unheated β-Lg-PP complexes (Figure 1.4C) has been focused on green tea PP, which consist mainly of four different types of catechin (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin gallate (ECG) and EGCG. For the preparation of these β-Lg-PP complex systems, β-Lg and green tea powder extract were generally dissolved

separately in a 0.01 M phosphate buffer of pH 6.0 at room temperature, stirred for 30 min, and mixed in the desired ratio (Rodríguez et al., 2015; von Staszewski et al., 2012, 2014).

Physicochemical Properties of Unheated β-Lactoglobulin

Kanakis et al., (2011), investigated the interactions between unheated β-Lg and the green tea catechins C, EC, ECG and EGCG, with the aid of different spectroscopy studies (Fourier transform infrared, circular dichroism and infrared) and molecular docking analysis. It was shown that higher levels of OH groups on the PP led to greater binding to β-Lg, in the order EGCG > ECG > EC > C. The types of binding between β-Lg and tea PP are both hydrophobic and hydrophilic, with the binding of green tea catechins leading to the formation of more β-sheets and α-helixes in β-Lg.

Binding of PP by unheated β-Lg resulted in an increase in particle size, with addition of 0.1% (w/w) PP to 0.3% (w/w) β-Lg at pH 6.0, resulting in a particle diameter increase from 6 to 33 nm (Oliveira et al., 2016; Rodríguez et al., 2015; von Staszewski et al., 2012, 2014). It was also found that pH, PP concentration and polysaccharide addition influenced the size of the complexes. At pH 3, the addition of 0.1% (w/w) PP to 0.3% (w/w) β-Lg increased the size from 3 to 142 nm and from 7 to 955 nm at pH 4.5. It is suggested that this large increase at pH 4.5, is associated with the isoelectric point of β-Lg. This point is at pH 4.5, in which β-Lg is mainly present in the form of oligomers which are more sensitive to association with PP (von Staszewski et al., 2012). The increase of PP concentration from 0.125 to 1% (w/w) increased the diameter of the β-Lg-PP complexes from 15 to 200 nm (Rodríguez et al., 2015). Addition of the polysaccharide pectin (0.38% w/w) to a system with 0.3% (w/w) β-Lg and 0.02% (w/w) PP at pH 4, resulted in a size increase from 5 to 458 nm. However, addition of the polysaccharide chitosan had no influence on the size distribution (Oliveira et al., 2016). At pH 4, β-Lg, pectin and chitosan have a positive, negative and positive net charge, respectively. This causes attraction between β-Lg and pectin, resulting in complex formation and the particle size increase. There will be a repulsion between β-Lg and chitosan preventing complex formation and

no influence on the particle size will be observed. The influence of PP concentration on the EE of 0.3% (w/w) β-Lg was studied by Rodríguez et al. (2015). An increase in PP concentration from 0.125 to 1.0% (w/w), decreased the EE from 85 to 62%. However, the quantity of PP bound on a protein basis increased with increasing PP concentration (Oliveira et al., 2016).

To use unheated β-Lg as a delivery system, the PP need to retain their antioxidant activity upon binding to β-Lg. This was studied by Oliveira et al. (2016), using a ABTS and oxygen radical absorbance capacity (ORAC) assay, who found that binding did not reduce the PP antioxidant activity. They also found that pectin or chitosan had no influence on the antioxidant activity of the PP in unheated β-Lg-PP complexes.

Effect of Unheated β-Lactoglobulin on the Antitumor Activity of EGCG

Von Staszewski et al. (2012) investigated the influence of β-Lg-TPP complexes on the antiproliferative activity of different tumour cell lines (breast, colon, glioma, kidney, lung, melanoma, ovarian and prostate cancer cells). The antiproliferative activity is expressed as the concentration of TPP needed to achieve total growth inhibition in the tumour cells. The concentration of TPP to achieve total growth inhibition in melanoma, kidney, and ovarian tumour cells was lower if the TPP were present in β-Lg-TPP complexes. For breast, colon, glioma, lung and prostate tumour cells the concentration was lower for free TPP.

1.4.2.2. Heated β-Lactoglobulin

Shpigelman et al., (2010) studied the properties of heat induced β-Lg-EGCG complexes (Figure 1.4D). Nanoaggregates were made by heating a solution of β-Lg to 75–85 °C for 20 min, after which a EGCG solution was added. Their size, EE, level of protection against EGCG oxidation and sensory properties were investigated. Once these were determined the potential influence of the β-Lg-EGCG complexes on the digestive release, bioaccessibility and bioavailability of EGCG were studied (Shpigelman et al.,

2012; Zagury, Kazir, et al., 2019). The influence of β-Lg-EGCG complexes on bio-functional properties, antitumor activity and biological efficacy in a high fat diet (HFD) obesity mice model, have also been studied (Wu et al., 2017; Zagury, Chen, et al., 2019).

Physicochemical Properties of Heated β-Lactoglobulin

Shpigelman et al. (2010) were the first to prepare heat induced β-Lg-EGCG complexes. In this study, binding of EGCG to heated and unheated β-Lg were compared using spectrofluorometric and UV absorbance binding. Unheated β-Lg had an K_b of 1.05 × 105 $M^{-1}$and heated β-Lg one of 3.70 × 105 M^{-1}, demonstrating that heated β-Lg bound more EGCG. This was likely due to unfolding of the β-Lg upon heating, resulting in increased exposure of hydrophobic amino acids, with these hydrophobic amino acids able to bind a greater number of EGCG molecules. The unfolding of β-Lg and binding of EGCG resulted in a colloidally stable system of which the particles had a size <50 nm (Shpigelman et al., 2010, 2012).

A β-Lg concentration of 0.5% (w/w) and 1% (w/w) had an EE of ~58.5% and ~70.5%, respectively. This was independent of the two tested ECGG:β-Lg molecular ratios, 2:1 and 8:1 (Shpigelman et al., 2012). It is suggested that this increase in EE at higher β-Lg concentrations was due to the higher probability of EGCG and β-Lg interacting in more crowded systems. However, the EE of the heat induced β-Lg nanoaggregates prepared in the study of (Zagury, Kazir, et al. (2019) was lower (40.1%), even though the nanoaggregates were made with a similar ECGC: β-Lg ratio. The binding of EGCG reduced EGCG oxidation 33-fold for the first 48 h and 3.2-fold after 8 d (Shpigelman et al., 2010).

Influence of Heated β-Lactoglobulin on bioaccessibility and Bioavailability of EGCG

Digestion was studied by Shpigelman et al. (2012) and Zagury et al. (2019b). Both articles studied *in vitro* gastric digestion by adding pepsin to the β-Lg nanoaggregates, lowering the pH to ~2.5 and analysing the intact β-Lg at different time points. An increase in digestion time reduced the β-Lg intensity on a polyacrylamide electrophoresis gel, from

100 to 68% after 2 h (Shpigelman et al., 2012). The study of Zagury et al. (2019b) showed no degradation of β-Lg during the first 60 min of gastric digestion. The difference in β-Lg digestion might be due to the different conditions used for digestion across the two studies. The study of Zagury et al. (2019b) found no release of EGCG after 60 min of digestion. When the release of EGCG during *in vitro* gastric digestion was studied by Shpigelman et al. (2012) it was shown that after 90 min, the β-Lg-EGCG complexes started to release EGCG, and after 2 h, 25% was released. The reason that EGCG was not released in the first 90 min was due to binding of EGCG to the β-Lg peptides; interestingly, only when peptides were digested to a molecular weight less than 3 kDa, EGCG started to be released (Shpigelman et al., 2012).

Zagury et al. (2019b) also studied the intestinal digestion, which was performed by adding simulated intestinal fluid, bile salts, taurocholic acid and sodium glycodeoxycholate to the gastric digested sample and adjusting the pH to 6.5, where it was maintained for 120 min at 37 °C. Rapid degradation of β-Lg occurred during the intestinal digestion, and compared to samples with free EGCG, the amount of intact EGCG was significantly higher for samples containing β-Lg-EGCG complexes. FRAP and ORAC assays also indicated that the antioxidant activity of β-Lg-EGCG complexes was significantly higher compared to free EGCG during intestinal digestion. These results support the hypothesis that binding of EGCG by heat induced β-Lg nanoaggregates protected EGCG from degradation and loss of its antioxidant activity during digestion.

The *in vitro* bioaccessibility and *in vivo* bioavailability of EGCG in β-Lg-EGCG complexes was also determined in the study of Zagury (2019a); the bioaccessibility was studied by analysing samples taken during *in vitro* gastric and intestinal digestion. The samples were centrifuged through a membrane with a molecular weight cut-off of 3 kDa, and the released EGCG concentration was quantified using RP-HPLC/UV. The bioaccessibility was expressed in Equation 1.1 as:

$$bioaccessible\ ECCG = \frac{E_f}{E_0} \times 100\% \quad (1.1)$$

where Ef represents the amount of free EGCG, measured with the RP-HPLC/UV at different points during the digestion and E0 represents the amount of EGCG before digestion. The bioaccessibility of free EGCG was higher during gastric digestion, but bound EGCG had a higher bioaccessibility during intestinal digestion. Overall, the *in vivo* results indicate that bound EGCG had a better bioavailability compared to free EGCG. The bioavailability was studied in a rat model and β-Lg-EGCG complexes, free EGCG, heat induced β-Lg nanoaggregates without EGCG and a blank were consumed by rats. After consumption, the EGCG content in the blood plasma of rats was analysed at different time intervals. It was shown that during the first 30 min of digestion, free EGCG resulted in the highest level of EGCG in the plasma. After the first 30 min, more EGCG was present in the plasma of rats fed β-Lg-EGCG complexes. Therefore, it can be concluded that heat induced β-Lg nanoaggregates increased the bioavailability of EGCG, mainly due to protection of degradation by β-Lg during digestion in the small intestine.

Effect of heated β-lactoglobulin on health benefits of EGCG

Wu et al. (2017) studied the antitumor activity of heat induced β-Lg-EGCG complexes. For this study, the inhibitory effect of free EGCG and β-Lg-EGCG complexes on different types of human cancer cells, 22RV1(prostate), 769-P (renal), A375 (melanocarcinoma), C33-A (cervical), CACO-2 (colon), HeLa (cervical), MSTO-211H (mesothelioma), NCI-N87 (gastric), SK-OV-3 (ovarian), SPC-A-1 (lung), TE-1 (oesophagus) and normal cells NIH3 (mouse embryo fibroblast cells), AML12 (alpha mouse liver cells), CCD-18CO (normal human colon cells) were studied. The inhibition of growth attributable to EGCG in normal cells was almost negligible, but significant in all cancer cells. The β-Lg-EGCG complexes were more effective in inhibiting growth compared to free EGCG, especially in A375 and TE-1 cells. In TE-1 cells there was more early apoptosis, whereas A375 showed more late apoptosis.

EGCG potentially reduces cardiovascular disease, obesity and diabetes (Crespy & Williamson, 2004; Kao et al., 2006; Sumpio et al., 2006). Because of this, Zagury et al. (2019a) studied if β-Lg-EGCG complexes improved the prevention of metabolic syndrome effects in a high fat diet induced obesity mouse model. The mice were split in two groups, one group had a normal diet (ND), and the other group had a high fat diet (HFD). These groups were again split into four groups, receiving β-Lg-EGCG complexes, EGCG with milk, EGCG with water or just water, creating a total of eight mice groups. The mice underwent different types of analyses: faecal lipid analysis, hepatic triglyceride analysis, body fat mass analysis, glucose tolerance and insulin tolerance. The bound EGCG did not reduce the body fat gained by the mice with a HFD. However, it did reduce the faecal lipid concentration, in both the mice with a HFD and ND, and the haptic triglyceride level in mice with a HFD. The glucose tolerance and insulin sensitivity were also greater in mice fed β-Lg-EGCG complexes. From this study, the authors concluded that heat induced β-Lg-EGCG complexes can improve the health effects of EGCG, due to delivery, and offer potential for use in preventive nutrition/medicine.

1.4.3. Blood Serum Albumin

The presence of blood serum albumin (BSA) in milk is associated with transportation of serum albumin in the blood through the maternal epithelial cells to the milk (Deeth & Bansal, 2018). BSA consists of 583 amino acids, has a molecular weight of 66 kDa, isoelectric point of 5.3 and denaturation temperature of 64 °C (Brodkorb et al., 2016; Deeth & Bansal, 2018). When quercetin dissolved in dimethyl sulfoxide was added to BSA, creating a solution with 10% (w/w) dimethyl sulfoxide, nanoparticles with a mean diameter of 9–12 nm were formed (Figure 1.4E) (Fang et al., 2011a; Fang et al., 2011b) and the binding of quercetin was studied with fluorescence spectrometry. An excitation wavelength of 280 nm was used, and the measurements were performed between an emission of 200–500 nm, at 27 °C. The K_b of BSA with quercetin was 7.34 x 104 M^{-1} and gave positive values for both the $\Delta H°$ (5.88 $kJ.mol^{-1}$) and $\Delta S°$ (112.80 $kJ.mol^{-1}$), which

indicated that the main type of interaction was hydrophobic (Fang et al., 2011b). In the study of Chanphai & Tajmir-Riahi (2019), tea catechins (C, ECG and EGCG) were added to BSA and dissolved in a 10 mM Tris-HCl buffer of pH 7.2. Their binding was studied with UV-visible spectroscopy between 255 and 350 nm and it was found that the K_b decreased with an increase in temperature from 298.15 K to 318.15 K, and an increase in molecular weight of the catechin increased the binding constant. With the lowest recorded K_b of 1.64 ×105 M^{-1} for catechin at 318.15 K and highest K_b of 3.2 × 105 M^{-1} for epigallocatechin gallate at 289.15 K. Further thermodynamic analysis of the results gave negative ΔH° values, indicating the presence of hydrogen bonds, and positive ΔS° values, indicating the presence of hydrophobic interactions. The study of Chanphai & Tajmir-Riahi (2019) also reported the EE of tea catechins, which was between 45 and 65%, and an increase of molecular weight of the catechin increased the EE.

In terms of functionality, a 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS study showed that binding of quercetin to BSA did not influence the antioxidant activity of quercetin (Fang et al., 2011a; Fang et al., 2011b). Free quercetin mostly oxidises in the first 24 h. Quercetin in BSA complexes only oxidised for 70% after 216 h, which shows that BSA partly protects quercetin from oxidation (Fang et al., 2011b). This protection against oxidation also occurred in intestinal fluid, indicating protection during digestion (Fang et al., 2011a).

1.5. Conclusion and Future Prospects

In summary, the caseins and whey proteins in milk both have the ability to interact with PP, with hydrophobic interactions being particularly important. Hydrogen bonds also play a role, especially with more polar PP, such as resveratrol and sesamol. There is considerable variation/choice in the size, structure, EE and functional properties of milk protein-PP complexes. These complexes are influenced by the type and concentration of PP, type and concentration of proteins and environmental (e.g., pH, temperature, salt concentration) and processing (e.g., choice of process operation) factors. The size of the

milk protein-PP complexes varies: WPI > WPI with pectin > β-Lg (unheated) > CNP > CM, r-CM > β-Lg (heated) > β-CM > BSA. The smallest complexes were BSA with quercetin (9 nm), with the largest being WPI aggregates with PP rich juice (100 μm). A smaller particle size might be preferred in terms of colloidal stability. The lowest recorded EE was 18% for CMs with EGCG, and the highest was >99% for curcumin and quercetin-casein complexes. In most systems, an increase in the PP and a decrease in protein content led to a decrease in EE. This was due to saturation of the hydrophobic regions of protein upon PP binding. The studies that focused on the digestion of milk protein-PP complexes indicated that the PP in the complexes were less prone to degradation during digestion, which resulted in higher bioaccessibility and bioavailability of PP. Binding of PP to milk protein systems does not decrease the antioxidant activity of PP. Indeed, it can help to increase the overall antioxidant activity due to the ability of the milk proteins to solubilise PP in a polar system. The effect of protein-PP complexes on the antitumor activity is dependent on the type of PP, type of encapsulation system and the type of tumour cells.

At this time, only limited research has been completed on the behaviour of PP during the digestion of milk protein-PP complexes. More digestion studies are needed on a range of milk protein systems with the same PP profile in order to compare the protective effect of these milk protein systems. The influence of PP on the gut microbiota may have a major influence on the positive health effects of PP (Rodríguez-Daza et al., 2021). However, no literature has focused on the effect of milk protein-PP binding on the effect of PP on the gut microbiota. Research has demonstrated that the size and morphology of whey protein aggregates influences the digestion behaviour (Zhang & Vardhanabhuti, 2014). The influence of morphology and size of casein systems has not been studied, and the influence this could potentially have on PP bound to protein systems is also not understood.

Even though certain areas of milk protein-PP systems need to be studied in more detail, this review concludes that casein and whey proteins have great potential for developing food-grade delivery systems for PP.

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Chapter 2

The influence of sodium caseinate and β-casein concentrate on the physicochemical properties of casein micelles and the role of tea polyphenols in mediating these interactions

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Declaration

This chapter was written by author TMvdL and reviewed by all co-authors. TMvdL co-designed the study and generated all data included in this chapter.

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2.1. Abstract

The influence of different casein-based ingredients on the physicochemical properties of native casein micelles (CMs) was studied. Sodium caseinate (SCN) and β-casein concentrate (BCC) were mixed with milk protein concentrate (MPC) at different ratios (1:3, 1:1, 3:1), at a total protein concentration of 20 g/L. Tea polyphenols (TPP) were added to select samples to investigate their effect on CM-SCN and CM-BCC interactions. The results demonstrated decreasing structural integrity of CMs and increased levels of non-micellar casein, due to addition of SCN to MPC, which led to a decreasing turbidity over time and an increase in surface hydrophobicity. Addition of BCC to MPC resulted in an increase in turbidity over time and a decrease in surface hydrophobicity of the system. TEM images suggested that small micelles from BCC interacted with the surface of CMs. The SDS-PAGE results show that CMs in samples with TPP dissociated less, indicating that TPP stabilized the CMs structure. In conclusion, the two casein ingredients, SCN and BCC, have a different effect on the colloidal and physicochemical properties of CMs in MPC suspensions. This study will contribute to a greater understanding of the effect of mixed protein formulation strategies on the functional properties of beverage systems.

2.2. Introduction

Blending of different protein ingredients is commonly practiced during beverage formulation in the food industry for economic and nutritional reasons. It has been shown, however, that blending sodium caseinate (SCN) with native casein micelles (CMs) - the major protein complex in milk and many nutritional beverages - causes dissociation of CMs (Thomar & Nicolai, 2015). As these structural changes may alter some functional properties of casein-based beverages, such as visual opacity and physical stability, the effects of using alternative casein-based ingredients to SCN warrants investigation.

Bovine milk contains approximately 3.3% (w/w) protein, of which 80% is casein and 20% whey protein (Li et al., 2019; van de Langerijt et al., 2021). The casein fraction consists of four different types of phosphoproteins, α_{s1}-, α_{s2}-, β- and κ-casein (ratio

≈4.0:1.0:3.5:1.5), of which 90% are present in the form of colloidal complexes of approximately 50–500 nm in diameter, called CMs (Dalgleish, 2011; Holt & Sawyer, 1993; Swaisgood, 2003). The CM maintains a high concentration of calcium phosphate as "colloidal calcium phosphate" (CCP) nanoclusters (de Kruif & Holt, 2003). The interaction between CCP and the α_{s1}-, α_{s2}-and β-caseins of the CMs stabilizes these micellar complexes and prevents precipitation and sedimentation of calcium phosphate (Griffin et al., 1988).There is a variety of casein ingredients, some with caseins present in their native form (e.g., milk protein concentrate/isolate, colloidal calcium concentrate/isolate), others without caseins present in their native form (e.g., different types of caseinates), or ingredients mainly consisting of one type of casein (e.g., α_{s1}-, α_{s2}-, β- or κ-casein concentrate) (Goulding et al., 2020).

In formulated nutritional products, such as beverages in the clinical nutrition and infant formula sectors, it is common for different casein-based ingredients to be blended. For example, sodium caseinate (SCN) might be blended with milk protein concentrate (MPC) to improve specific functional properties of the beverage (e.g., heat stability, viscosity). Depending on the process by which the casein ingredients are made, their structure and composition, different interactions may arise between the proteins in the formulation. Sodium caseinate (SCN) is made by acidification of skimmed milk to pH 4.6, which results in dissociation of CCP and precipitation of casein. The calcium phosphate is removed by washing, and the casein is resolubilized with sodium hydroxide to achieve a pH of 7 (Barraquio & van de Voort, 1991). When the pH of a SCN system is near neutral, aggregates with an approximate diameter of 20 nm are formed, with the size of the aggregates dependent on the ionic strength of the system (HadjSadok et al., 2008). Addition of SCN to CMs leads to an increase in soluble proteins, inhibited rennet-induced aggregation and increase in the reconstitution properties of casein powders (Bot et al., 2020; Gaygadzhiev et al., 2012; Schokker et al., 2011). Thomar & Nicolai (2015)

demonstrated that SCN has the capacity to solubilize CCP from the CMs, functioning as a calcium-chelating agent, thereby destabilizing the CMs.

The primary constituent of β-casein concentrate (BCC) is β-casein, which represents around 35% of the casein present in bovine milk. Each monomer of β-casein has 209 amino acids, which results in an approximate molecular weight of 24 kDa and an isoelectric point of 4.9. At pH 6.6, β-casein has a highly negatively charged hydrophilic N-terminus and a weakly positively charged hydrophobic C-terminus, which gives it a strong amphiphilic character (Huppertz, 2013; O'Mahony & Fox, 2013; Pfaff & Anderer, 1988). In water systems the hydrophobic interactions between β-caseins results in self-association and the formation of β-casein micelles with an average diameter of 20–30 nm, with a hydrophobic core and hydrophilic outer layer (O'Connell et al., 2003). Most studies in the literature have focused on the extraction of β-casein from milk and the influence of temperature on these processes, which are under active development (Crowley et al., 2015; Thienel et al., 2018). A decrease in temperature results in a reduction of the hydrophobic interactions within the CMs, which facilitates the release of β-casein into the serum phase. The fraction of total casein that is not associated with CMs, and present in the serum phase, is referred to as "non-micellar casein". Cold membrane filtration is applied to fractionate the non-micellar β-casein, creating a filtrate enriched in β-casein, which is used to produce BCC. Other than the study of Schwartz et al. (2015), who blended different ratios of a rapeseed protein – napin – with β-casein and found significant changes in turbidity due to aggregate formation, there have been limited investigations on the properties of mixed protein systems of this kind.

In addition to protein blending, micronutrients are often added to milk protein beverages and in particular fortification with polyphenols from a variety of sources is becoming more common. Tea contains a high content of tea polyphenols (TPP), which are largely flavanols, specifically catechins, of which the four main catechins are: (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechin gallate, and (–)-epigallocatechin gallate,

which represent 1–3, 3–6, 3–6, 7–13% of the dry weight of green tea leaves, respectively (Graham, 1992; Muto et al., 2001; Scalbert & Williamson, 2000). Catechins mainly interact with proteins through hydrophobic interactions, but also via van der Waals binding and hydrogen bonds (Chanphai et al., 2018). Yazdi et al. (2014) studied the binding of hydrophobic polyphenols to β-casein-depleted CMs. The interior of the β-casein depleted CMs was more hydrophobic than the native CMs, and the hydrodynamic diameter of the micelles increased due to swelling.

The aim of this study was to compare the physicochemical properties of CMs after blending with SCN or BCC, using measurements of turbidity over time, physical stability by analytical centrifugation, micellar dissociation by quantification of non-micellar casein, and morphological analysis using transmission electron microscopy. It will also be the first time that the effects of mixing native CMs with a novel protein ingredient, like BCC, is compared with the effects of SCN. This study will contribute to a better understanding of how ingredient choice affects the functional properties of blended protein systems.

2.3. Materials and methods

2.3.1. Materials

Milk protein concentrate (MPC) containing 83.5% (w/w) protein was kindly provided by Glanbia (Kilkenny, Ireland). Sodium caseinate (SCN) containing 87.9% (w/w) protein was kindly donated by Kerry Ingredients Limited (Listowel, Co. Kerry, Ireland). The integrated membrane filtration process of O'Mahony et al. (2014), with slight modifications, was used to produce the spray dried BCC. This concentrate (89.4% protein, with a casein purity of 74.4%, of which 95.6% is β-casein) (Crowley, 2016) was kindly provided by the Centre for Dairy Research at the University of Wisconsin-Madison, USA. The protein content of all ingredients was determined using the Kjeldahl method (IDF, 2014). Tea catechin extract (98% tea polyphenols) was obtained from Kinglong Natural

Plant Products Industry Ltd, Changsha, Hunan, China. All water used in this research was ultrapure water (PURELAB option-Q, ELGA, Veolia Water, Paris, France).

2.3.2. Sample preparation

MPC and SCN were prepared at 37.5 g·L^{-1} protein content at 22 °C and heated to 50 °C for 4 h, while being stirred using an overhead stirrer with a two-bladed propeller at 400 rpm. BCC was hydrated in water (40 g·L^{-1}) over 5 h at 22 °C, while being stirred magnetically at 300 rpm. All suspensions were stored for 18 h at 4 °C under gentle magnetic stirring. Afterwards, all suspensions were equilibrated to room temperature and the pH was adjusted to 6.8, using 0.1 M HCl for the MPC and SCN suspensions, and 0.01 M HCl for the β-casein suspension. The suspensions were reconstituted with ultrapure water to reach the desired protein stock suspensions with a concentration of 30 g·L^{-1}. The MPC dispersion was diluted to create samples with MPC concentrations of 5, 10, 15 and 20 g·L^{-1} protein, which will hereafter be referred to as M25, M50, M75 and M100 (Table 2.1). Other samples were prepared by blending the MPC with either SCN or BCC dispersion, to a final protein concentration of 20 g·L^{-1}, and consisted of 25, 50, 75 or 100% SCN or BCC, which hereafter will be referred to as S25, S50, S75 and S100 and B25, B50, B75 and B100 (Table 2.1).

Tea catechin extract was rehydrated in water (20 g·L^{-1} TPP) in the dark at 22 °C under magnetic stirring (300 rpm) for 2 h. Afterwards, the pH was adjusted to 6.8, using 0.1 M NaOH and the sample was further diluted to a concentration of 15 g·L^{-1}. Three samples with TPP concentration of 4 g·L^{-1} TPP and protein content of 20 g·L^{-1}, composed of 100% MPC, 75% SCN and 75% BCC, which hereafter will be referred to as M100T, S75T and B75T (Table 2.1), were prepared by mixing the stock dispersion, with the protein stock dispersion prepared in advance of TPP addition.

Table 2.1: Overview of codes used for the different samples and their protein content which consisted out of proteins from milk protein concentrate (MPC), sodium caseinate (SCN) and β-casein concentrate (BCC) and their polyphenol content from tea polyphenols (TPP). The abbreviation N.A. stands for not applicable.

Sample	Protein content ($g \cdot L^{-1}$)			Polyphenol content ($g \cdot L^{-1}$)
	MPC	SCN	BCC	TPP
M25	5	N.A.	N.A.	N.A.
M50	10	N.A.	N.A.	N.A.
M75	15	N.A.	N.A.	N.A.
M100	20	N.A.	N.A.	N.A.
S25	15	5	N.A.	N.A.
S50	10	10	N.A.	N.A.
S75	5	15	N.A.	N.A.
S100	N.A.	20	N.A.	N.A.
B25	15	N.A.	5	N.A.
B50	10	N.A.	10	N.A.
B75	5	N.A.	15	N.A.
B100	N.A.	N.A.	20	N.A.
M100T	20	N.A.	N.A.	4
S75T	5	15	N.A.	4
B75T	5	N.A.	15	4

2.3.3. Turbidity measurements

Caseins can be dissociated in the serum phase (non-micellar casein) or be present as stable aggregates (casein micelles). CMs scatter light, resulting in the white colour and turbidity of dispersions containing CMs, e.g., milk, yoghurt and redispersed skimmed milk powders (Culler et al., 2017). When CMs dissociate (corresponding to an increase in non-micellar casein) their turbidity decreases; Pitkowski et al. (2008) measured CM dissociation by determining the turbidity at 685 nm of dispersions with casein concentration up to 40 g L1. In the current study, the turbidity of the samples was calculated by measuring absorbance at 685 nm, using the method of Pitkowski et al. (2008), with a UV–visible spectrophotometer (Varian Cary 300 Bio, Varian Inc., Middelburg, The Netherlands). The influence of time on turbidity was monitored by performing multiple measurements between 1 and 450 min.

2.3.4. Analytical centrifugation

Separation rates of all samples 90 min after sample preparation were analysed with an analytical centrifuge (LUMiSizer®, L.U.M. GmbH, Berlin, Germany), according to the method of Crowley et al. (2019), with some slight modifications. A wavelength of 865 nm, temperature of 22 °C and a centrifugation speed of 200 RPM (5×g) for ~10.5 min for the first centrifugation and 4000 rpm (2300×g) for ~24.5 min for the second centrifugation was used. The first centrifugation measured the light transmission of the sample before sedimentation and the second centrifugation measured the light transmission during the sedimentation of CMs.

2.3.5. Surface hydrophobicity determination

The protein concentration of samples M100, S75, B75, both with and without TPP, were diluted from 20 to 10 g/L 90 min after sample preparation. Afterwards, the samples were diluted to 0.03, 0.06, 0.09, 0.12 and 0.15 g/L with a sample buffer at pH 6.8, made by mixing 0.1 M citric acid with 0.2 M sodium phosphate. The surface hydrophobicity of these samples was measured using the method of Joyce et al., (2017) which used the hydrophobic probe 1-anilino-naphthalene-8-sulphonic acid ammonium salt (ANS). An 80 μM ANS solution, in 0.1 M sodium phosphate buffer, pH 6.8, was prepared and 50 μL of this solution was added to 100 μL of the diluted samples in a 96 well plate and equilibrated for 15 min in the dark. Afterwards, the fluorescence of the samples at 22 °C was determined with an excitation/emission wavelengths of 390/470 nm, which was done with a Varioskan Flash plate reader (Thermo Fisher Scientific Oy, Finland). The sample and ANS blank were both extracted from the measured fluorescence intensity. The fluorescence intensity is plotted against the protein concentration; the initial (S_0) is used as a measure for the surface hydrophobicity (all slopes had a R2 >0.95).

2.3.6. Centrifugal membrane filtration and determination of protein content of filtrates

Samples were filtered 90 min after preparation through an Amicon® Ultra 15 mL centrifugal membrane filter with 100 kDa molecular weight cut-off (Merck Millipore Ltd. Cork, Ireland), at 5000 × g for 40 min with a RC 5C plus centrifuge (Sorvall, ThermoFischer scientific, Waltham, Massachusetts, USA). This membrane centrifugation separates the CMs, SCN aggregates, and β-casein micelles from the non-micellar caseins and whey proteins, present in the filtrate. The total protein content of the filtrates was measured using the Bradford assay (Bradford, 1976). More specifically, this was achieved by adding 2.7 mL of 5 ×diluted protein assay dye reagent concentrate to 0.3 mL of 2 × diluted filtrate, with the absorbance measured at 595 nm using a UV–visible spectrophotometer (Varian Cary 300 Bio, Varian Inc., Middelburg, the Netherlands) in 4 mL cuvettes. Blood serum albumin (BSA) was used as a standard (25–500 $mg \cdot L^{-1}$), and a blank was prepared by adding ultrapure water instead of diluted filtrate. The reagent and standard were both purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2.3.7. Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, under reducing conditions, on the filtrates using the method described by Laemmli (1970) with minor modifications. The different samples were loaded on Mini-PROTEAN TGX precast gels and run in a mini-PROTEAN® Tetra vertical Electrophoresis Cell, both from Bio-Rad Laboratories (Hercules, CA, USA). The gels were Coomassie-stained, scanned with a desktop scanner (HP Scanjet G4010, HP, Leixlip, Ireland) and the pixel density of the bands representing the different proteins were quantitatively analyzed with the software GelAnalyzer 19.1 (www.gelanalyzer.com) to determine the concentrations of individual proteins in the filtrates. Throughout this article "α_s-casein" refers to the sum of α_{s1}-casein and α_{s2}-casein.

2.3.8. Transmission electron microscopy

Transmission electron microscopy (TEM) was used to visualize the morphology of micelles and protein particles present in samples M100, S75 and B75. The samples were 100x diluted and negatively stained with 2% uranyl acetate on 400-mesh copper grids with carbon film (Agar Scientific, Essex, UK). The TEM used was a JEM 2000FXII (Jeol Ltd., Tokyo, Japan), operated at 160 kV and a Megaview-III digital camera and AnalySIS software were used for visualization of the micelles.

2.3.9. Statistical analysis

All samples were prepared and analysed in triplicate and the mean and standard deviations are presented in the manuscript. Differences between the mean values were analysed statistically with the analysis of variance (ANOVA) and Tukey test, and the significance level was set to p-value ≤ 0.05. The software used to perform the statistical analyses was SPSS Statistics for macOS version 26 (SPSS inc, Chicago, USA).

2.4. Results and discussion

2.4.1. The influence of total protein concentration on structure and stability of casein micelles

The influence of protein concentration on the turbidity over 420 min was studied, with the data normalized by the initial turbidity and the results are shown in Figure 2.1A. Sample M100 and M75 were stable whereas samples M50 and M25 showed instability, demonstrating a reduction in turbidity of 0.03 and 0.12, respectively. Decreasing the MPC concentration resulted in a greater reduction in turbidity over time. Similar observations were made by Thomar & Nicolai (2015) who attributed this behaviour to dissociation of CMs, which is caused by the release of CCP from the CMs into the serum, resulting in CM destabilization and dissociation. They also stated that samples with a higher protein concentration from CMs are less prone to dissociation of CMs. At these higher concentrations, less diffusion of calcium from individual CMs is needed to reach an

equilibrium of calcium between the CMs and the serum phase. Upon addition of TPP to M100 (sample M100T), the turbidity did not change over time, suggesting that TPP do not influence the stability of CMs, as measured by turbidity.

The analytical centrifugation profile of the different samples was studied to better understand the dissociation state and physical stability of the sample, the results are shown in Figure 2.1B. All samples were stable at the lower centrifugal speeds of 5×g, and at higher centrifugal speeds of 2300×g, an increase in integral transmission can be observed, which was due to sedimentation of the CMs. As expected, samples with less protein had a higher initial integral transmission, due to lower numbers of CMs. When the analytical centrifugation results of M100T were compared to M100, it was shown that TPP decreased the integral transmission. TPP bind to the hydrophobic sites of the CMs through hydrophobic interactions (Yuksel et al., 2010). These interactions most likely increased the attractive forces withing the CMs, which will make it harder for the CMs to dissociate, leading to the decrease in integral transmission.

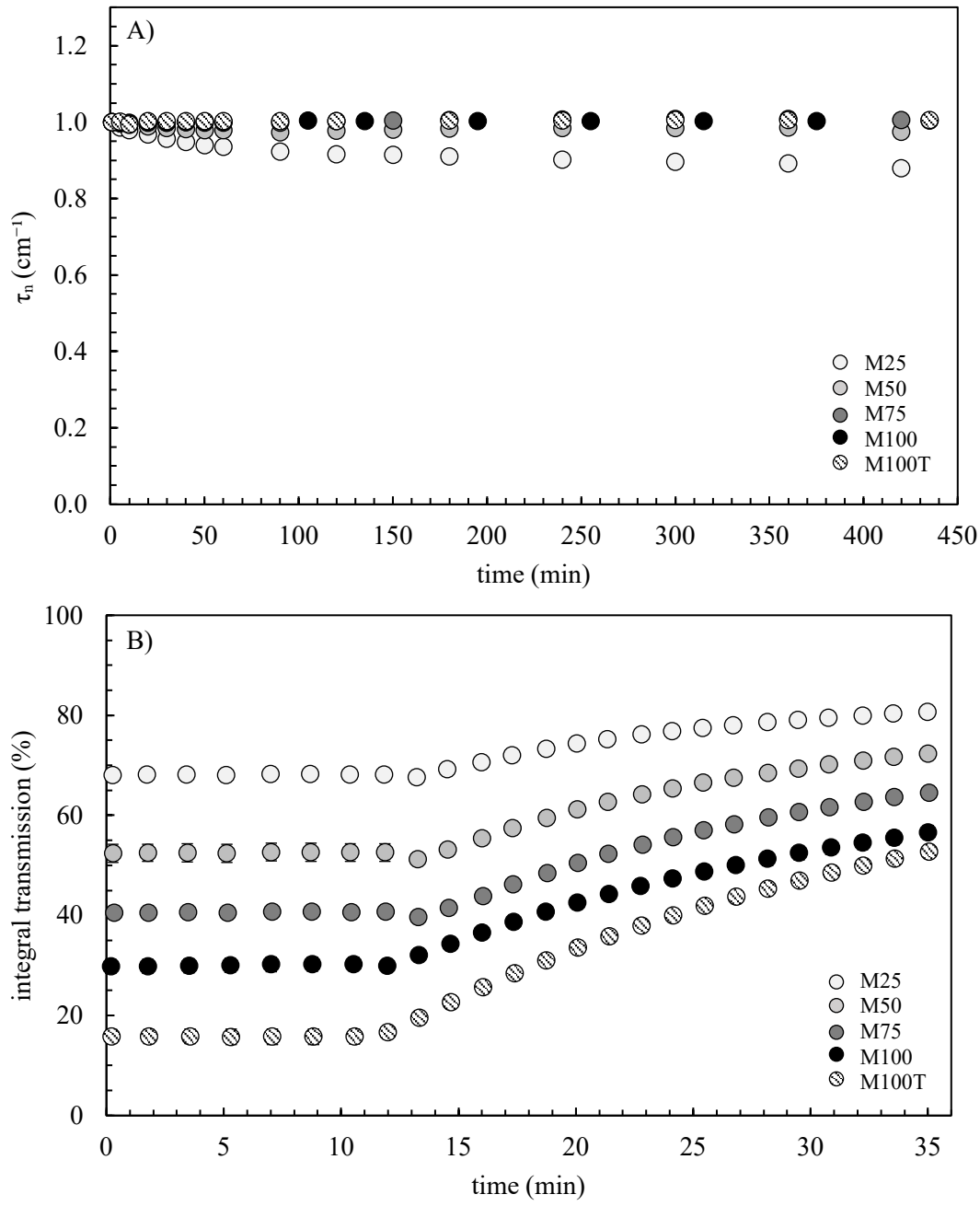

Figure 2.1. A) Turbidity (τ_n) of dispersions with different MPC concentrations (5, 10, 15 and 20 g·L^{-1} protein and 20 g·L^{-1} protein with 4 g·L^{-1} TPP, which is referred to as M25, M50, M75, M100 and M100T, respectively), normalized by their initial turbidity measured at 685 nm, as a function of time. Analysis was performed in triplicate; the curves are indicative of the trends observed. B) Sedimentation profiles of the dispersions, 90 min after preparation, in which the change in transmission of the NIR light through the sample cells is expressed over a time span of 35 min. The centrifugation speed in the first 10.5 min was 200 RPM (5×g) followed by 24.5 min with a centrifugation speed of 4000 RPM (2300×g). Mean values and standard deviations are presented (n = 3).

Yazdi et al. (2014) showed that hydrophobic polyphenols primarily bind to CMs via hydrophobic interactions at the CMs hydrophobic patches. ANS binding was performed on sample M100 and M100T to investigate the influence of TPP on the surface hydrophobicity of CMs. The binding of ANS gives information about the surface hydrophobicity of a system due to the difference in fluorescence behaviour of ANS in polar and apolar environments. In a polar environment, ANS has a low emission with a maximum at 520 nm, but in an apolar environments, e.g., when surrounded by hydrophobic amino acids, its fluorescence intensity increases and the maximum emission shifts to 470 nm (Liu & Guo, 2008). The surface hydrophobicity (S_0) value of M100 was significantly lower than M100T at 20.5 ± 1.4 and 7.7 ± 0.5 ($p \leq 0.05$), respectively. This is in line with the results of Yuksel et al. (2010), who studied the influence of TPP on ANS binding to skimmed milk and bovine casein. The authors also found a decrease in ANS binding upon TPP addition, resulting in a decrease in surface hydrophobicity S_0 value, which is probably the effect of binding TPP to the hydrophobic amino acids, preventing interaction with the ANS molecules, which reduces the fluorescence signal of the system.

In summary, an increase in MPC concentration results in a more stable CM structure, as also demonstrated by Thomar & Nicolai (2015). This phenomenon can be limited by addition of TPP, which was likely due to the hydrophobic bonds between TPP and the hydrophobic patches of CMs, which stabilize its structure and prevent dissociation.

2.4.2. Influence of sodium caseinate addition level on casein micelle structure and stability

It has been demonstrated that SCN increases the non-sedimentable/ non-micellar protein content of skimmed milk and MPC dispersions and facilitates solubilization of milk protein isolate (Bot et al., 2020; Lin et al., 2016; Thomar & Nicolai, 2015). In this study multiple experiments were performed to understand the effect of SCN addition and mixing ratio on the structure and stability of CMs.

The turbidity at different SCN:MPC ratios (S25, S50, S75) was measured over a time span of ~420 min (Figure 2.2A) with decreases of 0.04, 0.31 and 0.60 observed for S25, S50 and S75, respectively. An increase in SCN:MPC ratio resulted in a decrease in turbidity, which was even greater than the decrease in turbidity in samples without SCN, but at the same MPC concentration (Figure 2.1A). These results are in agreement with the results of Thomar & Nicolai (2015), who attributed this reduction in turbidity to the calcium chelation ability of SCN, which actively dissociates CCP from the CMs. This resulted in structural loss and causes CM dissociation, which lead to the turbidity decrease. This effect was most substantial for sample S75, as at this SCN concentration the largest decrease in turbidity was observed, which was likely the effect of greater calcium chelation. Likewise, Thomar & Nicolai (2015) showed that a SCN fraction of 75%, caused a significant reduction in turbidity and eventually complete dissociation of CMs. Sample S75T displayed a reduction in turbidity of 0.16 over 420 min (Figure 2.2A), which was significantly less compared to the 0.80 reduction in S75 ($p \leq 0.05$). This study indicates that altering the interactions present at the hydrophobic patches of the CMs, such as the addition of TPP, results in a reduction of the dissociation caused by SCN. The main interaction between polyphenols and CMs are hydrophobic interactions, which are present at the hydrophobic patches of CMs (Yazdi et al., 2014). A possible explanation could be that the TPP interacted with the hydrophobic patches of CMs, thereby stabilizing the CM structure and limiting the dissociation caused by SCN.

Analytical centrifugation was performed at 5×g and the sample was stable, while at 2300×g an increase in integral transmission is measured over time (Figure 2.2B). Increasing the SCN concentration resulted in a higher initial integral transmission and reduced the change between the initial and final integral transmission. The profile of S75 and S100 is similar, which was likely the effect of CM dissociation, creating a system with different particle properties which remain dispersed at the high centrifugal speed. These results align with the turbidity results in Figure 2.2A, together indicating that CMs

dissociated due to SCN addition and an increase in SCN concentration magnifies this effect. These sedimentation profile of S75T is similar to the one of S50 and has a lower initial and final integral transmission and a larger difference between them compared to S75, which indicates that there was less CM dissociation.

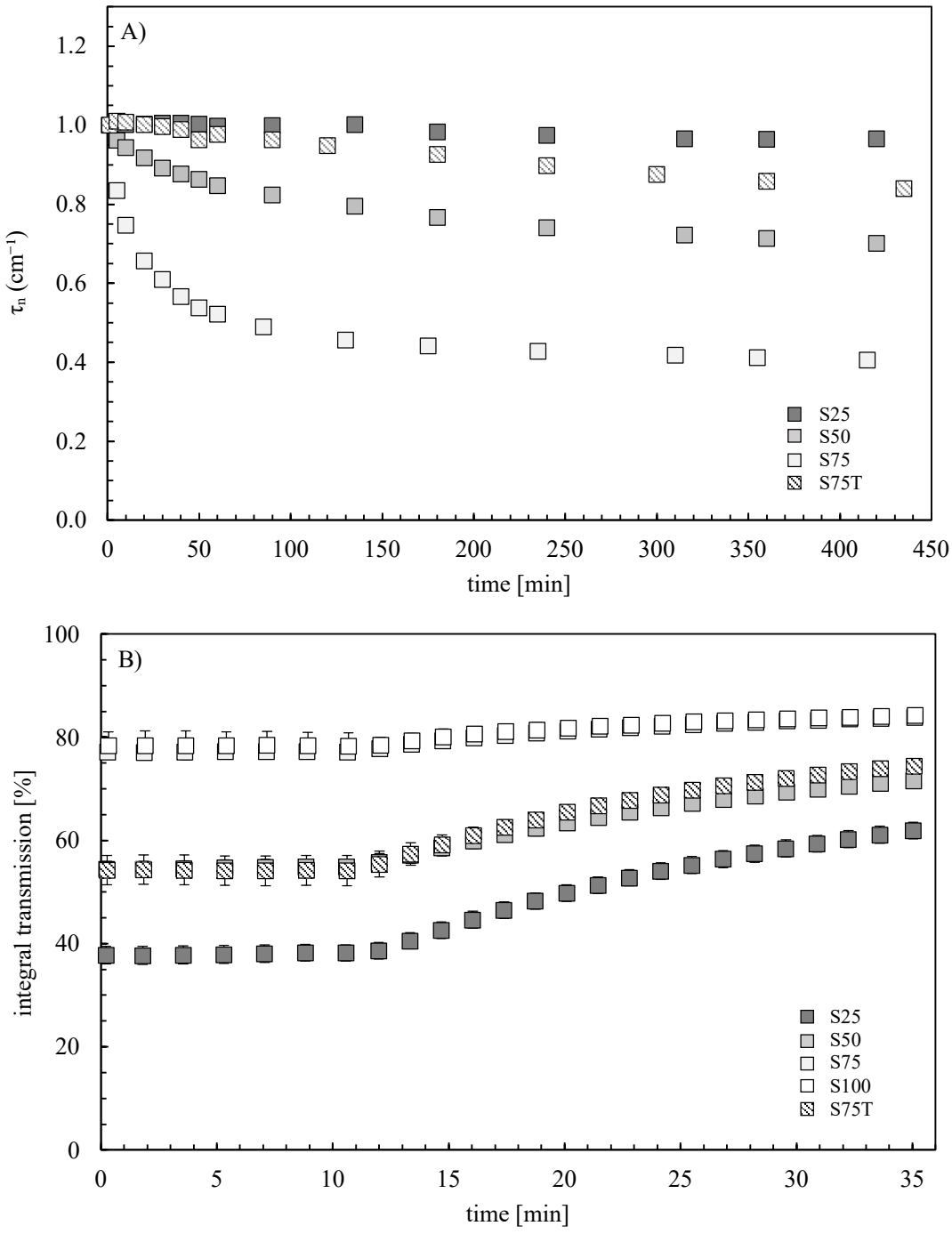

Figure 2.2: A) Turbidity (τ_n) of different dispersions, consisting out of different SCN and MPC blends (25, 50, 75 or 100% SCN and 75% SCN with 4 g·L^{-1} TPP, which is referred to as S25, S50, S75, S100 and S75T,

respectively), with a final protein concentration of 20 g·L^{-1}, normalized by their initial turbidity measured at 685 nm, as a function of time. Analysis was per-formed in triplicate; the curves are indicative of the trends observed. B) Sedimentation profiles of the dispersions, 90 min after preparation, in which the change in transmission of the NIR light through the sample cells is expressed over a time span of 35 min. The centrifugation speed in the first 10.5 min was 200 RPM (5×g) followed by 24.5 min with a centrifugation speed of 4000 RPM (2300×g). The mean values and standard deviations are presented (n = 3).

Both turbidity and analytical centrifugation analyses suggest that SCN addition to MPC causes dissociation of CMs, which is in agreement with previous results from Thomar & Nicolai (2015). Dissociation of CMs would be expected to increase the surface hydrophobicity due to exposure of hydrophobic amino acids, which were previously present in the core of the CMs. To study this the surface hydrophobicity was determined with an ANS binding study. The S_0 values of M100, S100, S75 and S75T are 20.5 ± 1.4, 21.0 ± 0.2, 23.4 ± 0.2 and 7.7 ± 0.5, respectively. There was no significant difference between the S_0 value of M100 and S100 ($p > 0.05$). This demonstrates that the increase in surface hydrophobicity S_0 value of S75 was due to a structural change and not to a higher surface hydrophobicity of SCN. The S_0 value of S75T was significantly lower in comparison to S75 ($p \leq 0.05$), suggesting that TPP binds to the hydrophobic surfaces, thereby preventing interaction of ANS with it, resulting in a lower S_0 value.

Dissociation of CMs should lead to a higher content of non-micellar casein and therefore non-sedimentable proteins (Lin et al., 2016; Thomar & Nicolai, 2015). This was demonstrated by separating the non-micellar fractions (whey proteins and dissociated caseins), from the micellar fractions (CMs and SCN aggregates) using centrifugal membranes (100 kDa cut-off) 90 min after sample preparation. The total amount of protein in the filtrate was quantified using a Bradford assay (Table 2.2). The permeates of M100, S25, S50, S75 and S100 were loaded on SDS-PAGE gels to identify the non-micellar proteins present in the filtrates, with the results found in Figure 2.3 and Table 2.2. In the SDS-PAGE gels the main proteins were found to be α_s-casein, β-casein, α-lactalbumin and β-lactoglobulin, with other milk proteins were only present at trace level. As expected, an

increase in SCN increased the amount of α_s-casein and β-casein in the permeate, indicating more non-micellar casein, thereby supporting the hypothesis that SCN promotes CM dissociation. Logically, it might be expected that the protein content of these filtrates would increase; however, there was no significant difference observed between the total protein content of permeates with and without SCN added ($p > 0.05$). This can be explained by the increase in whey protein content of samples with higher concentrations of MPC, as the two major whey proteins α-lactalbumin and β-lactoglobulin, which have a size of 14.2 and 18.3 kDa, are able to migrate through the membrane. Thus, all samples had roughly the same protein content, but an increase in SCN led to an increase of non-micellar α_s- and β-casein, which was most likely due to CM dissociation caused by SCN addition.

Table 2.2: Protein content and protein composition of filtrates from dispersions with different MPC with SCN or BCC blends (consisting out of 100% MPC, 25, 50, 75, 100% SCN and 25, 50, 75, 100% BCC, which is referred to as M100 and S25, S50, S75, S100 and B25, B50, B75, B100, respectively), after filtration through a 100 kDa centrifugal membrane. All dispersions had a final protein concentration of 20 $g \cdot L^{-1}$ before centrifugation.

Filtrate	Protein content of filtrate ($mg \cdot L^{-1}$)	Protein composition of filtrate (% of total intensity)			
		α_s-casein	β-casein	α-lactalbumin	β-lactoglobulin
M100	524.4 ± 26.2^{b}	N.D.	11.5 ± 0.5^{a}	$27.4 \pm 1.7^{c,d}$	61.1 ± 1.6^{d}
S25	$475.0 \pm 59.6^{a,b}$	N.D.	$12.6 \pm 1.8^{a,b}$	$26.4 \pm 0.6^{b,c,d}$	61.1 ± 2.5^{d}
S50	$419.8 \pm 47.93^{a,b}$	6.6 ± 0.9^{a}	$16.4 \pm 0.7^{a,b,c}$	$21.4 \pm 2.7^{a,b}$	$55.6 \pm 2.2^{c,d}$
S75	$404.2 \pm 73.7^{a,b}$	11.3 ± 1.1^{b}	$19.6 \pm 1.4^{c,d}$	$22.9 \pm 2.7^{b,c}$	46.3 ± 1.6^{b}
S100	311.0 ± 6.4^{a}	24.8 ± 1.7^{c}	35.6 ± 2.9^{e}	15.9 ± 2.3^{a}	23.8 ± 2.1^{a}
B25	$451.6 \pm 57.7^{a,b}$	N.D.	$14.8 \pm 1.8^{a,b,c}$	29.1 ± 2.0^{d}	$56.1 \pm 3.6^{c,d}$
B50	$426.7 \pm 91.7^{a,b}$	N.D.	$19.0 \pm 3.4^{c,d}$	$26.3 \pm 2.3^{b,c,d}$	$54.7 \pm 3.0^{c,d}$
B75	496.2 ± 36.6^{b}	N.D.	$17.1 \pm 1.2^{b,c}$	30.9 ± 1.1^{d}	$52.0 \pm 1.5^{b,c}$
B100	526.2 ± 14.9^{b}	N.D.	22.5 ± 1.1^{d}	$27.6 \pm 1.3^{c,d}$	$49.9 \pm 0.9^{b,c}$

Mean values and standard deviations are presented (n = 3) and the different superscript letters indicate statistical differences ($p \leq 0.05$). N.D. stands for not detected.

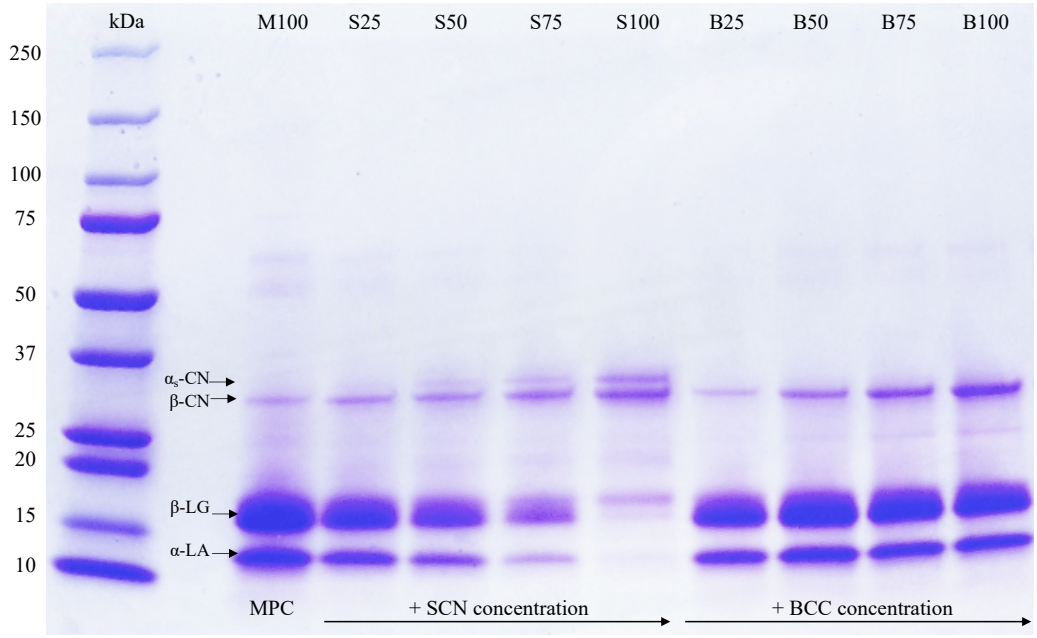

Figure 2.3: SDS-PAGE pattern of the filtrates made of the dispersions after filtration through a 100 kDa centrifugal membrane. M100 refers to the filtrate of the dispersion with 100% MPC. The dispersions with different MPC and SCN concentrations are referred to by S25, S50, S75 and S100, which consisted out of 25, 50, 75 and 100% SCN, respectively. The dispersions with different MPC and BCC concentrations are referred to by B25, B50, B75 and B100, which consisted out of 25, 50, 75 and 100% SCN, respectively. All dispersions had a final protein concentration of 20 g·L^{-1} before centrifugation. The abbreviations α_s- CN, β-CN, α-LA and β-LG represent α_s-casein, β-casein, α-lactalbumin and β-lactoglobulin, respectively.

The morphology of the CMs in M100 and S75 was visualized by TEM in order to study the impact of SCN on the CMs (Figure 2.4 A, B, C and D). The CMs of the MPC had a spherical shape, similar to that reported previously (Karlsson et al., 2007; McMahon & McManus, 1998; McMahon & Oommen, 2008). Addition of SCN to CMs appeared to fragment the CMs to smaller less spherical particles (Figure 2.4 C and D). Similar structures for CMs were visualized by Marchin et al. (2007) by the use of cryo-TEM after ethylenediaminetetra-acetic acid (EDTA) addition, which is a known calcium-chelating agent and the TEM images of this research support the hypothesis for the disassociation of CMs caused by SCN.

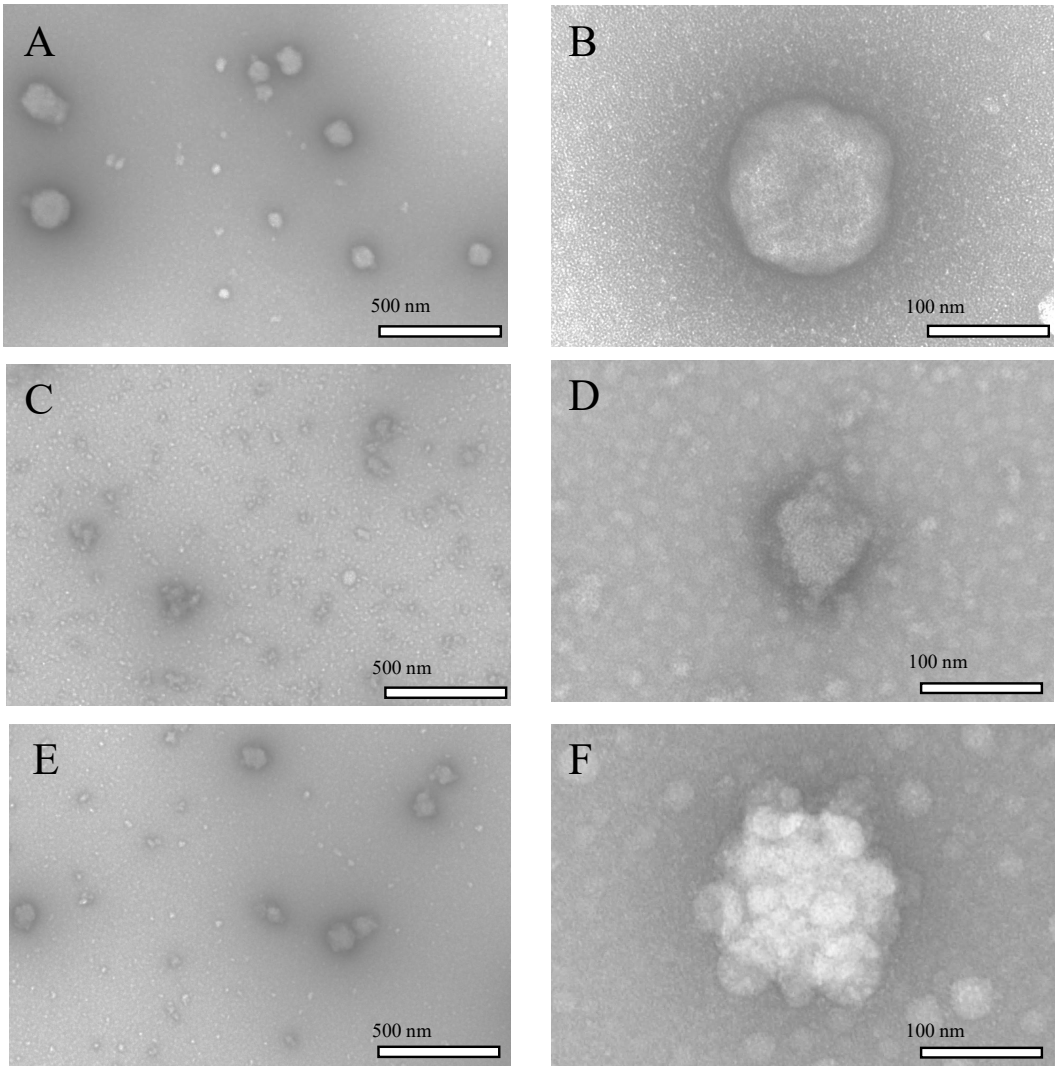

Figure 2.4: Transmission electron microscopy images of casein micelles in dispersions with different ingredients. A and B visualize casein micelles in a dispersion with MPC (M100), C and D visualize casein micelles in a dispersion with 75% SCN and 25% MPC (S75) and E and F visualize casein micelles in a dispersion with a 75% BCC and 25% MPC (B75). All dispersions had a final protein concentration of 20 g·L^{-1}.

It can be concluded that SCN addition promotes dissociation of CMs and that an increase in SCN:MPC ratio further increases this dissociation. In contrast, the addition of TPP seems to inhibit this effect, likely due to stabilization of the CM structure through increased hydrophobic interactions.

2.4.3. Influence of β-casein concentrate addition level on casein micelle structure and stability

This is, to our knowledge, the first study to investigate the effect of BCC on the stability and physicochemical properties of CMs. First, structural changes within the CMs caused by BCC addition were examined by measuring the turbidity of samples at different BCC:MPC ratio over 430 min and the turbidity results of B25, B50 and B75 are shown in Figure 2.5A. In contrast with the decrease in turbidity reported for samples with SCN, the addition of BCC resulted in an increase in turbidity of 0.05, 0.13 and 0.08, for B25, B50 and B75, respectively. Sample B75 reached a peak turbidity of 0.12 after 90 min. The reason for this increase in turbidity could be explained by the morphology of the CMs in sample B75, which were visualized by TEM (Figure 2.4E and F). Small spherical particles of roughly 30 nm, likely β-casein micelles, can be observed interacted with the surface of the CMs (O'Connell et al., 2003). While the influence of β-casein addition to CMs has not been studied previously using TEM analysis, there are reports on the use of cryo-TEM to visualize β-casein micelles (Bachar et al., 2012; Portnaya et al., 2006). The overall shape/conformation of β-casein micelles in these images is similar in size and shape to that of the small spherical particles shown to be interacting with the surface of CMs in Figure 2.4F. This change in morphology explains the increase in turbidity and the interaction between β-casein micelles and CMs might be due to the hydrogen bonds between the hydrophilic outer layer of β-casein micelles and the hydrophilic hairy layer of the CMs. The turbidity decrease observed in sample B75 after 90 min might be the effect of CM dissociation or detachment of β-casein micelles from the CMs. The second being more likely because the turbidity restabilizes after 320 min around its initial turbidity, which indicates that the structure of the CMs was the same as before the interaction with β-casein micelles and no dissociation occurred. Addition of TPP to B75 resulted in an increase in turbidity over time (Figure 2.5A), which indicated that the TPP prevents the detachment

of β-casein micelles; a possible explanation for this is that TPP increased the strength of interaction between the β-casein micelles and CMs.

The results of the analytical centrifugation of B25, B50, B75 and B100 are shown in Figure 2.5B. Like samples with SCN, the samples with BCC are stable at 5×g, but at 2300×g the increase in integral transmission indicates destabilization. Increasing the BCC:MPC ratio results in a higher integral transmission and reduced magnitude of change between the initial and final transmission values. The results for analytical centrifugation of the samples with B25, B50 and B75 have a lower initial transmission in comparison to their M and S counterparts (Figure 2.1B and 2.2B). Samples containing BCC transmitted less light, which is probably due the presence of larger particles compared to the samples without BCC or with SCN. This is in line with the turbidity results (Figure 2.1A and 2.2A) and also observed by the TEM pictures. Sample B75T has a lower initial integral transmission but the same final integral transmission compared to B75 (Figure 2.5B), which suggests that the supernatant of both samples has similar types of particles. The ANS binding study was used to understand the influence of BCC on the surface hydrophobicity of the CMs. The surface hydrophobicity S_0 value of 7.9 ± 0.1 for B100 was significantly different from M100 and S100 ($p \leq 0.05$), which is probably related to the hydrophilic outer layer of the β-casein micelles. The S_0 value of B75 was also considerably lower than M100 and S75, which could be due to better covering of the CMs surface by β-casein micelles. The surface hydrophobicity of S75T, at 7.7 ± 0.5 was significantly lower than S75 ($p \leq 0.05$), which suggests that TPP also has a higher binding affinity than ANS for the hydrophobic surface of β-casein micelles.

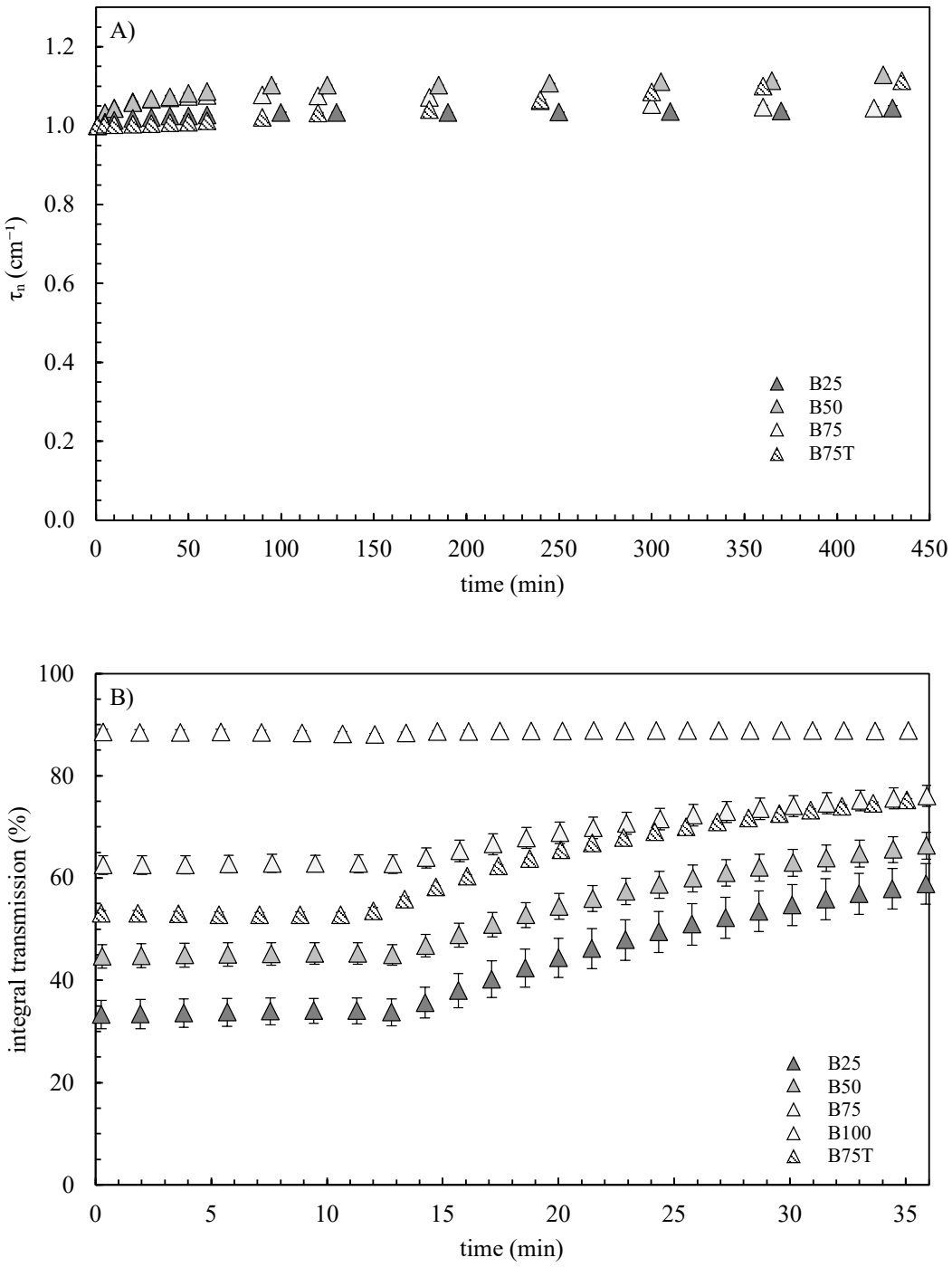

Figure 2.5: A) Turbidity (τ_n) of different dispersions, consisting out of different BCC and MPC blends (25, 50, 75 or 100% BCC and 75% BCC with 4 g·L^{-1} TPP, which is referred to as B25, B50, B75, B100 and B75T, respectively), with a final protein concentration of 20 g·L^{-1}, normalized by their initial turbidity measured at 685 nm, as a function of time. Analysis was per-formed in triplicate; the curves are indicative of the trends observed. B) Sedimentation profiles of the dispersions, 90 min after preparation, in which the change in transmission of the NIR light through the sample cells is expressed over a time span of 35 min. The

centrifugation speed in the first 10.5 min was 200 RPM (5×g) followed by 24.5 min with a centrifugation speed of 4000 RPM (2300×g). The mean values and standard deviations are presented (n = 3).

The samples with different BCC:MPC ratios were centrifugally filtered at 100 kDa and the total protein content of the filtrates and their protein profile analyzed (Figure 2.3, Table 2.2) to gain more information about any differences in the non-micellar casein fraction caused by different BCC:MPC ratios. The results demonstrated that there was no clear relationship between BCC concentration and the total protein content of the filtrates. Analysis of the SDS-PAGE patterns of the filtrates from sample B100 demonstrated that most β-casein was present in the form of β-casein micelles, with molecular weight greater than 100 kDa. The SDS-PAGE patterns also indicate that an increase in BCC results in an increase in non-micellar β-casein. However, there was no increase in the α_s-casein content, making it unlikely that the addition of BCC caused dissociation of CMs. The increase in β-casein content can be correlated to the higher content of non-micellar β-casein in BCC compared to MPC.

2.5. Conclusions

This study demonstrated that addition of SCN to MPC caused a decrease in turbidity over time and increased the integral transmission and surface hydrophobicity of CMs. This was likely the effect of dissociation of CMs, caused by SCN addition, which resulted in increases in the contents of non-micellar α_s- and β-casein in the suspensions. Addition of TPP reduced this effect, possibly due to structural stabilization of the CMs, by binding to the hydrophobic patches, making them less prone to disintegration on subsequent addition of SCN. BCC had the opposite effect, as its addition increased the turbidity over time and decreased the initial transmission of the centrifugation profile and the surface hydrophobicity of the sample. This was most likely the result of β-casein micelles interacting with the surface of the CMs, as visualized by TEM images; the results also indicated that addition of TPP stabilized this interaction. Mixing casein ingredients can result in different protein-protein interactions that affect functional properties.

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Chapter 3

The effect of blackberry polyphenols on the physicochemical properties and heat stability of skimmed milk and high protein milk

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Declaration

This chapter was written by author TMvdL and reviewed by all co-authors. TMvdL co-designed the study and generated all data included in this chapter. TRM assisted TMvdL with the rheology and particle size analysis.

This chapter was submitted to *International Dairy Journal* as: van de Langerijt, T. M., Murphy, T.R., O'Mahony, J. A., & Crowley, S. V. (2023) The influence of blackberry on the physicochemical properties and heat stability of skimmed milk and high protein milk.

3.1. Abstract

The influence of blackberry juice (BBJ) on the physicochemical properties and heat stability of skimmed milk with and without sodium caseinate or micellar casein concentrate (MCC) addition was investigated. This was performed by measuring the ionic calcium content, heat coagulation time, viscosity, particle size distribution and analytical centrifugation profiles of the different samples. BBJ decreased the ionic calcium of the samples, which resulted in a higher heat coagulation time at pH < 6.5. It's addition also prevented the increase in viscosity observed in the skimmed milk and skimmed milk with MCC at pH 6.2 after heating. The particle size distribution, turbidity and analytical centrifugation profiles indicated this was due to a prevention of protein aggregation. In conclusion, the blackberry juice improved the heat stability of different types of milk systems in the pH range between 6.2 and 6.4, which was likely due to binding of calcium by blackberry polyphenols.

3.2. Introduction

Functional foods are foods that are consumed on a regular basis in normal quantities with a proven health benefit (Doyon & Labrecque, 2008). The market for these types of foods is growing rapidly and was estimated at 280 billion U.S. dollars in 2021 (Grand View Research, 2023; Rodríguez-Pérez et al., 2019). Fruit-dairy beverages with high level of protein and polyphenols is an example of such functional foods (Yildirim-Elikoglu & Erdem, 2018). Polyphenols are secondary plant metabolites with an aromatic benzenoid ring and hydroxyl group (Belščak-Cvitanović et al., 2018). They are linked to positive health effects, such as reducing the risk of cancer, cardiovascular disease and anti-inflammation (Rodríguez-Daza et al., 2021; Scalbert et al., 2005). Both polyphenols, primarily anthocyanins, and milk proteins are positively linked to enhancement of muscle strength and recovery (Carey et al., 2021; Sousa et al., 2014). This makes beverages enriched in milk proteins and blackberry juice (BBJ) with associated high levels of anthocyanins (Oszmiański et al., 2015), ideal for sports nutrition.

Although fruit-dairy beverages have strong commercial potential, there is a lack of understanding on the effects of fruit juice on the physicochemical properties of milk systems, especially during heat treatment. He et al. (2015), investigated the effect of heat treatment on the antioxidant activity of polyphenols in fruit juice-dairy beverages and found that chlorogenic acid was more sensitive to processing then catechin. O'Connell & Fox (1999) studied the influence of polyphenols on the heat stability of milk and found a correlation between the type of polyphenol added to the system and heat stability. It is known that polyphenols stabilize the structure of casein micelles, with the exact mechanism being unclear (van de Langerijt et al., 2022). To our knowledge no research has focused on the influence of fruit juice on the heat stability of milk beverages fortified with milk protein, which can be achieved with a variety of ingredients in an industrial context, including sodium caseinate (SCN), micellar casein concentrate (MCC) and milk protein concentrate (MPC).

The aim of this study was to investigate the effect of BBJ on the physicochemical properties and heat stability of skimmed milk and two types of high protein milk, both made of skimmed milk to which sodium caseinate (SCN) or micellar casein concentrate (MCC) was added. SCN and MCC were selected because these ingredients have similar protein profiles (casein-dominant) but different protein structures. MCC contains native casein micelles and SCN small casein aggregates with an approximate diameter of 20 nm (HadjSadok et al., 2008; Hammam et al., 2021). Ingredients high in native micellar casein (like MCC and MPC) are associated with high levels of ionic calcium (Crowley et al., 2014) while SCN can reduce ionic calcium content (Thomar & Nicolai, 2015), which can be expected to impact heat stability. The effects of juice addition, pH, and total protein concentration on the physicochemical properties and heat stability of milk-BBJ systems was studied by means of heat coagulation time, analytical centrifugation, particle size analysis, ionic calcium and viscosity. This study aims to underpin the formulation of next generation health-promoting dairy beverages.

3.3. Materials and methods

3.3.1. Materials

Low-heat skimmed milk powder (LH-SMP) containing 36.5% (w/w) protein (IDF, 2014) was kindly donated by Arrabawn (Nenagh, Co. Tipperary, Ireland). SCN containing 85.8% protein was kindly donated by Kerry Ingredients Limited (Listowel, Co. Kerry, Ireland). MCC containing 83.3% protein was kindly provided by Milei (Leutkirch im Allgäu, Germany). Blackberry puree was obtained from Wild Orchard (Limerick, Ireland). All water used in this research was ultrapure water (PURELAB option-Q, ELGA, Veolia Water, Paris, France). All chemicals used in this research were obtained from Merck (Darmstadt, Germany).

3.3.2. Sample preparation

To create BBJ the blackberry puree was clarified according to the method of Kelly et al. (2016), with slight modifications. In short, a pectolytic enzyme (Pectinex® Ultra Color, Novozymes, Denmark) was diluted 1:10 with water and added to the puree at 0.25 mL/L. Afterwards, it was heated to 50 °C for 1 h in a water bath while being stirred using an overhead stirrer with a two-bladed propeller at 800 rpm, in a stainless-steel beaker covered with aluminium foil. Subsequently, the pectinase enzyme was deactivated by heating the puree to 80 °C in a water bath for 5 min, of which the puree was centrifuged in an RC-5C Plus centrifuge (Kendro Laboratory Products, Connecticut, USA) at 3000 RPM (1500×g) and 20 °C for 10 min. The supernatant was separated from the pellet and freeze dried, creating a powder with a polyphenol content of 5% (w/w). The polyphenol content was determined by the Folin-Ciocalteu assay (Singleton et al., 1999).

Three different samples were created, skimmed-milk (SM), sodium caseinate milk (SCM) and micellar casein milk (MCM) (Table 3.1). SM had a final protein content of 3.5 % (w/w) origination from LH-SMP. SCM and MCM had a final protein content of 5.5% of which 3.5 % originated from LH-SMP and 2% from SCN or MCC. The proteins were dissolved in 60% of the final water content at 20 ºC, after which they were heated to 50 ºC for 4 h, while being stirred magnetically. All suspensions were stored for 18 h at 4 ºC under gentle magnetic stirring, after which all suspensions were equilibrated at room temperature. For the milk samples, water was added to the samples providing a 5% water content for pH adjustment, after the pH adjustment with 0.1 N NaOH or HCl, water was added to reach the final protein content of 3.5 or 5.5% (w/w).

The freeze-dried blackberry powder was rehydrated in 65% of the water needed to make a dispersion with 7.0% freeze dried juice and a 0.35% polyphenol concentration. The pH of the solution was increased to 6.8 with 1N NaOH, after which water was added to reach the final weight. For the blackberry juice-dairy beverages (BJDB), the juice was added to the protein solutions, the pH was adjusted, and water was added to reach samples

with a final protein concentration of 3.5 or 5.5% (w/w). This created three different BJDB, blackberry milk (BM), blackberry sodium caseinate milk (SCM) and blackberry casein concentrate milk (Table 3.1).

Table 3.1: Overview of the different samples, along with sample codes and their protein content, which consist of low heat skimmed milk powder (LH-SMP), sodium caseinate (SCN) and micellar casein concentrate (MCC), and blackberry juice (BBJ) content.

Sample	Sample codes	Protein (%)			BBJ (%)
		LH-SMP	SCN	MCC	
Skimmed milk	SM	3.5	-	-	-
Blackberry milk	BM	3.5	-	-	10
Sodium caseinate milk	SCM	3.5	2	-	-
Blackberry sodium caseinate milk	BSCM	3.5	2	-	10
Micellar casein milk	MCM	3.5	-	2	-
Blackberry micellar casein milk	BMCM	3.5	-	2	10

All percentages are expressed as % (w/w) and the symbol (-) indicated that this ingredient was not present in the sample.

3.3.3. Heat stability at 140 ºC and 85 ºC

The method of White & Davies (1966) was used to determine the heat coagulation time (HCT) of the different samples at 140 ºC in a pH range between 6.2 and 7.2 (interval of 0.1). Glass tubes (12 cm length, 8 mm diameter, 1 mm wall thickness) were filled with 2 mL of sample, sealed with silicone bungs, placed in the oil bath, and rocked. The HCT was taken as the time between placing the sample in the oil bath and complete coagulation of the sample.

A sample volume of 28 mL was heated in an AR-G2 controlled-stress rheometer (TA Instruments, Crawley, UK) equipped with a starch pasting cell geometry. The sample was equilibrated at 20 °C for 2 min. A peak hold at 20 °C for 2 min at a shear rate of 100 s^{-1}, was followed by a temperature ramp of 10 °C / min to a temperature of 85 °C. The sample was then kept at 85 °C for 2 min and cooled at 10 °C / min to a temperature of 10

°C, after which it was held at 13 °C for 10 min. This was performed under a constant shear rate of 100 s^{-1}. The samples were analysed further after heating, as following.

3.3.4. Particle size

Samples before heating and after heating were diluted in simulated milk ultrafiltrate (SMUF) to a protein concentration of 0.35% (w/w). SMUF was prepared by the method of Jenness (1962). For each sample three scans were performed at 25 °C. The particle size of the samples before and after heating were measured by dynamic light scattering with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK), which was equipped with a He-Ne laser emitting at 633 nm. The refractive index of 1.45 was set for the particles and 1.33 for the dispersant. The dispersant viscosity was set at 0.8872 mPa·s. Before each measurement, the sample was equilibrated for 120 s at 25 °C, after which 3 measurements of 11 runs of 10 s each were taken, using a back-scattering configuration with a scattering angle of 173°.

3.3.5. Analytical centrifugation

Before and after heating to 85 °C for 2 min the samples, which were diluted in SMUF to a protein concentration of 2% (w/w), were analysed with analytical centrifugation (LUMiSizer®, L.U.M. GmbH, Berlin, Germany) according to the method of van de Langerijt et al. (2022). In short, the samples were centrifuged in two stages, stage one had a centrifugation speed of 200 RPM (5×g) for around 8 min and stage two a centrifugation speed of 4000 rpm (2300×g) for around 38 min.

3.3.6. Determination of ionic calcium

The concentration of ionic calcium was measured using a Titrando 907 auto titrator equipped with a polymer Ca2+ ion-selective electrode (Metrohm Ireland Ltd, Carlow, Ireland). This was performed using the method of Crowley et al., (2014), with slight modifications. Calcium standards of 0.1, 0.5, 1, 3 and 6 mM CaCl2 were used. These standards were prepared in a KCl and imidazole buffer. For the milk samples buffers with

an ionic strength of 40 mM/L were used and for the BJDB samples buffers with an ionic strength of 80 mM/L were used. Different ionic strengths were used in order to simulate the conductivity of the milk samples (± 6 µs/cm) and BJDB samples (± 10 µs/cm). Samples and standards were measured at 25 °C after a 240 s equilibrium.

3.3.7. Statistical analysis

Samples were analysed in triplicate, except for the rheology measurements which were performed in duplicate. The means and standard deviations are presented in the manuscript. Differences between the mean values of the triplicates were analysed statistically with the analysis of variance (ANOVA) and Tukey test. Differences between the mean values of the duplicates were analysed with an independent t-test. For both tests, the significance level was set to $p < 0.05$. The software used to perform the statistical analyses was SPSS Statistics for macOS version 26 (SPSS inc, Chicago, USA).

3.4. Results

3.4.1. Ionic calcium content

Figure 3.1 provides the results for amount of ionic calcium measured in the different samples. A decrease in pH increased the ionic calcium content in all samples. Samples containing SCN (SCM and BSCM) had the lowest ionic calcium content and samples with MCC (MCM and BMCM) had the highest ionic calcium content. Addition of BBJ also decreased the ionic calcium content of all samples.

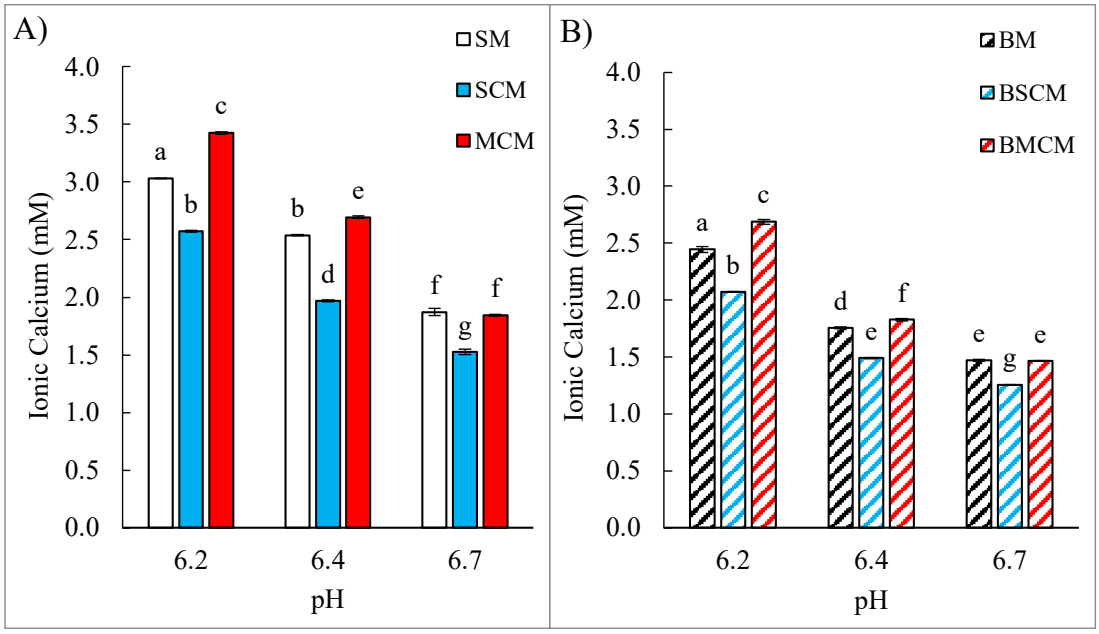

Figure 3.1: Ionic calcium at different pH values. A) Represents the milk samples and B) the blackberry juice dairy beverages. The different samples analysed were skimmed milk (SM), sodium caseinate milk (SCM) and micellar casein milk (MCM), blackberry milk (BM), blackberry sodium caseinate milk (BSCM) and blackberry micellar casein milk (BMCM). All samples contained 3.5% (w/w) protein from low heat skimmed milk powder, the sodium caseinate and casein milk also contained 2% protein from sodium caseinate or micellar casein concentrate, and the blackberry juice dairy beverages contained 7% freeze dried blackberry juice. The mean values and standard deviations are presented (n = 3), and the different superscript letters indicate statistical differences ($p < 0.05$).

3.4.2. Influence of pH on the heat coagulation time at 140 ºC

The HCT-pH profiles of the different milk and BJDB are presented in Figure 3.2. The HCT-pH profile of milk has a maximum at pH 6.7 and minimum at pH 6.9 (Figure 2A). SCM had a HCT-pH profile similar to milk but a higher HCT between pH 6.3-6.5 and pH 6.8-7.1. The HCT-pH profile of MCM was similar to that of SM, the order of HCT was SCM > SM and MCM.

BBJ had a substantial effect on the HCT-pH profile of all analysed milk systems (Figure 3.2B). At pH > 6.5, BBJ decreased the HCT, and pH < 6.5 BBJ increased the HCT with a peak at pH 6.4. The order in which BBJ increased the HCT at pH < 6.5 of the different BJDB was BM > BSCM > BMCM.

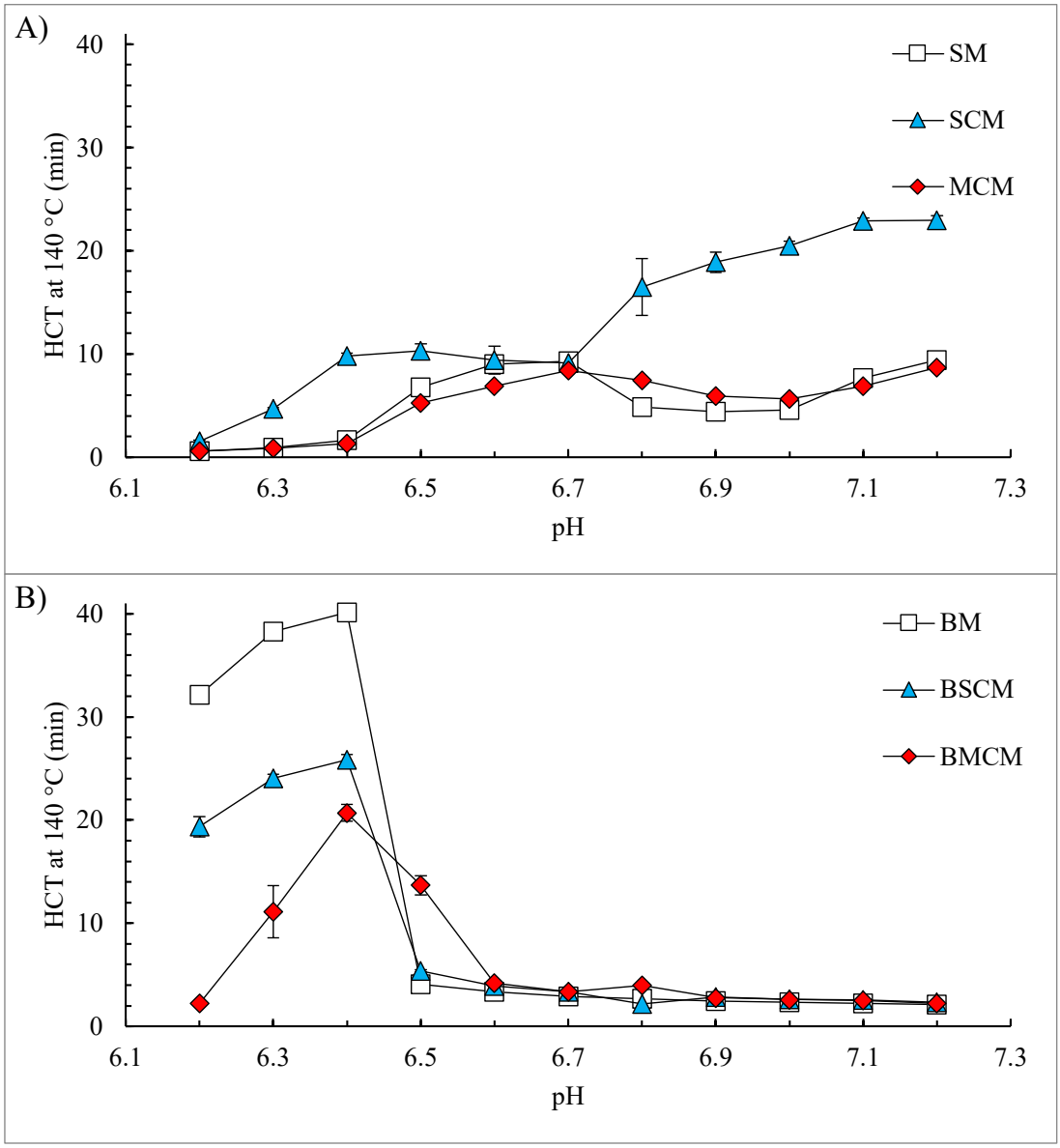

Figure 3.2: Heat coagulation time (HCT) – pH profiles at 140 °C, in which A) represents skimmed milk (SM), sodium caseinate milk (SCM), and micellar casein milk (MCM) and B) represents blackberry milk (BM), blackberry sodium caseinate milk (BSCM), and blackberry micellar casein milk (BMCM). All samples contained 3.5% (w/w) protein from low heat skimmed milk powder, the sodium caseinate and casein milk contained 2% protein from sodium caseinate or micellar casein concentrate. The blackberry milk samples contained 7% freeze dried blackberry juice. The mean values and standard deviations are presented (n = 3).

3.4.3. Viscosity during thermal processing

Before heating there was no significant (P>0.05) difference between the samples at pH 6.2, 6.4 and 6.7 at 20 °C. Addition of SCN, MCC and BBJ increased the initial viscosity of the samples (Figure 3.3, Table 3.2). During the heat treatment in the starch

pasting cell the viscosity of the samples was stable at 20 °C, decreased when the temperature increased to 85 °C, was stable when the temperature is 85 °C, increased when the temperature is decreased and became stable when the sample reached 13 °C. An exception was SM and MCM at pH 6.2. For SM at pH 6.2, viscosity increased during the cooling step compared to the other samples to 83.4 ± 1.0 mPa·s. In the case of MCM at pH 6.2 the viscosity increased during the heating step and had a large standard deviation, due to aggregate formation, which gradually decreased during the cooling step. The viscosity reached during the cooling step, 127.9 ± 0.6 mPa·s, was also significantly ($P<0.05$) higher than that of the other samples (Figure 3.3A, Table 3.2).

The viscosity of the samples containing BBJ all followed the same trend during heating in the starch pasting cell (Figure 3.3B), which was similar to the behaviour of the samples containing only milk protein (Figure 3.3A).

Table 3.2: Viscosity of samples during heat treatments in a starch pasting cell, z-average diameter (z.ave.d) and polydispersity index (PDI) before heat treatment (BH) and after the heat treatment (AH). The samples consisted of skimmed milk (SM), sodium caseinate milk (SCM), micellar casein milk (CM), blackberry milk (BM), blackberry sodium caseinate milk (BSCM) and blackberry micellar casein milk (BCM). All samples contained 3.5% (w/w) protein from low heat skimmed milk powder, the sodium caseinate and casein milk also contained 2% protein from sodium caseinate or micellar casein concentrate. The blackberry juice dairy beverages contained 7% freeze dried blackberry juice. The mean values and standard deviations of viscosity are presented (n = 2) and the different superscript letters indicate statistical differences ($p < 0.05$), calculated by independent t-test. The mean values and standard deviations of z.ave.d. and PDI are presented (n = 3) and the different superscripts letters indicate statistical differences ($p < 0.05$), calculated by analysis of variance and Tukey test.

Sample	pH	Viscosity (mPa·s)			z.ave.d (nm)		PDI	
		20 °C	85 °C	13 °C	BH	AH	BH	AH
SM	6.2	64.6±0.8^{a}	58.2±0.8^{b}	83.4±1.0^{c}	181±1^{a}	453±9^{j}	0.08±0.01^{a}	0.33±0.04^{b}
	6.4	64.7±0.1^{a}	50.3±0.1^{a}	67.0±0.1^{a}	182±1^{a,b}	189±2^{a,b}	0.10±0.04^{a}	0.09±0.01^{a}
	6.7	64.5±0.5^{a}	50.2±0.0^{a}	66.6±0.2^{a}	186±1^{a,b,c}	187±3^{a}	0.10±0.02^{a}	0.08±0.02^{a}
SCM	6.2	72.1±0.7^{c}	55.1±0.0^{a}	74.5±0.1^{b}	181±2^{a}	218±3^{g,h}	0.10±0.02^{a}	0.12±0.02^{a}
	6.4	70.8±3.2^{b,c}	57.5±1.0^{a}	72.1±2.8^{b}	183±1^{a,b}	193±1^{b,c}	0.09±0.03^{a}	0.12±0.02^{a}
	6.7	73.6±0.4^{c}	57.7±0.5^{a}	74.0±0.8^{b}	185±0^{a,b,c}	185±2^{a}	0.11±0.02^{a}	0.10±0.02^{a}
MCM	6.2	73.2±0.7^{c}	61.3±1.2^{b}	127.9±0.6^{d}	190±2^{a,b,c,d,e,f}	2853±326^{k}	0.11±0.02^{a}	0.50±0.02^{c}
	6.4	72.0±0.1^{c}	53.8±0.8^{a}	74.2±0.3^{b}	192±1^{a,b,c,d,e,f}	215±3^{f,g}	0.10±0.02^{a}	0.10±0.02^{a}
	6.7	72.2±0.3^{c}	53.2±1.7^{a}	74.3±0.2^{b}	198±3^{e}	197±3^{c}	0.10±0.03^{a}	0.10±0.02^{a}
BM	6.2	69.1±0.6^{b}	54.5±0.2^{a}	75.1±0.2^{b}	187±2^{a,b,c,d}	189±2^{a,b}	0.08±0.01^{a}	0.08±0.02^{a}
	6.4	69.0±0.0^{b}	54.3±0.2^{a}	74.7±0.4^{b}	186±2^{a,b,c}	192±3^{b,c}	0.09±0.02^{a}	0.09±0.02^{a}
	6.7	68.4±0.9^{b}	54.3±0.1^{a}	74.0±0.7^{b}	183±2^{a,b}	215±2^{f,g}	0.10±0.01^{a}	0.10±0.01^{a}
BSCM	6.2	75.9±0.8^{d}	56.4±0.1^{b}	81.6±0.5^{c}	194±1^{c,d,e,f}	213±2^{e,f,g}	0.11±0.01^{a}	0.08±0.03^{a}
	6.4	76.3±0.2^{d}	56.2±0.1^{b}	82.3±0.6^{c}	188±2^{a,b,c,d,e}	205±2^{d}	0.11±0.02^{a}	0.09±0.02^{a}
	6.7	76.5±0.2^{d}	56.2±0.0^{b}	80.6±1.0^{c}	183±2^{a,b}	209±2^{d,e}	0.11±0.02^{a}	0.10±0.02^{a}
BMCM	6.2	72.2±0.2^{c}	53.8±0.5^{a}	74.6±0.4^{b}	201±9^{g}	242±4^{i}	0.10±0.03^{a}	0.11±0.03^{a}
	6.4	71.3±1.0^{c}	54.3±1.1^{a}	75.8±2.0^{b}	200±9^{f,g}	222±9^{h}	0.10±0.03^{a}	0.10±0.02^{a}
	6.7	73.8±3.8^{c}	55.1±0.1^{a}	76.3±0.4^{b}	197±3^{d,e,f,g}	210±2^{e,f}	0.08±0.02^{a}	0.08±0.02^{a}

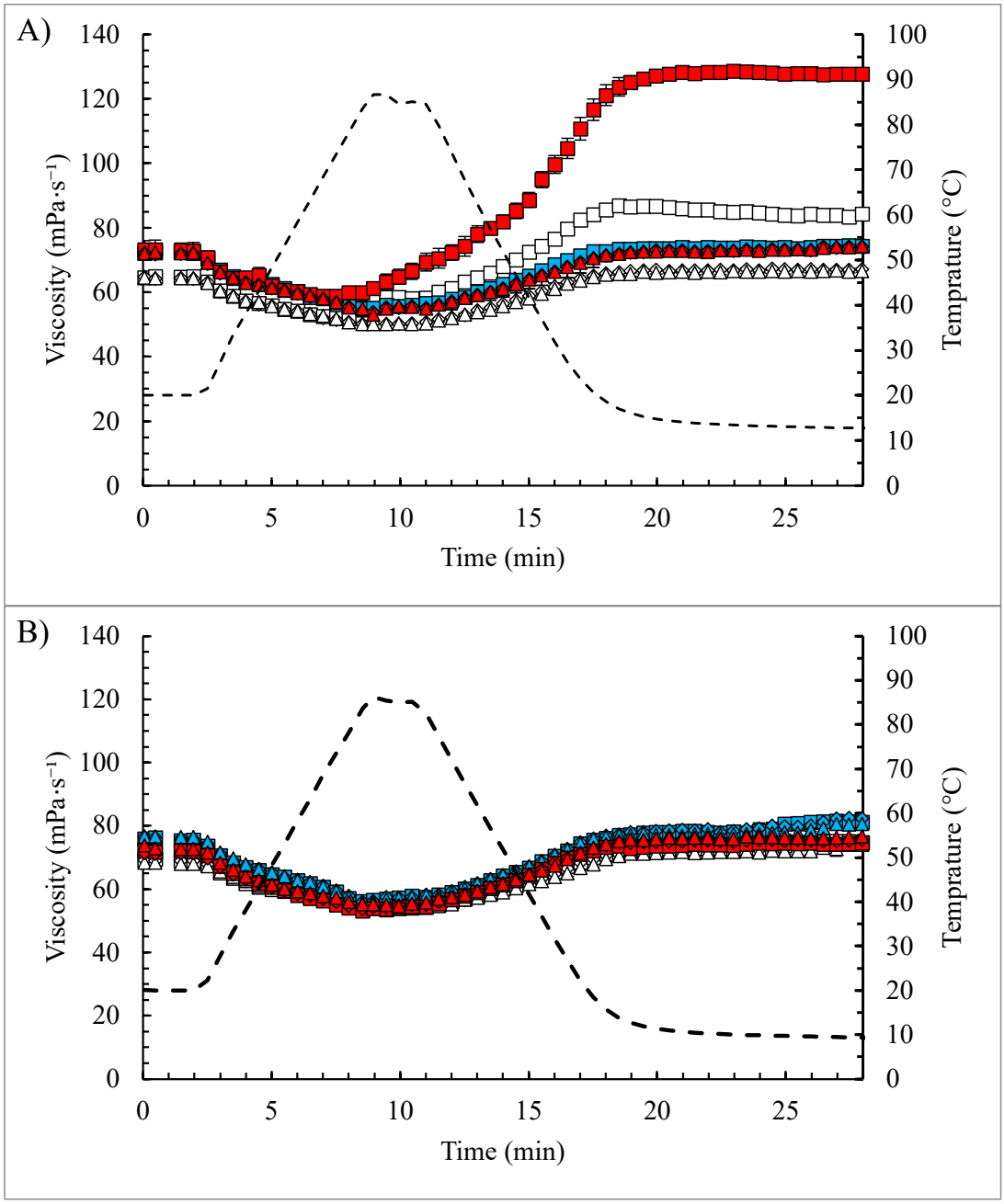

Figure 3.3: Viscosity (symbols) and temperature (dashed line) during heat treatments in a starch pasting cell with peak temperature hold of 85 °C. In which A) represents skimmed milk (white), sodium caseinate milk (blue), and micellar casein milk (red) and B) represents blackberry milk (white), blackberry sodium caseinate milk (blue), and blackberry micellar casein milk (red), pH 6.2 is represented by squares, pH 6.4 by rhombuses and pH 6.7 by triangles. All samples contained 3.5% (w/w) protein from low heat skimmed milk powder, the sodium caseinate and casein milk also contained 2% protein from sodium caseinate or micellar casein concentrate. The blackberry juice dairy beverages contained 7% freeze dried blackberry juice. The mean values and standard deviations are presented (n = 2).

3.4.4. Particle Size

Figure 3.4 shows the size distribution of the samples, measured by dynamic light scattering. The size distributions of all samples before heating was similar, with a monomodal shape and z-average diameter (z.ave.d.) between 180 and 217 nm (Figure 3.4, Table 3.2). Before heating, the pH of 6.2, 6.4 and 6.7 had no significant influence on the z.ave.d. of the samples, with the exception of BSCM at pH 6.2 and pH 6.7. In most samples a mild heat treatment of 85 °C for 2 min increased the z.ave.d. with 1 to 20 nm. Exceptions were observed in SM and MCM at pH 6.2, which size distributions became bimodal with z.ave.d. of milk becomes 453±9 nm and 2853±326 nm for SM and MCM, respectively, due to formation of protein aggregates.

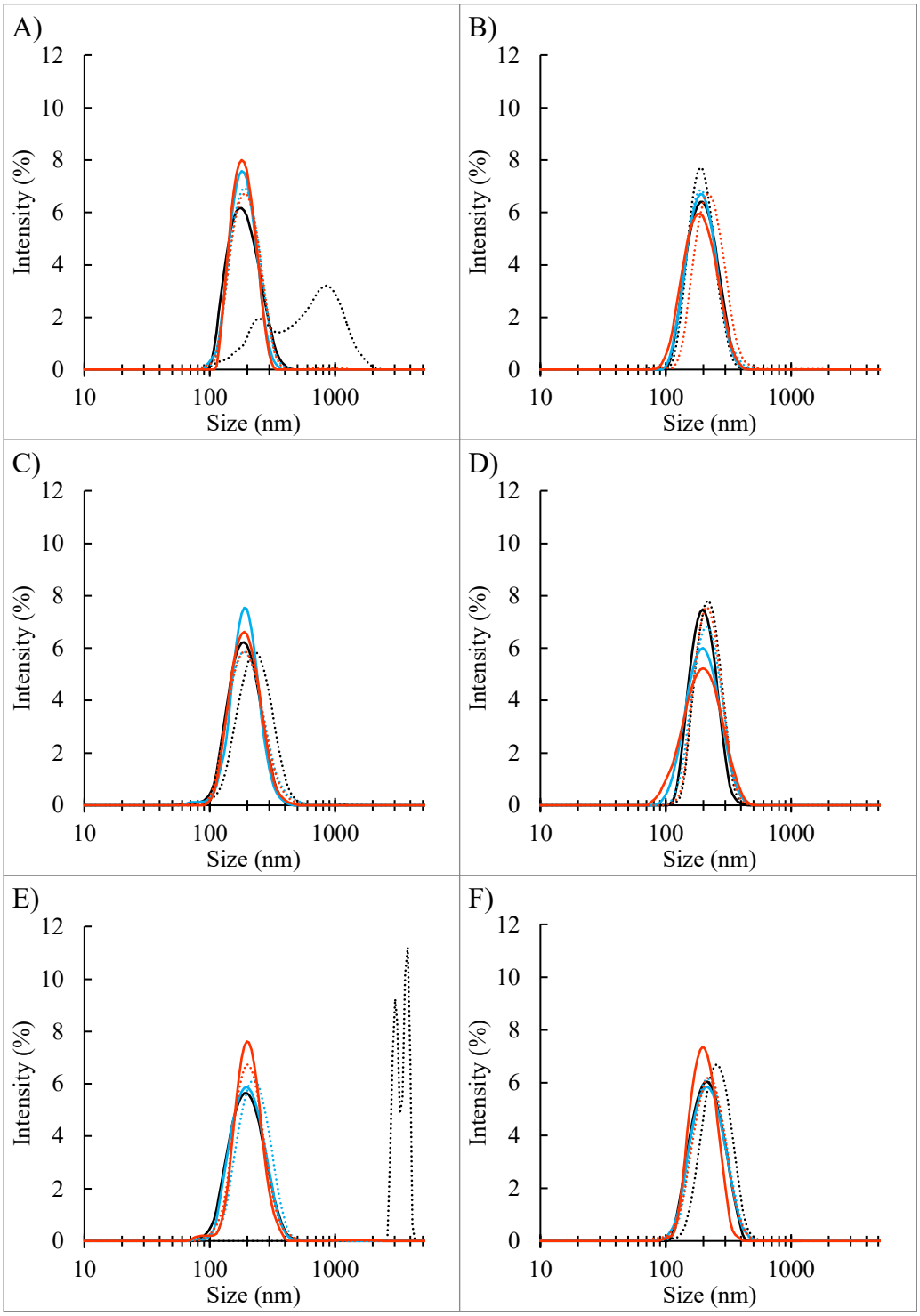

Figure 3.4: Dynamic light scattering measurements of different samples in which the solid lines represent before heating and the dotted lines after heating. Black represents pH 6.2, blue pH 6.4 and red 6.7. A) represents skimmed milk, B) blackberry milk, C) sodium caseinate milk, D) blackberry sodium caseinate milk, E) micellar casein milk, and D) blackberry micellar casein milk. All samples contained 3.5% (w/w) protein from low heat skimmed milk powder, the sodium caseinate and casein milk also contained 2% protein from sodium caseinate or micellar casein concentrate, and the blackberry juice dairy beverages contained 7% freeze dried blackberry juice. Values are the means of triplicate analysis.

3.4.5. Analytical centrifugation

The analytical centrifugation profiles of the samples are shown in Figure 3.5. All samples after heating were stable during the low centrifugation speed of 200 RPM (5×g) and all samples displayed an increase in integral transmission with the high centrifugation speed of 4000 RPM (2300×g). The low centrifugation speed allowed for measurements of the initial integral transmission of the samples, which was stable during this centrifugation speed. The high centrifugation speed demonstrated the separation rate of the samples and over time the samples displayed an increased integral transmission. Before heating, the initial integral transmission of SCM > SM > MCM and BSCM > BM > BCM. The separation rate of all samples before heating were similar to each other. After heating, there was a decrease in initial integral transmission in the milk samples in the order pH 6.7 > 6.4 > 6.2. Addition of BBJ seemed to limit the effect of pH on the initial integral transmission of BM and BMCM, with the exception of BMCM at pH 6.7 which had a lower initial integral transmission. In BSCM, the effect of pH on the integral transmission was the opposite to that of SCM, because the integral transmission of BSCM followed the order pH 6.2 > 6.4 > 6.7. The separation rates of the samples with and without BBJ were similar, except for SM and MCM at pH 6.2, which had a steeper slope and a higher final integral transmission.

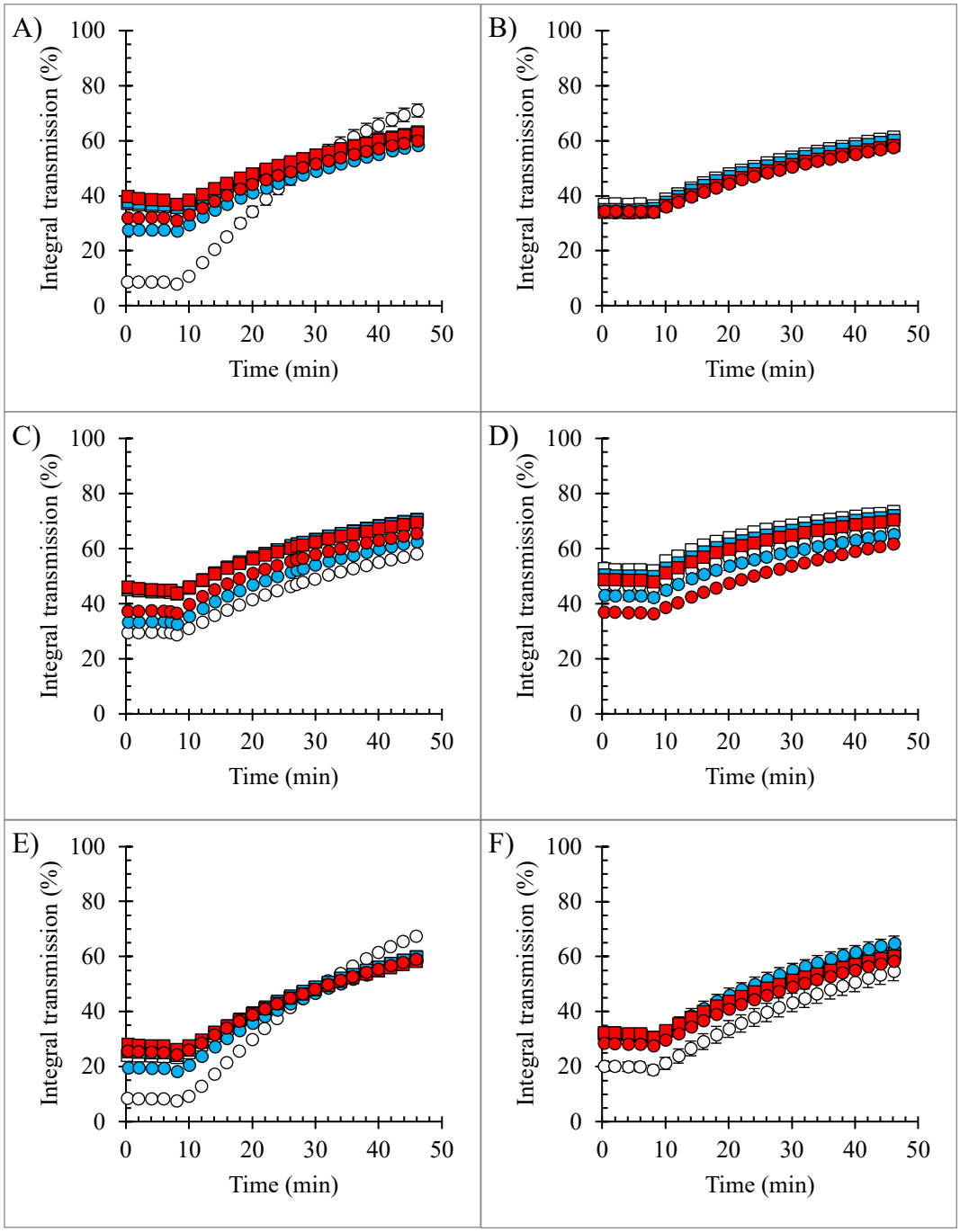

Figure 3.5: Sedimentation profiles of the dispersions in which the change in transmission of the NIR light through the sample cells is expressed over a time span of 46 min. The centrifugation speed in the first 8 min was 200 RPM (5×g) followed by 38 min with a centrifugation speed of 4000 RPM (2300×g). White represents pH 6.2, blue pH 6.4 and red 6.7, before heating is represented by squares and after heating with circles. A) represents skimmed milk, B) blackberry milk, C) sodium caseinate milk, D) blackberry sodium caseinate milk, E) micellar casein milk, and F) blackberry micellar casein milk. All samples contained 3.5% (w/w) protein from low heat skimmed milk powder, the sodium caseinate and casein milk also contained 2% protein from sodium caseinate or micellar casein concentrate, and the blackberry juice dairy beverages contained 7% freeze dried blackberry juice. The mean values and standard deviations are presented (n = 3).

3.5. Discussion

The different ingredients used in this study were SM, MCC, SCN and BBJ. SM, MCC and SCN are all rich in milk proteins and the main proteins present in MCC and SCN are α_{s1}-, α_{s2}-, β- and κ-casein. In MCC the caseins are present as casein micelles and in SCN the caseins are present in the form of small protein aggregates, which are able to act as calcium-chelating agents (Thomar & Nicolai, 2015; van de Langerijt et al., 2022). In this study, addition of SCN decreased the amount of ionic calcium present in milk systems (Figure 3.1), which confirms that SCN actively binds calcium. In MCC most calcium is present in the form of colloidal calcium phosphate (CCP) but also contains ionic calcium (Schäfer et al., 2021), which is responsible for the increase ion ionic calcium observed when MCC was added to SM (Figure 3.1). BBJ decreased the amount of ionic calcium in all systems (Figure 3.1). This is to our knowledge the first-time binding of calcium in milk protein blackberry systems has been reported. O'Connell et al. (1998a) found that extracts of food products rich in polyphenols, such as green tea, black tea, coffee and chocolate, decreased the calcium activity in skimmed milk. Therefore, it is assumed that the polyphenols are responsible for the decrease in ionic calcium observed in the samples with blackberry juice. Ionic calcium decreased progressively with increasing pH in the range 6.2-6.7 (Figure 3.1); it is suggested this is due to complex formation of calcium with phosphate, caused by a reduced solubility of calcium phosphate with increasing pH (Chow, 2001; Crowley, Megemont, et al., 2014; Vaia et al., 2006).

The different concentrations of ionic calcium in the samples might explain the differences in HCT-pH profiles between the samples. The HCT-pH profile of SM at 140 ºC followed a typical Type A profile, with a maximum at pH 6.7 and minimum at pH 6.9 (Huppertz, 2016; Singh, 2004a). Addition of SCN to SM increased the HCT-pH profile between pH 6.3-6.5 and pH 6.8-7.1. Similar observations in the pH 6.8-7.2 region, were reported by Lin et al. (2016, 2017), who changed the skimmed milk to SCN ratio and attributed the influence of SCN on the HCT on the reduction of ionic calcium. MCM had

a similar HCT-pH profile as SM, but at pH < 6.6 slightly decreased the HCT. The reason HCT of SCM > SM > MCM at pH < 6.6 is probably due to the differences of ionic calcium in these samples. Milk samples in this pH range generally have a low HCT because relatively high levels of ionic calcium facilitate the interaction between denatured whey proteins and casein micelles (Anema, 2007; O'Connell & Fox, 2003; Singh, 2004b). Because of the reduction of ionic calcium caused by SCN and the increase by MCC (Figure 3.1), the HCT at pH < 6.6 of SCM is larger than SM, and the HCT of MCM is lower than SM.

Addition of BBJ had a major effect on the HCT-pH profiles and increased the HCT between pH 6.2 and 6.4 (Figure 3.2B). The main soled components of BBJ are fructose, glucose, polyphenols, and sodium (internal communication), with the later originating from sodium hydroxide used to increase the pH of the juice from 2.94 to 6.80. Fructose and glucose both have limited effect on heat stability and are therefore unlikely to influence the changes in HCT due to the BBJ addition (Holt et al., 1978). Sodium reduces the net-negative charge of the κ-casein on the casein micelles, which can result in casein micelle aggregation and whey protein-casein aggregation. A concentration of 425 mM and above sodium reduced the heat stability of 2x concentrated milk at 120 °C (Huppertz & Fox, 2006); however, the amount of sodium added to the dispersion in this study in the form of NaOH is only around 30 mM and therefore expected to be minimal. Its effect on the heat stability of the systems with BBJ will therefore be limited. The other major compounds in the juice are polyphenols, mainly cyanidin-3-O-glucoside, lambertianin C, sanguiin H-6 and rutin (internal communication). O'Connell et al. (1998) and O'Connell & Fox (1999) investigated the influence of polyphenol rich extracts of tea, coffee and cocoa, and specific polyphenols like caffeic acid, epigallocatechin gallate and ferulic acid on the HCT-pH profile of skimmed milk and concentrated skimmed milk. It was found that the effect of the polyphenols on the HCT-pH profile depended very much on the type of polyphenol. Ferulic acid had a similar effect on the HCT as BBJ but is an organic acid and not an

anthocyanin like cyanidin-3-O-glucoside, or tannin like lambertianin C and sanguiin H-6 or flavonoid like rutin. O'Connell & Fox (1999) suggested that one of the factors contributing to the effect of polyphenols on the HCT was the binding of calcium by polyphenols. BBJ reduced the amount of ionic calcium in the samples at pH < 6.7 (Figure 3.1), which could explain why the HCT of the samples at the lower pH range was so high. The addition of BBJ also changed the order in which the samples were resistant to heat coagulation, the HCT of BM > BSC > BMCM. This indicates that ionic calcium is not the only factor influencing the HCT of the samples. The total protein content is also of influence because the sample with the lowest protein content, BM, had the highest HCT. For the BSM and BMCM, which have the same protein content and whey protein to casein ratio, the ionic calcium content is of influence, because the HCT of BSM > BMCM.

The heat treatment at 85 °C had a limited influence on the viscosity of SM at pH 6.4 and 6.7 but increased the viscosity at pH 6.2 (Figure 3.3A, Table 3.2), likely due to protein aggregation. Heating can increase the viscosity due to aggregation, which leads to an increase in the effective volume fraction and an increase in interactions between particles (Anema et al., 2004; Jeurnink & de Kruif, 1993). In skimmed milk, whey proteins are the main cause of heat induced protein aggregation and coagulation, due to their denaturation at temperatures > 65 ºC (Considine et al., 2007). Denaturation leads to the unexposed groups in the core of the whey protein to be exposed and these newly exposed groups can interact with other whey proteins and caseins (Schokker et al., 1999). Heat-induced aggregation at pH 6.2 is not due to increased whey protein-whey protein interactions but to a greater extent the association of whey proteins with casein micelles (Anema & Li, 2003). This is caused by the increased amount of ionic calcium present at lower pH values which can reduce the net-negative charge of κ-casein, resulting in casein micelle coagulation (Sievanen et al., 2008; Singh, 2004a). This could explain the results of this study, because the two samples with the highest level of ionic calcium, SM and MCM pH 6.2, displayed increases in viscosity on heating, indicators of protein aggregation in

these types of samples. The lower level of ionic calcium in SCM, BM, BSCM and BMCM is probably the reason that no large viscosity increase was observed after heating in these samples, due to a lack of aggregate formation.

The formation of aggregates was investigated in greater detail using DLS. Before heating, the z-average diameter of SM, SCM and MCM slightly decreased when the pH decreased from 6.7 to 6.4 to 6.2 (Table 3.2). Similar results were observed in milk by Sinaga et al. (2017), who attributed this effect to two factors. A reduction in net charge of the casein, causes a shrinkage of casein micelles, and a redistribution of CCP from the casein micelles to the serum phase. This leads to casein micelle disruption, resulting in a decrease in particle size. The solubilisation of CCP was confirmed by the increase in ionic calcium when the pH was decreased from pH 6.7 to 6.4 to 6.2 (Figure 3.1A). When BBJ was added to the different milk samples and the pH was decreased from 6.7 to 6.4 to 6.2, an increase of the z-average diameter was observed (Table 3.2). The ionic calcium content in the milk samples with BBJ was lower than the milk samples without BBJ, but followed the same general trend, with a decrease in pH causing an increase in ionic calcium. The interactions between polyphenols and casein micelles can stabilize the casein micelle structure, which can partially prevent casein micelle dissociation caused by CCP solubilising from the casein micelles into the serum phase (van de Langerijt et al., 2022). This could explain why the z-average diameter did not decrease with decreasing pH. The increase might be caused by the loosening of the structure of the casein micelles, due to CCP dissociation, which did not lead to disruption because of the stabilizing effect of polyphenols. Heating resulted in a slight increase in the z-average diameter of the different samples. Exceptions were SM, MCM and BCM at pH 6.2, which had large increase in z-average diameter after heating (Table 3.2, Figure 3.4). This increase in particle size in SM and MCM at pH 6.2 is most likely the result of protein aggregation. This protein aggregation would explain the increase in viscosity observed in these samples after heating (Figure 3.3A). Compared to SM and MCM at pH 6.2, BMCM at pH 6.2 had only a

relatively small z-average diameter increase of 45 nm. This could be due to attachment of whey proteins to the casein micelles. The lower concentration of ionic calcium in BMCM compared to SM and MCM, probably prevented formation of larger aggregates in the heating step.

During analytical centrifugation the initial integral transmission and separation rate of the samples were measured (Figure 3.5). The initial integral transmission during the low centrifugation speed provided information about the turbidity of the samples; a high integral transmission is related to a low turbidity and vice versa (de Kruif, 1993). Before heating samples SCM had a higher integral transmission and MCM a lower internal transmission compared to milk, similar results were found for BM, BSCM and BMCM. The turbidity in skimmed milk is mainly caused by the presence of casein micelles (Pitkowski et al., 2008). MCM and BMCM contain a higher ratio of casein micelles then SM and BM resulting in greater turbidity. The opposite was observed for SCM and BSCM due to the lower ratio of casein micelles in these samples compared to SM and BM. After heating, the initial integral transmission of the milk samples decreased with decreasing pH, especially SM and MCM at pH 6.2. An increase in particle size is related to an increase in turbidity (Orlien et al., 2006); therefore, it is assumed that this increase in turbidity is probably due to the slight increase in particle size after heating samples with a lower pH, and protein aggregation in SM and MCM at pH 6.2 (Table 3.2, Figure 3.4). BBJ limited the effect of pH on BM after heating, which is most likely due to the prevention of aggregate formation at pH 6.2. This is indicated by the size distribution results, which showed no influence of heating on the size distribution of BM at pH 6.2 (Table 3.2, Figure 3.4). The increase in BMCM turbidity at pH 6.2 after heating, is likely due to the increase in particle size caused by heating.

At higher centrifugation speeds the separation rate of casein micelles and protein aggregates occurred (Fig **3.**5). All samples had similar separation profiles, which shows gradual separation over time with a similar slope. However, there were two exceptions,

SMP and MCM at pH 6.2, which had a steeper slope, indicating more rapid and more extensive separation of particles, suggesting these samples can be expected to have reduced physical stability during storage.

3.6. Conclusions

Addition of blackberry juice to skimmed milk, skimmed milk with sodium caseinate and skimmed milk with micellar casein concentrate decreased the level of ionic calcium in these samples. This increased the heat stability of these samples at 140 °C especially at pH < 6.5. It also prevented the viscosity increases observed in the skimmed milk and skimmed milk with micellar casein concentrate at pH 6.2 after heating. The particle size increase observed for these two samples after heating was also limited by addition of blackberry juice. This indicates that blackberry juice prevented formation of milk protein aggregates, which was confirmed by the analytical centrifugation profiles. These profiles showed that these two milk systems with blackberry juice had similar turbidity and separation profiles compared to the respective samples before heating. This was not the case for the two samples without blackberry juice, which had a lower turbidity and greater separation, due to the presence of protein aggregates. It can be concluded that blackberry juice improved the heat stability of different types of milk systems at the pH range between 6.2 and 6.4, which was probably due to binding of calcium by blackberry polyphenols.

3.7. References

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Chapter 4

Co-concentration of milk proteins and polyphenols using ultrafiltration

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Declaration

This chapter was written by author TMvdL and reviewed by all co-authors. TMvdL co-designed the study and generated all the data with the exception of the chromatography work, which was carried out by KT.

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4.1 Abstract

In this study skimmed milk (SM) and skimmed milk with rutin (RM) was ultra-filtered at a processing temperature of 5, 25 or 50 °C. This was done to investigate the influence of processing temperature on the partitioning of rutin between the permeate and retentate. The concentration of rutin in the permeate stabilized after 10 min. A decrease in processing temperature increased the concentration of rutin in the retentate. Rutin concentrate only had a limited effect on the heat coagulation time-pH profiles SM and RM retentates. This research concludes that concentrating skimmed milk and rutin simultaneously can be achieved by ultrafiltration.

4.2. Introduction

A rapidly growing sector in the food industry is performance nutrition, which had a global market value of $42.9 billion (USD) in 2022 and is projected to grow with 7.4% by 2030 (Grand View Research, 2022). Products with great potential for sport and exercise nutrition, are beverages with a high polyphenol and milk protein content, due to the positive associations of polyphenols and milk proteins to enhancement of muscle strength and recovery (Carey et al., 2021; Sousa et al., 2014; Turck et al., 2018). However, polyphenols are rapidly oxidized in the gastrointestinal track, which can reduce their potential health benefits (Parada & Aguilera, 2007; Zhang & Tsao, 2016). The formation of milk protein-polyphenol complexes has been shown to reduce the oxidation in the gastrointestinal track (Lang et al., 2021). There are multiple approaches for the formation of milk protein-polyphenol complexes (van de Langerijt et al., 2023). Spray drying was used to isolate casein micelle–curcumin complexes, after which they could be rehydrated at higher concentrations (Khanji et al., 2018). However, to our knowledge, other techniques, such as membrane filtration, for the co-concentration of milk proteins and polyphenols by complex formation have not been studied.

Ultrafiltration (UF) is a technique commonly used in the dairy industry to concentrate milk proteins. It retains large molecules such as proteins, while letting small molecules, such as lactose and minerals permeate through the membrane (Chen et al., 2018). UF could potentially be used to concentrate milk protein-polyphenol complexes, while letting the free polyphenols permeate through the membrane. This would enable the formation of a high protein-polyphenol beverage, in which the polyphenols are partially protected by the proteins during digestion.

The polyphenol researched in this study is rutin, which is a flavonol glycoside, also known as quercetin-3-rhamnosylglucoside. Rutin is commonly found in fruit or vegetables (Gullón et al., 2017), among them blackberries (Chapter 5). It is also an affordable polyphenol and can be obtained in larger quantities. In previous studies, the

potential of milk protein–rutin complexes as delivery systems has been demonstrated (Rashidinejad et al., 2019, 2022).

One of the main factors influencing processing performance and partitioning achieved in membrane filtration of milk is the processing temperature. It can influence, among other things, the calcium content of the retentate and permeate, filtration performance and fouling (France, Bot, et al., 2021; France, Kelly, et al., 2021). A decrease in temperature reduces the hydrophobic interactions within and between casein micelles, which leads to dissociation of β-casein into the serum phase (Creamer et al., 1977). The temperature also influences the interactions between polyphenols and proteins and therefore the formation of milk protein-polyphenol complexes (van de Langerijt et al., 2023). The main interaction between the casein micelles present in milk and polyphenols are hydrophobic interactions and through hydrogen bonds, depending on the polarity of the specific polyphenol (Yazdi & Corredig, 2012; Yazdi et al., 2014). Increasing temperature increases the hydrophobic interactions but decreases the amount of hydrogen bonds (van de Langerijt et al., 2023), and at low temperature, β-casein depleted casein micelles have a higher binding affinity for polyphenols (Yazdi et al., 2014). Therefore, temperature differences could potentially influence the distribution of rutin between the protein rich retentate and protein depleted permeate during UF of such nutrients.

The aim of this study was to determine if UF of skimmed milk with the rutin could potentially influence the distribution of rutin between the retentate and permeate, and to assess the influence of temperature on its distribution.

4.3. Materials and methods

4.3.1. Materials

Low-heat skimmed milk powder with a protein content of 36.5% (w/w) (IDF, 2014) was kindly donated by Arrabawn (Nenagh, Co. Tipperary, Ireland). Rutin hydrate for adding to the samples was obtained from Merck (Berlington, MA, USA). Acetone,

dimethyl sulfoxide, Folin-Cioucalteu reagent, formic acid and gallic acid were also obtained from Merck (Berlington, MA, USA). The rutin standard was purchased from Extrasynthese (Extrasynthese Co., Genay Cedex, France). Acetonitrile of high purity was acquired from J.T. Baker® (Centre Valley, PA, US) and methanol from Romil (Lennox Laboratory Supplies LTD, Dublin, Ireland). C18 cartridges (3 mL, 200 mg) were acquired from Biotage, ISOLUTE® (Uppsala, Sweden). All water used in this research was ultrapure water (PURELAB option-Q, ELGA, Veolia Water, Paris, France).

4.3.2. Sample preparation

Skimmed milk (SM) with a final protein concentration of 3.5% (w/w) was prepared by adding low heat skimmed milk powder to water at room temperature and stirring overnight at 4 °C. To create the skimmed milk with rutin (RM), rutin hydrate, with an unknown amount of water molecules attached to the rutin, was dissolved in DMSO at a concentration of 0.2 g/mL and 5 mL was added to 995 mL of milk, creating a sample with a final rutin concentration of approximately 535 mg/L (determined by UPLC-MS/MS analysis, Section 4.3.8).

4.3.3. Ultrafiltration

The UF was performed using a lab-scale, pressure driven tangential-flow filtration device (Pellicon2 mini-holder, Merck Millipore, Tullagreen, Carrigtwohill, Ireland), using a 5 kDa molecular weight cut-off membrane, V-screen, polyethersulfone (PES) membrane (Bioma, Merck Millipore, Tullagreen, Carrigtwohill, Ireland). SM and RM were UF at a processing temperature of 5, 25 and 50 °C. A feed volume of 1 L was magnetically stirred continuously in a 1 L Schott Duran laboratory bottle. The feed was delivered to the feed port of the membrane unit using a Watson-Marlow 520 s variable speed tri-lobedperistaltic pump (Lennox Pump and Process, Dublin, Ireland) and the pump speed was set at 85 RPM. The temperature was controlled by allowing the retentate pass through a plate heat exchanger which was connected to a water bath set at the desired temperature (5, 25 or 50

°C). The transmembrane pressure (TMP) was calculated form pressure data collected, the two pressure gauges before and after the membrane module, and can be calculated using Equation 4.1:

$$TMP = \frac{P_i + P_o}{2} \quad (4.1)$$

in which P_i (bar) represents the inlet pressure and P_o (bar) the outlet pressure.

The retentate gauge was closed until an initial P_o of 0.8 bar was measured. For the first 30 min there was a total recirculation, both the retentate and permeate were recirculated to the feed (Figure 4.1A). After minute 1, 10, 20 and 30, the permeate flux was measured by capturing the permeate in a 25 mL graduated cylinder. The permeate colour was measured (Section 4.3.5.), and 2 mL was placed in an Eppendorf tube to estimate the rutin content by Folin-Ciocalteu method (Section 4.3.6.) and the left over permeate was carefully reunited with the feed. After 30 min the permeate was collected in a 1 L graduated cylinder, which resulted in concentrating the feed material (Figure 4.1B). This was done until a volume concentration factor of 2 was reached. For every 100 mL of permeate created, the permeate flow was determined and 2 mL was saved for analysis of polyphenols. There was also 2 mL of the total permeate sampled at a permeate volume of 100, 200, 300, 400 and 500 mL, for analysis of the rutin concentration by Folin-Ciocalteu method (Section 4.3.6.). The final retentate had a volume of 500 mL with a 7% (w/w) protein content. The final permeate had a volume of 500 mL, of which 28 mL was sampled for polyphenol analysis.

Cleaning was performed by flushing 5 L of 50 °C water through the system without backpressure. Afterwards, 1 L of water was recirculated for 15 min. Next, the TMP was set to 0.9 bar and 1 L of 1N NaOH, preheated in a water bath to 50 °C, was run through the system, followed by recirculation 1 L of 1N NaOH without TMP for 20 min. This step was repeated three times with fresh NaOH, followed by running 10 L of water through the

system after which the normalized water permeability (NWP) was measured. The NWP ($L \cdot m^{-2} \cdot h^{-1} \cdot bar^{-1}$)) can be calculated using Equation 4.2:

$$NWP = \frac{R \times F}{A \times TMP} \quad (4.2)$$

in which R represents the permeate flow ($L \cdot h^{-1}$), F the temperature correction factor based on water fluidity relative to 25 °C and A the membrane area (m^2). The membrane is considered to be clean when the NWP is maximally 60% different from the original NWP and maximally 20% different from the NWP at the start of the UF.

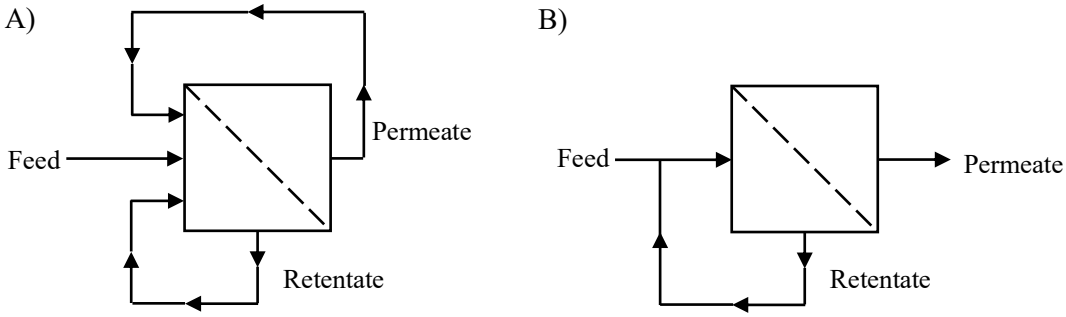

Figure 4.1. Schematic representation of membrane filtration process in which A) represents total recirculation during the first 30 min of the membrane filtration process and B) concentrating which occurred until a volume concentration factor of 2 was reached.

4.3.4. Conductivity and pH determination

The conductivity and pH were determined using a portable combined pH and conductivity meter (METTLER TOLEDO™, Columbus, Ohio, USA), fitted with an InLab Expert Go-ISM pH probe and an InLab 738 conductivity probe. The pH probe was calibrated with pH 4 and 7 buffer solutions (Scharlau, Barcelona, Spain) and the conductivity probe with a 12.88 mS/cm conductivity standard (InLab® Solutions, METTLER TOLEDO™, Columbus, Ohio, USA). All measurements were performed at 20 °C.

4.3.5. Colour determination

The colour was determined with a chromameter CR-400 (Konica Minolta Sensing Inc, Osaka, Japan) using CIELAB coordinates (L*, a*, b*). The chromameter was calibrated using a white tile (C: y:87.1, x:0.3163, z:0.3236). A sample volume of 13 mL was placed in a quartz cuvette, after which the colour of the sample in the cuvette was measured. Behind the cuvette, a white tile was used as a background. The L* indicates the brightness of the sample and has a value between 0 (black) and 100 (white). The a* value measures the greenness (negative) or redness (positive) and the b* value measures the yellowness (positive) or blueness (negative) of the sample.

4.3.6. Total rutin content estimated by Folin-Ciocalteu method

The concentration of rutin in the permeates was estimated using the Folin-Ciocalteu method (Singleton et al., 1999). Gallic acid was used as a standard (0-100 mg/L) and water was used as a blank. Folin Ciocalteu reagent was diluted 10 times and a volume of 125 µL was added to 20 µL of 5 times diluted permeate, standard or water in a Nunc™, F-bottom, polystyrene 96- MicroWell™ plate (Thermo Fisher Scientific, Oy, Finland). The 96-well plate was covered using aluminium foil to protect it from light and gently rocked for 5 min, after which 80 µL of 7.5 (v/w) % sodium carbonate was added to the wells. It was placed back in the tin foil and gently rocked for 120 min, after which its absorbance at 760 nm was measured using a Varioskan Flash plate reader (Thermo Fisher Scientific, Oy, Finland).

4.3.7. Solid phase extraction of rutin

Solid phase extraction (SPE), with ISOLUTE® C18 cartridges (3 mL, 200 mg) obtained from Biotage Sweden, was performed following the method of Zhang et al. (2018), with slight modifications. 2mL of 1% formic acid in methanol (v/v), followed by 2 mL of 1% aqueous formic acid (v/v) was used to pre-condition the cartridges. The samples were diluted 50 times in 1% aqueous formic acid to a total volume of 1 mL,

vortexed and loaded onto the SPE cartridge, after which it was filtered under gravity and washed with 1 mL of 1% aqueous formic acid. The final elution after the SPE clean-up was performed with 500 µL of 1% formic acid in methanol, followed by 500 µL of 1% formic acid in acetone. Following this, the extract was evaporated under nitrogen at 45 °C. The sample residue was reconstituted in 1 mL (initial samples were diluted 50 times) of 1% aqueous formic acid ensuring the sample was suitable for liquid chromatography-mass spectrometry analysis.

4.3.8. UPLC-MS/MS Quantification of rutin

The quantification of rutin was performed on a Waters Acquity ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-TQD, Waters Corporation, Milford, MA, USA) according to the method of Gangopadhyay et al. (2016) with slight modifications. An Acquity UPLC HSS T3 column (100 x 2.1 mm; 1.8 µm particle size) maintained at 40 °C with a binary mobile phase of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at 0.5 mL/min and was used. The UPLC-MS/MS data were acquired in negative electrospray ionisation mode and the quantification of rutin was determined using the software TargetLynxTM (Waters Corporation, Milford, MA, USA).

4.3.9. Evaluation of rutin in processing streams

The concentration of rutin in the feed, permeate and retentate quantified by UPLC-MS/MS analysis was used to determine the rejection (R), concentration factor (CF), sieving coefficient (S_o) of rutin, and proportion of total rutin absorbed the membrane. R was determined using Equation 4.3:

$$R = \left(1 - \frac{C_{p,rutin}}{C_{f,rutin}}\right) \times 100\% \quad (4.3)$$

in which C_p (mg/L) represents the concentration of rutin in the permeate and C_f (mg/L) the concentration of rutin in the initial feed material. The CF of rutin was determined using Equation 4.4:

$$CF = \frac{C_r,rutin}{C_{f,rutin}} \quad (4.4)$$

in which C_r (mg/L) represents the rutin concentration in the retentate. S_o was calculated using Equation 4.5:

$$S_o = \frac{C_{p,rutin}}{C_r,rutin} \quad (4.5)$$

The proportion of total rutin absorbed using the membrane was calculated based on a mass balance of which the formula can be found in Equation 4.6:

$$\%\ rutin\ attached\ to\ membrane = \frac{(C_{r,rutin} * V_r + C_{p,rutin} * V_p)}{C_{f,rutin} * V_f} \times 100\% \quad (4.6)$$

in which V_r represents the final retentate volume (L), V_p the final permeate volume (L) and V_f the initial feed volume (L).

4.3.10. Heat stability analysis

The heat coagulation time (HCT) of the retentate samples was determined using the method of White & Davies (1966) to obtain HCT-pH profiles. The temperature of the oil bath was set at 120 °C and the pH was adjusted to 0.1 pH unit intervals in the range 6.2 to 7.2. Glass tubes (12 cm length, 8 mm diameter, 1 mm wall thickness) were filled with a retentate volume of 2 mL and sealed with silicone bungs. The glass tubes were placed in the oil bath and rocked gently with HCT recorded as the time between placing the tubes in the oil bath and complete coagulation.

4.3.11. Statistical data analysis

Samples were prepared and analysed in duplicate and data is reported as the mean value ± standard error. Differences between the mean values were analysed statistically using a *t*-test, and the significance level was set to $p < 0.05$. The software used to perform the statistical analyses was SPSS Statistics version 28 (SPSS inc, Chicago, USA).

4.4. Results and discussion

Milk proteins were unable to permeate through the UF membrane, resulting in a retentate with a volume and protein concentration factor of 2 and a permeate depleted of

protein. An increase in processing temperature increased the permeate flux, and the rate of the flux declined over time (Figure 4.2A). Addition of rutin did not significantly influence ($p>0.05$) the initial or final permeate flux for membrane filtrations performed at the same temperature (Table 4.1). The results agree with the results of France et al. (2021) and McCarthy et al. (2017), who also found that an increase in processing temperature resulted in a higher initial permeate flux and a more rapid decline of permeate flux over time when processing skimmed milk. Both set of authors attributed these effects to an increase in fouling at higher temperatures. Similar results were found for fruit juice when comparing processing temperatures of 30, 40 and 50 °C (Saleh et al., 2006).

The transmembrane pressure (TMP) is a parameter which can be related to the process performance of the membrane. The initial TMP of SM and RM filtered at different processing temperatures was the same, i.e., 0.90 bar (Table 4.1). Over time, there was a slight increase in TMP, which was greatest for the samples processed at 50 °C (Figure 4.2B).

4.4.1. Process performance of ultrafiltration of skimmed milk with and without rutin

Table 4.1: Initial and final permeate flux and trans membrane pressure (TMP) of skimmed milk without (SM) and skimmed milk with rutin (RM), created at different processing temperatures (T)*.

T [°C]	Sample	Permeate flux ($L \cdot m^{-2} \cdot h^{-1}$)			TMP (bar)		
		Initial	Final	Slope	Initial	Final	Slope
5	SM	6.6 ± 0.6^{a}	$6.0 \pm 0.0^{a,b}$	-0.004	0.90 ± 0.00^{a}	1.13 ± 0.08^{a}	0.0023
	RM	7.2 ± 0.0^{a}	5.1 ± 0.3^{a}	-0.022	0.90 ± 0.00^{a}	1.10 ± 0.05^{a}	0.0031
25	SM	10.8 ± 0.0^{b}	8.7 ± 0.3^{d}	-0.028	0.90 ± 0.00^{a}	1.18 ± 0.03^{a}	0.0040
	RM	$9.9 \pm 0.9^{a,b,c}$	$8.4 \pm 0.6^{b,c}$	-0.016	0.90 ± 0.00^{a}	1.06 ± 0.06^{a}	0.0024
50	SM	14.1 ± 0.3^{d}	11.1 ± 0.9^{c}	-0.040	0.90 ± 0.00^{a}	1.25 ± 0.05^{a}	0.0063
	RM	$13.8 \pm 0.6^{c,d}$	10.8 ± 0.6^{c}	-0.063	0.90 ± 0.00^{a}	1.28 ± 0.02^{a}	0.0068

* Results are the mean and standard deviation of two independent trails and subscript letters indicate statistical differences in the column ($p \leq 0.05$).

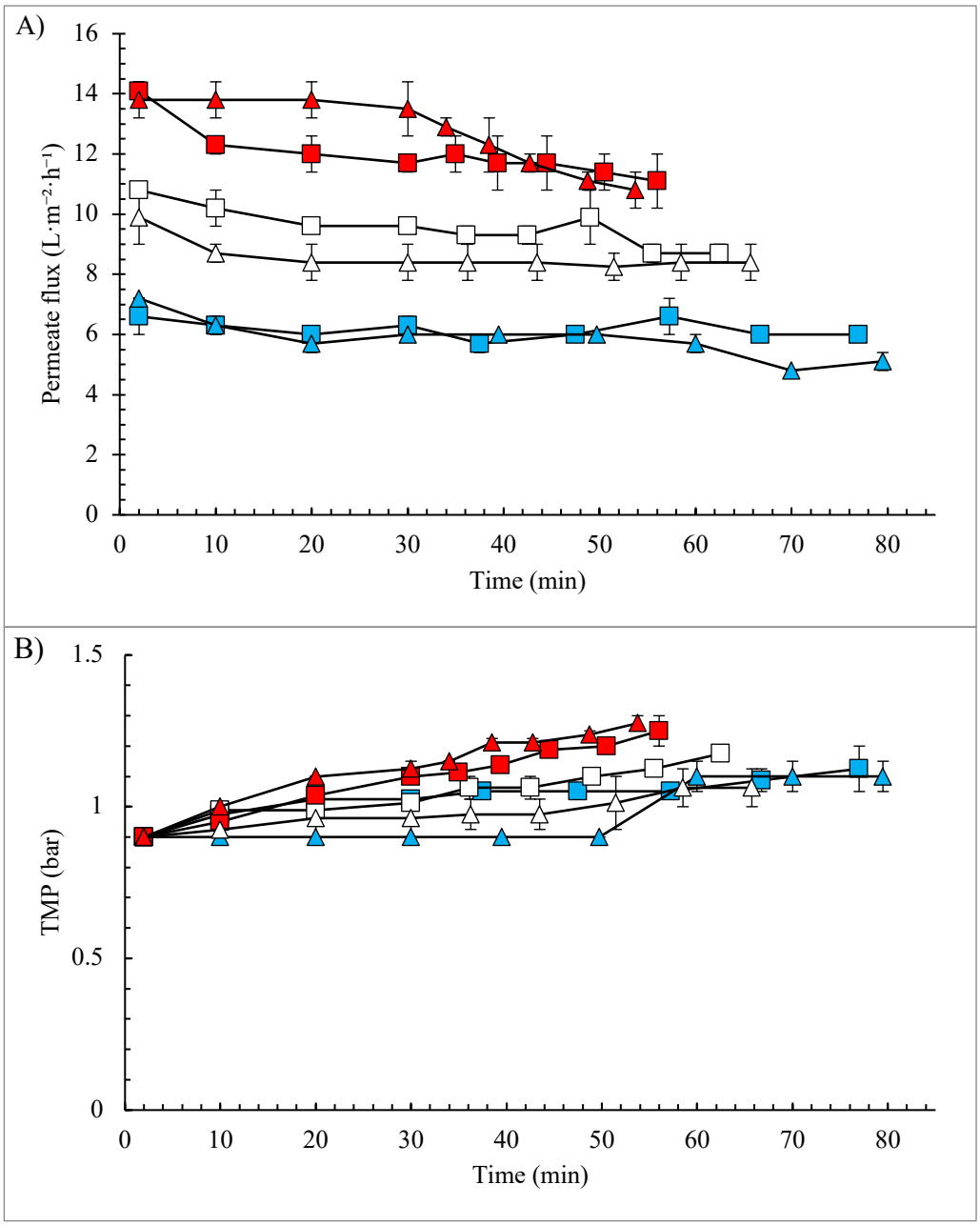

Figure 4.2: Permeate flux (A) and trans membrane pressure (B) as a function of processing time at 5 °C (blue), 25 °C (white) and 50 °C (red) for the different samples. The squares represent skimmed milk and the triangles skimmed milk with rutin.

4.4.2. Influence of temperature on conductivity, pH and colour of feed, retentate and permeate

The pH of all permeate and retentate samples was similar to the feed material (Table 4.2). The conductivity of milk is mainly determined by the ionic salt concentration (Mabrook & Petty, 2003). The conductivity of the feed material of RM and SM was not

significantly different from each other (P>0.05). The conductivity of the retentates decreased and the conductivity of the permeate remained similar to the conductivity of the feed material. Comparable results were found by Puri et al. (2020) for the retentates of UF skimmed milk. This change in conductivity is due to the redistribution of minerals between the retentate and permeate. The processing temperature had no influence on the ionic salt concentration of the retentate and therefore there were no significant differences (p>0.05) observed in the conductivity of the retentate samples (Table 4.2).

Table 4.2: pH and conductivity values of feed, retentate and permeate samples, of skimmed milk without (SM) and skimmed milk with rutin (RM), created at different processing temperatures (T)*.

T [°C]	Sample	pH			Conductivity (mS/cm)		
		Feed	Retentate	Permeate	Feed	Retentate	Permeate
5	SM	$6.78 \pm 0.02^{c,d}$	$6.78 \pm 0.03^{c,d}$	6.81 ± 0.00^{d}	5.10 ± 0.00^{b}	4.33 ± 0.04^{a}	5.14 ± 0.02^{b}
	RM	$6.77 \pm 0.00^{c,d}$	6.85 ± 0.01^{d}	6.85 ± 0.02^{d}	4.98 ± 0.01^{b}	4.21 ± 0.04^{a}	4.90 ± 0.05^{b}
25	SM	6.83 ± 0.00^{d}	6.82 ± 0.01^{d}	6.82 ± 0.01^{d}	5.05 ± 0.01^{b}	4.38 ± 0.05^{a}	4.99 ± 0.07^{b}
	RM	6.77 ± 0.00^{c}	$6.75 \pm 0.00^{b,c}$	6.74 ± 0.00^{b}	4.96 ± 0.01^{b}	4.25 ± 0.02^{a}	4.97 ± 0.02^{b}
50	SM	$6.75 \pm 0.00^{b,c}$	6.72 ± 0.00^{b}	6.51 ± 0.01^{a}	5.06 ± 0.04^{b}	4.39 ± 0.03^{a}	4.94 ± 0.00^{b}
	RM	$6.79 \pm 0.00^{c,d}$	$6.80 \pm 0.00^{c,d}$	6.58 ± 0.01^{a}	4.97 ± 0.00^{b}	4.35 ± 0.01^{a}	4.94 ± 0.03^{b}

*The values are the average and standard deviations of duplicate measurements. Values followed by different superscript letters are significantly different ($p \leq 0.05$) in pH or conductivity of all feeds, retentates and permeates, determined by *t*-test.

The colour of the feed, retentate and permeate was measured and the results are presented in Table 4.3. The L* value represents the brightness of a sample, a value of 0 is black and 100 white, with the brightness of skimmed milk mainly influenced by the presence of casein micelles (Milovanovic et al., 2021). The L* value of permeates are lower compared to the feed, due to exclusion of casein micelles in the permeates. The L* value of the retentates was significantly higher ($p \leq 0.05$) compared to the feed. An increase in the concentration of casein micelles increases the L* value of milk (Misawa et al., 2016). The retentates have a higher casein micelle concentration compared to the feed, which explains the higher L* value of the retentates compared to the feed.

The negative a* value indicates that all samples have some greenness. An increase in casein micelle concentration should decrease the greenness of milk according to Misawa et al. (2016). The same effect is observed in the retentates of SM, but not in the retentates of RM, likely due to the yellow-green colour of rutin. All feed, permeate and retentate samples had a positive value for b*, indicating their yellow-greenness. The b* value of all RM samples was higher than that of SM, due to the yellow colour of rutin.

Table 4.3: Colour space values of different feed, permeate and retentate samples of skimmed milk without (SM) and wit rutin (RM).*

Sample	Stream	Processing Temperature (°C)	L*	a*	b*
SM	Feed		76.89 ± 0.18^{g}	-5.64 ± 0.03^{d}	2.59 ± 0.03^{d}
	Retentate	5	79.92 ± 0.21^{h}	-5.28 ± 0.02^{e}	3.18 ± 0.09^{e}
	Retentate	25	81.46 ± 0.00^{i}	-5.06 ± 0.02^{f}	3.33 ± 0.07^{e}
	Retentate	50	82.74 ± 0.04^{j}	-4.65 ± 0.03^{g}	3.41 ± 0.03^{e}
	Permeate	5	64.92 ± 0.26^{e}	-3.15 ± 0.08^{i}	9.14 ± 0.13^{f}
	Permeate	25	64.84 ± 0.08^{e}	-3.66 ± 0.07^{h}	10.13 ± 0.18^{g}
	Permeate	50	64.80 ± 0.00^{e}	-3.80 ± 0.03^{h}	10.60 ± 0.08^{g}
RM	Feed		80.08 ± 0.00^{h}	-7.46 ± 0.00^{b}	11.03 ± 0.00^{h}
	Retentate	5	79.77 ± 0.13^{h}	$-7.72 \pm 0.14^{a,b}$	11.87 ± 0.17^{h}
	Retentate	25	80.32 ± 0.05^{h}	-7.78 ± 0.02^{a}	11.79 ± 0.01^{h}
	Retentate	50	80.17 ± 0.10^{h}	$-7.61 \pm 0.16^{a,b}$	11.82 ± 0.02^{h}
	Permeate	5	63.54 ± 0.68^{e}	-7.08 ± 0.37^{b}	19.90 ± 0.28^{i}
	Permeate	25	64.54 ± 0.04^{e}	-7.92 ± 0.01^{a}	21.96 ± 0.08^{i}
	Permeate	50	64.49 ± 0.16^{e}	-8.12 ± 0.12^{a}	22.67 ± 0.07^{i}

*The values are the average and standard deviations of duplicate measurements. Values followed by different superscript letters are significantly different ($p \leq 0.05$), determined by *t*-test.

4.4.3. Distribution of polyphenols between the retentate and permeate

The rutin content of the feed, permeate and retentate of RM was determined using UPLC-MS/MS and the results are presented in Figure 4.3A. From this data the retention (R), concentration factor (CF), sieving coefficient (S_o) and proportion of rutin attached to the membrane could be determined (Table 4.4). The R and CF of rutin increased with a decreasing processing temperature. Similar results were found by Saleh et al. (2006) for polyphenols in apple juice, separated using a 1 kDa spiral wound membrane, where a

processing temperature of 30 °C resulted in a higher polyphenol CF compared to a processing temperature of 50 °C. This was attributed to the polyphenols forming insoluble aggregates at lower temperatures. The reason for the lower R and CF at 50 °C, was probably not due to insoluble aggregate formation between the rutin molecules, but most likely due to the interactions between milk proteins and rutin. It should also be noted that the effect of temperature on the absorbance of rutin by the membrane could not explain the decrease of rutin concentration in the retentate, at higher processing temperatures (Table 4.4, Figure 4.3A). The rutin concentration of both the retentate and permeate should increase with a decreasing processing temperature, if rutin absorbance by the membrane was primarily responsible for the differences in rutin concentration. However, this was only the case for the permeate and not for the retentate, indicating that differences in binding of rutin by the milk proteins at different temperatures was responsible for the concentration of rutin in the retentate.

To our knowledge, no research has been done on the binding of rutin to different milk proteins, except for blood serum albumin (BSA). Hydrogen bonding, ionic interactions and hydrophobic interactions are of equal importance for the binding of rutin to BSA (Papadopoulou et al., 2005). An increase in processing temperature will increase the hydrophobic interactions but decrease the extent of hydrogen bonds between milk proteins and polyphenols (van de Langerijt et al., 2023). Glycosylated polyphenols, including rutin, are less hydrophobic compared to their aglycone counter parts (Xiao et al., 2009). This reduces the importance of hydrophobic interactions between rutin and milk proteins, compared to quercetin. A reduction of hydrogen bond interactions with increasing processing temperature might therefore not be compensated by the increase in hydrophobic interactions. This could have resulted in the lower concentration of rutin in the retentate obtained with a processing temperature of 50 °C. At temperatures of less than 20 °C, casein micelles are partially depleted from β-casein, due to a reduction of hydrophobic interactions (Creamer et al., 1977; France, Kelly, et al., 2021). Polyphenols which interact

with milk proteins through hydrogen bonds, such as rutin, are able to bind in higher quantities to these β-casein depleted micelles due to its more open structure compared to intact casein micelles (Yazdi et al., 2014). The higher concentration of rutin bound by the milk proteins at 5 °C could explain the higher R value at 5 °C compared to 50 °C. The conductivity of milk is mainly determined by the ionic salt concentration (Mabrook & Petty, 2003). The processing temperature had no influence on the conductivity, and therefore ionic salt concentration, of the retentate (Table 4.2). The permeation of rutin through the membrane is given by S_o and was higher at 50 °C, compared to 5 or 25 °C, indicating that more rutin permeates through the membrane at higher temperatures. The permeation is dependent on the amount of rutin bound to the proteins, which is less at 50 °C, resulting in the significantly higher ($p \leq 0.05$) S_o value of rutin at a processing temperature of 50 °C.

An important factor to note is the potential binding of polyphenols by PES membranes, which have a hydrophobic nature and can bind with polyphenols through hydrophobic interactions (Cassano et al., 2017). The proportion of rutin attached to the membrane, estimated by mass balance, is reported in Table 4.4. An increase in processing temperature resulted in a decrease in the percentage of rutin attached to the membrane. This is surprising since an increase in temperature should result in stronger hydrophobic interactions (van de Langerijt et al., 2023), which is expected to result in a higher percentage of rutin attached to the membrane. Other interactions like π-π stacking, which is the attractive interaction between a pi bond and aromatic ring, could also play a role in binding of polyphenols to the PES membrane (Susanto et al., 2009), which could potentially influence the binding of rutin to the membrane at different temperatures. Other membranes such as polypropylene membranes might be better suited for concentration of protein-polyphenol complexes, as they are less prone to polyphenol binding (Ulbricht et al., 2009).

Table 4.4: The retention (R), concentration factor (CF) and sieving coefficient (S_o) of rutin and percentage of rutin attached to the membrane.*

Processing temperature (°C)	5	25	50
R	73.2 ± 2.9^{a}	64.4 ± 1.1^{a}	53.5 ± 1.6^{b}
CF	1.28 ± 0.01^{a}	1.23 ± 0.04^{a}	1.21 ± 0.00^{b}
S_o	0.27 ± 0.03^{a}	0.36 ± 0.01^{a}	0.47 ± 0.02^{b}
% rutin attached to membrane	19.2 ± 0.9^{a}	$17.1 \pm 1.2^{a,b}$	11.7 ± 0.9^{b}

*The values are the average and standard deviations duplicate measurements. Values followed by different superscript letters are significantly different ($p \leq 0.05$), determined by *t*-test.

The rutin content in the permeate during the UF was estimated using the Folin-Ciocalteu method to study the rutin concentration in the permeate over time at different processing temperatures (Figure 4.3B). In the first 2 min, the rutin in the permeate at a processing temperature of 50 °C was significantly higher ($p \leq 0.05$) compared to 5 or 25 °C. This could be due to the higher permeate flux at 50 °C, resulting in a more rapid absorption of rutin by the membrane, due to a larger volume of permeate passing through the membrane in the first two min. However, the reduced binding affinity of the milk proteins and membrane absorbance of rutin at increasing processing temperature was probably the main cause.

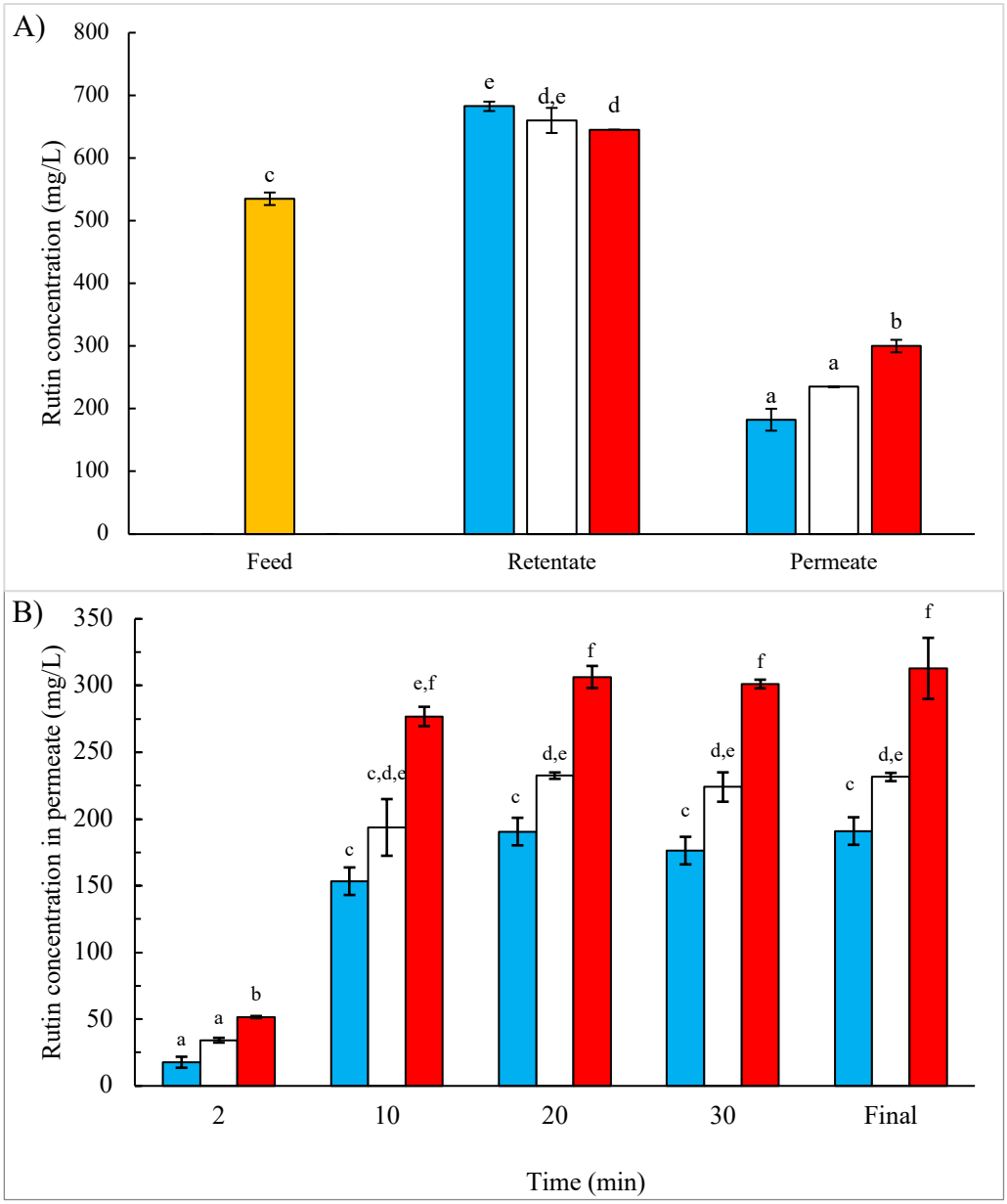

Figure 4.3: Rutin concentration of different streams. A) represents the rutin concentration in the feed, retentate and permeate, determined using UPLC-TQD-MS/MS. B) represents the rutin concentration in the permeate during the ultrafiltration process, determined using the Folin-Ciocalteu method. The retentate and permeate obtained at a processing temperature of 5 °C is represented by blue, 25 °C by white and 50 °C by red. Data represents 2 independent samples ± SD. The different subscript letters indicate a significant difference ($p \leq 0.05$) between the rutin concentration, analysed by an independent *t*-test.

4.4.4. Influence of polyphenols on the heat stability of retentate

The influence of processing temperature on the heat stability of the different retentates was investigated and the results are presented in Figure 4.4. The profiles of

retentates prepared using processing temperature of 5 and 25 °C were similar to each other, with a peak HCT at 6.7. For the retentates made with a processing temperature of 50 °C, the peak shifted to a pH value of 6.6. As alluded earlier, this effect of processing temperature, during UF on HCT-pH profiles has not been observed before. A reason for the shift in max peak could be due to a difference in heat induced association behavior of β-lactoglobulin to κ-casein. This is the main factor influencing the heat stability between pH 6.5 and 6.7 (O'Connell & Fox, 2003; Singh, 2004).

Rutin decreased the heat stability of the retentate at pH 7.2, independent of the processing temperature. The increase in heat stability above pH 6.9 is mainly caused by the binding of more calcium ions by the proteins, due to the increase in the net negative charges at a pH above 6.9 (O'Connell & Fox, 2003; Singh, 2004). Rutin has four hydroxyl groups with pKa values ranging from 7.1 to 11.65 (Jovanovic et al., 1994). The slight increase in negatively charges present in rutin at pH 7.2 could interfere with the calcium binding of casein, and slightly decrease the HCT compared to samples without rutin. O'Connell & Fox (1999) suggested that polyphenols could interfere with the ionic calcium in milk and thereby influence their pH-HCT profile.

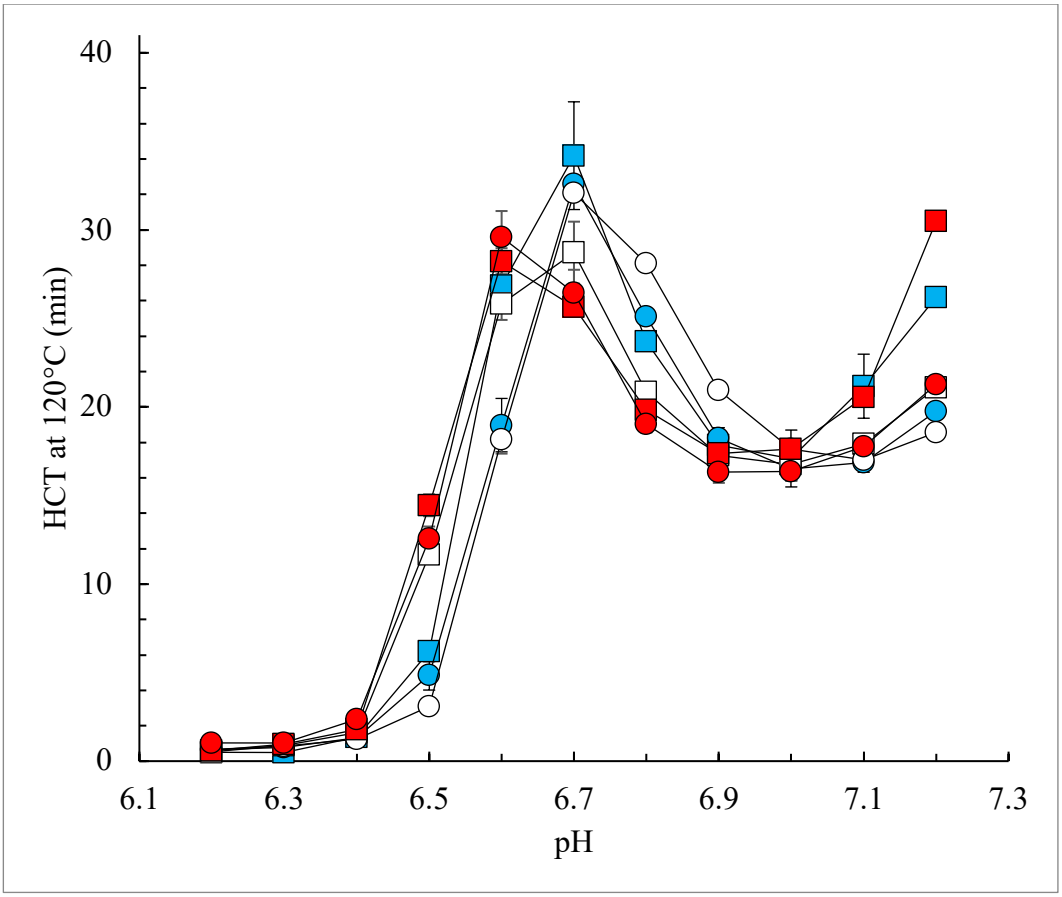

Figure 4.4: Heat coagulation time (HCT) pH profiles of retentate samples obtained at different temperatures and with different polyphenols. The squares represent retentates of skimmed milk without rutin, the circles represent retentates of skimmed milk with rutin. The retentates made at different processing temperatures of 5, 25 and 50 °C are represented by blue, white and red, respectively. Data represents 2 independent samples ± SD.

4.5. Conclusions

For a feed containing skimmed milk and rutin, ultrafiltration increased the rutin concentration in the retentate but decreased in the permeate. This effect on rutin concentrations increased with decreasing processing temperature. The formation of milk proteins-rutin complexes, mainly through hydrogen bonds, appears to be the main driving force for the increased concentration of rutin in the retentate, at lower processing temperatures. Binding of rutin by the PES membrane is substantial when skimmed milk with rutin is ultra-filtrated. Rutin had a minimal effect on the heat stability profiles of retentates. It can be stated that ultrafiltration could be used to concentrate rutin and milk proteins simultaneously, especially at lower temperatures.

4.6. References

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Chapter 5

The influence of milk with different compositions on the bioavailability of blackberry polyphenols in model performance nutrition beverages

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Declaration

This chapter was written by author TMvdL and reviewed by all co-authors. TMvdL co-designed the study, prepared all samples, determined particle size distribution and prepared SDS-page gels. The *in vitro* digestion and Caco-2 cell assay was performed by YCO. The chromatography work was carried out by KT.

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5.1. Abstract

This study aimed to determine if the fat and protein content in dairy-fruit blends prepared by full-fat, semi-skimmed, skimmed, and high-protein milk in combination with freeze-dried blackberry puree influenced the bioaccessibility and bioavailability of blackberry polyphenols and organic acids. This was done by *in vitro* digestion, after which bioavailability was analysed with a Caco-2 cell model. Puree addition to milk resulted in the formation of protein aggregates, which partially protected anthocyanins during digestion. Milk fat increased the bioaccessibility of anthocyanins but reduced the permeability through the Caco-2 cells leading to a lower bioavailability.

5.2 Introduction

In recent years, products that promote muscle synthesis and recovery have become more popular. These products include dairy-fruit blends that combine sources of high quality proteins and polyphenols, with the source of polyphenols often being berries, such as blackberries. Milk proteins are of importance due to their positive influence on skeletal muscle mass and strength (Gryson et al., 2014). Polyphenols, especially anthocyanins, are of interest for sports nutrition due to their role in enhancement of muscle recovery and muscle strength, which has been linked to their antioxidant properties (Carey et al. 2021). While as many as 10,000 different types of polyphenols have been reported so far (Eran Nagar et al. 2020), the main phenolic compounds in blackberries are anthocyanins, phenolic acids, flavonols and ellagitannins (Oszmiański et al. 2015). Polyphenols are prone to oxidise to a significant degree in the gastrointestinal tract, reducing their bio-accessibility, -availability and -functionality (Parada & Aguilera 2007; Zhang & Tsao 2016). A reduction in this oxidation during digestion could potentially amplify the positive physiological effect of polyphenols on muscle recovery and muscle strength. Milk proteins mainly interact with anthocyanins, the most abundant group of polyphenols in blackberries, through hydrophobic interactions and hydrogen bonding (He et al. 2016; Ren et al. 2021). The strength of these interactions mainly depends on the polarity of the polyphenols, with

more polar polyphenols having more hydrogen bonds and more apolar polyphenols having stronger hydrophobic interactions (van de Langerijt et al. 2023a). Anthocyanins can be partially protected by whey proteins, α-casein, and β-casein during *in vitro* digestion, which is likely due to encapsulation of the anthocyanins by the proteins (Lang et al. 2021; Wu et al. 2023). In this study, commercial milk types with different levels of milk fat were selected because previous research indicates that milk fat can prevent phenolic components of blueberries from being absorbed after *in vivo* digestion (Serafini et al. 2009). It is suggested this is due to the interactions between anthocyanins and the polypeptide chains present in the milk fat globule membrane (Zhang et al. 2012).

To our knowledge the effect of milk proteins and milk fat on polyphenols during digestion is poorly understood. Therefore, the aim of this study was to investigate the influence of different commercial milk types, with a variance in the level of milk fat and milk protein, on the bioaccessibility and bioavailability of polyphenols and organic acids present in blackberry puree.

5.3. Materials and methods

5.3.1. Materials

Pasteurized and homogenized whole milk, semi-skimmed milk, skimmed milk, and high-protein milk were purchased from a local retail outlet. Their protein and fat content are outlined in Table 5.1. Blackberry puree was obtained from Wild Orchard (Limerick, Ireland) and dried using a freeze dryer (Edwards vacuum, Burgess Hill, UK).

Acetone, sodium hydroxide, potassium chloride and foetal bovine serum were procured from Thermo Fisher Scientific (Waltham, MA, USA), Pefabloc from Roche (Basal, Switzerland), ethanol from OCON chemicals (Cork, Ireland), rutin from Extrasynthese (Genay, France), acetonitrile (MeCN) from J.T Baker® (Centre Valley, PA, USA) and methanol (MeOH) from Romil (Lennox Laboratory Supplies LTD, Dublin, Ireland). Formic acid (HCOOH) and the rest of the chemicals were purchased from Merck

(Darmstadt, Germany). All water used was ultrapure (PURELAB option-Q, ELGA, Veolia Water, Paris, France).

Table 5.1: Composition of different types of commercial milk. All fractions are in % (w/w). Data represent 3 independent samples ± SD. Abbr. stands for abbreviation.

Sample	Abbr.	Total solids %	Fat content %	Protein content %	Carbohydrate content %	Ash %
Whole Milk	WM	12.2 ± 0.01	2.96 ± 0.00	3.45 ± 0.03	5.25 ± 0.03	0.57 ± 0.01
Semi Skim Milk	SSM	9.94 ± 0.01	1.04 ± 0.06	3.52 ± 0.02	4.80 ± 0.06	0.58 ± 0.00
Skim Milk	SM	9.95 ± 0.01	0.08 ± 0.01	3.75 ± 0.03	5.48 ± 0.03	0.44 ± 0.01
High Protein Milk	HPM	11.9 ± 0.01	1.06 ± 0.04	5.31 ± 0.10	4.67 ± 0.11	0.82 ± 0.01

5.3.2. Sample preparation and compositional analysis

To create blackberry polyphenol DBBs, freeze-dried blackberry puree (FDBP) was manually stirred into four different types of commercial milk: whole milk (WM), semi-skimmed milk (SSM), skimmed milk (SM) and high-protein milk (HPM), creating samples with 10 % (w/w) FDBP. Addition of FDBP decreased the pH of the samples with WM, SM and SSM to 3.7 and HPM to 3.9. To compare the milk with the DBBs, milk with a similar pH to the DBBs named acidified milk (AM) was made by adding 7.3 mL of 1.0 N HCL to 100 mL WM, SSM, SM and HPM. This was done by adding the HCL dropwise under magnetic stirring. Standard methods were used to analyse the total solids,(IDF 2010) total protein (IDF 2014), total fat (IDF 2008a) and ash (IDF 2008b) content of the milk, while the carbohydrate content was calculated by difference (Table 5.1). A schematic overview of the experiments performed on the different samples can be found in Figure 5.1.

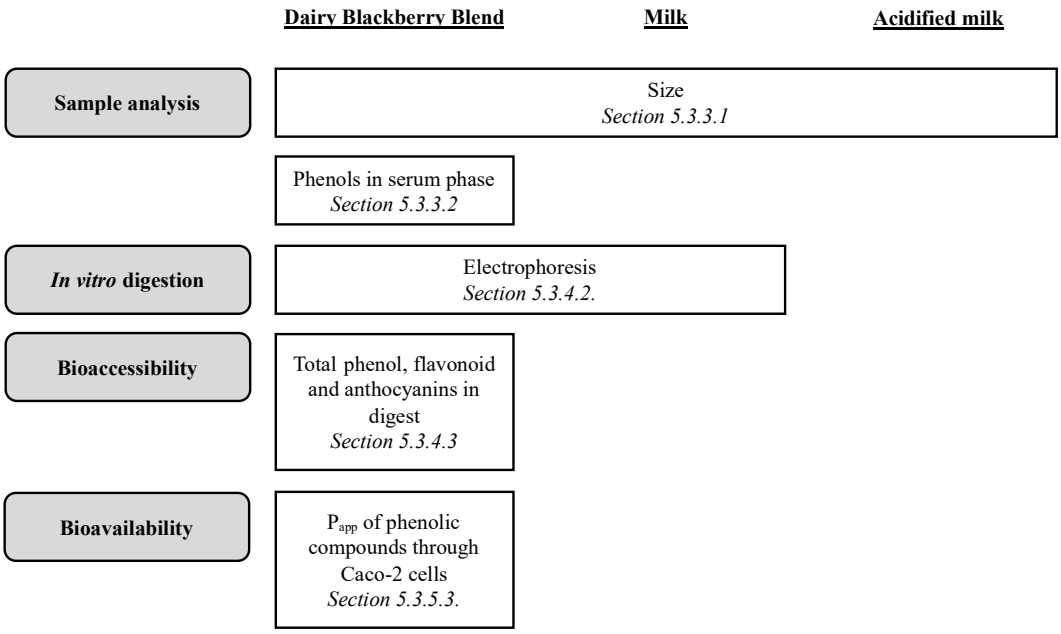

Figure 5.1: Schematic representation of different experiments performed on the samples in this study.

5.3.3. Sample analysis

5.3.3.1 Particle size distribution by light scattering

The particle size distribution (PSD) of the different milk samples was analysed in its original state, at pH 3.7 or 3.9 and with 10 % (w/w) FDBP using laser light diffraction with a Mastersizer 3000 (Malvern Instruments Ltd, Malvern, Worcestershire, UK). The different dispersions were added to ultrapure water in a Hydro SM until a laser obscuration of 8 ± 1% was reached for milk in its original state and 12 ± 1% for milk at pH 3.7 or 3.9 and with 10% FDBP. The PSD was determined using the spherical model and a particle and dispersant refractive index of 1.45 and 1.33, respectively.

5.3.3.2. Polyphenols in the serum phase of the milk

Samples with milk or water and 10% FDBP, were diluted with water to a protein content of 2% before centrifugation through an Amicon® Ultra 15 mL centrifugal membrane filter with molecular weight cut-off of 3 kDa (Merck Millipore Ltd. Cork, Ireland), at 4000 × g for 40 min with a RC 5C plus centrifuge (Sorvall, ThermoFischer scientific, Waltham, Massachusetts, USA). This centrifugal-filtration step separated the

polyphenols into those bound to the milk proteins and those present in the serum phase. The total phenol content (TPC) of the filtrates was determined with the Folin-Ciocalteu method (further discussed in Section 5.3.4.3.). The percentage of polyphenols in the serum phase was calculated using Equation 5.1:

$$Percentage\ in\ serum\ phase = \frac{TPC_{milk\ filtrate}}{TPC_{water\ filtrate}} \times 100\% \quad (5.1)$$

5.3.4. In vitro digestion and digest analysis

5.3.4.1. In vitro digestion

The *in vitro* digestion procedure followed the INFOGEST protocol (Brodkorb et al. 2019) with several adaptations to meet the specific requirements of the present study. The simulated gastric fluid (SGF) and the simulated intestinal fluid (SIF) were prepared as detailed in the INFOGEST method. For gastric digestion, sample (5 g) was placed in a 30 mL screw cap amber bottle and 5 mL SGF was added, the pH was adjusted to 2 using 1M HCl, after which pepsin was added to a final concentration of 2,000 units/mL. The samples were placed in a shaking water bath at 37 °C and 100 rpm for 1 h. The intestinal digestion followed with 8 mL SIF added to each sample and the pH was adjusted to approximately 7.4 using 1M NaOH. The purified bile salts (glycodeoxycholate, 0.85 mM; taurocholate, 0.75 mM and taurodeoxycholate, 0.5 mM) and pancreatin (100 units/mL) were added, with samples incubated for a further 2 h. The digested samples were then centrifuged at 5300 × g for 20 min with a 4K15 laboratory centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), the supernatant was collected and filtered with 0.45 μm syringe filters (Fisherbrand, Waltham, Massachusetts, USA). The filtrates were stored in the freezer for analysis of TPC, total flavonoid content (TFC), and total anthocyanin content (TAC) and the data obtained were used to calculate the samples required for assessment of bioavailability in the Caco-2 cell assays.

Samples were removed throughout the digestion at 0, 20 and 60 min gastric digestion and at 5, 10, 20, 40, 80 and 120 min of intestinal digestion for monitoring of

protein digestion. A sample (200 µL) was removed at each time point and 20 µL Pepstatin A (100 µM) and 20µL Pefabloc (100mM) were added to stop the enzyme activity during the gastric digestion and intestinal digestion, respectively. Samples were stored at -80 °C before running on gels to determine protein degradation.

5.3.4.2. Electrophoresis

The protein profile of WM, SSM, SM and HPM, both with and without blackberry polyphenols, during *in vitro* digestion were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The method of Laemmli (1970) was performed under reducing conditions and described in more detail by van de Langerijt et al. (2022). Throughout this article "α_s-casein" refers to the sum of α_{s1}-casein and α_{s2}-casein.

5.3.4.3. Total phenol, flavonoid and anthocyanin contents

For undigested FDBP beverages, 0.5 g of each sample was weighed, and 9.5 mL of 70% aqueous acetone was added; samples were vortexed and placed in a sonicator bath for 30 min. For digested samples, 200 µL of digest was mixed with 800 µL of 70% aqueous acetone and samples were sonicated for 30 min. Both undigested and digested samples were centrifuged at 5300 × g for 20 min with a 4K15 laboratory centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and the supernatants were used to measure TPC, TFC and TAC. Results were expressed per standard serving portion (250 mL). TPC was measured using the Folin-Ciocalteu method (Singleton & Rossi 1965), the TFC was determined according to a method described by Singleton & Rossi (1965), and the TAC was quantified using the pH differential method from Klopotek et al. (2005).

5.3.5. Cell culture and individual polyphenol identification and quantification in digested sample, apical chambers and basolateral chambers

5.3.5.1. Cell culture of Caco-2 cells

Caco-2 cells (passage 80-90) were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma D5796), supplemented with 10% (v/v) heat-inactivated foetal bovine

serum (FBS) and 1% (v/v) non-essential amino acids. Cells were incubated at 37 °C and 5% CO_2. The cells were seeded at a density of 6.0×10^4 cells cm^{-2} on polycarbonate transwell inserts (Corning product no. 3414) with a membrane pore size of 3 μm (Corning Inc, Lowell, MA). Caco-2 cells were maintained in transwells for 21 d, with media changing every 2-3 d, to ensure cell differentiation.

For bioavailability studies, digests were diluted (1:4) in Hanks' balanced salt solution (HBSS), filter sterilised (0.45 μm, Merck, Millipore) and added (1.5 mL) to the apical chamber (AC) of the transwell plate. HBSS (2 mL) was added to the bottom chamber and the cells were incubated for 4 h. Trans-epithelial electrical resistance values were recorded before and after incubation to ensure that the cell monolayer remained intact throughout the experiment. Following incubation, the AC and basolateral chambers (BC) were harvested and stored at -80 °C prior to phenolic characterisation.

5.3.5.2. Solid Phase Extraction of polyphenols

Prior to liquid chromatography-mass spectrometry analysis the polyphenols in digested, AC and BC samples were enriched by solid phase extraction (SPE) following the method of Zhang et al. (2018) with slight modifications. The SPE was performed on ISOLUTE® C18 cartridges (3 mL, 200 mg) obtained from Biotage Sweden. The cartridges were initially pre-conditioned with 2 mL of 1% HCOOH in MeOH followed by 2 mL of 1% aqueous HCOOH. Each sample was subjected to a 1:4 dilution with 1% aqueous HCOOH to a total volume of 1 mL, vortexed and loaded onto the SPE cartridge. The samples of the BC were subjected to a 1: 3 concentration prior to loading on to the SPE cartridges in order to maximize the possibility of phenolic detection and their presence in quantifiable levels. Subsequently, the loaded sample was filtered under gravity and washed with 1 mL of 1% aqueous HCOOH. The final elution after the SPE clean-up was performed with 500 μL 1% HCOOH in MeOH, followed by 500 μL of 1% HCOOH in acetone. The extract was then evaporated under nitrogen and at a temperature of 45oC, which represent suitable conditions to prevent degradation of the target compounds. The sample residue of

the BC, AC and digested samples was finally reconstituted in 200 µL (1:15 final concentration), 1,000 µL (initial concentration), and 6,000 µL (6-fold dilution) of 1% aqueous HCOOH, respectively, and subjected to liquid chromatography-mass spectrometry analysis.

5.3.5.2. Identification of polyphenol metabolites through HPLC-Q-ToF analysis

Untargeted high performance liquid chromatography coupled to quadrupole time of flight (HPLC-Q-ToF) mass spectrometer (Waters Corporation, Milford, MA, USA) was used for the identification of the predominant polyphenol metabolites in the *in vitro* digested samples. An in-house HPLC-Q-ToF method as described by Hossain et al. (2010) was employed with modifications. An Atlantis T3 C18 column (100 x 2.1 mm; 3.0 µm particle size) at 40 oC was used for the separation of analytes employing a binary mobile phase of 0.1% aqueous HCOOH (solvent A) and 0.1% HCOOH in MeCN (solvent B). A stepwise gradient ranging from 10 to 90% of solvent B was applied at a flow rate of 0.3 mL/min to a total run time of 25 min. Electrospray ionization (ESI) mass spectra were recorded in negative ion mode for a mass range (m/z) between 100 and 1500. Argon was used as collision gas (12-20 eV) to acquire collision-induced dissociation mass spectra.

5.3.5.3. Quantification of polyphenol metabolites using UPLC-MS/MS analysis

Targeted ultraperformance liquid chromatography-tandem mass spectrometry (UPLC- MS/MS) analysis was used for the quantification of the most abundant polyphenol metabolites in the *in vitro* digested samples, samples from AC and BC. A Waters Acquity UPLC coupled to tandem quadrupole mass spectrometer (UPLC-TQD, Waters Corporation, Milford, MA, USA) was used by adapting the method of Gangopadhyay et al. (2016). An Acquity UPLC HSS T3 (100 x 2.1 mm; 1.8 µm particle size) column at 40 °C involving a binary mobile phase of 0.1% aqueous HCOOH (solvent A) and 0.1% HCOOH in MeCN (solvent B) was used. The flow rate of the mobile phase was 0.5 mL/min for a total run time of 10 min. A multiple reaction monitoring (MRM) method was employed for the quantification. Waters IntellistartTM software (Waters Corp., Milford,

MA, USA) was used to acquire the MRM transitions for each targeted compound (Table 5.2). UPLC-MS/MS data were obtained in negative ESI mode for all the phenolic compounds except for cyanidin-3-O-glucoside, for which positive ESI was employed. The quantification of polyphenols was carried out using the TargetLynxTM software (Waters Corporation, Milford, MA, USA).

The apparent permeability (P_{app}) after quantification of the compounds present in the BC and AC (directed from AC to BC) was calculated using Equation 5.2 as described by Lee et al. (2013):

$$P_{app} = \left(\frac{V_A}{Area \times time}\right) \times \left(\frac{C_{basolateral}}{C_{apical}}\right) \quad (5.2)$$

Where: V_A is the volume in mL in the AC (2 mL), Area is the surface area of the membrane (2.161 cm^2), time is the total transport time in s (4 h equal 14,400 s), $C_{basolateral}$ is the concentration of compound quantified in the BC and C_{apical} is the concentration of each compound quantified in AC. Cell line models can exhibit a high variability, which may be particularly apparent after the assessment of individual compounds with a sensitive analytical technique such as UPLC-MS/MS.

Table 5.2: Retention time (t_R), MRM transitions, collision energy and cone voltage for the standards used and the phenolic compounds and organic acids that were quantified in the digested samples, as well as those of the apical and basolateral chambers.*

Reference Compound	t_R (min)	MRM Transition (*m/z*)	Cone Voltage (V)	Collision Energy (eV)
Malic acid	0.60	133.12 → 114.94	27	12
		70.92		14
Citric acid	0.78	191.12 → 110.93	29	10
		86.92		20
Gallic acid	1.49	169.12 → 124.94	37	12
		78.41		16
Protocatechuic acid	2.59	153.06 → 108.94	29	16
		80.88		18
Cyanidin-3-O-glucoside	3.60	448.61 → 287.20	30	15
		316.60		15
Ellagic acid	5.56	301.04 → 144.88	60	36
		283.88		28
Rutin	5.70	609.38 → 271.00	60	60
		300.20		34

* The first MRM transition (*m/z*) constitutes the quantifier and the second the qualifier.

5.3.6. Statistical analysis

Samples were prepared and analysed in triplicate for the TPC, TFC and TAC. The samples for polyphenol identification and quantification with HPLC-Q-ToF and UPLC-MS/MS were performed in duplicate. The data is reported as mean value ± standard error. Differences between the mean values of samples analyses of the triplicates were statistically analysed with the analysis of variance (ANOVA) and Tukey test, while duplicates were statistically analysed with an independent *t*-test, and the significance level was set to ($p \leq 0.05$). The software used to perform the statistical analyses was SPSS Statistics version 28 (SPSS inc., Chicago, USA).

5.4. Results and Discussion

5.4.1. Influence of freeze-dried blackberry puree on particle size and polyphenol distribution in milk

Dairy-blackberry blends (DBBs) were made by addition of freeze-dried blackberry puree (FDBP) to commercial milk (WM, SSM, SM and HPM). Upon FDBP addition, the pH of the milk decreased from 6.8 to 3.7 for WM, SSM and SM and to 3.9 for HPM, with

the particle size distribution of milk samples changing as a consequence. To investigate the effect of the decrease in pH on the size distribution of the DBBs, AM samples were made from the different types of commercial milk, whereby the pH of the AM samples was decreased from 6.8 to 3.7 for WH, SSM and SM and pH 3.9 for HPM with 1 N HCl. Dynamic light scattering was used to measure the size distribution of particles of the different types of commercial milk, AM and DBBs (Figure 5.2). The milk used in this study was commercial homogenized milk and had a size distribution with a peak at ~0.1 µm for WM and at ~0.4 µm for the SSM, SM and HPM, similar to reports in the literature (Ransmark et al. 2019). All the AM samples had a peak ~40 µm, this higher mean particle size was due to protein aggregation, caused by the acidification, and fat globules entrapped in these protein aggregates. The size distribution of the DBBs was bimodal, with peaks at ~13 and ~300 µm. FDBP in water had a size distribution which ranged from 3 to 1000 µm, with a shoulder at ~10 and a peak at ~120 µm (data not shown). The bimodal size distribution of blackberry milk is most likely a representation of aggregate formation in the milk systems.

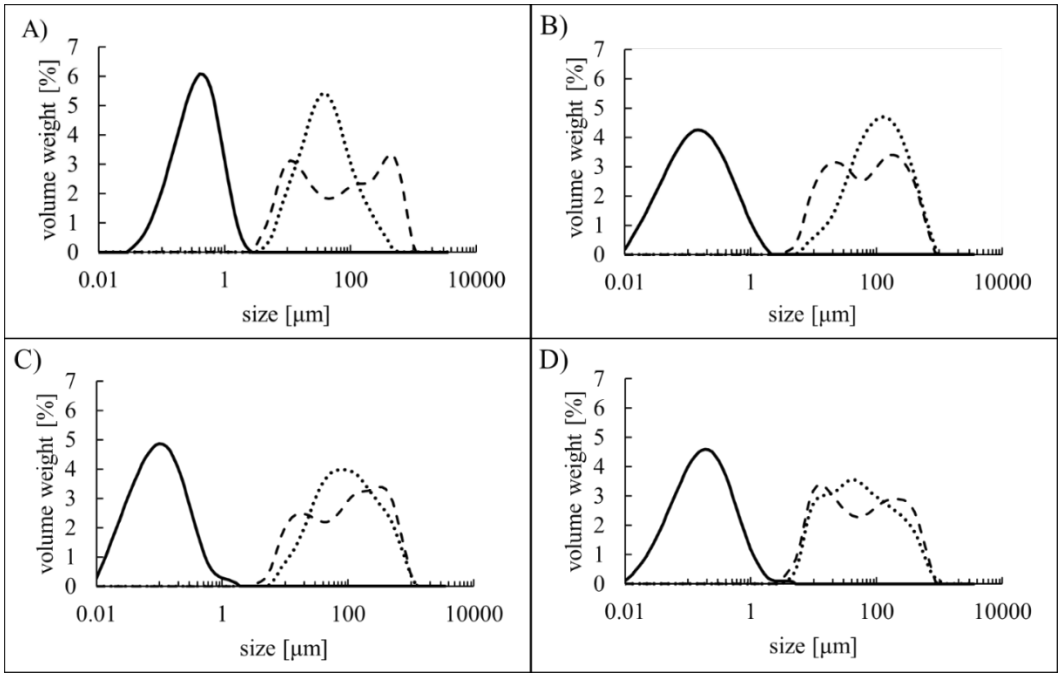

Figure 5.2: Particle size distribution in which A) represents samples made with whole milk (WM), B) samples made with semi-skimmed milk (SSM), C) samples made with skimmed milk (SM) and D) samples made with

high protein milk (HPM). Solid line represents the commercial milk, dotted line represents the acidified milk at pH 3.7 (for WM, SSM and SM) and 3.9 (for PM) and the striped line represents the blackberry polyphenol enriched dairy beverage with a pH of 3.7 (for WM, SSM and SM) and 3.9 (for PM). Mean values are presented (n = 3).

When the DBBs were centrifuged through a centrifugal membrane filter with molecular weight cut-off of 3 kDa the blackberry phenolics (BBP) in the serum was separated from the BBP that were interacting with milk proteins and fats. The amount of BBP present in the serum phase of WM, SM, SSM and HPM was 54.2 ± 1.5, 62.9 ± 5.1, 64.2 ± 6.7 and 55.0 ± 8.8%, respectively (Figure 5.3). The lower concentration of BBP in the serum phase of WM and HMP indicates that the high fat and/or protein contents of WM and HPM resulted in more fat/protein BBP interactions. However, the differences between WM and HPM were small and not significant ($p>0.05$).

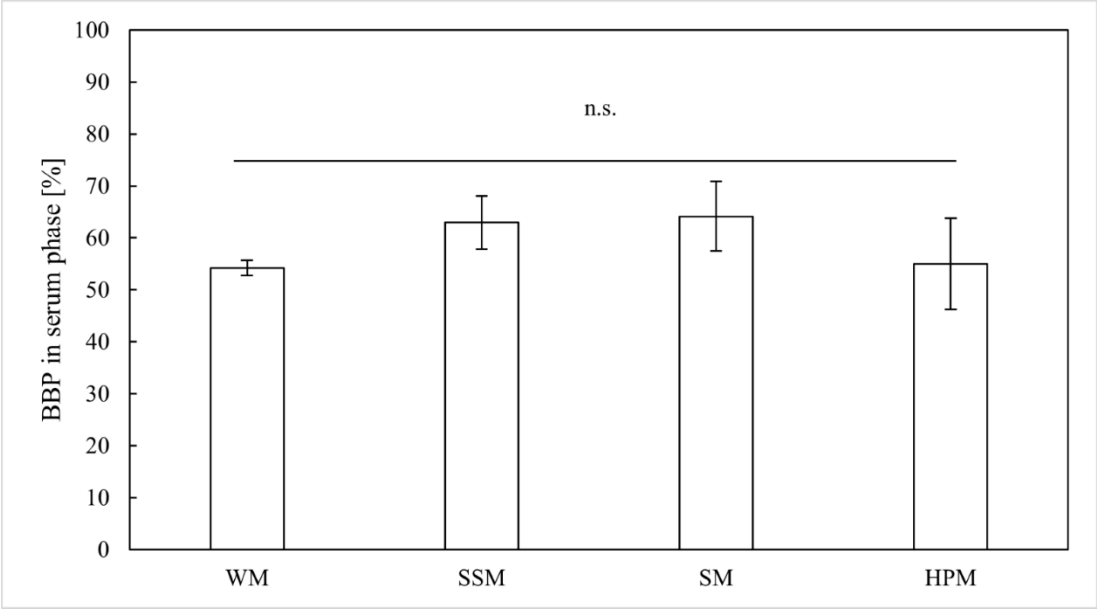

Figure 5.3: Blackberry phenolics (BBP) in the serum phase of blackberry polyphenol enriched dairy beverages made with whole milk (WM), semi skimmed milk (SSM), skimmed milk (SM), and high protein milk (HPM). Data represent 3 independent samples ± SD. The subscript letters n.s. indicate no significant difference ($p>0.05$) between the blackberry polyphenol enriched dairy and water beverages.

The interactions between polyphenols and milk proteins (e.g., β-lactoglobulin) or milk fat, may protect polyphenols from deterioration during digestion (Ortega et al. 2009;

Zagury et al. 2019). It should be noted that WM and HPM interacted with almost the same amount of BBP, but HPM contains ~25% less kilocalories (49 vs 64 kilocalories, respectively). This is an important consideration for athletes depending on whether they are participating in lean or non-lean sports, which influences the extent to which they need to maintain low bodyweight while developing muscle mass (Eck & Byrd-Bredbenner 2019); in any case milk composition should not have a significant effect on polyphenol protection.

5.4.2. Digestion of milk proteins

SDS-PAGE was used to assess the breakdown of different milk proteins during the *in vitro* digestion of SM and the DBB made with SM (Figure 5.4). The fat content had no influence on the bands visible on the SDS-PAGE and therefore the breakdown of SM and DBB in Figure 5.4 is representative for the digestion in all milk and DBB samples. This is similar to the findings of Ding et al. (2022), who found that fat content does not influence the digestion of casein micelles in milk.

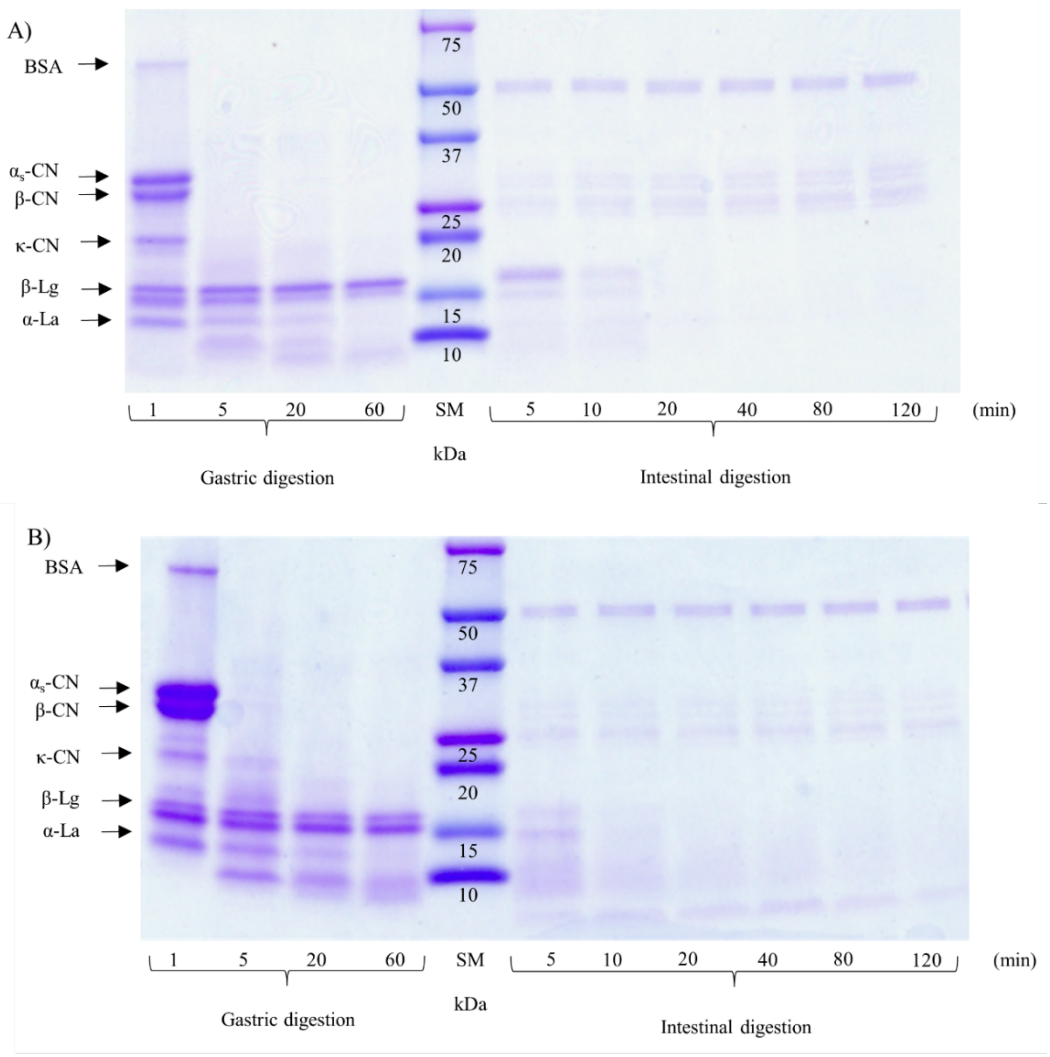

Figure 5.4: SDS-PAGE analysis of different time points in the gastric and intestinal *in vitro* digestion in which A) represents skimmed milk and B) blackberry polyphenol enriched dairy beverage made with skimmed milk and 10 % (w/w) frieze dried blackberry puree. The abbreviations α_s-CN, β-CN, κ-CN, β-LG and α-LA, stand for α_s-casein, β-casein, κ-casein, β-lactoglobulin and α-lactalbumin.

The caseins, α_s-, β- and κ-casein, were digested during the first 5 min of the gastric digestion. The whey protein α-lactalbumin was digested during the 60 min of gastric digestion and β-lactoglobulin seemed resistant during the gastric digestion and needed the intestinal digestion to be digested. These digestion patters were similar to the ones found by Egger et al. (2019), who also used the INFOGEST method for the *in vitro* digestion of milk proteins in skimmed milk powder. The SDS-PAGE gels showed no clear differences in bands during digestion for the milk and the DBB made with the same milk (Figure 5.4),

indicating that BBP did not affect the hydrolysis of milk proteins. Similar results were found for digestion of casein with the polyphenol epigallocatechin-3-gallate, which had no effect on casein hydrolysis (Guri et al. 2014). In the study of Cao & Xiong (2017) the effect of gallic acid and epigallocatechin-3-gallate on the *in vitro* digestion of α-lactalbumin and β-lactoglobulin was investigated. Gallic acid had no effect on the digestion rate of these, but epigallocatechin-3-gallate increased the protein digestion rate, which was attributed to structural unfolding, caused by binding of EGCG to the whey proteins. This indicates that the influence of polyphenols on the digestion of milk proteins depends on the type of protein and phenolic compound, and that the phenolic compounds in blackberry had no influence on the digestion rate of milk proteins.

5.4.3. Bioaccessibility

5.4.3.1. Bioaccessibility of phenolics, flavonoids and anthocyanins

The FDBP had a TPC of 3.14 ± 0.10% (w/w), a TFC of 0.71 ± 0.02% and a TAC of 0.50 ± 0.01%. Following reconstitution in either water or milk, the TPC did not differ significantly ($p>0.05$) between the samples and ranged from 926.8 ± 65.7 mg/serving (250 mL) in the DBB sample made with SM to 981.5 ± 82.4 mg/serving in the water sample (Figure 5.5A).

Following in-vitro digestion, the highest phenolic content of 693.2 ± 25.9 mg/serving was found in the water sample and had a bioaccessibility of approximately 72.8 ± 8.9% (Figure 5.5A and 5.6). The TPC of each of the DBB samples following digestion was significantly lower ($p\leq0.05$) than that of the FDBP reconstituted in water, but there were no significant differences ($p>0.05$) between the various DBB samples. The bioaccessibility of phenolic components in the DBB samples ranged from 45.0 ± 1.9% in HPM to 52.4 ± 5.8% in WM (Figure 5.6).

The TFC of the different samples containing FDBP did not differ significantly ($p>0.05$) and ranged from 171.6 ± 12.1 mg/serving in the DBB sample with SM to 195.2

± 3.0 mg/serving for the water sample (Figure 5.5B). After in-vitro digestion, the bioaccessibility ranged from 62.8 ± 9.4% in the HPM DBB sample to 75.5 ± 5.3% in the water sample (Figure 5.6), and the TFC was significantly lower ($p \leq 0.05$) in the samples after digestion. There was no significant difference ($p > 0.05$) between any of the TFC values for the digested samples.

Each of the samples had a similar TAC following reconstitution with values ranging from 134.2 ± 8.4 mg/serving for the DBB sample with SSM to 145.8 ± 3.4 mg/serving in the water sample. The anthocyanin content decreased to a greater extent following in-vitro digestion than either the flavonoid or the total phenolic contents (Figure 5.5C). This indicated that anthocyanins are particularly susceptible to degradation during digestion. In contrast to phenolics and flavonoids, the greatest decrease in anthocyanin content following digestion was observed when the FDBP was reconstituted in water, with a bioaccessibility of 11.4 ± 4.0%. This was significantly lower ($p \leq 0.05$) than the bioaccessibility in the samples reconstituted in milk which demonstrated values ranging from 31.5 ± 8.8% for SM to 48.2 ± 10.8% for SSM (Figure 5.6).

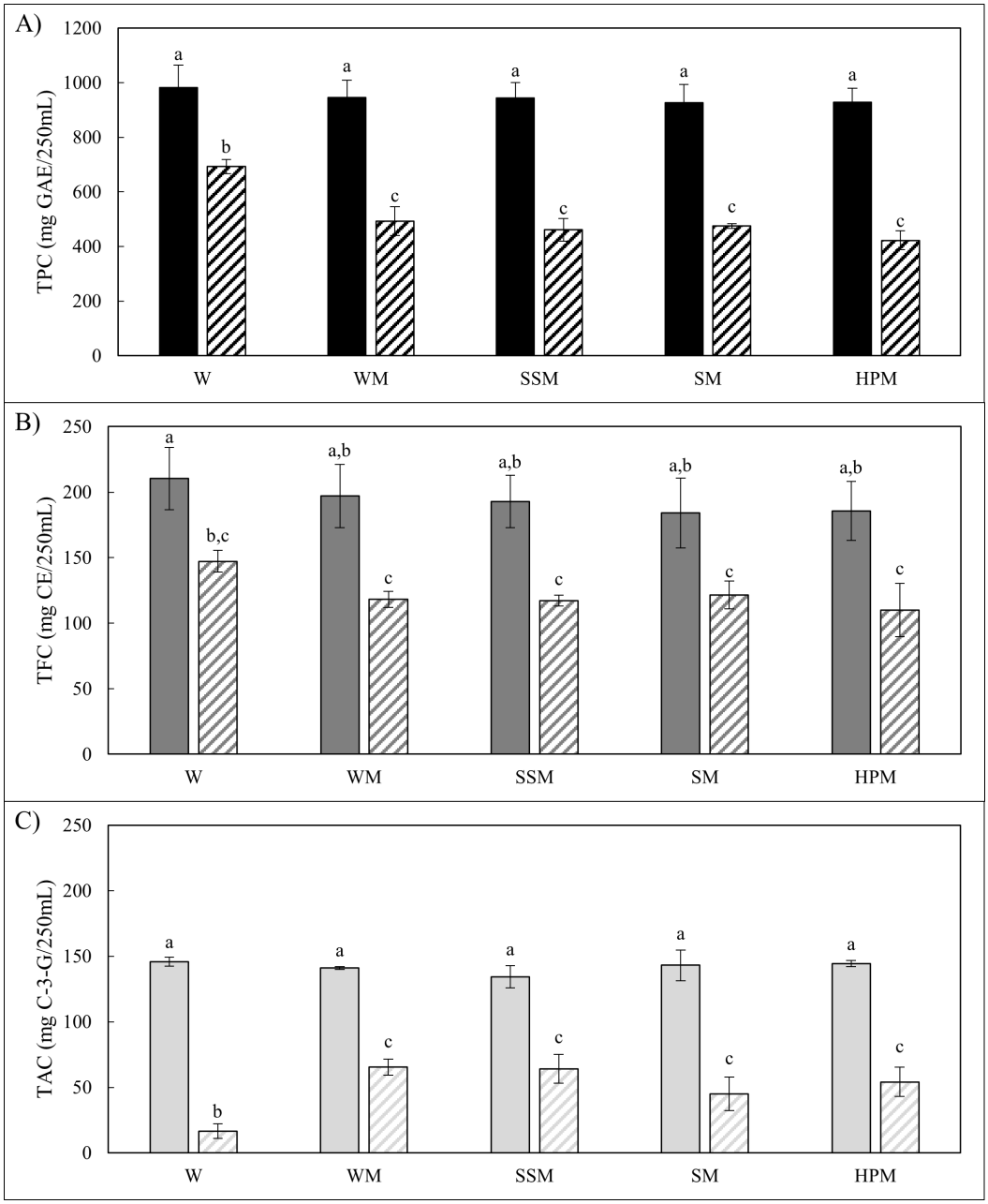

Figure 5.5: A) Total phenol content (TPC) (black), B) total flavonoid content (TFC) (dark grey) and C) total anthocyanin content (TAC) (light grey) of blackberry polyphenol enriched dairy and water beverages, containing 10 % (w/w) freeze-dried blackberry puree in water (W), whole milk (WM), semi skimmed milk (SSM), skimmed milk (SM) and high protein milk (HPM) samples before (solid fill) and after digestion (diagonal stripes). The abbreviations GAE stands for gallic acid equivalate, CE for catechin equivalent and C-3-G for cyanidin-3-glucoside equivalent. D) represents the bioaccessibility of the phenols, flavonoids and anthocyanins. Data represent 3 independent digestions ± SD. The subscript letters indicate a significant difference ($p \leq 0.05$) between the blackberry polyphenol enriched dairy and water beverages.

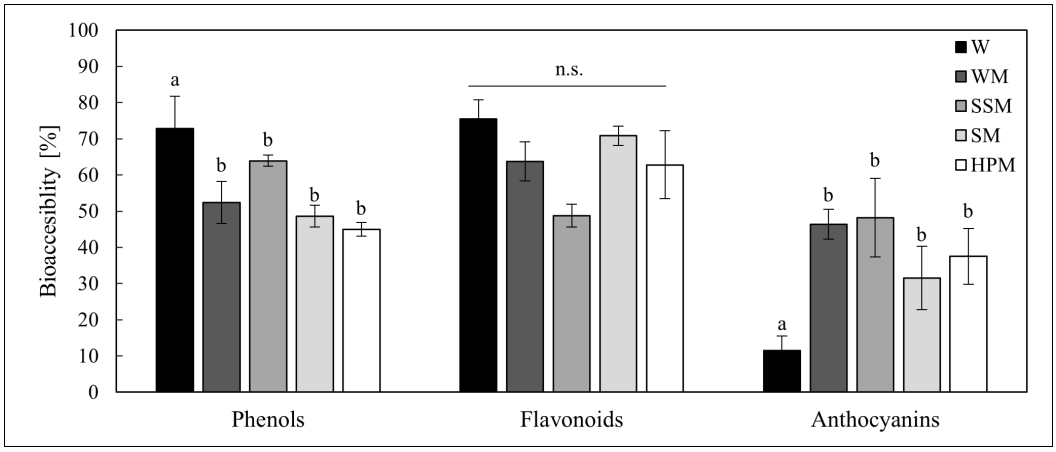

Figure 5.6: Bioaccessibility of phenols, flavonoids and anthocyanins in blackberry polyphenol-enriched dairy and water beverages, containing 10 % (w/w) freeze-dried blackberry puree in water (W), whole milk (WM), semi skimmed milk (SSM), skimmed milk (SM) and high protein milk (HPM). Data represent 3 independent digestions ± SD. The subscript letters indicate a significant difference ($p \leq 0.05$) and n.s. indicates no significant difference ($p > 0.05$) in the bioaccessibility of phenols, flavonoids or anthocyanins between samples.

The varying protein and fat contents within the commercial milk products (Table 5.1) did not impact the bioaccessibility of phenols, flavonoids or anthocyanins, and no significant difference ($p > 0.05$) was observed in TPC, TFC and TAC between the milk samples (Figure 5.5A-C). The combination of fat and protein in WM has been shown to reduce the phenolic bioavailability from a blend of jujube juice and milk (Zhang et al. 2012, 2013). This supports the findings of the current study, which showed a significant decrease ($p \leq 0.05$) in phenolic bioaccessibility in milk in comparison to water. Quan et al. (2020) found that the bioaccessibility of the phenolics in kiwi juice was higher in SM than WM, whereas the opposite effect was observed for the phenolics from pomelo juice. This indicates that the impact of fat in milk on the bioaccessibility of phenolics depends on the type of phenolics. In the current study, the blackberry polyphenols had similar bioaccessibility patterns in the different milk samples.

The content of anthocyanins in the digested samples was higher for the milk samples in comparison to the water sample. Anthocyanins are known to degrade during processing and digestion (Ge et al. 2019). Lang et al. (2021) showed that there was not a substantial

degradation of anthocyanins in anthocyanin-containing blueberry extract during the gastric phase of digestion, in contrast to a rapid decrease in the intestinal phase, which led to a 97% loss of its initial anthocyanin content. However, the addition of either α_s-casein or β-casein in the matrix resulted in an apparent protective effect on anthocyanins. This data led the authors to speculate that α_s/β-casein could encapsulate anthocyanins and thereby, improve their bioavailability. According to Pineda-Vadillo et al. (2016) the presence of food matrices, such as milk, impacted the release and solubility of anthocyanins during digestion, and protected the anthocyanins during the intestinal digestion. This would support the findings of the present study in which the bioaccessibility of anthocyanins was 3- to 4-fold higher in the milk samples compared to the water sample.

5.4.3.2. Bioaccessibility of individual compounds after digestion.

The polyphenols and organic acids in the *in vitro*-digested-FDBP in water and milk were identified by HPLC-Q-ToF analysis, which indicated the presence of fourteen different compounds (Table 5.3). The majority of compounds were identified through authentic standards as shown in Table 5.3, while identification of the remaining compounds was based on accurate mass measurements as well as fragmentation patterns and literature data. From the identified compounds, the first four, namely malic, aconitic, citric and furoic acid, were organic acids and not polyphenols. However, those compounds were included in Table 5.3 due to their high abundance in blackberries. For instance, malic and citric acids can be degradation products of major phenolic compounds, but they are also related to certain beneficial health effects (López-Bucio et al. 2000; Tembo et al. 2017) and have been reported in blackberries (Zafra-Rojas et al. 2018). However, to the best of our knowledge, aconitic and furoic acids have not been previously detected in blackberries. Aconitic acid has previously been reported in other fruits, including apricot and tomato (Marconi et al. 2007; Cirilli et al. 2019). Furoic acid is a metabolite formed after ascorbic acid degradation, which is one of the main acids present in blackberries (Zia-Ul-Haq et al. 2014). Furoic acid is not present in the blackberry puree itself (data not shown) but is

formed during *in vitro* digestion. In the case of identified phenolic acids, ellagic and gallic acids are known to be formed after ellagitannin degradation (Sójka et al. 2019), whereas protocatechuic acid is a known degradation product of anthocyanins (Seke et al. 2021).

Table 5.3. Compounds tentatively identified and their associated identification criteria following HPLC-Q-ToF analysis of the 10% freeze-dried blackberry sample reconstituted in water (Control). Identification of compounds were based on accurate mass measurement, fragmentation pattern and t_R of standards and literature sources.*

No	t_R (min)	Observed $[M-H]^-$ (*m/z*)	Calculated $[M-H]^-$ (*m/z*)	Empirical Formula	MS/MS fragment ions (*m/z*)	Compound	Reference
1	0.93	133.0126	133.0137	$C_4H_5O_5$	115.01; 71.02; 73.00; 133.02; 89.03	Malic acid*	Kim et al. (2019)
2	1.30	173.0147	173.0086	$C_6H_5O_6$	111.01; 85.03; 83.02; 73.00; 112.01; 99.01; 155.00	Aconitic acid	Hassanen et al. (2019)
3	1.33	191.0210	191.0192	$C_6H_7O_7$	111.02; 73.04; 73.00; 85.04; 117.02; 99.01	Citric acid*	al Kadhi et al. (2017) and Oszmiański et al. (2015)
4	1.34	111.0121	111.0082	$C_5H_3O_3$	67.03; 87.05	Furoic acid	Peixoto Araujo et al. (2020)
5	1.58	169.0158	169.0137	$C_7H_5O_5$	111.03; 96.98; 155.03	Gallic acid*	Chen et al. (2016) and Singh et al. (2016)
6	2.16	353.0822	353.0873	$C_{16}H_{17}O_7$	111.04; 135.08; 164.96; 191.09; 155.05; 179.07	Chlorogenic acid	Oszmiański et al. (2015)
7	2.22	447.0935	447.0927	$C_{21}H_{19}O_{11}$	284.05; 285.06; 256.05; 147.02	Cyanidin-3-O-glucoside*	Chen et al. (2016)
8	5.18	463.0876	463.0877	$C_{21}H_{19}O_{12}$	271.05; 300.06; 255.04; 301.06; 151.01	Quercetin-3-O-glucoside	Oszmiański et al. (2015)
9	6.51	153.0192	153.0188	$C_7H_5O_4$	109.03; 108.03; 110.04; 91.02; 126.90	Protocatechuic acid*	Huang et al. (2012) and Palafox-Carlos et al. (2012)
10	6.83	163.0445	163.0395	$C_9H_7O_3$	119.08; 108.05; 147.07; 167.06; 162.87; 152.04; 125.89; 93.05	Coumaric acid	Akin et al. (2016) and Gruz et al. (2008)
11	6.89	167.0391	167.0344	$C_8H_7O_4$	108.04; 109.04	Vanillic acid	Akin et al. (2016) and Gruz et al. (2008)
12	7.34	609.1461	609.1456	$C_{27}H_{29}O_{16}$	300.05; 271.04; 255.05; 301.05; 179.02; 151.02	Rutin*	Oszmiański et al. (2015)
13	7.42	463.0919	463.0877	$C_{21}H_{19}0_{12}$	300.03; 271.02; 255.03; 301.03	Quercetin-3-O-galactoside	Schulz et al. (2019) and Yu et al. (2018)
14	7.53	300.9999	300.9984	$C_{14}H_5O_8$	301.02; 301.01; 117.05; 284.02; 229.03; 111.03	Ellagic acid [1]	Chen et al. (2016)

* Identification confirmed with the use of authentic standards, and these compounds were present in the basolateral chamber.

Seven of the identified organic acids and polyphenols (citric acid, cyanidin-3-O-glucoside, ellagic acid, gallic acid, malic acid, protocatechuic acid and rutin) in the digested milk and water samples were quantified through UPLC-MS/MS, to determine their bioaccessibility (Figure 5.7). Upon comparison of the different compounds in the water sample with the milk samples, it was found that the bioaccessibility of citric acid, cyanidin-3-O-glucoside and gallic acid increased, whereas ellagic acid, rutin and malic acid concentrations remained unaltered and the protocatechuic acid was decreased (Figure 5.7). The highest bioaccessibility was observed for cyanidin-3-O-glucoside, especially in WM. These data are in accordance with the high concentration of anthocyanins found in the digested milk samples (Figure 5.5C). As stated previously, anthocyanins of berries in the Rubus family, namely blueberries, can be partly protected during intestinal digestion by the presence of milk proteins (Pineda-Vadillo et al. 2016; Lang et al. 2021). In parallel, the fat content may have increased the bioaccessibility of anthocyanins, including cyanidin-3-O-glucoside (Eker et al. 2020). This could potentially explain the higher content of cyanidin-3-O-glucoside in the different DBB samples, particularly samples prepared with WM, which had the highest fat content. The cyanidin-3-O-glucoside content in the SM sample was not significantly different ($p>0.05$) from that of the water sample, a result that could be potentially attributed to the low fat content of SM. As this data demonstrates, the degradation of the anthocyanins, and specifically cyanidin-3-O-glucoside, led to the formation of protocatechuic acid upon digestion (Dai et al. 2009). This suggests milk had a protective effect on cyanidin-3-O-glucoside from degradation, which could explain the presence of lower concentrations of protocatechuic acid in the milk samples compared to the water sample (Figure 5.7).

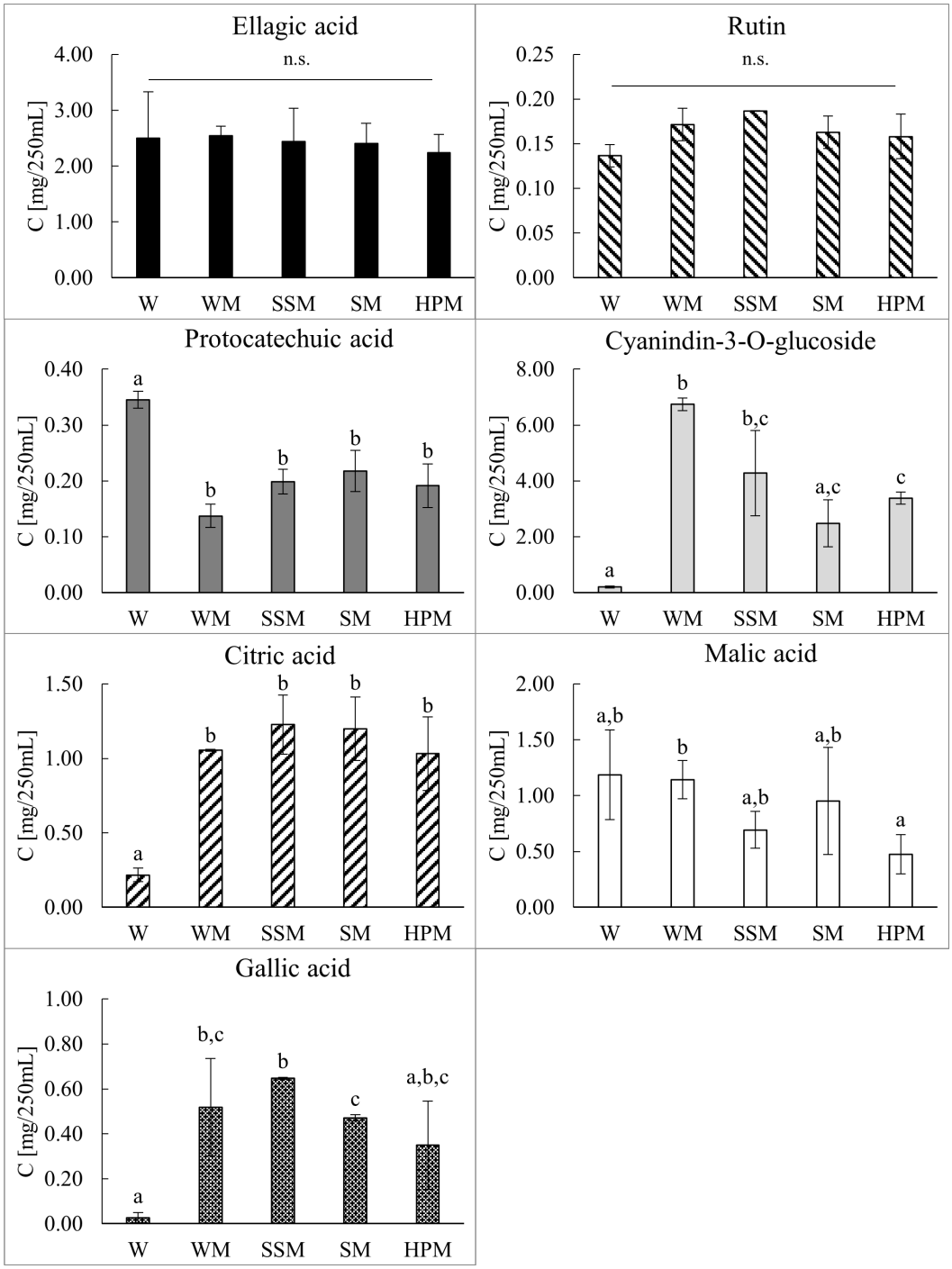

Figure 5.7: The concentration of different phenolic components and organic acids in one portion (250 mL) of *in vitro* digested water (W), whole milk (WM), semi skimmed milk (SSM), skimmed milk (SM) and high protein milk (HPM) with 10% (w/w) freeze-dried blackberry puree. Data represent 2 independent digestions ± SD. The subscript letters indicate a significant difference ($p \leq 0.05$) and n.s. indicates no significant difference ($p > 0.05$) between the blackberry polyphenol enriched dairy and water beverages.

5.4.4. Bioavailability and permeability of individual components in Caco-2 cells

Among the fourteen compounds detected through HPLC-Q-ToF mass spectrometry in the digested sample, seven were also detected in the BC (citric acid, cyanidin-3-O-glucoside, ellagic acid, gallic acid, malic acid, protocatechuic acid and rutin) and of these seven, six were detected in the AC (all except for gallic acid). These compounds were quantified by UPLC−MS/MS. Based on the quantification data the effect of milk on Papp, which is directly related to bioavailability, of the polyphenols and organic acids was determined.

The transfer of phenolic compounds polyphenols from the AC to the BC exhibited a high variability and relatively low Papp values (Figure 5.8). According to Gómez-Juaristi et al. 2020, high variability can be observed due to differences in the matrix and lipophilicity of the compounds and their structure. A non-significant difference ($p>0.05$) was observed in the Papp values of citric acid (Figure 5.8). There were no clear correlations for rutin, protocatechuic acid and malic acid between the Papp values of the water sample and the milk sample with different fat and protein contents (Figure 5.8). Cyanidin-3-O-glucoside had a significantly lower ($p\leq0.05$) Papp value in WM compared to water. Even though the differences between the milk types are not always significant, the Papp values of cyanidin-3-O-glucoside, malic acid and protocatechuic acid are lower in WM compared to the milk samples with less fat and roughly the same protein content, SM and SSM. The results align with previous research about the phenolic compounds of blueberries and jujube fruit. The phenolic compounds of these fruits in whole milk had a lower bioavailability compared to water and skimmed milk, which is attributed to the binding of phenolic compounds to the polypeptide chains present in the milk fat globule membrane (Serafini et al. 2009; Zhang et al. 2012).

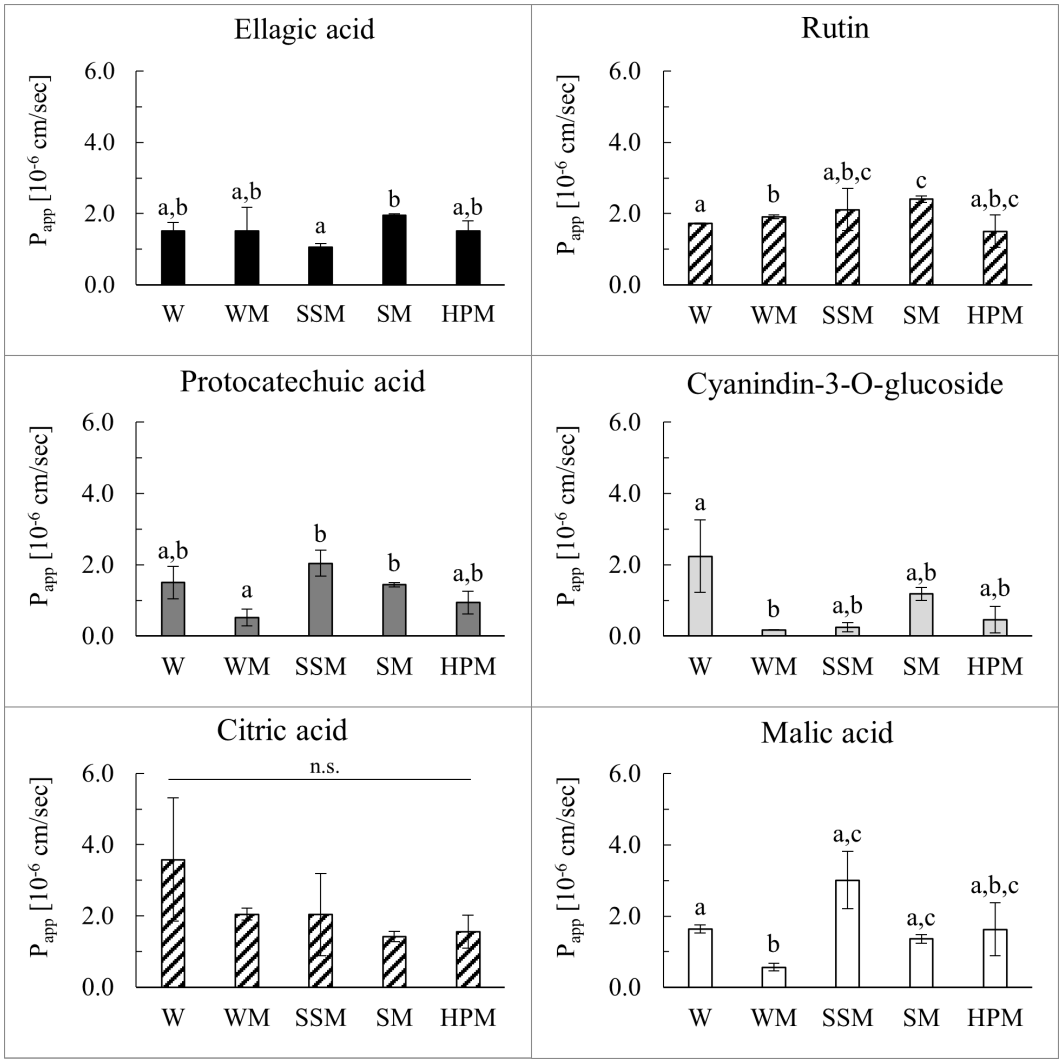

Figure 5.8: The estimated apparent permeability coefficient (P_{app}) of Caco-2 cells of different phenolic components and organic acids in water (W), whole milk (WM), semi skimmed milk (SSM), skimmed milk (SM) and high protein milk (HPM) after *in vitro* digestion. Data represent 2 independent digestions ± SD. The subscript letters indicate a significant difference ($p \leq 0.05$) and n.s. indicates no significant difference ($p > 0.05$) between the blackberry polyphenol enriched dairy and water beverages.

During *in vitro* digestion, glycosylated polyphenols (e.g., rutin and cyanidin-3-O-glucoside) need to be first hydrolysed in order to be absorbed by the epithelial cells (Eran Nagar et al. 2020), which could be why rutin and cyanidin-3-O-glucoside were detected in the AC of the Caco-2 model but not in the BC. *In vitro* models such as the Caco-2 model may differ from *in vivo* models (Gómez-Juaristi et al. 2020); for example, glycosylated polyphenols are commonly detected in the BC of Caco-2 models but are not absorbed in *in vivo* models. This is probably related to enzymatic and bacterial hydrolysis of the sugar

molecules in *in vivo* models (Fang et al. 2017; Gómez-Juaristi et al. 2020). Polyphenols are transported through the Caco-2 cells by passive diffusion (Farrell et al. 2012), but the presence of 3-OH or glycosidic groups on flavonoids decrease the absorption of these flavonoids (Fang et al. 2017). It should also be noted that Caco-2 models can exclusively evaluate the absorption in the upper gut (Redan et al. 2017). Therefore, the polyphenols metabolites produced in the large intestine by the gut microbiota could not be studied. The assessment of compounds highly cited in the literature, including urolithins, which can only be produced after colonic microbial action on ellagic acid (Espín et al. 2013), was not feasible in the current study. However, the Caco-2 cell model provides indicative results pertaining to the influence of different commercial milk systems on the bioavailability of blackberry polyphenols.

5.5. Conclusions

Addition of freeze-dried blackberry puree to the different types of commercial milk resulted in the formation of protein aggregates, which incorporated a part of the polyphenols in their structure. The puree had no influence on the digestion rate of the different milk proteins when the samples were *in vitro* digested. The dairy-blackberry blends, especially the sample made with whole milk, had a protective effect on anthocyanins (e.g., cyanidin-3-O-glucoside) during *in vitro* digestion, thus improving their bioaccessibility. This does not relate to a higher permeability of polyphenols and organic acids through the Caco-2 cells and the bioavailability of the polyphenols depended both on the type of phenolic compound or organic acid in the milk product. Milk fat in a dairy-blackberry matrix had a protective effect on polyphenols during *in vitro* digestion but reduced their permeability through the Caco-2 cells leading to a lower bioavailability. The study indicates that the composition of milk may be an important consideration when designing sports nutrition products that combine milk protein and plant polyphenols.

5.6. References

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Chapter 6

The effect of protein and leucine concentration on the in vitro accessibility and apparent permeability of protein hydrolysate and phenolic compounds in blackberry dairy blends

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Declaration

This chapter was written by author TMvdL and reviewed by all co-authors. TMvdL co-designed the study, prepared all samples, determined particle size distribution and prepared SDS-page gels. The *in vitro* digestion and Caco-2 cell assay was performed by YCO. The chromatography work was carried out by KT.

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6.1. Abstract

There is increasing consumer interest in performance nutrition beverages that improve muscle recovery, endurance and strength, and a desired product type would be dairy-blackberry beverages. However, the effect of phenolic compounds on the digestion of milk proteins, and the effect of milk proteins on the bioaccessibility and permeability of phenolic compounds through the intestinal wall is poorly understood. In this study, dairy-blackberry beverages were made by adding milk protein concentrate and/or leucine and freeze-dried blackberry puree to skimmed milk, to formulate beverages with different protein contents and two different leucine contents. These beverages were subjected to in vitro digestion, and their protein breakdown was analysed with electrophoresis. The accessibility of flavonoids, anthocyanins and individual phenolic compounds was determined and the apparent permeability (Papp) of individual phenolic compounds through Caco-2 cells was analysed. It was found that β-lactoglobulin in samples with a higher protein content was more resistant to digestion. An increase in protein had no influence on the bioaccessibility of flavonoids as a whole but increased the bioaccessibility of the subgroup anthocyanins from 13% in water to 64% in high protein milk. The Papp of cyanidin-3-O-glucoside, gallic acid, protocatechuic acid and rutin was determined. Milk proteins improved the Papp of gallic acid and cyanidin-3-O-glucoside, to a maximum value of 4.26 and 0.36×10^{-6} cm/s but had no clear influence on protocatechuic acid and rutin. In conclusion, an increase in milk protein content resulted in higher bioavailability of anthocyanins and their metabolites, which suggests that these higher protein beverages are well suited for performance nutrition.

6.2. Introduction

In recent years the market for performance nutrition has been growing rapidly (Grand View Research, 2022). The study of Carey et al. (2023) found that athletes and active individuals interested in performance nutrition seek and prioritise functional foods that enhance muscle recovery, endurance and strength, preferably in the form of a

beverage. Fruit-dairy beverages could potentially be a product to meet these consumer requirements, due to the milk proteins and polyphenols present in these beverages.

Milk proteins rich in the amino acid leucine are positively linked to enhancing skeletal muscle mass and strength (Gryson et al., 2014). The recommended daily allowance (RDA) of proteins is 0.83 g per kg body weight per day (EFSA Panel on Dietetic Products, 2012). While the RDA may be sufficient for the general healthy population, those undergoing endurance or strength training may benefit from consuming 1.2-1.6 g/kg/day split over 3-4 equal meals (Phillips, 2012). These high levels of protein consumption are also of importance for the elderly population for maintaining their muscle mass (Deutz et al., 2014). The study of Churchward-Venne et al. (2014) showed that part of the protein content could be replaced by the amino acid leucine, while maintaining similar levels of enhanced myofibrillar protein synthesis in young men. This demonstrates the potential for low protein-high leucine beverages, targeting muscle recovery. Milk protein is comprised of 80% casein and 20% whey protein. The whey proteins generally consist of 60% β-lactoglobulin (β-Lg), 20% α-lactalbumin (α-La), 10% blood serum albumin (BSA) and 10% immunoglobulins (Igs) (van de Langerijt et al., 2022). The caseins consist of four different phosphoproteins called α_{s1}-, α_{s2}-, β- and κ-casein (ratio ≈ 4.0:1.0:3.5:1.5). These proteins are mostly present as colloidal complexes called casein micelles, which have diameters of 50-500 nm (Dalgleish, 2011; Huppertz et al., 2017; Swaisgood, 2003). Casein micelles have the ability to stabilise a high concentration of calcium phosphate, in stable suspension, in the form of colloidal calcium phosphate (CCP) nanoclusters. The CCP stabilizes the casein micelle structure by interacting with α_{s1}-, α_{s2}- and β-casein, which also prevents precipitation and sedimentation of calcium phosphate (de Kruif & Holt, 2003; Griffin et al., 1988). After consumption, the acidic conditions of the stomach cause coagulation of casein and formation of a gastric curd. The whey proteins remain in solution and will therefore be emptied from the stomach more quickly than caseins, resulting in a

gradual release of proteins to the intestine, optimizing protein digestion and amino acid absorption (Boirie et al., 1997; Huppertz & Chia, 2021).

Polyphenols are secondary plant metabolites consisting of an aromatic benzenoid ring and hydroxyl group (Belščak-Cvitanović et al., 2018). Blackberries contain high levels of polyphenols, mainly anthocyanins, phenolic acids, flavonols and ellagitannins (Oszmiański, Nowicka, et al., 2015). While an exact polyphenol dose remains to be established, a review in the area by Bowtell & Kelly (2019) and recent supporting data from a comprehensive systematic review and meta-analysis (Carey et al., 2021) indicated that consumption of more than 1 g polyphenols per day for 3 d prior and following exercise is linked to muscle recovery. Berry polyphenols have also been positively linked to a reduction of cardiovascular disease, which can be of importance for the elderly population (Heneghan et al., 2018). In the gastrointestinal tract, polyphenols are prone to oxidation, which lowers their bioavailability, bioaccessibility and functionality (Parada & Aguilera, 2007; Zhang & Tsao, 2016). Polyphenols can interact with milk proteins through hydrophobic interactions and hydrogen bonds, with the strength of bonds depending on the polarity of the polyphenols present (van de Langerijt et al., 2023a). Due to this interaction, milk proteins could partially protect polyphenols from oxidation (Lang et al., 2021) and thereby improve the bioavailability, bioaccessibility and functional properties of the polyphenols. However, only limited data is available on the influence of milk protein concentration on the protection of polyphenols during the digestive process. With the exception of a recent study with different types of commercial milk (van de Langerijt, 2023b), the protective effect of milk proteins on the polyphenols of blackberries during digestion has, to our knowledge, not been studied. It is also unknown how replacing part of the protein with leucine will impact the polyphenols during digestion. Therefore, the aim of this study was to, firstly, investigate the influence of milk protein concentration in different blackberry-dairy beverages on the bioaccessibility of phenolic compounds present in blackberries after *in vitro* digestion. Secondly, to investigate the influence of the

beverage macronutrient composition, targeting a specific leucine and polyphenol content, on the digestion of proteins and the permeability of phenolic compounds through Caco-2 cells was also investigated.

6.3. Materials and methods

6.3.1. Materials

Low-heat skimmed milk powder containing 36.5% (w/w) protein (IDF, 2014), was kindly donated by Arrabawn (Nenagh, Co. Tipperary, Ireland). Milk protein concentrate (MPC) containing 83.5% protein (IDF, 2014), was kindly provided by Glanbia (Kilkenny, Ireland). Leucine powder was obtained from Merck (Darmstadt, Germany). Blackberry puree was purchased from Wild Orchard (Limerick, Ireland) and dried using a freeze dryer (Edwards vacuum, Burgess Hill, UK). The polyphenol content of the freeze-dried blackberry puree (FDBP) was 3.0% (w/w), as determined using the Folin-Ciocalteu method (Singleton & Rossi, 1965). Acetone, sodium hydroxide, potassium chloride and foetal bovine serum were purchased from Thermo Fisher Scientific (Waltham, MA, USA), Pefabloc from Roche (Basal, Switzerland), ethanol from OCON chemicals (Cork, Ireland), rutin from Extrasynthese (Genay, France), acetonitrile from J.T. Baker® (Centre Valley, PA, USA) and methanol from Romil (Lennox Laboratory Supplies LTD, Dublin, Ireland). Formic acid and all other chemicals were obtained from Merck (Wicklow, Ireland). All water used in this research was ultrapure water (PURELAB option-Q, ELGA, Veolia Water, Paris, France).

6.3.2. Preparation of dairy blackberry beverages with different leucine contents

Milk was prepared at 3.5% (w/w) protein from low-heat skimmed milk powder at 22 °C for 30 min under magnetic stirring, which had an innate leucine content of 0.29% in the final beverage. The milk was fortified to create a beverage with: 0.60% leucine (0.31% leucine fortification) or 1.20% leucine (0.94% leucine fortification). This fortification was achieved with MPC, a 2:1 MPC:leucine supplement ratio, a 1:2 MPC:leucine supplement

ratio, or leucine supplement. The protein and leucine supplement content can be found in Table 6.1. The MPC and/or leucine supplement was added to the milk at 22 °C. Afterwards, the suspensions were heated to 50 °C for 4 h, while being stirred magnetically. All suspensions were stored for 18 h at 4 °C under gentle magnetic stirring. The different types of milk were blended with 10% freeze-dried blackberry puree (FDBP) at 22 °C to create the final samples. A pure blackberry sample without milk proteins was prepared by adding 10% FDBP to water. The sample names, leucine content from milk, MPC and leucine supplement, final leucine content and final protein content of the samples are provided in Table 6.1.

Table 6.1: Leucine (Leu) content of the different milk samples, originated from different sources, milk, milk protein concentrate (MPC) or leucine supplement, and final protein content of the samples containing 10% (w/w) freeze-dried blackberry puree.*

Sample (#)	Sample name	Abbreviation	Leu from milk (%)	Leu from MPC (%)	Leu from supplement (%)	Total leucine (%)	Final protein content (%)
1	water	W	N.A.	N.A.	N.A.	0.00	N.A.
2	milk	M	0.30	N.A.	N.A.	0.30	3.15
3	protein milk	PM	0.29	0.31	N.A.	0.60	6.33
4	0.1 leucine milk	0.1LM	0.29	0.21	0.10	0.60	5.22
5	0.2 leucine milk	0.2LM	0.29	0.10	0.21	0.60	4.12
6	leucine milk	LM	0.29	N.A.	0.31	0.60	3.01
7	high protein milk	HPM	0.26	0.94	N.A.	1.20	12.65
8	0.3 high leucine milk	0.3HLM	0.26	0.63	0.31	1.20	9.34
9	0.6 high leucine milk	0.6HLM	0.26	0.31	0.63	1.20	6.04
10	high leucine milk	HLM	0.26	N.A.	0.94	1.20	2.73

*All % in the table represent % (w/w). The abbreviation N.A. stands for not applicable.

6.3.3. Particle size distribution analyses by light scattering

A Mastersizer 3000 (Malvern Instruments Ltd, Malvern, Worcestershire, UK) was used to analyse the particle size distribution (PSD) of the different samples by laser light diffraction. The sample was added to a Hydro SM dispersion unit containing water until a laser obscuration of 10 ± 1% was reached. A refractive index of 1.33 was used for the dispersant and a refractive index of 1.45 was used for the particles. The spherical model was used to determine the PSD. The Sauter mean diameter (D[3,2]), the volume weighted

diameters (D[4,3]) and the span are given to analyse the differences between the PSD of the samples.

6.3.4 In vitro digestion and analysis of digests

6.3.4.1. In vitro digestion

The INFOGEST method of Brodkorb et al. (2019) was used for the in-vitro digestion and some slight adaptations were made to fit the requirements of this study. The simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the INFOGEST method. The gastric digestion was performed by placing 5 g sample in a 30 mL screw cap amber bottle. A volume of 5 mL SGF and 1M HCl was used to adjust the pH to 2. Pepsin was added to reach a final concentration of 2,000 units/mL. Afterwards, the samples were shaken at 100 rpm in a water bath at 37 °C for 1 h. This was followed by the intestinal digestion, involving addition of a volume of 8 mL SIF, and the pH was adjusted with 1M NaOH to approximately 7.4. Purified bile salts (glycodeoxycholate, 0.85 mM; taurocholate, 0.75 mM and taurodeoxycholate, 0.5 mM) and pancreatin (100 units/mL) were added, and the samples were shaken for another 2 h at 37 °C in a water bath. A 4K15 laboratory centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) was used to centrifuge the digested samples at 5300 × g for 20 min. After this, the supernatant was filtered with a 0.45 μm syringe filter (Fisherbrand, Waltham, Massachusetts, USA), with the filtrates stored in a -80 °C until the total flavonoid content (TFC) and total anthocyanin content (TAC) were determined (Section 6.3.4.3) and used to calculate the bioaccessibility of flavonoids and their subgroup anthocyanins. The TFC and TAC were also used to determine the amount of digested sample required for the assessment of the apparent permeability (P_{app}) in the Caco-2 cell assay (Section 6.3.5.).

A volume of 200 μL was removed after gastric and intestinal digestion for monitoring of protein digestion. To stop the enzyme activity during digestion, 20 μL of Pepstatin A (100 μM) was added to stop the gastric digestion and 20 μL of Pefabloc (100

mM) was added to stop the intestinal digestion. Samples were stored at -80 °C before running on gels to determine protein hydrolyses.

6.3.4.2. Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse the protein hydrolyses of the samples during *in vitro* digestion. This was performed under reducing conditions according to the method of Laemmli (1970), which has previously been described in detail by van de Langerijt et al. (2022). The samples before and after gastric digestion were diluted to 0.3% (w/w) protein content and a volume of 5 µL was loaded on the gels. The sample after intestinal digestion was diluted to 0.7% protein content and a volume of 15 µL was loaded on the gel. A desktop scanner (HP Scanjet G4010, HP, Leixlip, Ireland) was used to scan the gels. Samples were loaded in a Mini-PROTEAN TGX pre-cast gels and run in a mini-PROTEAN® Tetra vertical Electrophoresis Cell, both from Bio-Rad Laboratories (Hercules, CA, USA). The gels were Coomassie-stained and scanned with a desktop scanner (HP Scanjet G4010, HP, Leixlip, Ireland). Throughout this article "α_s-casein" refers to the sum of α_{s1}-casein and α_{s2}-casein. Digestions were performed in triplicate and one gel was prepared for each individual digestion.

6.3.4.3. Bioaccessibility of flavonoid and anthocyanin

For the undigested sample, 0.5 g of sample was added to 70% aqueous acetone, to achieve a final volume of 5 mL. For the digested samples, 200 µL of the sample was mixed with 800 µL of 70% aqueous acetone. Both the digested and undigested samples were sonicated for 30 min and centrifuged at 5300 × g for 20 min with a 4K15 laboratory centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The supernatant was used to determine the TFC and TAC.

The method of Jia et al. (1999) was used to determine the total flavonoid content. In brief, a volume of 300 µL of the acetone extracted supernatant of the digested and

undigested sample was mixed with 2 mL water, 100 µL of 5% sodium nitrite and 100 µL of 10% aluminium chloride. After 6 min, 700 µL of 1 M NaOH was added. The sample was shaken, and the absorbance was measured at 510 nm. A standard curve was prepared of catechin in 20% aqueous ethanol with a concentration between 5-200 µg/mL. A sample blank was made with water, and results are expressed as g catechin equivalent in 1 L sample.

The TAC was determined by the pH differential method of Klopotek et al. (2005). A buffer of pH 1.0 was prepared by mixing 125 mL of 0.2 N potassium chloride with 0.2 N HCl, which was added until a pH of 1.0 was reached. The volume was adjusted to 1 L with water. A pH 4.5 buffer was prepared by mixing 400 mL of 1 N sodium acetate with 1 N HCl, which was added until a pH of 4.5 was reached. The volume was adjusted to 1 L with water. The samples were diluted 1:4 with buffer of pH 1.0 and the absorbance was measured at 510 and 700 nm. The sample was also diluted 1:4 with the buffer of pH 4.5 and the absorbance was measured at the same wave lengths. The absorbance measured at 700 nm gave a background, which was subtracted from the absorbance at 510 nm. The difference between the absorbance of pH 1.0 and 4.5 was determined and used to estimate the anthocyanin concentration. A standard curve of cyanidin-3-glucoside (0-50 µg/mL) was made by diluting a stock solution of 10 mg/mL cyanidin-3-glucoside in DMSO, which was diluted in water to reach the concentration of the standard curve. The results are expressed as g cyanidin-3-glucoside equivalent in 1 L sample.

The concentration of flavonoids and their subgroup anthocyanins in this study was expressed as the content available in 1 L of initial sample, both before and after digestion. The bioaccessibility (%) is calculated through the following Equation (6.1):

$$Bioaccessibility = \frac{C_{digest}}{C_{sample}} \times 100\% \quad (6.1)$$

in which C_{digest} (g/L) is the concentration of flavonoids or anthocyanins in the digested

sample, C_{sample} (g/L) is the concentration of flavonoids or anthocyanins present in the sample before digestion.

6.3.5. Cell culture and individual phenolic compound identification and quantification in freeze-dried blackberry puree, digested samples, apical chambers and basolateral chambers

6.3.5.1. Cell culture of Caco-2 cells

A Dulbecco's modified Eagle's medium (DMEM; Sigma D5796), supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) and 1% (v/v) non-essential amino acids was used to grow Caco-2 cells (passage 80-90). The cells were seeded at a density of 6.0×10^4 cells cm^{-2} on polycarbonate transwell inserts (Corning product no. 3414) with a membrane pore size of 3 μm (Corning Inc, Lowell, MA) and incubated at 37 °C and 5% CO_2 for 21 d. The media was changed every 2-3 d, to remove waste build up and replenish nutrients.

The Caco-2 cell model was used to determine the P_{app} of polyphenols. The digests were diluted (1:4) in a Hank's balanced salt solution and filter sterilised (0.45 μm, Merck, Millipore). A volume of 2 mL Hank's balanced salt solution was added to the basolateral chamber (BC) and 1.5 mL of the diluted digest was added to the apical chamber (AC) of the transwell plate, after which the cells were incubated for 4 h. To ensure that the monolayer remained intact throughout the experiment trans-epithelial electrical resistance values were recorded before and after the incubation. After the incubation the AC and BC were harvested and stored at -80 °C until further analysis.

6.3.5.2. Solid phase extraction of polyphenols

The method of Zhang et al. (2018) was used with slight modifications to purify the FDBP, digest, AC and BC using solid phase extraction. C18 cartridges (3 mL, 200 mg) were purchased from Biotage, ISOLUTE® (Uppsala, Sweden). The phenolic compounds of FDBP were extracted with 70% acetone (1:10 w/v) at 60 °C over a period of 60 min.

The cartridges were pre-conditioned with 2 mL of 1% formic acid in methanol and subsequently with 2 mL of 1% aqueous formic acid. The FDBP extract, digested samples and AC samples were diluted 1:4 with 1% aqueous formic acid, vortexed and 1 mL was loaded onto the SPE cartridges. The BC samples were concentrated 1:4 with nitrogen, to maximize the detection of phenolics in quantifiable levels, with 1 mL loaded on the SPE cartridge. The loaded samples were filtered under gravity, after which they were washed with 1 mL of 1% aqueous formic acid. For the FDBP extract and digested samples, this was followed by the elution step using 1 mL of 1% formic acid in methanol. The elution step was performed twice because of the high level of phenolic compounds in the sample, which could be retained in the SPE cartridges. The eluted fractions were dried under nitrogen at 40 °C. The dried samples were reconstituted in 1% aqueous formic acid. The rehydration was performed for three different concentrations: 200 µL (1 to 20 final concentration), 1 mL (initial concentration) and 5 mL (5-fold dilution), after which the samples were subjected to chromatographic-mass spectrometry analysis.

6.3.5.3. Identification of phenolic compounds through HPLC-Q-ToF-MS/MS analysis

To identify the phenolic compounds and metabolites present in the FDBP, in-vitro digested samples, AC and BC samples, an untargeted metabolite analysis was performed on the high-performance liquid chromatography quadrupole-time of flight (HPLC-Q-ToF) mass spectrometry (Waters Corporation, Milford, MA, USA) by adapting the method described by Hossain et al. (2010). An Atlantis T3 C18 column (100 mm x 2.1 mm; 3.0 µm particle size) maintained at 40 °C was used to separate the analytes using a binary mobile phase of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Electrospray ionization mass spectra were recorded in negative ion mode for a mass range (m/z) between 100 and 1500. Argon was used as a collision gas (12-20 eV) to acquire collision-induced dissociation mass spectra.

6.3.5.4. Quantification of phenolic compounds using UPLC-TQD-MS/MS analysis

The most abundant phenolic compounds in the FDBP, *in vitro* digested samples, AC and BC samples were analysed and quantified using an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (Waters Corporation, Milford, MA, USA) system, according to the method of Gangopadhyay et al. (2016) with modifications. An Acquity UPLC HSS T3 column (100 x 2.1 mm; 1.8 µm particle size) at 40 °C and a binary mobile phase of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B) was employed at a flow rate of 0.5 mL/min for 10 min. The gradient elution used was 0–2.5 min at 2% solvent B, 2.5–3 min at 10% solvent B, 3–7.5 min 15% solvent B, 7.5–8.5 min at 35% solvent B, 8.5–9.5 min at 98% solvent B and 9.5–10.0 min at 2% solvent B. To quantify the targeted compounds (Table 6.2), a multiple reaction monitoring (MRM) method was used in which MRM transition ions were generated by Waters IntellistartTM software (Waters Corp., Milford, MA, USA). The UPLC-MS/MS data were recorded in the negative electrospray ionization mode, except for cyanidin-3-O-glucoside for which positive ion mode was used. The quantification of standards was performed by the TargetLynx™ software (Waters Corporation, Milford, MA, USA). The P_{app} (cm/s) could be determined by Equation 6.2 described by Lee et al. (2013), after quantifying the amount of compound present in the BC and AC:

$$P_{app} = \left(\frac{V_A}{Area \times time}\right) \times \left(\frac{C_{basolateral}}{C_{apical}}\right) \quad (6.2)$$

In which V_A is the volume in mL in the AC (2 mL), Area is the surface area of the membrane (2.161 cm²), time is the total transport time in s (4 h equal to 14,400 s), $C_{basolateral}$ is the concentration of compound quantified in the BC and C_{apical} is the concentration of each compound quantified in AC.

Table 6.2: Retention time (t_R), multiple reaction monitoring (MRM) transitions, collision energy and cone voltage for the standards used and the phenolic compounds and organic acids that were quantified in the digested samples, as well as those of the apical and basolateral chambers[1,2].

Reference Compound	t_R (min)	MRM Transition (*m/z*)	Cone Voltage (V)	Collision Energy (eV)
Gallic acid	1.48	169.12 → 124.94	37	14
		78.41		16
Protocatechuic acid	2.56	153.06 → 108.94	29	16
		90.97		24
Cyanidin-3-O-glucoside	3.60	448.61 → 287.20	30	15
		316.60		15
Cyanidin-3-O-rutinoside	3.65	594.92 → 286.80	30	20
		449.20		20
Procyanidin B2	3.75	577.30 → 288.94	55	26
		406.90		28
(-)-Epicatechin	3.96	289.24 → 244.95	47	14
		202.74		16
Lambertianin C	4.20	1401.87 → 300.09	65	35
		632.61		35
Sanguiin H-6 [2]	4.46	633.44 → 300.93	55	27
		275.28		27
Ellagic acid	5.68	301.04 → 283.88	60	28
		185.02		26
Rutin	5.86	609.38 → 300.20	60	34
		271.00		60
Quercetin-3-O-glucoside	6.30	463.19 → 270.95	57	44
		300.27		22
Quercetin	8.11	301.27 → 150.94	47	20
		120.95		32

[1]The first MRM transition (*m/z*) constitutes the quantifier and the second the qualifier. [2]Sanguiin H-6 A was quantified using the transitions of Galloyl-HHPD glucose isomer, which is a dominant fragment of the compound.

6.3.6. Statistical data analysis

All samples were made in triplicate after which they were individually analysed. The data is reported as mean value ± standard error. Differences between the mean values between different samples were statistically analysed with the analysis of variance (ANOVA) and Tukey test. The differences between the mean values within one sample, such as before and after digestion were statistically analysed with an independent t-test. For both statistical analysis the significance level was set to ($p \leq 0.05$). The software used to execute the statistical analyses was SPSS Statistics version 28 (SPSS inc., Chicago, USA).

6.4. Results and Discussion

6.4.1. Influence of blackberry puree on milk proteins

Due to the importance of leucine for muscle recovery, the target was to create a beverage, facilitating daily consumption of 6 g leucine, consumed in two 250 g portions with 1.20% (w/w) leucine or two 500 g portions with 0.60% leucine. Ideally this leucine will originate from beverages with 12.65 or 6.33% milk proteins, but high protein beverages can be challenging in terms of product processing (e.g., heat stability, fouling in heat exchanger, high viscosity, etc.) and consumer acceptability (e.g., dry mouth feeling due to high viscosity) (Singh et al., 2019; Withers et al., 2014). Therefore, multiple beverages in which part of the protein content was replaced by a leucine supplement were formulated (Table 6.1). With respect to total polyphenols, the target was a consumption level of at least 1.5 g per day. FDBP consisted of 3% (w/w) polyphenols, and to reach this target of 1.5 g per day in two portions of 250 mL, the beverage needed to have a FDBP content of 10% (w/w).

Addition of FDBP to water and different milk protein systems resulted in a pH reduction to between pH 3.29 and 4.80 (Table 6.3). Samples with more milk proteins had a smaller reduction in pH, which was due to the buffering capacity of milk fat and proteins (Salaün et al., 2005). The particle size distribution of the different samples was measured and the sample which consisted of FDBP and water (W) had its main peak around 130 μm and a shoulder to the right (Figure 6.1), which most likely originated from the blackberry skins present in the FDBP. The particle size distribution of the different milk samples were similar to each other, also in terms of their D[4,3] and span, and showed multiple peaks between 2 and 1300 μm (Figure 6.1, Table 6.3). The FDBP was partially responsible for the overall shape of the particle size distribution in the milk samples, but the milk protein aggregates formed due to the reduction in pH were also contributed. At a pH close to the isoelectric point (pH 4.6) of the milk proteins the electrostatic repulsion between the proteins decreases, resulting in protein aggregation (Lucey & Singh, 1998).

Table 6.3: Static light scattering results of different samples expressed as D[4,3], D[3,2] and Span *.

Samples	pH	D [4,3]	D [3,2]	Span
			(µm)	
W	3.29	196 ± 20^{a}	58 ± 3^{a,b}	3.20 ± 0.15^{a}
M	4.07	204 ± 18^{a}	45 ± 3^{a}	4.92 ± 0.58^{b}
PM	4.45	247 ± 48^{a}	44 ± 3^{a}	4.62 ± 0.50^{a,b}
0.1LM	4.35	204 ± 15^{a}	45 ± 4^{a}	4.49 ± 0.24^{a,b}
0.2LM	4.20	222 ± 31^{a}	49 ± 6^{a}	5.27 ± 0.92^{b}
LM	4.05	234 ± 29^{a}	59 ± 11^{a,b}	4.70 ± 0.74^{a,b}
HPM	4.80	247 ± 46^{a}	45 ± 6^{a}	4.88 ± 0.69^{b}
0.3HLM	4.56	227 ± 14^{a}	47 ± 2^{a}	4.87 ± 0.24^{b}
0.6HLM	4.19	237 ± 4^{a}	53 ± 4^{a,b}	4.37 ± 0.1^{a,b}
HLM	3.83	242 ± 29^{a}	66 ± 9^{b}	4.01 ± 0.72^{a,b}

*Data represents mean of 3 independent samples ± SD. The subscript letters indicate a significant difference ($p \leq 0.05$), which was determined by an analysis of variance (ANOVA) and Tukey test.

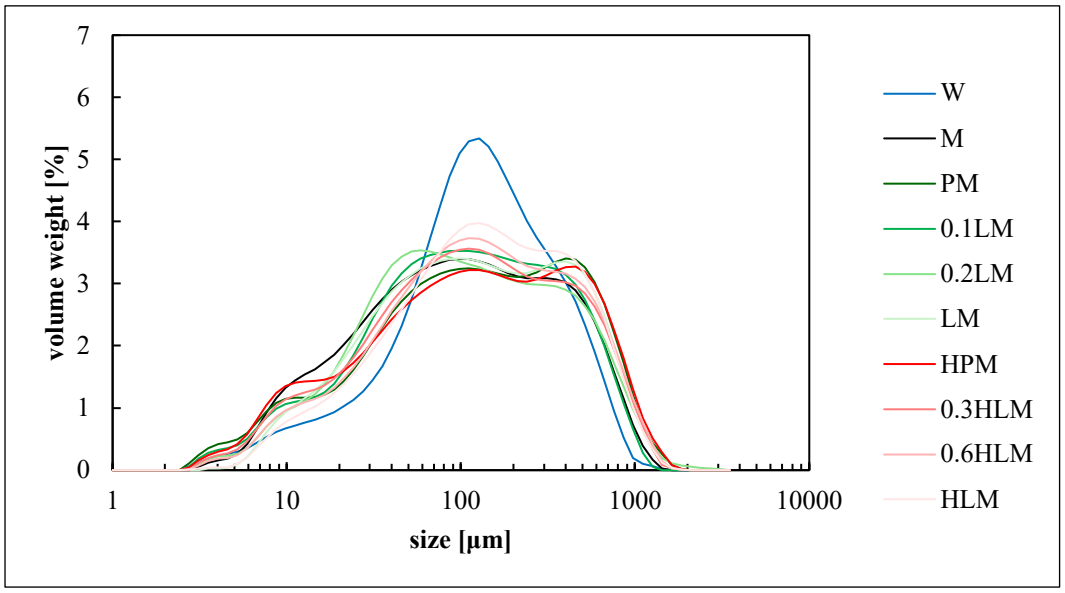
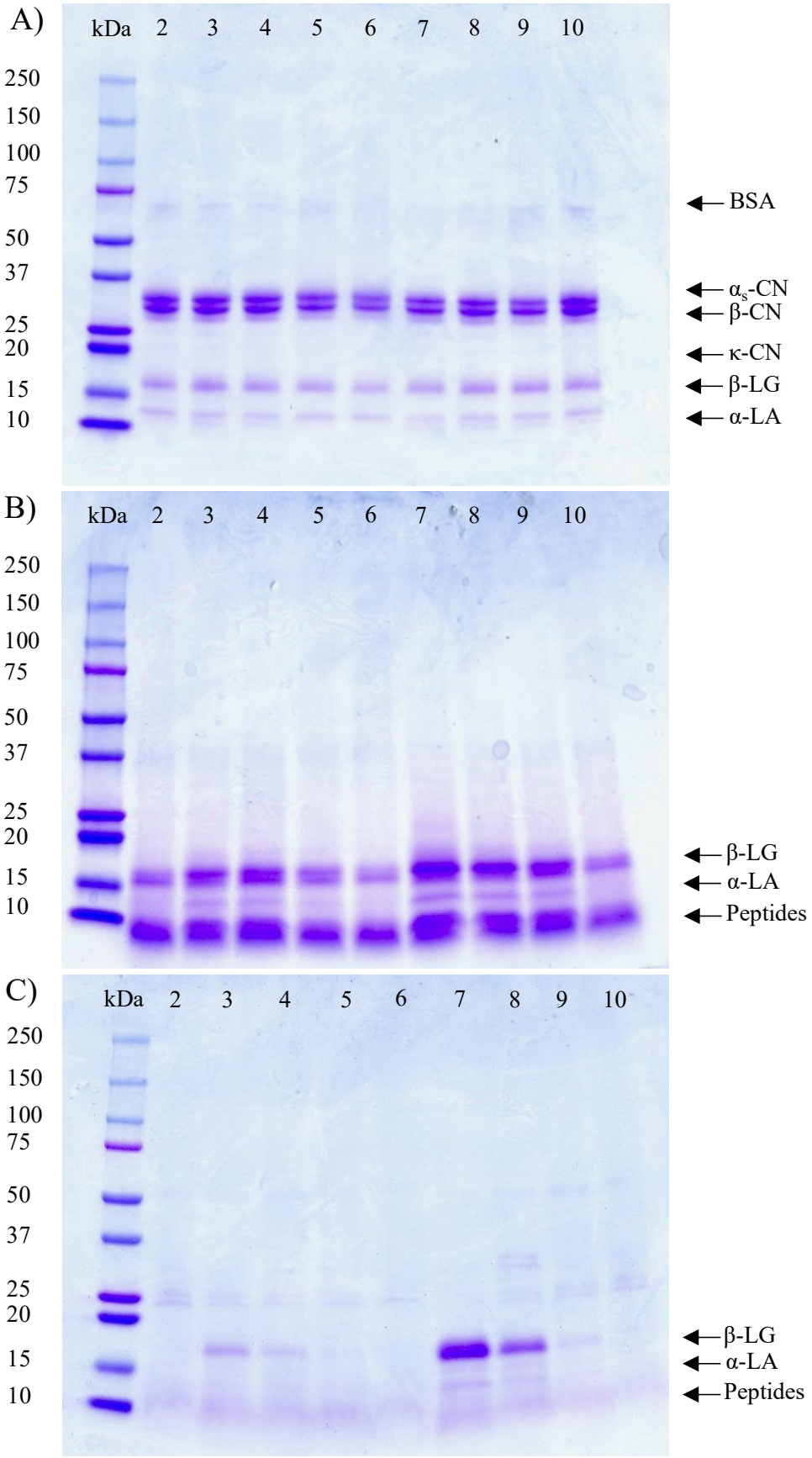


Figure 6.1: The particle size distribution of the different samples. The meaning of abbreviation W, M, PM, 0.1LM, 0.2LM, LM, HPM, 0.3HPM, 0.6HPM and HLM used to describe the samples, which contained a variety of different protein and leucine concentrations originating from milk protein and/or a leucine supplement can be found in Table 6.1. Data represents mean of 3 independent samples.

6.4.2. Influence of milk protein content in the beverage samples on the protein digestion

The SDS-PAGE results show that, even though the protein content in the different samples varied, there was no difference in milk protein composition before digestion (Figure 6.2A). After gastric digestion most of the bovine serum albumin, α_s-casein, β-casein, κ-casein, and α-lactalbumin in the different samples was digested (Figure 6.2B). An exception was β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), for which a higher initial protein content resulted in less digestion of them during the gastric phase. The same effect was found after intestinal digestion, where more β-LG and α-LA was present in the samples with a higher protein content (Figure 6.2C). The study of Wang et al. (2018) found that β-LG originating from skimmed milk was digested more rapidly compared to β-LG from MPC. Samples with a higher original protein content have a higher ratio of β-LG from MPC compared to skimmed milk, which is probably the reason for their slower digestion rate (Wang et al., 2018). It could also be that the heating during MPC production, caused structural and chemical changed to β-LG and α-LA, which reduced their digestibility (Accardo et al., 2022).


A)
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250
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Figure 6.2: SDS-PAGE gels of different samples, in which A) represents the samples before digestion, B) the samples after gastric digestion and C) the samples after intestinal digestion. Lane 2 was loaded with M, lane 3 with PM, lane 4 with 0.1LM, lane 5 with 0.2LM, lane 6 with LM, lane 7 with HPM, lane 8 with 0.3HLM, lane 9 with 0.6HPM, and lane 10 with HLM. The abbreviations of the different samples can be found in Table 6.1. All samples had 10% freeze-dried blackberry puree. The proteins found in the gels were bovine serum albumin (BSA), α_s-casein (α_s-CN), β-casein (β-CN), κ-casein (κ-CN), β-lactoglobulin (β-LG) and α-lactalbumin (α-LA).

6.4.3. Bioaccessibility

6.4.3.1. Bioaccessibility of flavonoids and anthocyanins

The FDBP had a TFC of 0.71 ± 0.02% (w/w) and there was no significant difference ($p>0.05$) in TFC when FDBP was dissolved in water or milk samples, which values ranging from 0.74 ± 0.13 g/L in W to 0.48 ± 0.04 g/L in HPM (Figure 6.3A). After *in vitro* digestion, the TFC decreased (Figure 6.3A) and for W, M, LM and HLM the TFC was significantly lower ($p\leq0.05$) compared to the undigested sample. The total flavonoid bioaccessibility of the different samples ranged from 55.6 ± 5.9% in LM to 78.7 ± 8.8% in HPM (Figure 6.3C). There was a very weak linear correlation ($R^2 = 0.217$) between the protein content in the samples and the bioaccessibility of flavonoids (Figure 6.3D). Even though the TFC decreased upon digestion, the influence of protein concentration was minimal. Similar results were found in previous research, where commercial milk with different protein and fat contents had no influence on the TFC after digestion (van de Langerijt et al., 2023b). Previous work has shown that milk had a negative influence on the bioaccessibility of flavonoids originating from a fruit blend made of orange, kiwi, pineapple and mango (Rodríguez-Roque et al., 2014). This indicates that the influence of milk proteins on the bioaccessibility of flavonoids was dependent on the origin of the flavonoids.

Anthocyanins are a flavonoid subgroup and the total anthocyanin content (TAC) of FDBP was 0.50 ± 0.01% (w/w). After reconstitution there was no significant difference

($p>0.05$) in TAC between the different samples (Figure 6.3B), and the TAC ranged from 0.34 ± 0.04 g/L in M to 0.48 ± 0.04 g/L in HLM. The TAC of all samples, except for HPM, was significantly lower after *in vitro* digestion (Figure 6.3B). The bioaccessibility was highest in HPM ($64.5 \pm 3.4\%$) and lowest ($26.5 \pm 4.8\%$) in LM (Figure 6.3C). There was a strong positive linear correlation ($R^2 = 0.856$) between the protein content and bioaccessibility of anthocyanins in the sample (Figure 6.3D). The results agree with the study of Sánchez-Velázquez et al. (2021), who showed that most anthocyanins present in blackberries are degraded during the first 30 min of *in vitro* intestinal digestion. A linear correlation between the milk protein concentration and bioaccessibility of anthocyanins has, to our knowledge, not been observed before. However, the study of Lang et al. (2021) found that addition of α_s-casein or β-casein to blueberry anthocyanins resulted in a partial protection of the anthocyanins during *in vitro* digestion, which was probably due to encapsulation of polyphenol in the protein structure. Whey proteins are also able to protect anthocyanins during *in vitro* digestion (Wu et al., 2023), and a study of the influence of different types of commercial milk on blackberries also indicated that milk proteins had a protective effect on the anthocyanin content of blackberries during *in vitro* digestion (van de Langerijt et al., 2023b). The reason that a high protein content resulted in a higher bioaccessibility of anthocyanins could be due to greater encapsulation of anthocyanins in the protein structure, affording protection during digestion. Another reason could be the slower digestion rate of β-LG (Figure 6.2), which could offer greater protection to anthocyanins.

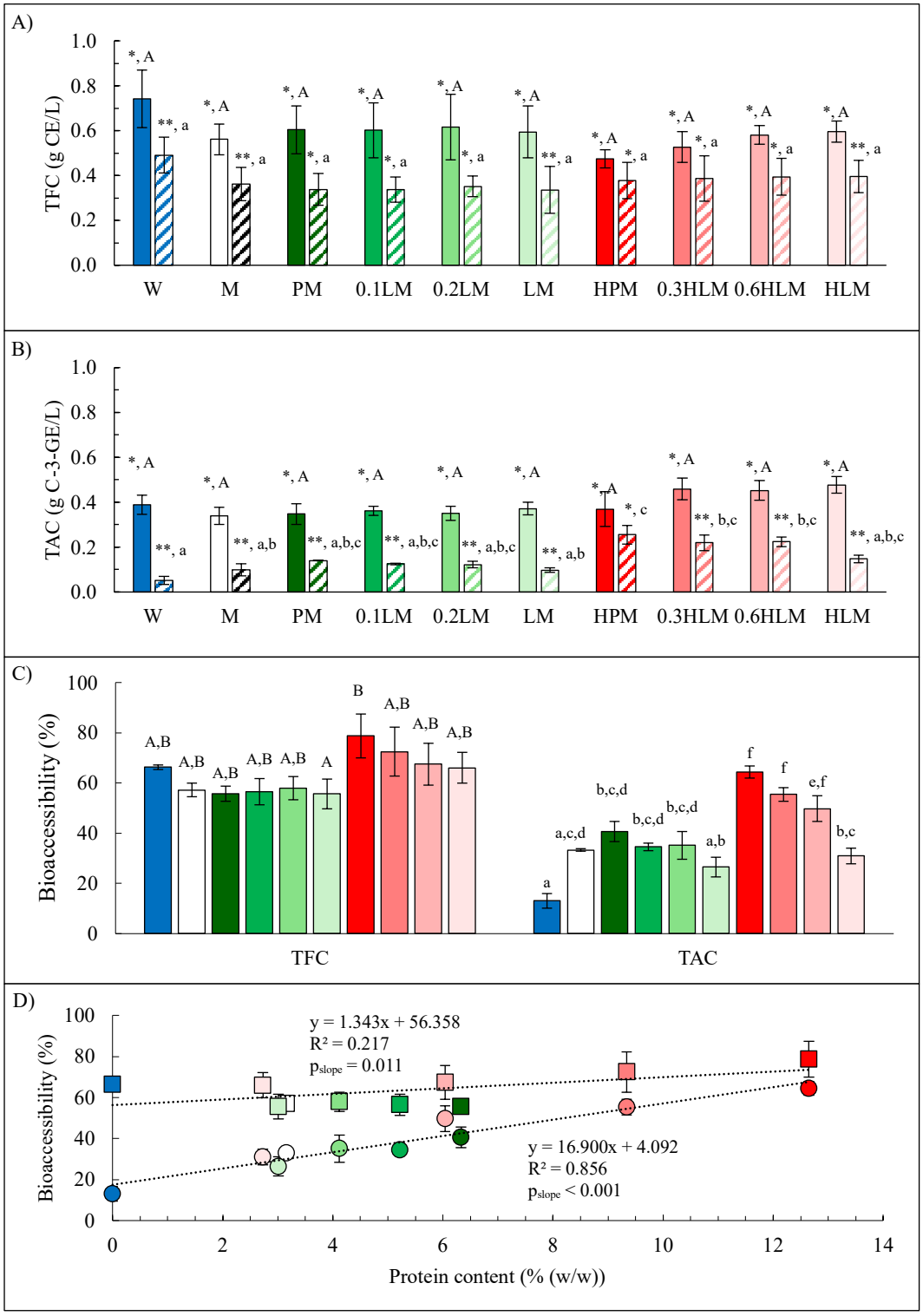

Figure 6.3: The influence of digestion and protein content on the total flavonoid and anthocyanin concentration. A) represents the total flavonoid content (TFC) expressed as catechin equivalent (CE), before digestion (solid bar) and after digestion (striped bar). B) Represents the total anthocyanin content (TAC) expressed as cyanidin-3-glucoside equivalent (C-3-GE), both before digestion (solid bar) and after digestion (striped bar). C) Represents the bioaccessibility of flavonoids and anthocyanins in different samples. D) Represents the influence of protein content on the bioaccessibility of flavonoids (squares) and anthocyanins

(circles). The colours in C and D, relate to the colours of the samples in A) and B). The meaning of abbreviation W, M, PM, 0.1LM, 0.2LM, LM, HPM, 0.3HPM, 0.6HPM and HLM used to describe the samples can be found in Table 6.1. The protein contents of the samples can be found in figure 6.3D. All samples had 10% freeze-dried blackberry puree. Data represent mean of 3 independent samples ± SD. Uppercase-subscript letters in Figure 6.3A and 6.3B indicate a significant difference ($p \leq 0.05$), between the samples before digestion, the lowercase subscript letters indicate a significant difference ($p \leq 0.05$) between the samples after digestion, both determined by analysis of variance (ANOVA) and Tukey test. The asterisks represent the significant difference ($p \leq 0.05$) between the sample before and after digestion, analysed with an independent t-test. In Figure 6.3C the uppercase subscript letters indicate a significant difference ($p \leq 0.05$) in flavonoid bioaccessibility between the different samples and the lowercase subscript letters indicate a significant difference ($p \leq 0.05$) in anthocyanin bioaccessibility between the different samples, both determined by ANOVA and Tukey test.

6.4.3.2. Bioaccessibility of individual phenolic compounds after digestion

The fifteen different polyphenols in FDBP, identified using HPLC-Q-ToF mass spectrometry, can be found in Table 6.4. All compounds were identified by use of commercial standards, except for Lambertianin C, Sanguiin H-6 and Procyanidin trimer B type, for which the identification was based on accurate mass measurements, fragmentation patterns and literature data. The presence of these polyphenols in blackberries has been confirmed by previous research (D'Angelo et al., 2020; Hager et al., 2008; Liao et al., 2020; Oszmiański, Nowicka, et al., 2015; Schulz et al., 2019). Twelve polyphenols of the fifteen present in the FDBP were quantified; the W sample was used as a representative for the concentration of phenolic compounds in all samples (Table 6.5).

Table 6.4: Identified phenolic compounds of freeze-dried blackberry puree using HPLC-Q-ToF-MS/MS. Identification of compounds were based on retention time (t_R), accurate mass measurement, fragmentation pattern, commercial standards where available, and literature.

No.	Name	t_R (min)	Molecular Formula	Observed $[M-H]^-$	Calculated $[M-H]^-$	MS/MS (*m/z*)	Literature
1	Gallic acid	1.52	$C_7H_6O_5$	169.0143	169.0137	111.03; 96.98; 155.03	(Chen et al., 2016; Singh et al., 2016)*
2	Protocatechuic acid	1.78	$C_7H_6O_4$	153.0185	153.0188	109.03; 91.02	(D'Angelo et al., 2020)*
3	5-Chlorogenic acid	2.16	$C_{16}H_{18}O_9$	353.0866	353.0873	191.09; 155.05; 179.07	(D'Angelo et al., 2020)*
4	Lambertianin C	2.21	$[C_{123}H_{80}O_{78}]^{2-}$	1401.1112	1401.1068	301.01; 633.09; 1250.64; 935.11	(Hager et al., 2008)
5	Sanguiin H-6	2.26	$C_{82}H_{54}O_{52}$	1869.1470	1869.1503	935.09; 1235.16; 1567.15; 633.08	(Hager et al., 2008)
6	Cyanidin-3-O-glucoside	2.42	$[C_{21}H_{21}O_{11}]^+$	447.0929	447.0927	285.05; 269.06; 125.03	(Chen et al., 2016)*
7	Cyanidin-3-O-rutinoside	2.58	$[C_{27}H_{31}O_{15}]^+$	593.1597	593.1506	285.06; 284.05; 269.06; 287.07	(de Gomes et al., 2019; Vagiri et al., 2012)*
8	(-)-Epicatechin	2.90	$C_{15}H_{14}O_6$	289.0723	289.0712	245.09; 203.08; 125.04	(Mikulic-Petkovsek et al., 2012; Yuzuak et al., 2018)*
9	Procyanidin B2	3.06	$C_{30}H_{26}O_{12}$	577.1339	577.1346	289.15; 407.18; 125.07; 408.19	(Bystrom et al., 2008; De Pascual-Teresa et al., 1998)*
10	Procyanidin trimer C	6.83	$C_{45}H_{38}O_{18}$	865.1976	865.1980	287.13; 125.06; 407.18; 243.10	(Czyżowska et al., 2020; Rockenbach et al., 2012)
11	Ellagic acid pentoside	7.10	$C_{19}H_{14}O_{12}$	433.0403	433.0407	300.98; 153.03; 78.98	(Oszmiański, Wojdyło, et al., 2015)*
12	Rutin	7.32	$C_{27}H_{30}O_{16}$	609.1430	609.1456	301.05; 271.04; 255.05; 151.02	(Oszmiański, Wojdyło, et al., 2015)*
13	Ellagic acid	7.53	$C_{14}H_6O_8$	300.9998	300.9984	301.01; 117.05; 284.02; 229.03	(Hager et al., 2008)*
14	Quercetin-3-O-glucoside	7.74	$C_{21}H_{20}O_{12}$	463.0867	463.0877	301.03; 271.02; 255.03	(Oszmiański, Wojdyło, et al., 2015)*
15	Quercetin	9.18	$C_{15}H_{10}O_7$	301.0357	301.0348	178.05; 125.05; 137.05; 147.00	(Kolniak-Ostek et al., 2015)*

* Identification confirmed with the use of authentic standards. In which the abbreviations t_R stands for retention time.

Table 6.5: Concentration of phenolic compounds in undigested sample consisting of water and 10% (w/w) FDBP determined by UPLC-TQD-MS/MS.

Phenolic compound	g/100 mL sample
Cyanidin-3-O-glucoside	22.28 ± 1.09
Cyanidin-3-O-rutinoside	0.14 ± 0.01
Ellagic acid	60.81 ± 4.71
Epicatechin	0.18 ± 0.06
Gallic acid	9.72 ± 0.76
Galloyl-HHDP glucose isomer	33.35 ± 1.17
Lambertianin C isomer	141.40 ± 9.02
Procyanidin B2	0.44 ± 0.03
Protocatechuic acid	0.84 ± 0.07
Quercetin	31.37 ± 1.24
Quercetin-3-O-glucoside	0.36 ± 0.04
Rutin	0.21 ± 0.04

*Data represent mean of 3 independent samples ± standard deviation.

The individual polyphenols for which the bioaccessibility could be determined were: cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, ellagic acid, gallic acid, protocatechuic acid, quercetin-3-O-glucoside and rutin (Table 6.6). The bioaccessibility of cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, gallic acid and protocatechuic acid in PM was significantly different ($p \leq 0.05$) compared to LM, which was also the case for HPM compared to HLM (Table 6.6). This demonstrated that, not the leucine content, but the protein content was responsible for the differences in bioaccessibility between the samples. The bioaccessibility of the anthocyanin cyanidin-3-O-glucoside ($R^2 = 0.877$, $p_{slope} < 0.05$) increased with increasing protein concentration (Figure 6.4A). For gallic acid, in all milk samples, except for HLM, there was a significantly higher ($p \leq 0.05$) bioaccessibility than for W (Table 6.6, Figure 6.4C). For rutin there was no significant difference ($p > 0.05$) in bioaccessibility between the samples (Table 6.6, Figure 6.4F) and the anthocyanin cyanidin-3-O-rutinoside could only be quantified in the digested samples with high protein content, like PM and HPM (Table 6.6). The relationship between the individual anthocyanins, namely cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside, and protein was similar to the relationship between the total group of anthocyanins and protein, in

which proteins seemed to have a protective effect on the anthocyanin during *in vitro* digestion (Figure 6.3D, 6.4A and 6.4E, Table 6.6).

For both ellagic acid and protocatechuic acid, the bioaccessibility from W was significantly higher ($p \leq 0.05$) compared to the samples containing milk proteins (Table 6.6). For protocatechuic acid there was also a linear relationship between protein content and bioaccessibility ($R^2 = 0.672$, $p_{slope} < 0.05$) (Figure 6.4D). Ellagic acid is the main hydrolysis product of the Lambertian C and Galloyl-HHDP glucose isomers (Kool et al., 2010; Sójka et al., 2019) and protocatechuic acid is a metabolite of anthocyanins, especially cyanidin-3-O-glucoside (Ozdal et al., 2016; Vitaglione et al., 2007). Milk proteins might partially protect these tannins and anthocyanins from being completely hydrolysed to ellagic acid and protocatechuic acid during their digestion. This may have resulted in the lower concentrations of ellagic acid and protocatechuic acid in the samples with more milk protein, explaining the lower bioaccessibility in these samples.

Table 6.6: Bioaccessibility of phenolic compounds in the digested milk. The abbreviations W, M, PM, LM, HPM and HLM used to describe the different samples together with their protein and leucine content can be found in table 6.1.*

Phenolic compound	Bioaccessibility [%]					
	W	M	PM	LM	HPM	HLM
Cyanidin-3-O-glucoside	0.22 ± 0.01^{a}	1.12 ± 0.05^{b}	3.67 ± 0.17^{c}	1.06 ± 0.05^{b}	4.67 ± 0.22^{d}	0.39 ± 0.02^{a}
Cyanidin-3-O-rutinoside	n.q.	n.q.	11.74 ± 0.67^{a}	n.q.	11.79 ± 0.67^{a}	n.q.
Ellagic acid	9.55 ± 0.70^{c}	5.67 ± 0.42^{b}	3.35 ± 0.25^{a}	$4.37 \pm 0.32^{a,b}$	3.95 ± 0.29^{a}	4.14 ± 0.30^{a}
Gallic acid	1.14 ± 0.08^{a}	2.75 ± 0.20^{b}	4.74 ± 0.35^{c}	2.77 ± 0.20^{b}	2.80 ± 0.20^{b}	1.66 ± 0.12^{a}
Protocatechuic acid	50.45 ± 3.96^{c}	32.41 ± 2.55^{b}	$27.28 \pm 2.14^{a,b}$	33.97 ± 2.67^{b}	21.83 ± 1.72^{a}	34.45 ± 2.71^{b}
Quercetin-3-O-glucoside	16.43 ± 1.73^{a}	18.80 ± 1.99^{a}	21.15 ± 2.23^{a}	20.72 ± 2.19^{a}	21.74 ± 2.30^{a}	16.76 ± 1.77^{a}
Rutin	23.00 ± 3.56^{a}	21.80 ± 3.37^{a}	28.49 ± 4.41^{a}	34.01 ± 5.26^{a}	33.07 ± 5.12^{a}	24.15 ± 3.74^{a}

*Data represents mean of 3 independent samples ± SD. The subscript letters indicate a significant difference ($p \leq 0.05$) between the bioaccessibility of individual phenolic compounds between samples, which was determined by an analysis of variance (ANOVA) and Tukey test. The abbreviation n.q. stands for not quantified.

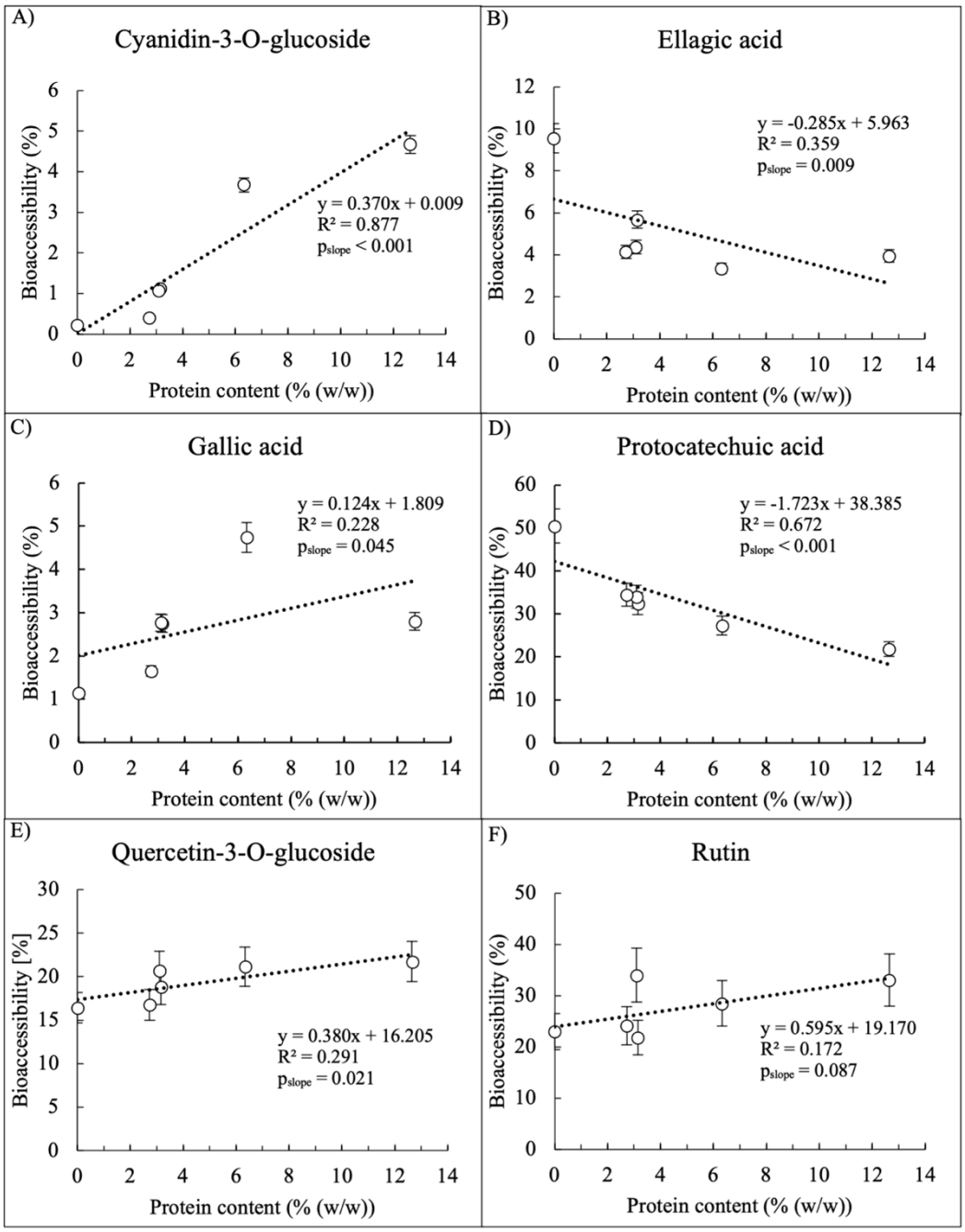

Figure 6.4: The influence of protein content on the bioaccessibility of different phenolic compounds. In which A) represents cyanidin-3-O-glucoside, B) ellagic acid, C) gallic acid, D) protocatechuic acid, E) quercetin-3-O-glucoside and F) rutin. Data represents mean of 3 independent samples ± SD.

6.4.4. Apparent permeability of individual polyphenols through Caco-2 cells

Of the phenolic compounds present in the digested samples, four could be quantified in the BC of the Caco-2 cells, using UPLC-TQD-MS/MS. These compounds were cyanidin-3-O-glucoside, gallic acid, protocatechuic acid and rutin. Previous studies with Caco-2 cell models have also found that they could permeate through the Caco-2-cells (Hithamani et al., 2017; Pollini et al., 2022; Rastogi & Jana, 2016; Zou et al., 2014). The quantification of the phenolic compounds in the BC and AC was used to determine the influence of the sample and protein content of the apparent permeability (P_{app}) of the different phenolic compounds (Figure 6.5).

Current studies suggest that absorption of most polyphenols in the small intestine occurs after polyphenols are broken down to phenolic acids, such as gallic acid and protocatechuic acid (Eran Nagar et al., 2020). Besides our study (van de Langerijt et al., 2023b), there has, to our knowledge, not been another study investigating the influence of milk proteins on the P_{app} of phenolic compounds originating from blackberries. The P_{app} value of gallic acid was highest in PM, but the P_{app} values for W and HLM could not be determined due to levels of gallic acid in the AC of the Caco-2 cells being under the detection limit (Figure 6.5A). An increase of protein content seemed to increase the P_{app} of gallic acid, but this relationship was not linear ($R^2 = 0.195$ and $p_{slope}>0.05$). Gallic acid is transported through the Caco-2 cells by passive diffusion and protein mediated transport (Mao et al., 2016). There was a direct link between the bioaccessibility and P_{app} of gallic acid, so the more gallic acid was available after digestion the more could be transported through the Caco-2 cell layer (Figure 6.4C, Figure 6.5A and 6.5B). According to literature, gallic acid is the phenolic acid that is absorbed most excessively by the large intestine (Eran Nagar et al., 2020), which if the concentration of gallic acid after digestion was over a certain level, as was the case for M, PM, LM and HPM, agrees with the results of this study (Figure 6.5A and 6.5B).

The P_{app} of protocatechuic acid was determined for all samples (Figure 6.5A and C). There was no significant difference ($p>0.05$) in the P_{app} of protocatechuic acid between the different samples and the bioaccessibility seemed to have no strong correlation with the P_{app} (Figure 6.4D, Figure 6.5A and C). Furthermore, the P_{app} of protocatechuic acid was not influenced by the protein content (Figure 6.5C). Similarly, rutin showed no significant difference ($p>0.05$) in P_{app} between the BCs of all samples (Figure 6.5A), and that the protein content had no influence on the P_{app} of rutin (Figure 6.5D). Previous research showed that the absorption of rutin is modest, similar to our present study, and its trans-epithelial transport is predominantly through passive diffusion (Rastogi & Jana, 2016). The P_{app} values of protocatechuic acid and rutin in the different samples were similar to each other, except for W, M and HLM, where rutin was higher (Figure 6.5A). This indicates that rutin, unlike protocatechuic acid, was influenced more by the presence of milk proteins to permeate through the Caco-2-cell layer.

The P_{app} of cyanidin-3-O-glucoside could only be determined for HPM, because the level of cyanidin-3-O-glucoside in the BC of the other samples was too low to be determined (Figure 6.5A). Only a limited number of anthocyanins can be transported from the AC to the BC of Caco-2 cells and the more hydrophilic the anthocyanin the poorer its absorption through the Caco-2 cells (Herrera-Balandrano et al., 2023; Kamiloglu et al., 2015). The study of Zou et al. (2014) demonstrated that glucose-transporters are mainly responsible for the transport of cyanidin-3-O-glucoside from the AC to the BC. However, these transporters easily become saturated, limiting the amount of cyanidin-3-O-glucoside. In conclusion, cyanidin-3-O-glucoside, a relatively hydrophilic anthocyanin, will only be transported through the Caco-2 cells in limited amounts. This could explain why only HMP, which had the highest bioaccessibility, had high levels of cyanidin 3-O-glucoside in the BC.

It should be noted that results obtained by *in vitro* studies can differ from *in vivo* studies. For example, the absorbance of glycosylated polyphenols, such as rutin and

cyanindin-3-O-glucoside, is rarely exhibited in *in vivo* models, which is probably because of enzymatic and bacterial hydrolysis of sugar molecules (Fang et al., 2017; Gómez-Juaristi et al., 2020). Another limitation is that Caco-2 models can only assess absorption in the upper intestine (Redan et al., 2017) and therefore the gut microbiota's polyphenol metabolites produced by the gut microbiota, in the large intestine could not be investigated. However, in terms of ethics, time and cost, an *in vitro* model like the Caco-2 cell model is a useful tool for preliminary investigations into the influence of different milk systems on the absorbance of phenolic compounds from blackberries.

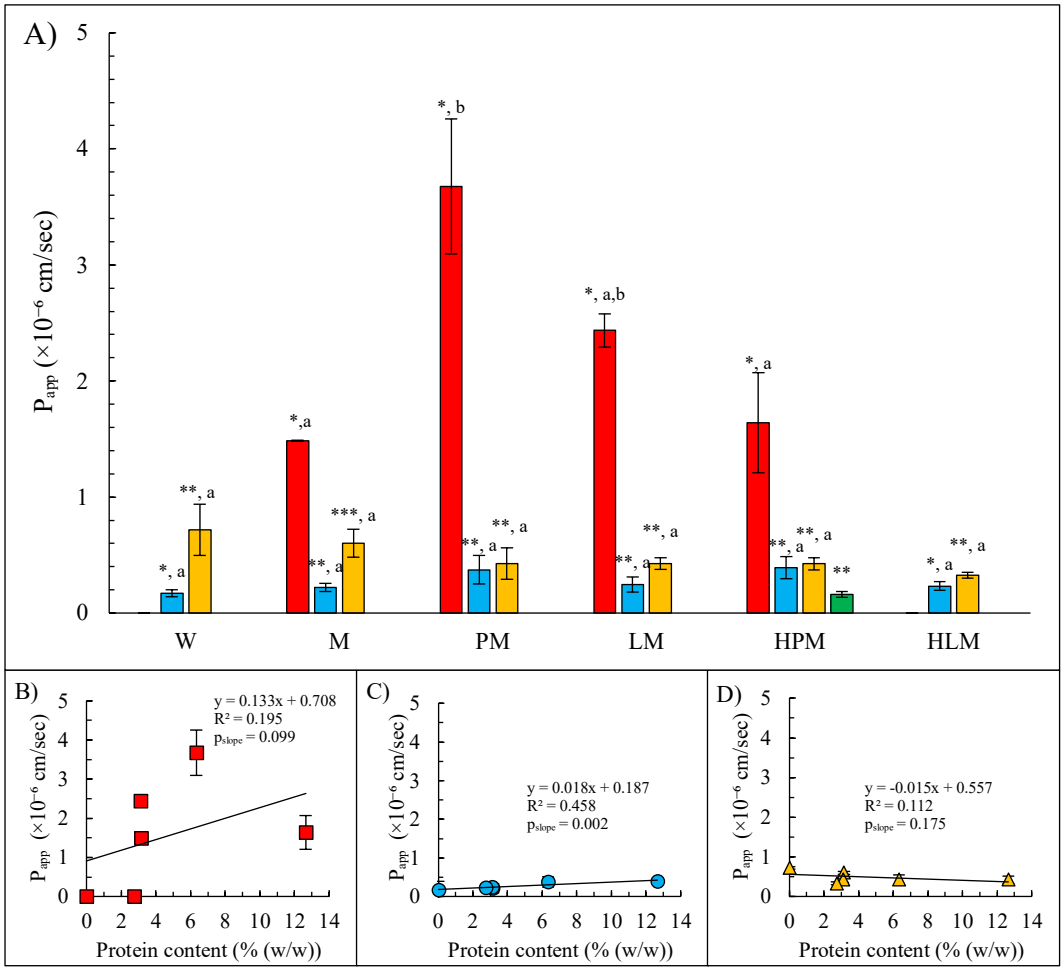

Figure 6.5: The apparent permeability (P_{app}) of gallic acid (red), protocatechuic acid (blue), rutin (yellow) and cyanidin-3-O-glucoside (green). A) Compares the P_{app} values of the different phenolic compounds in the different samples to each other. B) Relationship between the protein content of the P_{app} of gallic acid, C) relationship between the protein content of the P_{app} of protocatechuic acid and D) relationship between the protein content of the P_{app} of rutin. The meaning of abbreviation W, M, PM, 0.1LM, 0.2LM, LM, HPM,

0.3HPM, 0.6HPM and HLM used to describe the samples can be found in Table 6.1. All samples had 10% freeze-dried blackberry puree. Data represent mean of 3 independent samples ± SD. The subscript letters indicate a significant difference ($p \leq 0.05$) between the P_{app} of a phenol between samples, which was determined by an analysis of variance (ANOVA) and Tukey test. The asterisks represent the significant difference ($p \leq 0.05$) between the P_{app} of different phenols in a sample analysed with an independent *t-test*.

6.5. Conclusions

Freeze-dried blackberry puree decreased the pH of the milk systems, which resulted in milk protein aggregation. The digestion of these milk protein aggregates was influenced by the protein content of the milk systems, with increasing protein concentration resulted in decreased proteolysis of β-lactoglobulin. Milk proteins had a limited impact on the bioaccessibility of the total flavonoids. For the total anthocyanins, which are a flavonoids subgroup, milk proteins, and not the leucine concentration, had a positive influence on their bioaccessibility, which was also observed for the individual anthocyanin, cyanidin-3-O-glucoside. The bioaccessibility of the phenolic acids, such as, ellagic acid and protocatechuic acid, decreased with an increasing protein content. These low molecular weight phenolic acids are breakdown products of polyphenols, on which proteins had a protective effect during digestion. This resulted in relatively lower concentrations of these phenolic acids at higher protein concentrations, thereby reducing their bioaccessibility. The apparent permeability of the polyphenols through the Caco-2 cell layer and its relationship to bioaccessibility depended on the individual compounds. For ellagic acid, the apparent permeability was directly related to its bioaccessibility. It could therefore be concluded that milk proteins have a protective effect on polyphenols, which resulted in more polyphenols permeating through the Caco-2 cells. Therefore, a beverage with a high protein content, and not necessarily a high leucine content, would be best when designing performance nutrition beverages from the perspective of high polyphenol bioavailability.

6.6. References

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Chapter 7

General discussion and suggestions for future research

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Declaration

This chapter was written by author TMvdL.

7.1 General discussion

Hybrid dairy-plant beverages, rich in milk proteins and polyphenols are gaining traction in the market for performance nutrition applications (Carey et al., 2023). In the past, research mainly focused on the interactions in complexes of individual milk proteins and polyphenols, and the influence of these complexes on the antioxidant and antitumour activity of the polyphenol as reviewed in van de Langerijt et al. (2023) (**Chapter 1**). However, little is known about the influence of complex formation between a variety of polyphenols and milk proteins, which are more representative of commercial applications, on the techno-functional properties of milk systems and bio-functional properties of polyphenols, in more complex materials. Therefore, the objective of this thesis was to investigate the influence of these complexes on techno-functional properties (**Chapter 1**, **Chapter 2** and **Chapter 3**) and bio-functional properties (**Chapter 4** and **Chapter 5**) of different milk protein-polyphenol systems.

High-protein beverages are often made with a variety of different milk protein ingredients. The effect of mixing different milk protein ingredients on the stability of casein micelles and the influence of polyphenols on this effect is largely unknown. Therefore, **Chapter 2** investigated the influence of sodium caseinate and β-casein concentrate and polyphenols on the turbidity of milk systems containing casein micelles. Sodium caseinate decreased the turbidity over time, likely due to casein micelle dissociation, caused by the calcium chelation effect of sodium caseinate. β-Casein concentrate did the opposite and resulted in increased turbidity over time, likely due to attachment of β-casein micelles to the surface of the native casein micelles. When polyphenols were added to the two different systems, turbidity was more stable over time, indicating that polyphenols increased the strength of the interactions and stabilized the systems.

Most commercial milk and dairy products undergo a heat treatment to reduce microbiological activity and extent shelf life (van den Oever & Mayer, 2021). However, little is known about the influence of polyphenols, originating from polyphenol-rich fruits,

on the heat stability of milk and protein-enriched milk systems, with poor heat stability making heat processing of fruit-dairy beverages often challenging. **Chapter 3** tried to address this knowledge gap by studying the influence of polyphenol mixtures originating from blackberries on the heat stability of a variety of milk protein systems. It was found that blackberry juice, rich in polyphenols, generally increased the heat stability in the pH range between 6.2 and 6.4. The data in **Chapter 3** suggests this is most likely due to the binding of calcium by blackberry polyphenols, which restricted milk protein aggregate formation.

Production of protein-polyphenol beverages can also be challenging due to poor rehydration properties of both milk protein and polyphenol powder (Crowley et al., 2015; Neves et al., 2019). Dissolving them at lower concentrations and co-concentrating them with ultrafiltration could be an alternative approach and its potential was studied in **Chapter 4**. It was shown that ultrafiltration could simultaneously concentrate milk proteins and rutin, especially at chilled temperatures. This is most likely due to complex formation between β-casein depleted micelles and rutin. Which according to the literature review in **Chapter 1** is most likely due to hydrogen bonds between milk proteins and rutin (van de Langerijt et al., 2023).

In **Chapter 5** and **Chapter 6** the influence of milk proteins, milk fat and leucine concentration on the bioavailability and bioaccessibility of blackberry polyphenols in a Caco-2 cell model after *in vitro* digestion was studied. Milk proteins and milk fat have been recorded to influence the bioavailability and bioaccessibility of certain polyphenols, such as, epigallocatechin gallate and the polyphenols in blueberry and jujube juice (Serafini et al., 2009; van de Langerijt et al., 2023; Zhang et al., 2012). **Chapter 5** demonstrated that both milk proteins and milk fat had a protective effect on polyphenols of blackberry puree, especially on the anthocyanins, during *in vitro* digestion; thereby improving their bioaccessibility. However, the bioavailability of polyphenols was negatively influenced by fat in a dairy-blackberry matrix. **Chapter 6** demonstrated that the leucine content of milk

had no clear influence on the bioaccessibility and bioavailability of blackberry polyphenols. The protein content was of more importance, proteins increased the bioaccessibility of certain polyphenols and decreased production of their breakdown products. An increase in protein content also resulted in a higher number of polyphenols permeating through the Caco-2 cell membrane, proving an increase in bioavailability.

In summary, the studies suggest the formation of milk protein-polyphenol complexes improves casein micelle stability and the heat stability between pH 6.2 and 6.4. These complexes can be used to co-enrich milk proteins and polyphenols with ultrafiltration. From a bio-functional perspective beverages with a high protein content, not necessarily a high leucine content, and low-fat content will result in the highest polyphenol bioavailability and will therefore be the recommended formulation from a performance nutrition perspective. It is important to keep in mind, that when developing milk protein-polyphenol beverages, selecting the appropriate protein content is crucial from both a tecno- and bio-functional perspective. A high milk protein content is desirable for the bioaccessibility and bioavailability of polyphenols, but creates challenges in terms of processability. There is a delicate balance between achieving a sufficient protein content to protect polyphenols, while still creating a beverage that can be processed. All in all, the development of milk protein-polyphenol beverages for performance nutrition, even though technically challenging, can be achieved and remains a promising researcher avenue.

7.2 Suggestions for future research

Suggestions for future research that would be complementary to the studies in this thesis include:

Testing a larger variety of polyphenols and polyphenol mixes on the stability of casein micelles in more dilute systems

In **Chapter 2** the influence of tea polyphenols on the stability of casein micelles in different milk protein systems, containing milk protein concentrate with and without sodium caseinate or β-casein concentrate was investigated. It was found that tea polyphenols stabilized protein interactions preventing casein micelle dissociation. This dissociation is observed at low protein concentrations or when calcium chelators like sodium caseinate are added, due to calcium dissociation from the casein micelles (Thomar & Nicolai, 2015). The influence of tea polyphenols on the stability of casein micelles in a system made with milk protein concentrate, was investigated for a concentration of 20 $g \cdot L^{-1}$ protein. It would be interesting to test the influence of tea polyphenols on a variety of lower protein concentrations. Preferably as low as 0.4 $g \cdot L^{-1}$, to find out how significant the influence of tea polyphenols can be, and to compare the results with data obtained in the study of Thomar & Nicolai (2015). Another suggestion would be to add different polyphenols, for instance more hydrophobic polyphenols like curcumin or a polyphenol rich extract like blackberry juice. Curcumin would have more hydrophobic interactions and less hydrogen bonds with the casein micelles compared to tea polyphenols, which might result in differences in the stabilizing effects of polyphenols on casein micelles. A polyphenol-rich extract contains more components than just polyphenols, such as, salts and sugar, which might also influence the casein micelle stability differently.

Influence of carbohydrates, such as, pectin on the heat stability of acid milk protein polyphenol systems

In **Chapter 3** the influence of blackberry juice, rich in polyphenols, on the heat stability of skimmed milk and protein rich milk in the pH range between 6.2 and 7.2 was investigated. Questions remain about the influence of polyphenols on the heat stability of milk systems at a lower pH range. Addition of fruits, such as berries, to milk can reduce its pH significantly, making pasteurization and sterilization challenging due to heat stability problems. Addition of stabilizing carbohydrates, such as pectin, have been positively linked to improving the heat stability of skimmed milk (Lucey et al., 1999). Investigating

how different stabilizing carbohydrates could influence the heat stability of acid fruit-dairy milk systems would further support beverage development technology.

Using different membranes and a mixture of different polyphenols for the co-enrichment of polyphenols and milk proteins with ultra-filtration

The membrane used for the ultra-filtration in **Chapter 4** was a polyethersulfone (PES) membrane, which has been suggested to bind polyphenols (Cassano et al., 2017). The study of Ulbricht et al. (2009) showed that polypropylene membranes bind less polyphenols and might therefore be more suitable for co-concentration of milk protein and rutin.

Chapter 4 solely focused on the polyphenol rutin. It would therefore be interesting to repeat this experiment with a more apolar polyphenol, such as quercetin. More apolar polyphenols will favour to attach to the hydrophobic areas of the milk proteins, potentially creating an even larger difference between the polyphenol concentration in the retentate and permeate. The possibility to concentrate different polyphenols simultaneously by protein-polyphenol complex formation during ultra-filtration would be valuable to investigate, from a beverage development perspective. The proteins will probably favour to interact with one polyphenol over the other, resulting in the concentration of one but the depletion of another in the retentate.

Investigating the potential of β-casein depleted casein micelles for co-enrichment of milk proteins and rutin by microfiltration

A decrease in temperature results in a reduction of hydrophobic interactions, which causes depletion of β-casein from casein micelles below 20 °C (Creamer et al., 1977; France et al., 2021). The study in **Chapter 4** demonstrated that cold filtration at 5 °C had the most potential for co-concentration of milk proteins and rutin. This was most likely due to binding of more rutin by β-casein depleted casein micelles, compared to native casein

micelles. Ultrafiltration which uses a membrane pore size of 5 kDa, retains all proteins in the retentate. Thus, the β-casein released from the casein micelles at 5 °C is preserved in the retentate. Microfiltration with a pore size of 1000 kDa could separate the β-casein and whey proteins in the serum phase of the milk, from the β-casein depleted casein micelles at 5 °C. This might result in a different distribution of polyphenols between the retentate and permeate. It would also facilitate the study of techno-functional and bio-functional properties of β-casein depleted casein micelle-polyphenol complexes.

Use different milk fat ingredients to investigate the influence of milk fat into more detail

In **Chapter 5** the influence of milk fat in commercial milk on the bioaccesbility and bioavailability of blackberry polyphenols was investigated. Serafini et al. (2009) suggested that the binding of polyphenols to the polypeptide chains present in the milk fat globule membrane reduced the bioavailability of polyphenols. Therefore, the bioaccessibility of polyphenols in homogenized and unhomogenized milk might be different. The disruptions in the globule membrane caused by homogenization might result in differences in binding of polyphenols to the polypeptide chains, causing differences in polyphenol bioavailability. It would also be interesting to investigate the influence of milk fat in samples with a larger variety of milk fat concentrations, for instance 0 up to 30 % (w/w) milk fat, the concentration of fat in cream. These concentrations might potentially result in more significant differences in polyphenol bioavailability between the samples. Milk fat is often replaced by vegetable oil emulsions, which are stabilized by emulsifiers, such as lecithin (Huang et al., 2019). Therefore, the influence of vegetable oil emulsions stabilized by different types of emulsifiers on the bioaccessibility, and bioavailability of polyphenols should also be investigated.

Investigate the influence of the gut microbiota on polyphenol digestion

In **Chapter 5** and **Chapter 6** the influence of milk on the absorbance of blackberry polyphenols by Caco-2 cells after *in vitro* digestion was investigated. The Caco-2 cell

model is an *in vitro* model, which was used to give information about the potential absorbance of polyphenols in the small intestine. A restriction of this model is that absorbance is limited to the small intestine and the influence of the gut microbiota on the absorbance of polyphenols in the large intestine cannot be studied. Investigating this in more detail is important because polyphenols can be transformed by the gut microbes to bioactive components, which can be absorbed in the large intestine (Williamson & Clifford, 2017). Recently it has also been shown that polyphenols can influence the gut microbiota itself and can function as a prebiotic, positively influencing beneficial bacterial strains and inhibiting pathogenic species (Rodríguez-Daza et al., 2021). The influence of milk proteins on the absorbance and prebiotic activity of polyphenols has to our knowledge not yet been studied and could be interesting to investigate in future research.

Influence of leucine fortification on rheological properties and sensory properties

In **Chapter 6** leucine was added to samples, to lower overall protein content, while maintaining leucine levels. This was done to limit the viscosity of the samples and prevent a dry mouthfeel. Visually this viscosity reduction was observed but time and machine limitations did not permit viscosity and sensory analysis of the samples. Future research could analyse this to support the visual observations.

Determining total phenol content of samples with different leucine levels after digestion

Time restrictions prevented us from measuring the total phenol content of the digests in **Chapter 6**. Even though the data of **Chapter 5** suggests that milk proteins have no influence on the bioaccessibility of the total group of phenolic compounds, it would be beneficial confirm this for the samples generated in **Chapter 6**.

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