DIGESTION OF MILK PROTEINS IN INFANTS

Influence of processing and implications for intestinal transport and immunoreactivity

Julie Miltenburg

Propositions

1. The food matrix plays a more important role than heat treatment in affecting the digestion of milk proteins.
   (this thesis)

2. The immunoreactivity of non-human milk proteins in infant nutrition is only to be evaluated after heat treatment and digestion.
   (this thesis)

3. The division between fundamental and applied research is completely arbitrary since knowledge gained through fundamental research often leads to practical applications.

4. A PhD defense is an unsuitable moment to discuss scientific and socially relevant statements.

5. While positive discrimination of women promotes reaching gender balance, it hinders reaching gender equality.

6. Strict hierarchical structures, such as those in universities, create risks for social safety.

Propositions belonging to the thesis, entitled

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Thesis

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Table of contents

Chapter 1

General introduction

1.1 Human milk and infant health

Human milk is a complex and fascinating fluid, which contains all nutrients that infants need for healthy growth and development during the first six months of life. Human milk is mainly composed of lipids, proteins, and lactose, but it also contains many minor components, such as vitamins, hormones, growth factors, antibodies, and immune cells [1]. The combination of all these components with different functions makes human milk the best nutrition for infants, but it also makes human milk a very complex fluid. Drinking human milk is important for infants, both for health during infancy and for health later in life. Human milk consumption is for instance associated with a lower risk for necrotizing enterocolitis (NEC), respiratory infections, sepsis, asthma development, and allergy development during infancy [2–4]. Long-term health outcomes include a reduced risk of developing obesity, diabetes type 2, leukemia, and a higher cognitive ability [2,4]. These health benefits are the strongest for infants who have been fed exclusively human milk during the first six months of life, but also infants who have received human milk for a shorter duration benefit from human milk but to a lower extent. Several studies have been performed to link these health outcomes to human milk composition or specific components in human milk [5–7].

The composition of human milk can vary between individuals, ethnicities, time during the day, fore- and hindmilk, and lactation stages [8–10]. Although the abundances of different components in human milk vary, the presence of specific components is largely similar. Human milk contains proteins that can be divided into two types: caseins and whey proteins. Caseins contain a low level of secondary and tertiary structure, and consist of three types in human milk: α_{s1}-casein, β-casein, and κ-casein. They cluster together to form casein micelles in which α_{s1}-casein and β-casein are located in the interior, whereas amphiphilic κ-casein is located more at the outside and stabilizes the micelle [11]. The micelles are further stabilized due to hydrophobic interactions and the binding of calcium phosphate nanoclusters [12]. In contrast, whey proteins are highly structured proteins with many secondary and tertiary structures [13]. The most abundant whey protein in human milk is α-lactalbumin, but human milk contains several other whey proteins including lactoferrin, osteopontin, lysozyme, and secretory immunoglobulin A (sIgA). In particular, lactoferrin and sIgA have been shown to contribute to infant health [14–18]. In total, human milk can contain more than 1500 proteins, although many are present in very low quantities [19]. The lipids in human milk are largely composed of triacylglycerides (TAGs), which consist of three fatty acids that are bound to a glycerol molecule via an ester binding. The fatty acids range from short (C8) to long fatty acids (C22), of which palmitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2) are the most abundant ones [20]. The high levels of palmitic acid at the sn-2 position in TAGs in human milk are thought to play an important role in bone health, calcium absorption and gut microbiome development [21]. Regarding carbohydrates, lactose is the most abundant but not the only one in human milk. Many different human oligosaccharides (HMOs) have been identified, of which many have been linked to infant health [22,23].

The changing human milk composition during lactation, which suits the changing needs of the growing and developing infant, may partly be responsible for the health benefits of breastfed infants compared to infant formula-fed infants [24]. During the first days after birth, the mother produces very concentrated milk, which is called colostrum. Colostrum

contains a high concentration of proteins (20 mg/ml) and HMOs (20 mg/ml), and a low ratio of casein to whey proteins (11:89) compared to later lactation stages [25–27]. The human milk composition changes tremendously during the first month, which is called transition milk. After the first month, the milk composition becomes more stable and is considered mature [28]. During this first month, the protein concentration decreases to 12 mg/ml, the casein:whey ratio increases to 40:60, and the lipid concentration increases from 18 mg/ml in colostrum to 34 mg/ml in mature milk [8,26,27]. The human milk composition still changes after the first month but more gradually than before, and the composition of whey [29] and lipids [30] in human milk have been shown to change considerably during prolonged lactation.

1.2 Infant formula production

1.2.1 Differences between human and bovine milk

Whereas human milk is the best nutrition for the growing infant, infant formula is often given as an alternative. The choice to feed infants infant formula instead of human milk can be made because of different reasons [31]. For instance, the mother does not produce enough milk, the mother uses medicines that the infant is not allowed to receive, or simply because of inconvenience. Since human milk is considered the best nutrition for infants, its composition is attempted to be mimicked as closely as possible during the production of infant formula. However, human milk is a complex mixture of many different components, which differ between individuals and during lactation, which makes this a challenging task.

Infant formula is usually made from bovine milk, whose composition has many similarities but also many differences compared to human milk (Figure 1.1). Like human milk, bovine milk contains different caseins and whey proteins, contains lipids that mainly consist of TAGs, and with lactose being the most abundant carbohydrate. However, bovine milk has higher protein and salt concentrations, and lower lactose, oligosaccharide, and immunoglobulin concentrations than human milk [32]. Moreover, the composition of several components differs between bovine and human milk. Bovine milk has a casein:whey ratio of 80:20, whereas mature human milk has a casein:whey ratio of 40:60. Bovine milk contains α_{s2}-casein and the whey protein β-lactoglobulin, which are lacking in human milk. In addition, bovine milk contains more short and more saturated fatty acids (SFAs) and less long chain polyunsaturated fatty acids (LC-PUFAs) compared to human milk [32]. The composition of the minor components in milk, such as oligosaccharides and immunoglobulins (Igs), also differ between human and bovine milk. More than 200 different oligosaccharides have been identified in human milk, whereas only 40 have been identified in bovine milk [33]. In addition, bovine milk contains mainly IgG, whereas human milk contains mainly IgA [34]. Due to these differences in composition between bovine and human milk, the composition of bovine milk is adapted during infant formula production, and ingredients from other sources are used to supplement bovine milk [35]. Although the human milk composition is attempted to be closely resembled during the production of infant formula, the compositions of human milk and infant formula are not identical.

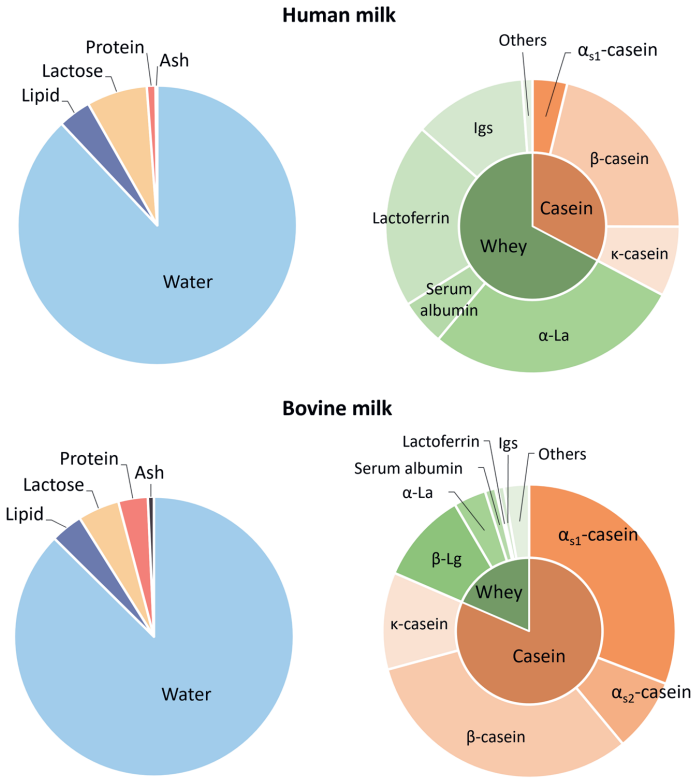

Figure 1.1: Overall composition (left) and protein composition (right) of mature human and bovine milk. Values from Hailu et al. [36] were used. β-Lg: β-lactoglobulin, α-La: α-lactalbumin, Igs: immunoglobulins.

1.2.2 Infant formula production process

Infant formula production is a process that consists of many different steps, and can vary between different countries and manufacturers. Despite these differences, infant formula production generally contains similar steps (Figure 1.2) [35,37]. Usually, infant formula is made from bovine milk, but its composition is altered to more closely mimic the composition of human milk. Therefore, skim bovine milk is mixed with a whey protein concentrate or isolate, plant oils, lactose, and other minor components such as oligosaccharides, vitamins, and salts. Depending on what materials are chosen, they have undergone different heat loads, already changing infant formula composition before the production process has even started. After the raw materials are mixed, the mixture is pasteurized to kill pathogens, followed by homogenization to decrease the size of the lipid droplet and to form a stable emulsion. This emulsion is partly evaporated to lose some of the water before the mixture is spray-dried. Finally, the infant formula is packaged, so it can be transported and sold to the customer.

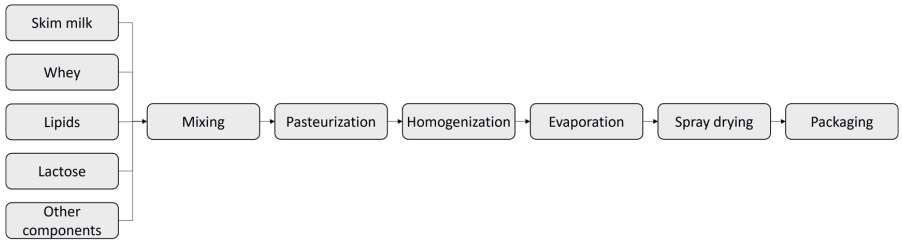

Figure 1.2: Overview of the infant formula production process.

1.2.3 Protein modifications during infant formula production

Whereas the infant formula production process is designed to obtain a product that closely resembles human milk, it also induces changes to some of the components in infant formula. In particular, the heating steps can alter the proteins in infant formula, resulting in various protein modifications including denaturation, aggregation, glycation, cross-linking, oxidation, and isopeptide formation [38,39]. Protein denaturation and glycation, which both can result in aggregation, are the most studied heat-induced protein modifications in milk.

1.2.3.1 Denaturation

Whey protein denaturation occurs during the pasteurization of infant formula. When whey proteins are in their native form, they contain many secondary structures, such as α-helices and β-sheets, and tertiary structures, such as disulfide bridges and hydrophobic interactions. The presence of these structures in whey proteins makes them heat-labile, and these structures are lost upon heating above their denaturation temperature. The denaturation temperature of whey proteins ranges from 62°C for α-La to 78°C for β-Lg at pH 6.0 [40]. After unfolding, whey proteins can interact via thiol-disulfide bond reshuffling or hydrophobic interactions, which was extensively studied for isolated β-Lg [41–43] and β-Lg in the presence of α-La [44,45]. The denaturation of β-Lg plays an important role in protein aggregation via the reshuffling of thiol-disulfide bonds because this protein contains a free cysteine residue hidden in its interior, which is exposed upon denaturation. Moreover, denatured whey protein can bind to k-casein on the outside of the casein micelles via thiol-disulfide bond interactions [38,46,47]. Therefore, whey protein denaturation usually leads to the formation of protein aggregates. In contrast, caseins contain very few secondary and tertiary structures and are therefore heat-stable proteins. Hence, caseins are not susceptible to denaturation induced by pasteurization, although structural changes can occur during more severe processing steps than pasteurization, such as heating at 140°C, heating after lowering the pH or heating after changing the salt composition [48]. Despite the heat stability of the caseins during pasteurization, casein-whey aggregates can be formed during this heating step. The interaction between whey proteins and caseins and between different whey proteins upon heat-induced protein denaturation shows the importance of the matrix in which milk proteins are heated.

1.2.3.2 Glycation

Spray drying as well as storage of dry milk products mainly leads to glycation, which happens via the Maillard Reaction. The Maillard reaction starts with the binding of a reducing sugar via its carbonyl group to a free amino group of a protein. This results in the unstable Schiff base that rearranges into the Amadori product. In infant formula, this reaction usually occurs with lactose, since it is the most abundant reducing sugar in milk. After the formation of the Amadori product, the Maillard reaction can continue and can be divided into an early, advanced, and final stage. Each stage contains specific molecular markers that can be used to determine to which stage the Maillard reaction has proceeded [49]. The early stage is characterized by the reduction in available lysine, which contains a free amino group, and the formation of furosine. Several different products are formed during the advanced stage of the reaction, which are called advanced glycation endproducts (AGEs). These AGEs include molecules such as carboxymethyl lysine (CML), carboxyethyl lysine (CEL), pyrraline, and pentosidine. In the final stage of the Maillard reaction, polymers are formed, resulting in high molecular weight compounds. These compounds are called melanoidins and cause the brown color in the final stage of the Maillard reaction. The Maillard reaction can also induce protein aggregation, which was shown for multiple individual milk proteins as well as for mixtures of different milk proteins [50,51]. The occurrence and proceeding of the Maillard reaction depends on several factors, including the concentration and type of reducing sugar, the number and accessibility of free amino groups in the proteins, the pH value, the water activity and the heating temperature and duration [52,53]. In milk, the reaction happens slowly during storage at room temperature and happens within seconds during heat treatment at high temperatures [49,54,55]. A lower water activity was shown to increase the proceeding of the Maillard reaction in casein-lactose mixtures, with its optimum at a water activity of 0.52 [56]. At the processing conditions of commercial infant formulas, the Maillard reaction results in the formation of different AGEs including CML, CEL and pyrraline [57], which can change the immunoreactive properties of proteins [58].

1.3 Digestion of milk proteins

1.3.1 Native milk proteins

Before the nutrients in milk can be used by infants for growth and development, the milk needs to be digested, and the nutrients need to be taken up through the intestinal barrier. When babies drink milk, they quickly swallow it, after which the milk enters the stomach via the esophagus. In the stomach, the milk is mixed with gastric juice due to gastric contractions. This gastric juice has a pH ranging from 4 to 6 in infants and mainly consists of water, salts, acid, pepsin, and gastric lipase [59]. Gastric mucus is also present in the stomach and protects the stomach wall from the acidic gastric juice. The digestion of milk proteins starts in the stomach due to pepsin activity (Figure 1.3). Pepsin cleaves κ-casein on the outside of the casein micelle, leading to destabilization of the micelle. This destabilization leads to coagulation of casein micelles during gastric digestion, resulting in a clot [60,61]. The formation of this gastric clot ensures a slow gastric emptying of caseins into the duodenum. Therefore, caseins are often called 'slow proteins'. The presence and structure of a gastric clot are important for infants to ensure a gradual release of amino acids

[62]. The absence of a clot can result in too fast gastric emptying, which overloads the duodenum, leading to a lower uptake of amino acids by infants [63]. A hard gastric clot, however, can prevent digestive enzymes from reaching their cleavage sites, resulting in incomplete digestion of the caseins in the clots [60,62]. This may also lead to a reduced amino acid uptake since parts of undigested clots were found in the stool of some infants [64]. Therefore, a soft gastric clot is desired, which ensures a slow and gradual digestion of caseins and maximizes amino acid uptake. In contrast to caseins, whey proteins are largely resistant to pepsin cleavage due to their globular structure, in which some cleavage sites for pepsin are buried in the interior of the proteins [65,66]. This results in a quick gastric emptying of whey proteins in the duodenum, which is why whey proteins are often called 'fast proteins' [67]. Whey proteins and caseins thus complement each other: whey proteins provide a quick supply and caseins provide a slower and more gradual supply of amino acids. Due to muscular contractions, the clot slowly moves to the lower part of the stomach, and empties in the duodenum via the pylorus. Gastric milk protein digestion happens slower in infants compared to adults due to the lower pepsin concentration and higher stomach pH in infants, which both affect the pepsin activity [61]. Therefore, information on gastric clot formation, protein digestion, and gastric emptying obtained in adults cannot directly be translated to infants.

Small intestinal digestion starts in the duodenum, in which the intestinal juice neutralizes the acidic gastric juice, resulting in a suitable environment for the intestinal digestive enzymes. In addition, bile and pancreatin are released in the duodenum. Bile is important for lipid digestion since it facilitates the disruption of large lipid droplets into smaller ones, thereby enabling easier digestion by intestinal lipase. Moreover, protein hydrolysis happens faster in the presence of bile [68]. Pancreatin consists of multiple digestive enzymes, including amylase, lipases, and proteases such as trypsin and chymotrypsin. After milk proteins are emptied in the duodenum, trypsin and chymotrypsin further hydrolyze intact proteins and peptides into smaller peptides or free amino acids. Whey proteins are quickly digested in the small intestine, resulting in a supply of amino acids. The residual of the casein clot, which is gradually emptied in the small intestine, quickly disappears due to the proteolytic digestive enzymes in the small intestine [69]. The formed small peptides can be digested further by brush-border enzymes that are secreted by epithelial cells in the intestinal barrier [70]. Thereafter, the peptides and free amino acids can be transported through the intestinal barrier, which is further described in section 1.4. Due to peristaltic contractions, unabsorbed milk components enter the large intestine. The largest part of the large intestine is the colon, in which the last water and salts are absorbed, and unabsorbed milk components are fermented by the intestinal microbiota [71]. Finally, the unabsorbed material, which includes unabsorbed milk peptides, is excreted via the rectum [72].

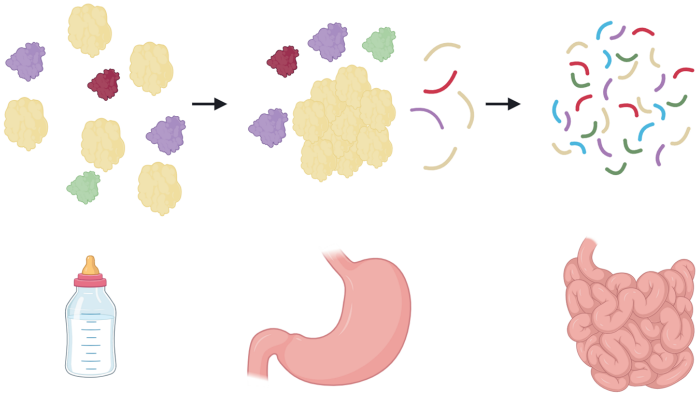

Figure 1.3: Digestion of milk proteins in the stomach and the small intestine. Figure was created with BioRender.

1.3.2 Denatured milk proteins

The digestion of milk proteins is affected by whey protein denaturation, which happens during heat treatments such as pasteurization or sterilization. Denatured whey proteins are unfolded, which improves the accessibility of cleavage sites for digestive enzymes. Therefore, whey proteins are hydrolyzed by pepsin during gastric digestion when they are denatured in contrast to native whey proteins [73]. Furthermore, the composition and structure of the gastric clot changes in the presence of denatured whey proteins. Whey proteins can interact with κ-casein on the outside of casein micelles during heat treatment. This results in the formation of a gastric clot that consists of both caseins and whey proteins when whey proteins are denatured instead of a gastric clot that consists only of caseins [74,75]. In case only caseins are present in the gastric clot, caseins tightly coagulate due to hydrophobic interactions. Denatured whey proteins prevent the casein micelles from tightly coagulating, leading to a softer and opener structure, which can also affect the gastric emptying rate [60]. Due to the higher gastric digestion of denatured whey proteins, they are already more digested at the start of intestinal digestion, which can change the intestinal digestion kinetics. The overall intestinal digestion of denatured milk proteins was reported to be either lower or higher compared to native milk proteins and seemed to depend on the exact heating conditions and the matrix in which milk proteins were heated [73]. An increased protein digestion was found for isolated β-Lg after heat treatment, probably due to unfolding which increases the accessibility of cleavage sites for pepsin and pancreatic enzymes [76]. In contrast, a decreased protein digestion upon heating was found different whey proteins heated in a mixture, which may be caused by the formation of aggregates [77]. The intestinal digestion of caseins can also be affected by the presence of denatured whey proteins, although an increased as well as a decreased digestion of caseins has been described upon heat treatment [78,79]. Denatured whey proteins can bind to α_{s2}-casein and

κ-casein, which either may result in a higher casein digestion due to an easier breakdown of the residual gastric clot, or in a lower casein digestion due to blocking of cleavage sites in caseins by the bound whey proteins. Hence, the influence of denatured whey proteins on intestinal casein digestion is not completely clear.

1.3.3 Glycated milk proteins

Glycation of milk proteins generally results in a reduced digestibility for both caseins and whey proteins. The reduced digestibility of glycated milk proteins may be caused by: (1) glycation at a cleavage site for digestive enzymes, which blocks the cleavage site, (2) glycation close to a cleavage site, resulting in either blocking or decreasing the recognizability of the cleavage site for digestive enzymes, (3) cross-linking of glycated milk proteins, leading to either blocking or reduced accessibility of cleavage sites [73]. Whereas most studies report reduced digestion of milk proteins due to glycation [80–84], a few studies found increased digestion after glycation [85,86]. Many of these studies that investigated the effect of glycation on the digestion of milk proteins used heating conditions that result in glycation but may also result in other protein modifications, such as denaturation and aggregation. Therefore, separating the effect of glycation and other protein modifications is difficult in several of these studies. In addition, many studies do not report till what stage of the Maillard reaction the glycation has proceeded. The few studies that were performed regarding the effect of glycation on milk protein digestion without inducing denaturation found that glycation reduced milk protein digestion [80,82]. At low glycation levels and in the early stage of the Maillard reaction, a reduced digestibility was found only during intestinal digestion, whereas at higher glycation levels, which reached the advanced stage of the Maillard reaction, a reduced milk protein digestibility was found during both gastric and intestinal digestion [80].

1.3.4 Matrix effects

The matrix in which milk proteins are processed and digested plays an important role in their digestion behavior. For instance, denatured whey proteins can interact with caseins upon heating, which affects the gastric clot structure, and the presence of a reducing sugar during heat treatment can result in glycation, which reduces protein digestion, as described in sections 1.3.2 and 1.3.3. However, also other components in milk can influence the digestion of milk proteins. In particular, lipids are known to affect milk protein digestion. A slower milk protein digestion was observed in whole milk compared to skim milk when the milk was unheated, pasteurized, or ultra-high temperature (UHT) treated [87]. In addition, homogenization was reported to affect the digestion of milk proteins due to the higher surface area of lipids upon homogenization, which increases the interaction between lipids and proteins [87,88]. During gastric digestion, this interaction was found to result in a gastric clot with a more open structure in which proteins are more easily accessible for pepsin, increasing gastric milk protein digestion [88]. In contrast, homogenization was reported to decrease milk protein digestion during intestinal digestion, which was probably caused by the increased interaction between lipids and peptides after the increase in surface area of the lipid droplets upon homogenization [87].

1.4 Intestinal transport and immune response

1.4.1 Intestinal barrier

Before nutrients can be used by the body, they need to be taken up through the intestinal barrier. The intestinal barrier is shaped in villi and microvilli, which increases its surface area and enhances the possibility of nutrient absorption. However, not all substances that enter the small intestine should be taken up. Therefore, the intestinal barrier's task is to transport nutrients across the barrier and to protect the body from harmful substances by preventing their transport [89]. The first protective layer of the intestinal barrier is provided by Goblet cells, which produce a mucus layer. Mucus is composed of water, lipids, salts, and proteins, of which mucin glycoproteins are the most abundant ones. This gel-like layer on top of the intestinal barrier cells prevents pathogens from reaching the intestinal cells [90]. After the nutrients have passed the mucus layer, they reach the cells of the intestinal barrier. Different types of cells are present in the barrier, which all have a specific function, and together they form a monolayer that separates the lumen from the *lamina propria* [89]. Most of these cells are absorptive enterocytes, which produce brush border enzymes that further digest nutrients before they can cross the intestinal barrier. Next to enterocytes, the intestinal barrier also contains other cells, including mucus-producing goblet cells, microfold cells (M-cells), antimicrobial peptide-producing Paneth cells, and hormone-producing enteroendocrine cells. M-cells have a lower activity of brush border enzymes and shorter microvilli compared to enterocytes, and they are located close to Peyer's patches in the *lamina propria* [91]. These Peyer's patches are enriched in antigen-presenting dendritic cells and macrophages, but also in T-cells and B-cells, enabling the immune system to quickly respond. Due to this close contact with immune cells, M-cells are thought to play an important role in transporting antigens through the intestinal barrier to enable immune cells to adequately respond to these antigens [91]. In addition, dendritic cells can sample through the intestinal barrier via their membrane extensions, which allows them to recognize antigens directly after gastric emptying. Therefore, dendritic cells are considered to play an important role in the development of either a tolerogenic or an inflammatory response towards food antigens [92].

1.4.2 Routes of uptake through the intestinal barrier

Peptides can cross the intestinal barrier in different manners: paracellularly (crossing in between the intestinal cells) and transcellularly (crossing through the intestinal cells) [93], and is shown in Figure 1.4. Paracellular transport is determined by the permeability of the intestinal barrier, which is controlled by tight junctions located between the intestinal cells [94]. Transcellular transport can happen in multiple ways. Peptides can be inactively transported through enterocytes via passive diffusion or they can be actively transported through enterocytes via carrier-mediated transport or via endocytosis. During endocytosis, peptides are further broken down in endosomes by enzymes, and thereby the peptide composition changes during this type of transport. In addition, peptides can cross the intestinal barrier via follicle-associated epithelium (FAE) transport routes through M-cells. Since M-cells have a lower enzymatic brush border activity than enterocytes, peptides are digested to a lesser extent in this pathway compared to transport via enterocytes [91].

After transport through M-cells, peptides reach a Peyer's patch in which different types of immune cells can immediately respond to the transported peptides. These different manners of peptide transport through the intestinal barrier thus influence to what extent the peptides are further broken down, and how they are eventually presented to immune cells. In infants, the intestinal barrier is generally considered to be more permeable than in adults, enabling larger components to cross the barrier [93].

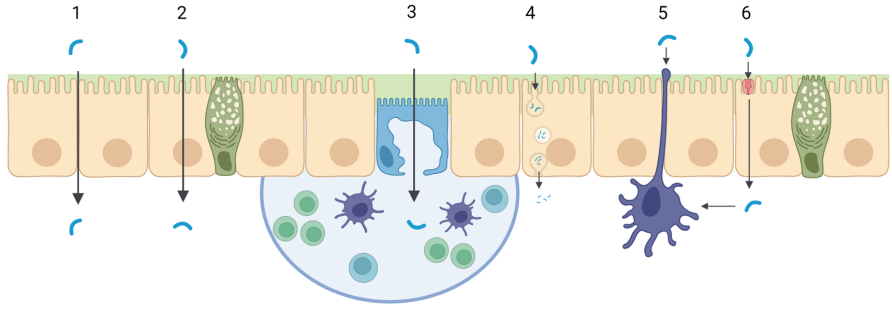

Figure 1.4: Overview of the intestinal barrier and intestinal transport routes. (1) paracellular transport, (2) passive diffusion, (3) transport through microfold (M) cells, (4) transport via endocytosis, (5) sampling by dendritic cells through the intestinal barrier, (6) carrier-mediated transport. Figure was created with BioRender.

1.4.3 Intestinal immune response

The immune response usually starts with antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, that sample their surroundings and present what they have taken up on their major histocompatibility complex class II (MHC-II) molecules. They recognize the taken-up component either as unharmful and stimulate a tolerogenic response, or they recognize the component as harmful and stimulate a pro-inflammatory response [95]. The presented compounds on MHC-II molecules of APCs can be recognized by naïve T-cells via its T-cell receptor (TCR). This MHC-II-TCR interaction together with the interaction of co-stimulation molecules on the surface of APCs and T-cells and cytokine release by the APCs can activate and differentiate naïve T-cells into different types of T-cells, including the T-helper (Th) cells Th1, Th2 and Th17, and regulatory T-cells (Treg) [96]. Differentiation into Th1 and Treg subtypes leads to a tolerogenic immune response, whereas differentiation into Th2 and Th17 subtypes results in a pro-inflammatory response [58]. These Th2 cells play an important role in the development of food allergies. They present antigens to naïve B-cells, resulting in the activation of B-cells, which change their Ig class from IgM to IgE. The released IgE by the activated B-cells binds to basophils and mast cells via the FcεR1 receptor, which sensitizes these cells [97]. Upon re-exposure to the same component, allergen-specific IgE molecules on the surface of the sensitized basophils and mast cells crosslink by binding to the allergen. The activated basophils and mast cells degranulate and release pro-inflammatory mediators, which results in an allergic response. In addition, food allergies can also develop via a non-IgE mechanism, but this mechanism is poorly understood [98]. The balance between a tolerogenic and pro-inflammatory response is important for the

immune system to adequately function. The immune system of infants is generally more balanced towards Th2 activity compared to the one of adults, making infants more prone to develop allergies [99]. Therefore, it is especially important to study the immunoreactivity of milk proteins, which is the only source of protein that infants receive during the first six months of life, in combination with processing conditions applied to infant formula and infant digestion conditions.

1.4.4 Effect of processing on intestinal transport and immune response to milk proteins

Processing of milk proteins can affect their digestion, intestinal transport and the type of immune response they induce. During the production of infant formula, different milk protein modifications arise, including denaturation, glycation, and aggregation, as described in section 1.2.3. These modifications can result in hiding or destroying epitopes, but also in forming new epitopes or in exposing epitopes that are usually hidden in the interior of proteins, resulting in a changed immunoreactivity of milk proteins. Heat-induced denaturation was shown to result in a reduced allergenicity of milk proteins, probably because of the increased digestion of milk proteins during digestion which disrupts epitopes [100], the loss of conformational epitopes upon unfolding, or the shielding of epitopes in denaturation-induced aggregates [101]. In contrast, denaturation was also shown to result in an increased allergenicity of milk proteins, which was probably caused by the exposure of hidden epitopes after unfolding [101] or the increased uptake of denaturation-induced aggregates by Peyer's patches [102]. Increases in hydrophobicity and fibril structure upon heating of β-Lg were also linked to an enhanced uptake by DCs and macrophages [103,104]. Moreover, glycation of milk proteins has been reported to influence the immunoreactivity of milk proteins. Especially AGEs, which are formed during the advanced stage of the Maillard reaction, are thought to play an important role in increasing the immunoreactivity of milk proteins upon glycation. Milk proteins with AGEs resulted in an increased binding to several receptors on DCs and macrophages, such as RAGE, scavenger receptors, and galectin-3, which are involved in the uptake of antigens, and maturation and differentiation of DCs and macrophages [58,105,106]. However, glycation was also shown to decrease the IgE and IgG binding capacity of milk proteins, probably due to masking of epitopes [107–109]. Moreover, the digestion and intestinal transport of milk proteins can also affect the induced immune response. Since smaller peptides are less likely to contain epitope structures, a higher extent of protein digestion generally decreases the immunoreactivity of milk proteins. The digestion of milk proteins is affected by different factors, including heating steps during milk processing and the digestion conditions. Many studies only investigated the effect of heat treatment on the immunoreactivity of milk proteins without considering the effect of digestion or intestinal transport. A couple of studies included both heat treatment and digestion to evaluate the immunoreactivity of milk proteins [100,102,107–111]. These studies showed that both denaturation and glycation change the immunoreactivity of milk proteins after digestion, but increases as well as decreases in immunoreactivity were reported upon denaturation and glycation. Moreover, the differences in the digestion of milk proteins due to heat treatment can result in subsequent changes in intestinal uptake of milk proteins. Dry heating was shown to lower the intestinal transport of milk peptides

and to decrease the intestinal transport of immunoreactive structures [110], whereas wet heating was shown to decrease the intestinal transport of β-Lg [112]. Whether these differences in intestinal transport also result in a changed intestinal immune response is, however, not clear. To summarize, processing has been shown to induce changes in digestion, intestinal transport, and immunoreactivity of milk proteins. However, unraveling a link between milk protein processing and subsequent changes in digestion, intestinal transport and immune response remains difficult since this depends on several factors, including the used milk proteins, heating conditions, digestion conditions, and immunological readouts.

1.5 Research aim and thesis outline

Milk protein processing as well as digestion conditions are known to influence the digestion of milk proteins, which can impact their intestinal uptake and immune response. Digestion and intestinal uptake of milk proteins are important for infants to provide them with the right nutrients for growth and development. In addition, a healthy immune response towards milk proteins is important both during infancy and later in life. However, the relationship between processing, and subsequent changes in digestion, intestinal uptake, and immunoreactivity of milk proteins is not well understood. **Therefore, this thesis aims to investigate how processing as well as the changing digestion conditions of the developing infant impact the digestion of milk proteins and how this affects subsequent intestinal transport and immunoreactivity of the digested milk proteins.**

The digestive system is immature upon birth and quickly develops towards more adult conditions during the first months of life. However, how the changes in digestion conditions with infant age affect milk protein digestion has not been investigated. In **Chapter 2**, unheated and wet heated skim milk was digested by use of *in vitro* gastric digestion models representing 1, 3, and 6-month-old infants. The effect of infant age and heat treatment on the gastric clot and milk protein digestion was determined.

Wet heating and dry heating during the production of infant formula are known to result in milk protein denaturation and glycation, which can affect the digestion of milk proteins. **Chapter 3** provides insights into this by wet and dry heating milk proteins at different conditions, followed by digesting the heated milk proteins under infant digestion conditions. Changes in physico-chemical properties of milk proteins after heat treatment were measured as well as changes in hydrolysis of milk proteins and formation of peptides during gastric and intestinal digestion. Moreover, differences in the effect of these intestinal peptides after wet and dry heating on intestinal mucus production, intestinal transport, and immunoreactivity were studied in **Chapter 4**. To this end, the intestinal digests were applied to Caco-2 cells and Caco-2/HT29-MTX-E12 cocultures to determine the effect of wet and dry heating on the intestinal transport of milk peptides in the presence and absence of a mucus layer. Lastly, the immunoreactivity of the peptides was investigated by identifying immunoreactive structures and by determining changes in dendritic cell response upon stimulation with the transported milk peptides.

Whereas infant formula is usually made from bovine milk, the milk lipids are replaced by plant lipids to better resemble the lipid composition of human milk. However, little is known about how milk protein digestion is affected by the presence and type of lipids. Hence,

Chapter 5 aimed to investigate how milk lipids and plant lipids influence the gastric and intestinal digestion of milk proteins. In addition, the effect of different types and concentrations of free fatty acids on the digestion of milk proteins was studied to elucidate a link between lipid composition and milk protein digestion.

Finally, the findings in **Chapters 2 to 5** are integrated and discussed in **Chapter 6**. This chapter ends with an overall conclusion and provides prospects for future research.

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Chapter 2

Gastric clot formation and digestion of milk proteins in static *in vitro* infant gastric digestion models representing different ages

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Abstract

Gastric digestion conditions change during infancy from newborn towards more adult digestion conditions, which can change gastric digestion kinetics. However, how these changes in gastric digestion conditions during infancy affect milk protein digestion has not been investigated. Therefore, we aimed to investigate milk protein digestion with static *in vitro* gastric digestion models representing one-, three- and six-month-old infants. With increasing age, gastric clots and soluble proteins were digested more extensively, which may partly be attributed to the looser gastric clot structure. Larger differences with increasing age were found for heated than unheated milk proteins, which might be caused by the presence of denatured whey proteins. Taken together, these findings show that gastric milk protein digestion increases during infancy. These *in vitro* gastric digestion models could be used to study how milk protein digestion changes with infant age, which may aid in developing infant formulas for different age stages.

2.1 Introduction

Mother's milk is considered the best nutrition for infants, and drinking mother's milk is associated with several health benefits, including a reduced occurrence of respiratory and gastrointestinal infectious diseases [1]. These mother's milk-related health benefits may partially be explained by the differences in composition of mother's milk during lactation, which were shown to coincide with the development of the digestive and immune system of infants [2]. In contrast, no different types of infant formula are available that are designed for different ages of infants within the first six months of life.

The digestive system changes tremendously during infancy [3], which may affect the digestion of infant nutrition. After birth, activities of digestive enzymes, such as pepsin and trypsin, are low, and the pH in the stomach is relatively high compared to adults. In full-term newborns, the pH in the stomach ranges from 3.5 before to 6.5 after drinking mother's milk, and it takes more than 3h before the preprandial pH values are reached again [4,5], whereas in adults, the pH in the stomach ranges from 1.5 before to 6.5 after drinking casein or whey protein solutions, and it takes only 1h before the pH is below 3.5 again [5,6]. During the first months of life, the pH in the stomach slowly decreases from newborn towards more adult values and digestive enzyme activities increase. Although some digestive parameters have already reached adult values directly after birth, such as gastric lipase activity [3], other parameters take years before they reach adult values, such as the gastric acid profile [7]. Understanding the effect of age-related digestion conditions on the protein digestion of infant nutrition may aid in optimizing infant formula to promote infant growth and health.

Nowadays, two widely used static *in vitro* digestion models exist that mimic adult and infant conditions: the consensus INFOGEST adult digestion model [1], and the infant model that represents one-month-old infants [8]. By use of these digestion models, caseins were shown to be digested faster and whey proteins more extensively during gastric digestion in the adult compared to the infant model [8]. After *in vitro* intestinal digestion of milk proteins, smaller peptides and a lower variety of β-lactoglobulin (β-Lg) and β-casein peptides were detected in adults compared to infants [9]. The milder digestion conditions in infants thus lead to differences in both gastric and intestinal digestion compared to adults. However, obtaining information on how milk protein digestion changes with infant age is difficult since static *in vitro* models for other infant ages are either lacking, do not indicate what infant age they are representing, or are not in line with the current consensus adult and one-month infant digestion models [10].

To set up *in vitro* digestion models representing different ages, *in vivo* data are needed on the digestion conditions of infants at different ages, which has been reviewed by Bourlieu et al. [3] and has been described in brief above. Such age-related *in vivo* data are available on gastric conditions but are extremely scarce on intestinal conditions because of the invasiveness of obtaining these data. Since this lack of data makes it currently impossible to include accurate intestinal digestion conditions for different age stages during infancy, we focused only on the gastric digestion conditions. Changes in gastric digestion conditions likely influence both gastric and intestinal digestion kinetics and subsequent uptake of amino acids. In addition, the gastric clot may be affected by infant age since the gastric clot was shown to depend on both pH and pepsin concentration [11], which change during infancy. The presence and structure of the gastric clot is important for infants to enable a

constant release of amino acids into the bloodstream and to avoid nitrogen loss [12–14].

In addition to digestion conditions, milk protein digestion is also affected by heat treatment [15]. While mother's milk is usually drunk raw by children, infant formula undergoes heat treatment before consumption to ensure microbiological safety. In raw bovine milk, a firm clot is formed in the stomach, which consists only of caseins. Upon heating, whey proteins unfold and bind to the outside of the casein micelle, resulting in a looser clot that contains both caseins and whey proteins [16]. Furthermore, native whey proteins are not susceptible to pepsin digestion, whereas denatured whey proteins are digested by pepsin because of an increased accessibility of cleavage sites after unfolding [17]. Protein denaturation can therefore alter digestion kinetics and amino acid levels in the bloodstream [18].

This study aimed to investigate the effect of age-dependent changes in gastric digestion conditions on milk protein digestion in infants during the first six months of life. As protein denaturation and aggregation are known to play a major role in milk protein digestion, changes in digestion of both unheated and heated milk proteins during different stages of infancy were studied. We hypothesized that milk proteins are more extensively digested with increasing age due to maturation of the gastric digestion conditions, and that heated milk proteins form a looser gastric clot and are therefore digested faster than unheated milk proteins at all age stages because of the previously shown increase in whey protein digestion after denaturation.

2.2 Materials and methods

2.2.1 Materials and chemicals

Raw cow's milk was provided by FrieslandCampina (Wageningen, The Netherlands). Pierce BCA protein assay kit, NuPAGE LDS sample buffer (4x), NuPAGE sample reducing agent (10X), NuPAGE 4 to 12% Bis-Tris protein gel, and NuPAGE MES SDS running buffer (20X) were purchased from Thermo Fisher Scientific (Massachusets, USA). BlueRay prestained protein marker was obtained from Jena Bioscience (Jena, Germany). Pepsin (P6887), pepstatin A (P5318) and all other chemicals were purchased from Sigma Aldrich (Missouri, USA).

2.2.2 Processing of milk

As human milk is usually only drunk raw and commercial infant formulas have undergone heat treatments, which will result in variable levels of whey protein denaturation, raw cow's milk was chosen as starting material to enable studying differences in digestion between native and denatured proteins. To skim the raw cow's milk, it was centrifuged at 6000g at 4°C for 20 min, after which the cream layer was removed. Thereafter, part of the skim milk was heated in a water bath at 80°C to induce protein denaturation. Once the skim milk had reached this temperature, which took about 15 min, heating was continued for 30 min. This heating temperature and duration was chosen to induce denaturation and aggregation, while avoiding the formation of other protein modifications, such as glycation as shown by Wu et al. [19]. After heating, the skim milk was cooled down by placing it in ice water. The

skim milk was stored at -20°C until further use.

2.2.3 *In vitro* gastric digestion models

2.2.3.1 One-month infant gastric digestion model

The gastric phase of the infant static digestion model of Ménard et al. [8] was used to mimic the gastric digestion of one-month-old infants without the addition of gastric lipase since skim milk was used for the digestion experiments. This one-month (1M) model uses a meal:simulated gastric fluid (SGF) ratio of 63:37, a pH value of 5.3, and a pepsin activity of 268 U/ml. These meal:SGF ratio and pH value are based on the gastric conditions at the emptying half-time of infants after drinking infant formula (78 min) [20] since the gastric emptying half-time is usually considered more relevant than the final gastric digestion timepoint. The duration of the gastric digestion was set to 60 min, which is close to the gastric emptying half-time. The pepsin activity of 268 U/ml in the final mixture of meal and SGF was calculated using the mean body weight of one-month-old infants and the average pepsin activity (63 U/ml gastric content/kg body weight) measured in infant gastric aspirates [21]. SGF consisted of 94 mM sodium chloride and 13 mM potassium chloride, and was based on the gastric fluid composition of full-term infants [22].

2.2.3.2 Three-month and six-month infant gastric digestion models

The 1M digestion model was used as a basis, and its parameters were adapted to set up gastric digestion models representing three-month-old and six-month-old infants (Table 2.1). The parameters of the two models were determined based on the same gastric emptying half-time as used in the 1M model (78 min) because the half-time does not change during the first year of life [23]. In addition, it is still unknown how the gastric acid composition and production develop during the first two years of life. Therefore, the SGF composition, meal:SGF ratio, and the duration of the gastric digestion were used according to the 1M model in all three digestion models. For the three-month (3M) and six-month (6M) models, pH was set to 4.7 and 4.4 and pepsin activity to 385 U/ml and 480 U/ml in the final mixture of meal and SGF, respectively. The chosen pH values are the averages that were found in an *in vivo* study in which the pH value was measured in the stomach of 2-3 month-olds and 4-6 month-olds one hour after they were fed resolubilized dried skim milk [24]. The used pepsin activities of 385 and 480 U/ml were calculated in the same manner as for the 1M model [8], in which the mean body weight and average pepsin activity (63 U/ml gastric content/kg body weight) measured in infant gastric aspirates [21] were used. With a mean body weight of 6.11 kg for three-month-old infants and 7.62 kg for six-month-old infants [25], this results in a pepsin activity of 385 U/ml and 480 U/ml in the final mixture of meal and SGF, respectively. Gastric lipase was not added in this study since skim milk was digested but a lipase activity of 21 U/ml would be suitable for three- and six-month-old infants. This activity is identical to the lipase activity in the INFOGEST adult model [1] because gastric lipase has already reached adult activity levels upon birth [3].

Table 2.1: Parameters of the *in vitro* infant gastric digestion models representing one-month-old (1M), three-month-old (3M) and six-month-old (6M) infant digestion.

Parameter	Digestion model		
	1M	**3M**	**6M**
Ratio meal: SGF	63:37	63:37	63:37
pH	5.3	4.7	4.4
Pepsin activity (U/ml)	268	385	480
Duration (min)	60	60	60

2.2.4 *In vitro* gastric digestion

In vitro gastric digestions were performed using the three digestion models representing one-month, three-month and six-month-old infants as described above. Unheated skim milk (USM), heated skim milk (HSM) and SGF were preheated at 37°C for 10 min. Thereafter, SGF was added to the milk, the pH was adjusted using 1 M hydrochloric acid, and pepsin was added. These mixtures were incubated at 37°C with continuous shaking of 20 rpm. Digestions were stopped after 0, 5, 15, 30 and 60 min (G0, G5, G15, G30, and G60) by the addition of 10 µl of 7.2 µM pepstatin per ml digest. After digestion, samples for soluble protein content, protein composition, and free amino groups measurements were centrifuged at 4500g, 4°C for 30 min. The supernatant (soluble digest) was poured into a new tube, and the supernatant and the remaining pellet (gastric clot) were stored separately at -20°C until further use. Different digestion samples were prepared for confocal scanning laser microscopy (CLSM) and dry matter content, which were only digested for 60 min. These samples were not centrifuged after digestion to retain the gastric clot structure but were placed on ice and were used on the same day for further preparation for analysis. Digestions were performed in triplicate.

2.2.5 Soluble protein

Protein concentration of the soluble digests was measured with the BCA assay following the manufacturer's protocol. Percentage soluble protein was calculated with the following formula:

$$\text{Soluble protein (\%)} = \frac{\text{Measured soluble protein concentration}}{\text{Theoretical protein concentration}} \cdot 100\%$$

in which measured soluble protein concentration is the protein concentration measured with the BCA assay and theoretical protein concentration is the protein concentration in milk before digestion.

2.2.6 Dry matter content of clots

Dry matter content of the clots at G60 was determined by measuring the wet and dry weight of the clots. Approximately 1 gram of clot was weighed and was dried at 100°C for 24h. After drying, the clot was weighed to determine the dry weight. Dry matter content of the clot was calculated by use of the following formula:

$$\text{Dry matter (\%)} = \frac{\text{Dry weight}}{\text{Wet weight}} \cdot 100\%$$

in which dry weight is the weight of the clot after drying and wet weight is the weight of the clot before drying.

2.2.7 Microstructure of clots

The microstructure of the clots at G60 was visualized by use of confocal laser scanning microscopy (CLSM). After digestion, clots were washed twice by pouring off the liquid and adding fresh SGF at the same pH as the corresponding digestion model. Proteins in the clots were stained overnight by the addition of Rhodamine B (0.2% w/v). The next day, the residual dye was removed and proteins in the clots were visualized by use of an LSM510 microscope (Zeiss) with excitation wavelengths of 542 nm, and emitted light was collected between 540 and 750 nm. Images of the clots were taken with a magnification factor of 20 using Zen 2009 software.

2.2.8 SDS-PAGE

SDS-PAGE was performed to monitor the disappearance of intact protein in the clots and soluble digests during gastric digestion. Clots were first freeze-dried. Approximately 0.22 mg powder was mixed with 25 µl 4x LDS sample buffer, 10 µl 10x reducing agent, and 65 µl Milli-Q water. Supernatants of the digestion samples were mixed with 4x LDS sample buffer, 10x reducing agent, and Milli-Q water in a 1/5/2/12 (v/v/v/v) ratio. Subsequently, both supernatant and clot samples were heated at 70°C for 10 min. After heating, a volume corresponding to 5 µg protein of clots and soluble digests, as measured with the BCA assay, and 3 µl marker were loaded on a 4-12% Bis-Tris polyacrylamide precast gel. Gels were run at 120V for 75 min with MES running buffer, stained with Coomassie brilliant blue solution, and destained with 10% (v/v) ethanol and 7.5% (v/v) acetic acid solution. Bands on the gels were visualized by use of a GS-900 Calibrated Densitometer (Bio-Rad) with Image Lab software.

2.2.9 Proteomics

Bands of interest on the SDS-PAGE gels were analyzed further on protein composition by use of LC-MS/MS as described previously [26] with some modifications. After destaining, gels were washed three times with Milli-Q water. Then, gels were reduced by 15 mM dithiothreitol for 1h and alkylated with 20 mM acrylamide for 30 min. Gels were washed five times with Milli-Q water, and bands were excised from the gel and cut into small pieces. Gel pieces were digested with 50 µl trypsin (5 ng/µl) overnight at room temperature. Then, 10% trifluoro acetic acid was added to lower the pH to 2-4. Thereafter, samples were cleaned up with solid phase extraction (SPE) C8 stage tip columns, which were made in-house. Samples were loaded onto the C8 stage tip columns, and peptides were eluted into low-binding Eppendorf tubes. To extract more peptides from the remaining gel pieces, 100 µl of 50% acetonitrile/ 50% 1 ml/l formic acid was added to the remaining gel pieces, mixed and the liquid was added to the C8 filter. Peptides were eluted into the same low-binding Eppendorf

tube and were concentrated to a volume of less than 15 µl with the Eppendorf concentrator at 45°C. The volume of the sample was adjusted to 50 µl with 1 ml/l formic acid. Peptides were analyzed on a Thermo nLC 1000 system (Thermo Fisher Scientific) coupled to an Orbitrap Exploris 480 (Thermo Fisher Scientific). LC-MS/MS parameters were used, and data were analyzed as described previously [26] with a few modifications. Obtained LC-MS/MS data were analyzed with MaxQuant [27] (v2.0.3.0) with the trypsin semi-specific digestion mode and further default settings in the Andromeda search engine. Modifications that were included in protein quantifications were M-oxidation, protein N-terminal acetylation, and N- and Q-deamidation. Maximum number of modifications in one peptide was set to 3. Peptides were identified with the UP000009136 bovine database and a contaminant database that includes common contaminants such as trypsin and human keratin. Intensity values were used to calculate percentages of milk proteins in the SDS-PAGE bands.

2.2.10 Protein hydrolysis in soluble digests

The o-phthaldialdehyde (OPA) assay was performed to measure free amino groups in the soluble digests, as described previously [28]. In brief, soluble digests were diluted in Milli-Q water till a protein concentration between 1 and 5 mg/ml, and an L-leucine standard curve ranging from 5-50 µM was prepared. Then, 200 µl of freshly prepared OPA reagent was added to 10 µl diluted sample, or L-leucine standard in a transparent 96-well polystyrene plate. After incubation in the dark for 15 min, absorbance was measured at 340 nm with a Spectramax M2 microplate reader (Molecular Devices). Finally, protein hydrolysis was calculated using the following formula:

$$\text{Protein hydrolysis} = \frac{\text{Free } NH_2 \text{ groups in soluble digest (mM)}}{\text{Protein concentration in soluble digest (mg/ml)}}$$

in which NH_2 groups in soluble digests is the concentration of free amino groups in the soluble digest as determined by the OPA assay, and protein concentration in soluble digest is the protein concentration in the soluble digests as determined by the BCA assay.

2.2.11 Statistical analysis

Statistical analysis was performed in GraphPad prism v8.0.2 (GraphPad Software). As not enough data were obtained to perform a normality test, normality of the data was assumed. Comparison between 1M and 3M and between 1M and 6M models was done using one-way ANOVA and Dunnett's multiple comparisons test. Comparison between different heat treatments was done using unpaired two-tailed t-test. Differences were considered significant if $p<0.05$.

2.3 Results

2.3.1 Clot formation and structure during gastric digestion

Formation and proteolysis of gastric clots from USM and HSM during digestion with 1M, 3M and 6M *in vitro* digestion models were monitored. Changes in percentage soluble protein during gastric digestion compared to undigested skim milk were measured with the BCA

assay (Figure 2.1), which detected both intact proteins and large peptides. At G0, digests from all age models had a soluble protein percentage lower than 100%, and digests from the 3M and 6M models had a lower soluble protein percentage than digests from the 1M model. During the first 5 min of gastric digestion, soluble protein concentration decreased in USM in all models and in HSM in the 1M model, followed by a gradual increase in soluble protein concentration till 60 min. Whereas the percentage soluble protein only slightly increased in the 1M model between 5 and 60 min of digestion in both USM and HSM, it increased to a greater extent in the 3M and 6M models. At the end of gastric digestion, USM and HSM digests in the 3M and 6M models contained significantly more soluble protein than in the 1M model, and USM digests contained significantly more soluble protein compared to HSM digests in the same digestion model (Figure 2.1C).

More information about the gastric clots was obtained by studying their structure and dry matter content at G60 (Figure 2.S1). Clots from USM in the 1M model seemed firmer than all other clots by visual observation and had the highest dry matter content (19.6%). Compared to the clot from USM in the 1M model, lower dry matter contents were found in the 3M and 6M models (16.2% and 17.9% respectively), although this difference was only significant compared to the 3M model. Clots from HSM had similar dry matter contents in all digestion models, which ranged from 16.8 to 17.2%. Only in the 1M model, clots from USM had a significantly higher dry matter content than those from HSM.

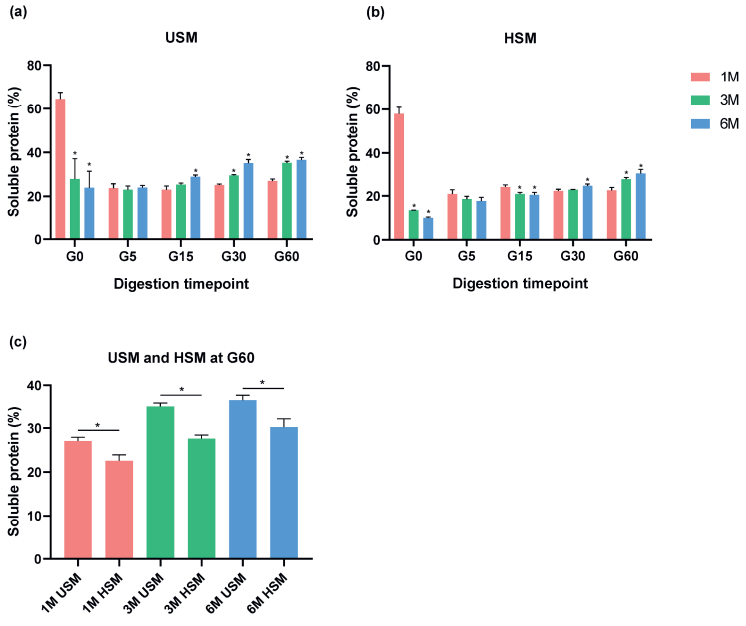

Figure 2.1: Percentage soluble protein after gastric digestion for 0, 5, 15, 30 and 60 min (G0, G5, G15, G30, and G60) of (a) unheated skim milk (USM), (b) heated skim milk (HSM), (c) comparison of percentage soluble protein at G60 between USM and HSM. Gastric digestion was conducted by use of *in vitro* infant gastric digestion models representing one-month-old (1M), three-month-old (3M) and six-month-old (6M) infants. Significant differences between 1M and 3M, between 1M and 6M, and between USM and HSM are indicated with * ($p<0.05$).

The microstructure of the gastric clots at G60 was analyzed with CLSM (Figure 2.2). Microscopy images revealed that clots from USM consisted of large aggregated structures in the 1M and 3M models. The clot structure seemed denser with smaller pores in the 1M model than in the 3M model, which is in line with the higher dry matter content of the USM clots in the 1M model. In the 6M model, small aggregates were observed in USM with a size between 50 and 150 µm. In HSM, clots in the 1M and 3M models also had large aggregated microstructures, whereas clots in the 6M model consisted of many small aggregates with sizes ranging from 20 to 100 µm. Differences between the clots from USM and HSM were found in the 1M and 6M models. In the 1M model, clots from USM showed a higher protein density than clots from HSM, and in the 6M model, clots from USM consisted of larger aggregates than clots from HSM. No clear differences could be observed between the clots from USM and HSM in the 3M model.

2.3.2 Protein composition of gastric clots

The protein composition of the gastric clots was determined by use of SDS-PAGE, and ten bands were analyzed for protein identification with LC-MS/MS (Figure 2.3). At G0, casein bands in the 3M and 6M models were more intense than in the 1M model, showing that relatively more casein was incorporated in the clots in the 3M and 6M models. β- and κ-casein were digested the quickest, as nearly no intact protein was detected with LC-MS/MS

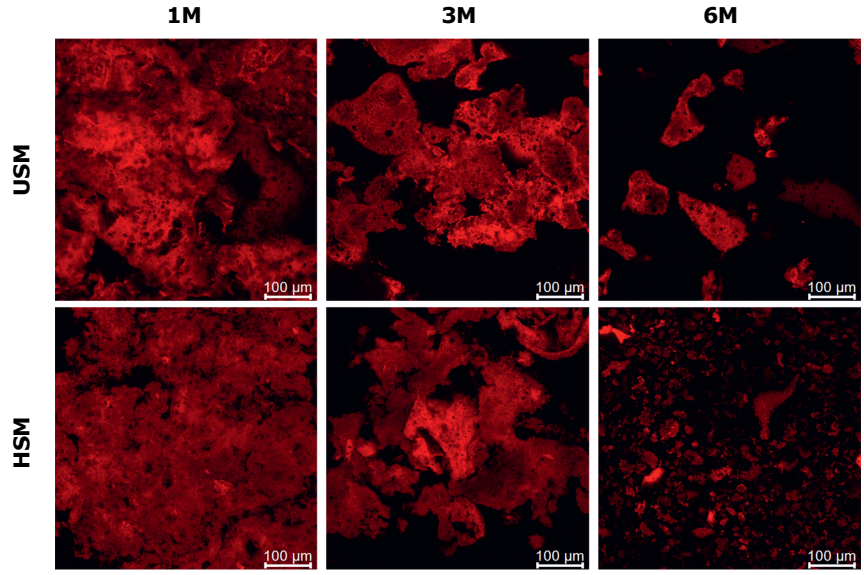

Figure 2.2: Confocal Laser Scanning Microscopy (CLSM) images of gastric clots from unheated (USM) and heated skim milk (HSM) after 60 min of gastric *in vitro* infant digestion with models representing one-month-old (1M), three-month-old (3M) and six-month-old (6M) infant digestion. Proteins were stained with 0.2% Rhodamine B. Clots from USM after 60 min gastric digestion with (a) 1M, (b) 3M, (c) 6M infant digestion models, and clots from HSM after digestion with (d) 1M, (e) 3M, (f) 6M infant digestion models.

after 5 min of digestion (band 3), whereas α_{s2}-casein (band 1) was the most persistent during digestion, as some intact casein was still present after 60 min of digestion in all digestion models. Intact caseins disappeared faster from the clots in the 3M and 6M models than in the 1M model on the SDS-PAGE gel. Caseins were digested into large peptides with varying sizes (bands 2-4, 7, 9 and 10), which showed a higher intensity in the 3M and 6M models compared to the 1M model. The bands with a higher molecular weight (bands 2-4) consisted of a mixture of α_{s2}-, α_{s1}- and β-caseins, whereas bands with a smaller molecular weight (bands 7, 9 and 10) consisted of a mixture of α_{s2}-, α_{s1}- and κ-casein and β-Lg peptides. The total composition and intensities of the proteins in the bands analyzed with LC-MS/MS are shown in Table 2.S1.

In addition to caseins, also intact whey proteins were detected in the clots in all digestion models, which remained largely intact during gastric digestion. Relative band intensities of β-Lg (band 5) and α-lactalbumin (α-La; band 8) at G60 were either similar or increased compared to at G5, showing that they were digested slower than caseins. Only in the 6M model, band intensities of β-Lg and α-La slightly decreased during digestion. The

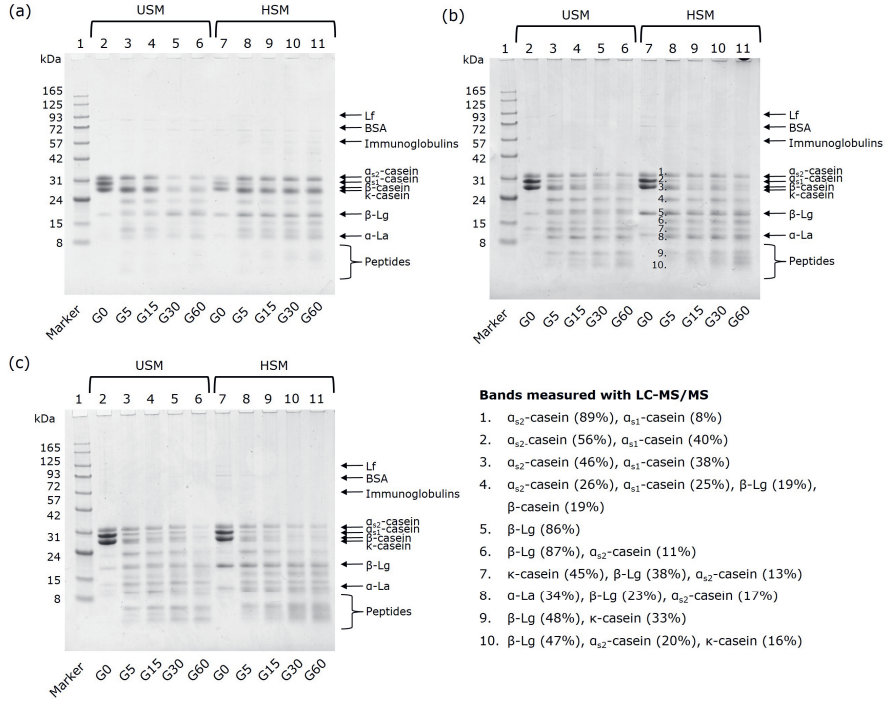

Figure 2.3: SDS-PAGE gels of gastric clots from unheated (USM) and heated skim milk (HSM) after gastric digestion for 0, 5, 15, 30 and 60 min (G0, G5, G15, G30, and G60) with digestion models representing (a) one-month-old (1M), (b) three-month-old (3M), (c) six-month-old (6M) infant digestion. The numbered bands in the gels were analyzed on protein composition with LC-MS/MS. Lf: lactoferrin, BSA: bovine serum albumin, β-Lg: β-lactoglobulin, α-La: α-lactalbumin.

digestion of β-Lg resulted in peptides ranging from <8 to 17 kDa (bands 6–10), whereas no α-La peptides were detected in the clots. The effect of heating could also be observed in the protein composition of the clots. Clots from HSM contained relatively more whey proteins (bands 5 and 8) than clots from USM already at G0, and this difference persisted till the end of gastric digestion. Heating also resulted in a slower disappearance of intact α_{s2}-casein but this effect was more pronounced in the 1M model than in the 3M and 6M models. Moreover, clots from HSM showed higher band intensities of small peptides (bands 9 and 10) in all digestion models.

2.3.3 Soluble protein composition during gastric digestion

Intact caseins disappeared from all soluble digests within the first 5 min of digestion due to clot formation (Figure 2.4). With increasing age, whey proteins such as β-Lg, α-La, lactoferrin (Lf), bovine serum albumin (BSA) and immunoglobulins, were digested faster in both USM and HSM, although intact whey proteins were still present at the end of gastric digestion in all models. Furthermore, an increased intensity of small peptides was observed with increasing age. Heating resulted in a decreased amount of whey protein in the soluble digests at G0 in the 3M and 6M models and at G5 in the 1M model, and in a larger decrease in band intensities of the whey proteins during digestion.

Five bands on the SDS-PAGE gel were analyzed with LC-MS/MS to analyze their protein composition (Figure 2.4, Table 2.S2). At G0, two bands (bands 11 and 12) were selected that did not match the molecular mass of intact milk proteins. These bands consisted of α_{s1}-, α_{s2}- and β-casein as well as β-Lg. At G5, a faint band with a size of 25 kDa (band 13) became visible in USM and remained present till the end of gastric digestion, which contained Ig-like domain-containing protein and β-Lg. Peptides with a lower molecular weight (bands 14 and 15) were present from G5 onwards and consisted of α_{s1}-, α_{s2}-, β-casein and β-Lg. Peptides smaller than 8 kDa, originating from α_{s1}- and α_{s2}-casein and β-Lg, were thus detected in both the clot and soluble digest, whereas small peptides from β-casein were mainly detected in the soluble digest, and small peptides from κ-casein were mainly detected in the clot.

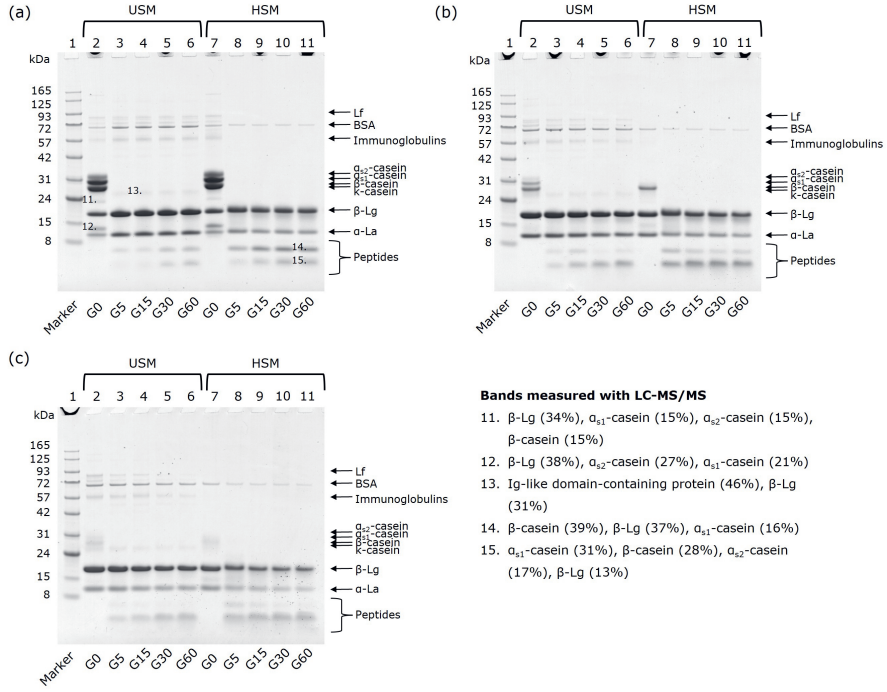

Figure 2.4: SDS-PAGE gels of soluble digests from unheated (USM) and heated skim milk (HSM) after gastric digestion for 0, 5, 15, 30 and 60 min (G0, G5, G15, G30, and G60) with digestion models representing (a) one-month-old (1M), (b) three-month-old (3M), (c) six-month-old (6M) infant digestion. The numbered bands in the gels were analyzed on protein composition with LC-MS/MS. Lf: lactoferrin, BSA: bovine serum albumin, β-Lg: β-lactoglobulin, α-La: α-lactalbumin.

2.3.4 Hydrolysis of soluble protein during gastric digestion

Free amino groups were determined in the soluble digests and were corrected for soluble protein concentration as a measure of protein hydrolysis (Figure 2.5). Protein hydrolysis increased quickly during the first 15 min and more slowly during the following 45 min of gastric digestion. Compared to the 1M model, protein hydrolysis was increased in the 3M and 6M models for both USM and HSM. Although significant differences between the different digestion models were observed in USM digests at G15 and in HSM digests at G5, G15 and G30, only between the 1M and 3M models in HSM digests the protein hydrolysis significantly differed at the end of gastric digestion. However, no differences in protein hydrolysis at G60 were found between soluble digests from USM and HSM that were digested with the same digestion model (Figure 2.5C).

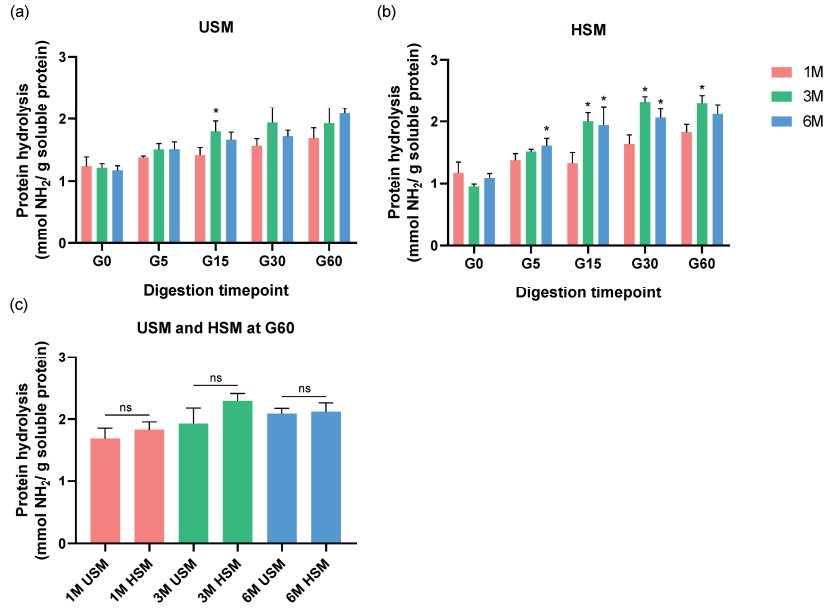

Figure 2.5: Protein hydrolysis measured as free amino groups (NH_2) per gram soluble protein after gastric digestion for 0, 5, 15, 30 and 60 min (G0, G5, G15, G30, and G60) of (a) unheated skim milk (USM), (b) heated skim milk (HSM), (c) comparison of protein hydrolysis at G60 between USM and HSM. Gastric digestion was conducted by use of *in vitro* infant gastric digestion models representing one-month-old (1M), three-month-old (3M), and six-month-old (6M) infants. Significant differences between 1M and 3M, between 1M and 6M, and between USM and HSM are indicated with * ($p<0.05$) with ns meaning non-significant.

2.4 Discussion

This study aimed to determine age-dependent changes in gastric clot formation and digestion of milk proteins in infants by use of *in vitro* gastric digestion models representing one-month, three-month and six-month-old infants. Results showed that gastric clot formation and clot structure differed between the models and that milk proteins were digested faster with increasing age due to age-dependent maturation of gastric digestion conditions.

2.4.1 Age-dependent effect on gastric clot formation and breakdown

Age-dependent differences were observed for the formation and breakdown of the clots. Less protein was incorporated in the clots from USM and HSM in the 1M model at G0 compared to the 3M and 6M models (Figure 2.1). According to the SDS-PAGE results of the gastric clots (Figure 2.3) and the soluble digests (Figure 2.4), the clots in the 1M model also had a lower casein content and the soluble digests had a higher casein content than the 3M and 6M models. At G0, gastric clot formation was only acid-induced as no pepsin was present at this time point. More acid-induced coagulation took place in the 3M and 6M models because

their pH values (4.7 and 4.4) were closer to the isoelectric point (pI) of caseins (4.6), whereas the pH value of the 1M model (5.3) deviated more from the pI of caseins. Clots from HSM contained more protein than those from USM at G0, which can probably be attributed to a higher content of whey protein in these clots as shown by SDS-PAGE (Figure 2.3). During heat treatment, whey proteins denature and bind to the outside of casein micelles, resulting in gastric clots that consist of both caseins and whey proteins [29]. However, clots from USM also contained some whey protein at G0, demonstrating that also native whey proteins were present in gastric clots. Whereas a very low level of glycation may have occurred during wet heating at 80°C for 30 min [19], such low glycation levels have been shown not to influence gastric milk protein digestion [30]. Therefore, changed milk protein digestion after heat treatment was considered to be caused solely by protein denaturation and aggregation.

Further clot formation and breakdown of the clot occurred between 5 and 60 min due to pepsin activity. The soluble protein content was similar for all digestion models at G5 (Figure 2.1). Thereafter, the amount remained similar till the end of digestion in the 1M model, whereas it increased for the 3M and 6M models. This indicates that gastric clots were broken down faster, but gradually, in the 3M and 6M models, which may be caused by the faster digestion of caseins as visualized with SDS-PAGE (Figure 2.3). A gradual digestion of the clot was shown to be important in infants to provide a constant release of amino acids after intestinal digestion without leading to nitrogen loss [13,14]. The faster clot breakdown in the 3M and 6M models was probably caused by the higher pepsin concentrations and lower pH values than the 1M model, which both contribute to a higher pepsin activity, leading to a quicker digestion of the caseins into peptides and subsequent breakdown of the clot. While the pepsin concentration of the 6M model (480 U/ml) was not even twice as high as the one of the 1M model (268 U/ml), the pepsin activity differed more. At the pH of the 6M model (4.4), pepsin is at 65% of its maximal activity, whereas at the pH of the 1M model (5.3), this is only 10% [31]. Therefore, the pepsin activity was about twelve times higher in the 6M model compared to the 1M model, resulting in a faster gastric protein digestion with increasing age.

The difference in clot structure and hydration might also have contributed to the quicker breakdown of the clot with increasing age. The clot from USM in the 1M model had a higher dry matter content than clots in the 3M and 6M models, meaning a lower hydration (Figure 2.S1). This was in agreement with the higher protein density that was observed for the USM clot in the 1M model with CLSM (Figure 2.2). This lower hydration can lower the accessibility for pepsin, which may result in a slower breakdown of the clot. Clots from the 6M model consisted of smaller aggregates as determined by CLSM (Figure 2.2), which thus have an increased relative surface area compared to larger aggregates, increasing the accessibility for pepsin and enhancing the breakdown of the clot. Microstructures of pepsin-induced milk clots were previously shown to depend on pH value, as clots formed at the same pepsin activity had larger pore sizes at a pH of 5.3 than at higher pH values [11]. The faster digestion of gastric clots with a higher hydration and a more fragmented microstructure was in line with Ye et al. [29], although in their study it was caused by heat treatment of milk prior to digestion instead of by different pH values and pepsin activities during digestion.

The faster digestion of milk proteins with increasing age was also shown by the difference in protein and peptide composition in the clot between the three age models (Figure 2.3). Intact caseins disappeared faster but still gradually from the clots with increasing age, resulting in more peptides with different sizes in the 3M and 6M models compared to the 1M model. In addition, whey proteins were digested faster in the clots from the 6M model than in those from the other models, although still to a small extent. In contrast to caseins, whey proteins are largely resistant to pepsin cleavage. Therefore, caseins were affected to a greater extent by the increased pepsin activity with increasing age. This is in agreement with Ménard et al. [8], who reported a gradual decrease of intact casein during infant gastric digestion and a quick decrease during adult gastric digestion. Peptides that were detected in the clots originated from α_{s1}-, α_{s2}-, β- and κ-casein and β-Lg. These peptides were probably hydrophobic as they did not solubilize. In a previous study on the gastric clot of bovine skim milk, also peptides with different sizes were detected with SDS-PAGE including para-κ-casein [29]. However, by use of SDS-PAGE not all peptides in the clot could be identified in that study. In one study, the protein composition of gastric clots from goat skim milk was analyzed with SDS-PAGE in combination with LC-MS/MS [32]. They also found that large peptides in the gastric clots were mainly originating from α_{s1}-, α_{s2}- and β-casein, whereas the smaller peptides were from α_{s1}-, α_{s2}- and κ-casein as well as whey proteins.

Relatively larger differences were observed between the digestion models for HSM than USM with regard to casein digestion and peptide formation (Figure 2.3). The higher increase in peptide intensity in HSM might be caused by the presence of denatured whey protein. Denatured whey protein is more easily digested by pepsin than native whey protein because of unfolding, leading to an easier access to the cleavage sites [33]. The increased whey protein digestion after heating might cause bigger differences in peptide formation with age-dependent increasing pepsin activity. Heating also led to a slower casein digestion but only in the 1M model. The clot of USM visibly had a firmer clot structure, a higher dry matter content (Figure 2.S1), and a higher protein density (Figure 2.2) than the clot of HSM in the 1M model, whereas no differences were observed between USM and HSM in terms of dry matter content and protein density in the other models. The higher resistance of casein to infant gastric digestion in HSM than USM is in line with a previous study, which was suggested to be due to the formation of casein-whey aggregates blocking the casein cleavage sites [34]. In another study, also firmer gastric clots were reported for unheated milk than for heated milk [29]. However, they found a faster instead of a slower casein digestion after heating due to the looser clot structure, which was more susceptible to pepsin cleavage. This difference may be explained by the use of a dynamic digestion model that included mechanical disruption of the gastric clot, which is not included in static digestion models as used in our study. Overall, gastric digestion of both USM and HSM increased with age but the difference was larger for HSM than USM, which was most likely caused by differences in pepsin activity and gastric clot structure.

2.4.2 Age-dependent effect on hydrolysis of soluble protein

Differences in milk protein digestion between the different age models were also observed in the soluble digests. With increasing age, clots were digested faster, leading to a higher soluble protein content (Figure 2.1). In addition, whey proteins were more easily digested and smaller peptides were formed (Figure 2.4), indicating a more extensive protein digestion with increasing age. The soluble protein composition of USM digested with the 1M model was in agreement with Ménard et al. [8], in which at least 80% of the whey proteins were still intact after 60 min of gastric digestion. The protein compositions of the 3M and 6M digests were in between the protein compositions of digests from the 1M model of Ménard et al. [8] and the adult INFOGEST model [8], showing that the gastric milk protein digestion increases during infancy but does not reach adult conditions yet. Whereas nearly all whey proteins had been digested after 60 min in the adult model, a large part of the whey proteins was still intact after digestion with the 3M and 6M models. Peptides from α_{s1}-, α_{s2}- and β-casein were detected in the soluble digests. As no intact caseins were present in the soluble digests after 5 min of digestion, these peptides most likely moved from the gastric clot to the soluble digests, showing a gradual release of casein peptides from the clot during gastric digestion. Peptides from β-Lg were also detected in the soluble digests and could have originated from both β-Lg in the clot as from soluble β-Lg.

An increased digestibility with increasing age was also seen in protein hydrolysis for HSM but not for USM (Figure 2.5), while an increased percentage soluble protein with increasing age was observed for both USM and HSM (Figure 2.1). The higher percentage soluble protein with increasing age was probably due to the difference in pepsin activity between the age models, resulting in more protein solubilizing from the gastric clot from both USM and HSM to the soluble digest. An increased protein hydrolysis with increasing age was, however, only observed in HSM, which means relatively more free NH_2 groups per gram soluble protein. This is probably caused by the larger increase in whey protein digestion with age in HSM than in USM, due to the easier digestion of denatured compared to native whey protein [17], resulting in relatively more small peptides with more free NH_2 groups in HSM. This is in agreement with the faster breakdown of denatured whey protein in the digests and the higher increase in intensity of peptides with age in HSM compared to USM (Figure 2.4). Although the HSM digests in the 3M model had a higher protein hydrolysis at some timepoints than in the 6M model (Figure 2.5), they did not significantly differ, indicating that the digestion of milk proteins was more affected by the changes in gastric digestion conditions in the first three months of life.

2.4.3 Relevance and limitations of gastric digestion models

In this study, the effect of infant age on clot formation and digestion of milk proteins during gastric digestion was investigated by use of gastric digestion models representing one-, three-, and six-month-old infants. Studying gastric clot formation and breakdown provides insights into the complete digestion process, as it is known to influence intestinal protein digestion and subsequent uptake of amino acids [12– 14]. The 1M model is a model that is commonly used and is in line with *in vivo* gastric data from preterm infants [35], although a direct validation of the *in vitro* model with *in vivo* data from full-term infants is still lacking.

The parameters of this 1M model were changed to mimic the digestion of three- and six-month-old infants, which were based on *in vivo* data. However, it remains unknown how well the 3M and 6M models mimic *in vivo* gastric digestion of milk proteins as to our knowledge no *in vivo* studies have been performed that investigated milk protein digestion at different infant ages. Validating the *in vitro* data with *in vivo* data in future studies is needed to determine the accuracy of the gastric digestion models and should be done with either human milk or infant formula as a meal. Obtained *in vivo* data could also be used to improve the gastric digestion models, for instance with age-dependent gastric fluid composition and meal:gastric fluid ratio. Moreover, *in vivo* kinetics of gastric clot formation and breakdown could be more closely mimicked by developing dynamic infant gastric digestion models representing different ages as dynamic gastric acid and enzyme secretions and gastric peristalsis, which change with infant age, may influence the formation, structure and breakdown of the gastric clot. *In vivo* data on digestion kinetics of milk proteins at different infant ages are, however, even more scarcely available, which limits the development of such dynamic *in vitro* digestion models. In addition, no intestinal phase was included in the 3M and 6M models because too little data were available on intestinal digestion conditions at these ages, as explained in the introduction (section 2.1). When more intestinal data would become available, an intestinal phase could be added to the gastric digestion models, and could be used to determine how the found age-related differences in gastric milk protein digestion influence the overall gastro-intestinal digestion at different age stages during infancy.

2.5 Conclusion

Parameters of an existing static *in vitro* infant gastric digestion model representing one-month-old infants were changed to set up digestion models representing three- and six-month-old infants. Digestion of USM and HSM with these gastric digestion models showed that milk proteins in gastric clots and soluble digests were more intensively digested with increasing age, and this difference with increasing age was larger for HSM than for USM. Both the higher pepsin activity and looser clot structure may have contributed to the faster casein and whey protein digestion with increasing age. The larger difference in protein digestion with increasing age for HSM than for USM may have been caused by a larger difference in the digestion of denatured whey proteins than native whey proteins between the different age models. Together, this demonstrates that milk protein digestion in infants is affected by age-related gastric digestion conditions and that heat treatment influences milk protein digestion differently depending on the gastric pH and pepsin activity. By use of these gastric digestion models, information could be obtained on age-dependent differences in milk protein digestion in infants to optimize infant formulas for specific ages, which better resemble human milk of different lactation stages and its digestion, and better support the infant's development in the first six months of life. However, further investigation on *in vivo* validation of digestion models representing different infant ages is needed.

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Supplementary information

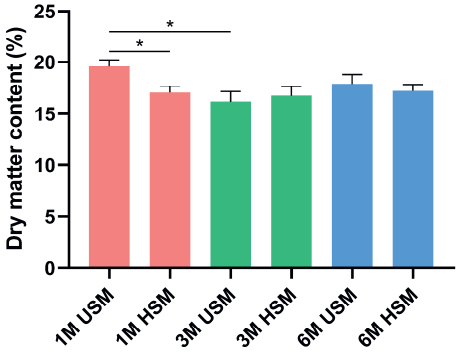

Figure 2.S1: Dry matter content of gastric clots from unheated (USM) and heated skim milk (HSM) after gastric digestion for 60 min with models representing one-month-old (1M), three-month-old (3M) and six-month-old (6M) infants. Significant differences between 1M and 3M, between 1M and 6M, and between USM and HSM are indicated with * ($p<0.05$).

Table 2.S1: Intensities of proteins detected in bands in SDS-PAGE gel of gastric clots (Figure 2.3) with LC-MS/MS. BSA: bovine serum albumin, α-La: α-lactalbumin, β-Lg: β-lactoglobulin, Glycam-1: glycosylation-dependent cell adhesion molecule 1, pIgR: polymeric immunoglobulin receptor, ND: not detected.

Protein	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Band 8	Band 9	Band 10
BSA	9.6E+08	1.1E+08	1.0E+09	1.1E+09	9.0E+08	2.7E+08	3.1E+08	3.6E+08	3.6E+08	1.7E+08
κ-casein	6.4E+08	2.6E+08	2.7E+09	2.7E+09	1.4E+09	8.3E+08	2.3E+10	2.2E+09	3.4E+10	2.9E+09
Ig-like domain containing protein	6.3E+06	1.4E+07	5.5E+08	5.5E+08	1.9E+08	1.0E+08	2.4E+08	3.2E+08	3.3E+08	7.3E+07
Uncharacterized protein	1.3E+08	6.2E+07	8.1E+07	1.2E+08	4.3E+08	7.6E+07	2.1E+08	1.6E+08	3.6E+07	2.1E+07
β-casein	2.7E+08	4.4E+08	1.0E+10	1.1E+10	3.8E+09	2.3E+08	3.3E+08	5.1E+08	5.3E+09	1.6E+09
α-La	7.1E+07	1.7E+06	8.0E+07	1.3E+08	6.5E+08	3.1E+07	2.2E+08	8.1E+09	1.5E+09	1.4E+07
Lactadherin	1.3E+08	6.3E+07	2.9E+08	1.4E+08	2.6E+07	1.4E+07	4.1E+07	1.7E+07	1.9E+07	2.1E+06
α_{s1}-casein	1.3E+10	2.4E+10	4.5E+10	1.4E+10	1.9E+09	3.6E+08	3.7E+08	2.5E+09	4.6E+09	1.3E+09
α_{s2}-casein	1.5E+11	3.4E+10	5.5E+10	1.5E+10	1.5E+10	1.0E+10	6.9E+09	4.1E+09	6.7E+09	3.8E+09
β-Lg	3.1E+09	1.4E+09	3.0E+09	1.1E+10	1.5E+11	7.9E+10	2.0E+10	5.5E+09	5.0E+10	8.8E+09
Lipoprotein lipase	1.8E+08	6.0E+07	1.1E+08	1.4E+08	1.3E+07	8.2E+06	8.9E+05	1.0E+07	4.5E+07	2.9E+07
Lactotransferrin	6.9E+08	2.0E+08	3.3E+08	9.3E+08	2.2E+08	1.1E+08	1.0E+08	1.6E+08	9.6E+07	4.9E+07
Glycam-1	2.3E+06	1.5E+08	1.2E+08	8.4E+08	1.2E+07	4.7E+05	ND	7.0E+05	4.0E+06	ND
pIgR	4.4E+07	1.9E+07	4.8E+07	2.2E+07	7.1E+06	1.5E+07	5.1E+06	7.0E+05	6.2E+07	8.5E+05
Complement C3	7.4E+07	7.0E+07	4.9E+08	1.6E+08	6.0E+07	4.2E+06	5.9E+06	6.9E+06	3.7E+06	1.7E+06
Immunoglobulin J	ND	ND	ND	3.8E+07	ND	ND	ND	ND	ND	ND

Table 2.S2: Intensities of proteins detected in bands in SDS-PAGE gel of soluble digests (Figure 2.4) with LC-MS/MS. BSA: bovine serum albumin, α-La: α-lactalbumin, β-Lg: β-lactoglobulin, Glycam-1: glycosylation-dependent cell adhesion molecule 1, pIgR: polymeric immunoglobulin receptor, ND: not detected.

Protein	Band 11	Band 12	Band 13	Band 14	Band 15
BSA	1.1E+08	3.9E+07	2.0E+07	3.2E+08	5.8E+07
κ-casein	9.0E+07	1.1E+08	6.8E+07	1.5E+09	3.3E+08
Ig-like domain containing protein	3.1E+08	6.1E+06	4.5E+09	4.9E+07	8.7E+06
Uncharacterized protein	ND	2.0E+05	1.7E+07	4.3E+06	1.7E+07
β-casein	7.0E+08	3.8E+08	1.7E+08	1.6E+10	1.4E+09
α-La	2.2E+07	1.1E+08	4.2E+06	6.5E+08	8.3E+06
Lactadherin	1.6E+06	1.2E+05	1.3E+06	2.6E+05	ND
α_{s1}-casein	7.4E+08	1.1E+09	4.7E+08	6.5E+09	1.5E+09
α_{s2}-casein	7.3E+08	1.4E+09	3.9E+08	5.3E+08	8.5E+08
β-Lg	1.6E+09	2.1E+09	3.0E+09	1.5E+10	6.2E+08
Lipoprotein lipase	ND	7.8E+05	ND	1.5E+07	8.1E+06
Lactotransferrin	2.5E+06	ND	6.3E+07	6.5E+05	1.9E+07
Glycam-1	3.7E+08	1.1E+08	7.0E+08	9.8E+07	7.2E+07
pIgR	ND	ND	2.7E+07	1.9E+07	2.6E+05
Complement C3	1.4E+05	ND	2.4E+08	ND	ND
Immunoglobulin J	8.8E+07	ND	8.1E+07	4.5E+05	ND

Chapter 3

The effect of wet versus dry heating of an infant formula model system on *in vitro* milk protein digestion

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Abstract

Infant formula is usually heated under both wet and dry conditions to ensure microbiological safety and to prolong shelf life. These heat treatments induce, amongst others, protein denaturation and glycation, which can influence the digestion of milk proteins, and can eventually lead to a reduced amino acid uptake. However, a direct comparison of how wet and dry heating affect milk protein digestion is lacking, especially under infant digestion conditions. An infant formula model system was made from raw bovine milk with a 40:60 casein:whey ratio and a 12:70 protein:lactose ratio. This model system either remained unheated, was wet heated to induce protein denaturation, or was dry heated to induce glycation. Wet heating resulted in increased protein denaturation levels with increasing heating duration but it did not affect glycation levels. Dry heating led to increased glycation levels, which were either in the early or the advanced stage of the Maillard reaction depending on the heating duration. During infant gastric digestion, casein hydrolysis was decreased after wet heating, possibly due to casein-whey protein complexes, whereas gastric protein digestion was unchanged after dry heating. Wet heating increased intestinal protein digestion, as observed by lower peptide intensities, and was most likely caused by an increased accessibility of cleavage sites after denaturation. In contrast, dry heating decreased protein digestion, which was shown by a lower degree of hydrolysis, longer peptide lengths, and higher peptide intensities. The high intensity of peptides containing glycated amino acids close to cleavage sites indicated that this decreased hydrolysis of dry heated milk proteins was probably due to glycation, which blocked the accessibility of cleavage sites for digestive enzymes. Together, digestion differed more between wet and dry heated proteins than between unheated and either wet or dry heated proteins under infant digestion conditions.

3.1 Introduction

Infant formula (IF) is an alternative for mother's milk, which supplies infants with the needed nutrients for healthy growth and development. Before IF can be consumed, it needs to undergo heat treatment to ensure safety and to prolong shelf life. However, heating of IF also results in the formation of milk protein modifications, such as denaturation, glycation and aggregation [1]. These protein modifications can influence the digestibility of proteins and are generally undesirable because they can reduce the nutritional quality and amino acid uptake [2].

Production of IF usually includes at least two heating steps: pasteurization and spray drying. Pasteurization is performed to destroy pathogens and takes place at a temperature above the denaturation temperature of whey proteins. This process of denaturation has been extensively studied for the two most abundant whey proteins in bovine milk, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) [3–5]. During this heat treatment, the globular whey proteins first unfold, followed by aggregation via sulfhydryl interactions, and eventually, larger aggregates can be formed by non-specific interactions.

As a final step of IF production, it is spray dried into a powder, which prolongs shelf life and enables easy packaging, transportation and storage. During spray drying, glycation of milk proteins occurs via the Maillard reaction (MR) [6]. In milk, this reaction starts with the binding of lactose to the free amino group of lysine, resulting in a reduction of available lysine and the formation of lactulosyllysine [7]. Thereafter, the MR can proceed, resulting first in early MR products (MRPs) and later in advanced MRPs. A decrease in available lysine and an increase in furosine are indicators for the early MR, whereas an increase of carboxymethyllysine (CML) and pyrraline are indicators for the advanced MR.

Milk protein denaturation and glycation have been shown to affect the digestion of milk proteins [2]. Denaturation increases the digestion of whey proteins during gastric digestion because their cleavage sites are more easily accessible for pepsin after unfolding. Furthermore, denatured whey proteins that are associated to caseins can end up in the gastric clot, changing the gastric emptying rate [8]. Although a changed gastric emptying rate leads to altered gastric and intestinal digestion kinetics, multiple studies reported a similar overall digestibility of milk proteins after intestinal digestion regardless of protein denaturation levels [9,10]. In contrast to denaturation, most studies reported that glycation led to a reduced digestibility of milk proteins [2]. This reduction can be caused by blocking of lysine at or close to cleavage sites for digestive enzymes, or by cross-linking, resulting in a decreased recognition or a decreased accessibility of cleavage sites. In contrast, some studies reported an increased digestibility after industrial heating that induced glycation [11,12]. Industrial heating can, however, also lead to other protein modifications next to glycation, such as denaturation, which could have caused this increased digestibility. The changes in digestion and digestion kinetics of milk proteins due to denaturation and glycation can subsequently result in several changed physiological outcomes, including intestinal transport of milk peptides, immune response towards milk proteins and support of the intestinal barrier homeostasis [2].

Whereas wet and dry heating have been shown to affect milk protein digestion, a direct comparison of these two heating conditions on milk protein digestion is lacking. In addition, most studies have been performed under adult instead of infant digestion

conditions and used wet or dry heating conditions that induced both denaturation and glycation. Since the digestive capacity of infants is lower than adults, these modifications might have a larger impact on protein hydrolysis under infant than adult digestion conditions. Therefore, this study aimed to directly compare the effect of wet and dry heating on the digestion of milk proteins in infants. An IF model system made from raw bovine milk was either wet heated or dry heated to induce either only protein denaturation or only glycation. After heat treatment, the IF model system was digested with an *in vitro* infant digestion model, and protein hydrolysis and peptide formation were determined.

3.2 Materials and methods

3.2.1 Materials

Raw cow's milk was provided by FrieslandCampina (Wageningen, The Netherlands). NuPAGE 4 to 12% Bis-Tris protein gel, NuPAGE LDS sample buffer (4x), NuPAGE sample reducing agent (10X) and NuPAGE MES SDS running buffer (20X) were purchased from Thermo Fisher Scientific (Massachusets, USA). BlueRay prestained protein marker was purchased from Jena Bioscience (Jena, Germany). Sodium hydroxide, sodium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Merck (Darmstadt, Germany). Analytical standards lysine, lysine-d4, furosine, furosine-d4, N-ε-carboxymethyllysine (CML) and N-ε-carboxymethyllysine-d2 (CML-d2) were bought from from Polypeptide laboratories (Strasbourg, France). HPLC ultra-gradient grade acetonitrile was obtained from Actu-All Chemicals (Oss, The Netherlands). Pepsin (P6887), pancreatin (P7545), porcine bile extract (B8631), a dialysis cellulose tube with a cut-off of 12 kDa, and all other chemicals were purchased from Sigma Aldrich (Missouri, USA).

3.2.2 Infant formula model system

Raw cow's milk was centrifuged at 6000g, 4°C for 20 min and the cream was discarded. A part of the skim milk was acidified to precipitate the casein micelles by adding a volume of 1 M hydrochloric acid till pH 4.6 was reached. Subsequently, the acidified skim milk was centrifuged at 4500g, 4°C for 20 min. The supernatant with soluble whey proteins was dialyzed overnight at 4°C by use of a dialysis membrane with a 12 kDa molecular weight cut off against a lactic acid in MilliQ water solution with a pH of 4.6. Thereafter, the demineralized whey was neutralized with 1 M sodium hydroxide to a pH of 6.6. The casein:whey ratios and protein contents of the demineralized whey and the skim milk were measured with reverse-phase high performance liquid chromatography (RP-HPLC) and DUMAS as described in sections 3.2.3 and 3.2.4. By use of this information, the demineralized supernatant was mixed with skim milk to obtain a 40:60 casein:whey ratio. The lactose concentration of this mixture was measured with a lactose/galactose assay kit (Megazyme) according to the manufacturer's instruction. Thereafter, lactose was added to obtain an infant formula (IF) model system with a 12:70 (w:w) protein:lactose ratio. The IF model system was lyophilized, cryomilled, and was stored at -20°C till further use.

3.2.3 RP-HPLC

The casein:whey ratios of skim milk and demineralized whey were measured with RP-HPLC (Thermo Scientific™ UltiMate 3000, Thermo Fisher Scientific) as described previously [13]. The RP-HPLC was equipped with an Aeris Widepore 3.6 µm XB-C18 column, 250 × 4.6 mm (Phenomenex), and two solvents, consisting of 0.1% TFA in MilliQ water and 0.1% TFA in acetonitrile were used for protein elution. Chromatograms were analyzed with Chromeleon 7.1.2. (Thermo Fisher Scientific). The sum of the peak areas of α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein, and of β-Lg and α-La were determined. Thereafter, the ratio of the sum of the casein peak areas and the sum of the whey protein peak areas was taken to determine the casein:whey ratios of skim milk and demineralized whey.

3.2.4 Protein content

Total nitrogen contents of skim milk and demineralized whey were determined by use of a DUMAS Flash EA 1112 Protein analyzer (Thermo Fisher Scientific). Total nitrogen content was converted to protein content with a conversion factor of 6.38.

3.2.5 Heat treatment of the IF model system

The IF model system remained either unheated (UH) as control, was dry heated (DH) to induce glycation or was wet heated (WH) to induce protein denaturation. For dry heating, the IF model system was incubated at 60°C in a desiccator with a saturated potassium iodide solution to obtain a water activity of 0.6. The IF model system was dry heated for 4, 24, or 72h to obtain DH samples with different glycation levels, which were named DH-4, DH-24 and DH-72. For wet heating, the IF model system was dissolved in MilliQ water at a protein concentration of 12 mg protein/ml, which is similar to the recommended protein concentration of commercial IF, and was placed in a water bath at 80°C. Once the solution had reached this temperature, heating was continued for 5, 15, or 40 min to obtain WH samples with different protein denaturation levels, and were named WH-5, WH-15 and WH-40. After wet heating, the samples were cooled in ice water. UH, DH and WH samples were stored at -20°C till further use.

3.2.6 SDS-PAGE of undigested samples and whey protein denaturation

After wet and dry heating, SDS-PAGE under both reducing and non-reducing conditions was performed to determine the protein composition and whey protein denaturation level of the samples. For reducing SDS-PAGE, 0.30 mg UH or DH sample, or 4 µl WH sample was mixed with 10 µl sample buffer, 4 µl sample reducing agent and 26 µl MilliQ water. For non-reducing SDS-PAGE, the volume of sample reducing agent was replaced by MilliQ water. Samples were centrifuged at 2000g for 1 min, heated at 70°C for 10 min, and centrifuged again at 2000g for 1 min. Thereafter, 10 µg protein of each sample was loaded on 4-12% Bis-Tris gels along with 4 µl marker. Gels were run at 120V for 75 min with MES running buffer. Proteins on the gel were stained with Coomassie brilliant blue and were destained with a solution of 10% ethanol and 7.5% acetic acid in demi water. Bands were visualized with a GS-900 Calibrated Densitometer (Bio-Rad) with Image Lab software (v6.0.1). Percentage whey protein

denaturation in the WH samples was determined by use of the non-reducing SDS-PAGE gel. The intensity of the β-Lg band in the UH sample was set to 1.00 and the relative decrease in band intensity of β-Lg in the WH samples compared to the UH sample was used as a measure for percentage whey protein denaturation.

3.2.7 Maillard reaction products

The Maillard reaction markers lysine, furosine and CML were determined using uHPLC-ESI-MS/MS as described previously [14]. In brief, IF model system samples were mixed with 4 ml 6 M hydrochloric acid, saturated with nitrogen, and heated at 110°C for 22h. Thereafter, the samples were filtered through an 0.2 µm polytetrafluoroethylene (PTFE) syringe filter (Phenomenex), were completely dried under a nitrogen flow, and were reconstituted in 1 ml MilliQ water. Solid phase extraction was performed to clean up the samples by use of Oasis HLB 3 cc cartridges (Waters). The cartridge was activated using 1 ml methanol and conditioned using 1 ml 10 mM nonafluoropentanoic acid (NFPA). The sample was loaded on the cartridge, was washed with 1 ml 10 mM NFPA/methanol (95:5), and was eluted with 2 ml 10 mM NFPA/methanol (50:50). The sample was completely dried under a nitrogen flow, reconstituted in 190 µl 50% acetonitrile and 10 µl internal standard was added, containing 25 ppm d4-Lys, d4-furosine and d2-CML. Additionally, calibration curves of lysine, furosine and CML ranging from 20 ppb to 2 ppm were prepared, which were also spiked with 10 µl internal standard. Lysine, furosine and CML were separated on a Kinetex 2.6 µm HILIC 100A, 100 × 2.1 mm (Phenomenex) and further settings according to Troise et al. [14]. Data were analyzed with LabSolutions software (Shimadzu). Lysine blockage was calculated from lysine and furosine contents with the following formula as described previously [15]:

$$\text{Lysine blockage (\%)} = \frac{3.1 \cdot \text{furosine}}{\text{total lysine} + 1.87 \cdot \text{furosine}} \cdot 100\%$$

3.2.8 Surface hydrophobicity

Surface hydrophobicity was measured with the 8-anilino-1-naphthalenesulfonic acid (ANS) assay as described previously [16]. In brief, 1 ml 0.25 mg/ml protein solution in 100 mM sodium phosphate buffer pH 7.4 was mixed with 5 µL ANS stock solution (8 mM in sodium phosphate buffer). After incubation in the dark for 15 min, 100 µL sample was transferred to a 96-well black polystyrene plate (Greiner bio-one) and measured with a plate reader (Tecan Spark, Tecan) with Infinite 200 PRO NanoQuant and i-control software using 390 nm as excitation wavelength and 470 nm as emission wavelength. The fluorescence intensity was corrected for the sample without the addition of ANS.

3.2.9 Fibril structure

The thioflavin T (ThT) assay was performed to determine changes in fibril structures [17]. For this, 0.25 mg/ml protein solution in 100 mM sodium phosphate buffer pH 7.4 was mixed with 4 mM ThT solution in a 7:1 ratio, and was incubated for 10 min in the dark. Thereafter, 100 µL sample was transferred to a 96-well black polystyrene plate (Greiner Bio-One) and fluorescence was measured using 440 nm as excitation wavelength and 490 nm as emission

wavelength. The fluorescence intensity was corrected for the sample without the addition of ThT.

3.2.10 *In vitro* infant digestion

UH, DH and WH IF model system samples were digested according to the *in vitro* infant digestion model by Ménard et al. [18]. Protein concentrations of UH and DH samples were adjusted to 12 mg/ml by the addition of MilliQ water. Thereafter, UH, DH and WH samples were preheated at 37°C for 10 min, followed by *in vitro* gastric and intestinal digestion. In the gastric phase, simulated gastric fluid (SGF), which consisted of 94 mM sodium chloride and 13 mM potassium chloride, was added to the samples in a 63:37 meal:SGF ratio. The pH was adjusted to 5.3 with 1 M hydrochloric acid, and pepsin was added to reach an activity of 268 U/ml in the final mixture. No gastric lipase was added since the samples did not contain lipids. Digestions were performed at 37°C for 60 min with continuous shaking at 20 rpm. After 60 min of gastric digestion, the pH was raised to 6.6 with 1 M sodium hydroxide, followed by the addition of simulated intestinal fluid (SIF), which was composed of 164 mM sodium chloride, 10 mM potassium chloride and 85 mM sodium bicarbonate, in a 62:38 gastric phase:SIF ratio. Then, calcium chloride, bile salts and pancreatin were added to reach a concentration of 3 mM calcium chloride, 3.1 mM bile salts, and 16 U/ml trypsin in the final mixture, and intestinal digestions were performed at 37°C for 60 min with continuous shaking of 20 rpm. Digestions were stopped after 60 min of gastric digestion (G60) by raising the pH to 6.6 with 1 M sodium hydroxide and after 10 and 60 min of intestinal digestion (I10, I60) by adding 50 µl of 0.1 M Pefabloc per ml digest. In addition, gastric digests without pepsin were prepared as a reference (G0). Digestions were performed in triplicate.

3.2.11 SDS-PAGE of digested samples

Reducing SDS-PAGE was conducted after digestion to monitor the disappearance of intact protein. Samples were prepared and gels were run as described in section 3.2.6, with the exception that 7 µg protein was loaded on the gels.

3.2.12 Degree of hydrolysis

The o-phthaldialdehyde (OPA) assay was performed to determine the degree of hydrolysis as described previously [19]. An acid hydrolysate was created by heating 4 mg protein per ml 6 M hydrochloric acid at 110°C for 22h, which was used to determine the total content of free amino groups in the sample. After the pH of the acid hydrolysate was neutralized with 1 M sodium hydroxide, 0.5 ml neutralized acid hydrolysate, undigested (G0) and digested (G60, I10, I60) samples were precipitated with 0.83 ml of 5% trichloroacetic acid (TCA). In addition, an L-leucine standard curve ranging from 5-50 µM was prepared. Intestinal digestion samples were three times diluted before measurement, whereas the undigested samples, G60 samples and acid hydrolysate remained undiluted. A mixture of 200 µl freshly prepared OPA reagent and either 10 µl sample, acid hydrolysate or L-leucine standard was incubated in the dark at RT for 15 min. The absorbance was measured at 340 nm using a

Spectramax M2 microplate reader (Molecular Devices). The degree of hydrolysis was calculated using the following formula:

$$\text{Degree of hydrolysis} = \frac{(NH_2 \text{ final} - NH_2 \text{ initial})}{(NH_2 \text{ acid} - NH_2 \text{ initial})} \cdot 100\%$$

in which NH_2 final, NH_2 initial and NH_2 acid are the concentrations of free amino groups in the digested sample, undigested sample and acid hydrolysate, respectively.

3.2.13 Peptidomics

Peptides in the intestinal digests from UH, WH-40 and DH-72 were analyzed with LC-MS/MS. Samples were prepared and measured according to Zenker et al. [20]. In brief, 75 µl digest was mixed with 75 µl 20% TCA, and this mixture was centrifuged at 3000g at 4°C for 10 min. Supernatants were cleaned up with in-house made solid phase extraction (SPE) C18 stage tip columns [21]. After SPE, samples were concentrated to 15 µl or less with an Eppendorf concentrator at 45°C, and the volume was adjusted to exactly 50 µl with 1 ml/l HCOOH to compensate for the dilution during TCA precipitation. The samples were analyzed with a Thermo nLC 1000 system (Thermo Fisher Scientific) coupled to an Orbitrap Exploris 480 (Thermo Fisher Scientific) with settings according to Zenker et al. [20]. LC-MS/MS data were analyzed with MaxQuant v2.2.0.0 with the Andromeda search engine [22]. Digestion mode was set to unspecific. A first run was performed with a bovine milk peptide database, which is available on ProteomeXchange with idenitification number PXD003011 [23], and a common contaminant database. Peptides with a length between 6 and 50 amino acids (AAs) were identified, and no modifications were included. Milk proteins that were detected in the first run were included in the second run, together with the whole common contaminant database. In the second run, peptides with a length between 6 and 25 AAs were identified since no peptides larger than 25 AAs were detected in the first run. Variable modifications were set for ST-phosphorylation, NQ-deamidation and M-oxidation. To identify glycated peptides, also variable modifications were included for K-lactosylation (+324 Da), K-furosine (+108 Da), K-pyrraline (+108 Da) and K-carboxymethyllysine (+58 Da). Peptides that were detected in at least two out of three biological replicates were included, and average peptide count (number of different peptides) and intensities (abundancies) according to the MaxQuant output were presented. Peptide alignment over the proteins was investigated by use of the peptide alignment tool Peptigram [24]. Peptides that were identical to peptides in the milk bioactive peptide database [25] were considered bioactive peptides.

3.2.14 Statistical analysis

Statistical analysis was performed with GraphPad prism v8.0.2 (GraphPad Software). Differences between heat treatments were analyzed through one-way ANOVA and Tukey's multiple comparisons test. Differences were considered significant if $p<0.05$.

3.3 Results

3.3.1 Characterization of milk proteins after heat treatment

An infant formula (IF) model system was made from raw bovine milk, which had a 40:60 casein:whey ratio, and a 12:70 protein:lactose ratio. After the model system was either wet or dry heated, the protein composition was visualized by use of both reducing and non-reducing SDS-PAGE (Figure 3.1). After wet heating, all bands on the reducing gel remained on the same position compared to unheated IF model system, whereas after dry heating, the bands of all proteins shifted upwards under both reducing and non-reducing conditions. No large aggregates were detected after dry heating on the non-reducing and reducing gel, whereas aggregates and a reduced band intensity for all whey proteins were observed with increasing wet heating duration, especially under non-reducing conditions.

With increasing wet heating duration, the whey protein denaturation percentage increased, resulting in samples with different whey protein denaturation levels (Table 3.1). Lysine blockage, furosine and CML levels increased with increasing dry heating duration but not with increasing wet heating duration. Whereas lysine blockage and furosine levels were significantly increased for all DH samples compared to UH and WH samples, CML levels were only significantly increased in the DH-24 and DH-72 samples, which were dry heated for a longer duration. In addition, changes in surface hydrophobicity and fibril structure were determined. After wet heating, both the surface hydrophobicity and fibril structure significantly increased compared to both unheated and dry heated IF model system. Dry heating did not change the surface hydrophobicity or fibril structure of the proteins.

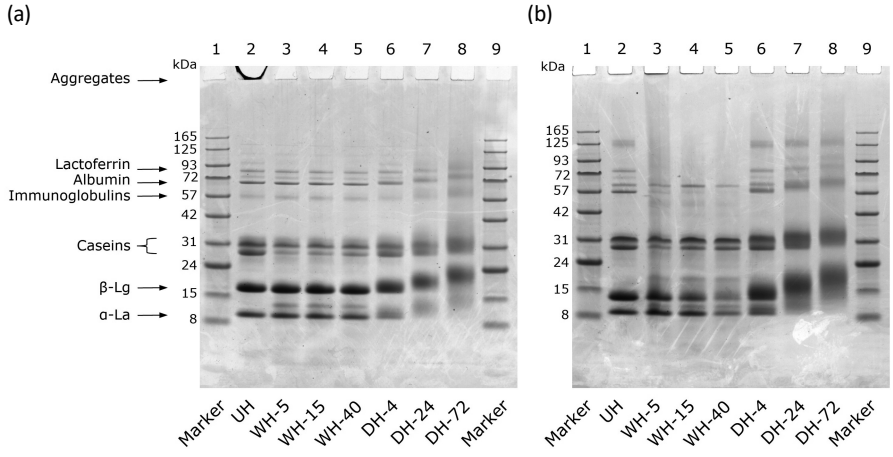

Figure 3.1: SDS-PAGE of unheated (UH), wet heated (WH) and dry heated (DH) infant formula (IF) model system under (a) reducing conditions, (b) non-reducing conditions. Lane 1 and 9: marker. Lane 2: UH IF model system. Lane 3-5: WH IF model system, which was heated at 80°C for 5 (WH-5), 15 (WH-15) and 40 min (WH-40). Lane 6-8: DH IF model system, which was heated at 60°C for 4 (DH-4), 24 (DH-24) and 72h (DH-72). Identification of the protein bands was done by comparison with literature [26] and the UniProt database. β-Lg: β-lactoglobulin, α-La: α-lactalbumin.

Table 3.1: Characterization of the unheated (UH), wet heated (WH) and dry heated (DH) infant formula (IF) model system. WH IF model system was heated at 80°C for 5 (WH-5), 15 (WH-15) and 40 min (WH-40), and DH IF model system was heated at 60°C for 4 (DH-4), 24 (DH-24) and 72h (DH-72). Whey protein denaturation after wet heating was determined based on the relative decrease in band intensity of β-Lg band compared to the UH sample on the non-reducing SDS-PAGE (Figure 3.1B) with ND meaning not detected. Surface hydrophobicity was determined with the ANS assay, and fibril structure formation was determined with the ThT assay. Lysine blockage, furosine concentration and carboxymethyllysine (CML) concentration were measured with LC-MS/MS. Different letters indicate significant differences ($p<0.05$). RFU: Relative fluorescence units.

IF model system	Whey protein denaturation (%)	Lysine blockage (%)	Furosine (mg/g protein)	CML (µg/g protein)	Surface hydrophobicity (RFU x 10^3)	Fibril structure (RFU x 10^3)
UH	0	1.34 ± 0.28^a	0.34 ± 0.03^a	7.18 ± 4.88^a	19.6 ± 1.1^a	4.6 ± 0.4^a
WH-5	26	0.64 ± 0.13^a	0.19 ± 0.06^a	6.75 ± 4.90^a	32.1 ± 0.4^b	8.1 ± 0.3^b
WH-15	45	0.86 ± 0.05^a	0.25 ± 0.02^a	6.77 ± 5.10^a	35.6 ± 3.3^b	9.8 ± 0.6^c
WH-40	60	1.52 ± 0.32^a	0.43 ± 0.08^a	13.06 ± 4.28^a	39.1 ± 5.8^b	12.3 ± 1.2^d
DH-4	ND	17.9 ± 3.7^b	5.49 ± 1.31	29.29 ± 9.04^a	19.4 ± 1.2^a	43.3 ± 0.5^a
DH-24	ND	41.9 ± 3.2^c	13.47 ± 2.40^c	131.4 ± 70.1^b	20.2 ± 0.7^a	49.9 ± 0.2^a
DH-72	ND	68.3 ± 3.1^d	22.29± 1.88^d	265.3 ± 49.9^c	19.3 ± 0.3^a	50.6 ± 0.2^a

3.3.2 Protein hydrolysis during digestion

After the IF model system samples were characterized, they were digested with an *in vitro* infant digestion model. The disappearance of intact proteins during digestion was monitored with SDS-PAGE under reducing conditions (Figure 3.2). At the end of gastric digestion, whey proteins were still largely intact in all samples. Wet heated samples still contained intact caseins, and contained more intact casein with increasing wet heating duration. Especially

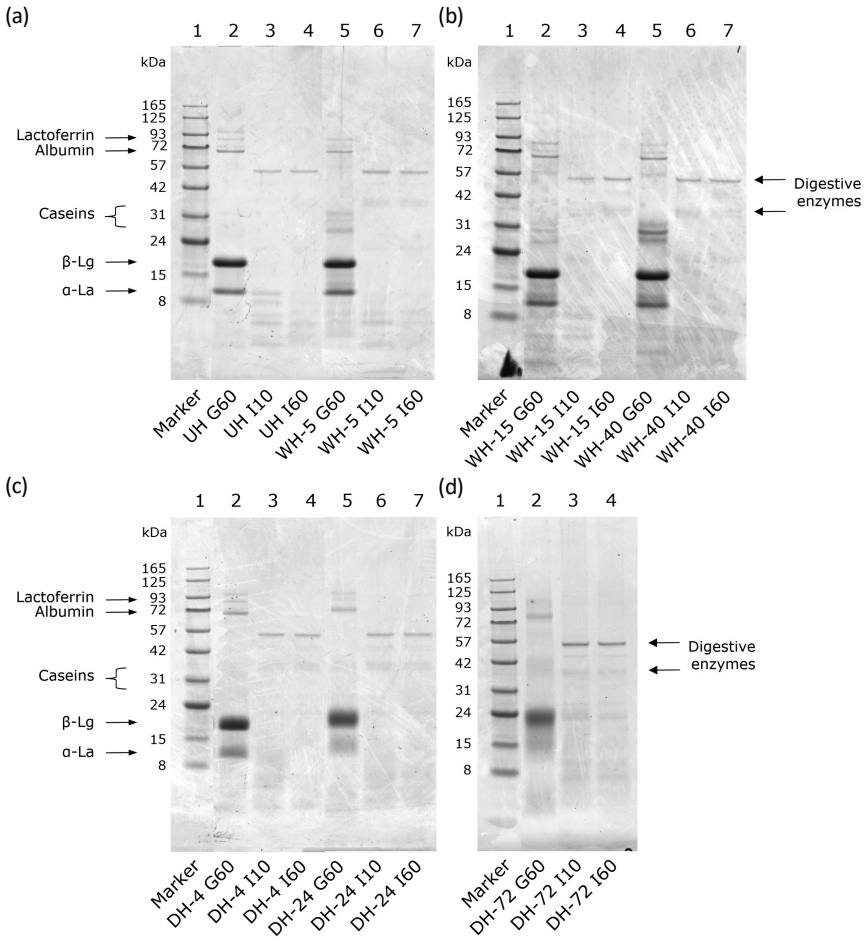

Figure 3.2: Reducing SDS-PAGE of infant formula (IF) model system after *in vitro* infant digestion. (a) unheated IF model system (UH) and wet heated IF model system at 80°C for 5 min (WH-5), (b) wet heated IF model system at 80°C for 15 min (WH-15) and 40 min (WH-40), (c) dry heated IF model system at 60°C for 4h (DH-4) and 24h (DH-24), (d) dry heated IF model system at 60°C for 72h (DH-72). Samples were taken after gastric digestion for 60 min (G60), and after intestinal digestion for 10 min (I10) and 60 min (I60). Lane 1: marker, lane 2 and 5: G60, lane 3 and 6: I10, lane 4 and 7: I60. Identification of the protein bands was done by comparison with literature [26] and the UniProt database. β-Lg: β-lactoglobulin, α-La: α-lactalbumin.

more α_{s1}-casein was intact in WH-40, whereas this protein was largely digested in WH-5 and WH-15 samples at G60. In contrast, no intact caseins were detected at G60 after dry heating. In addition, wet heated samples showed vague peptide bands with a size smaller than 8 kDa, especially in the WH-15 and WH-40 samples. In the intestinal phase, no intact proteins were detected in all samples but some light peptide bands of various sizes smaller than 8 kDa were present.

The degree of hydrolysis during *in vitro* infant gastro-intestinal digestion was measured with the OPA assay (Figure 3.3), and soluble protein content was measured with DUMAS (Figure 3.S1). At G60, all WH samples had a lower degree of hydrolysis but also a lower soluble protein concentration than UH and DH IF model systems. Dry heating did not significantly change the degree of hydrolysis at G60 compared to the unheated sample. During intestinal digestion, no differences were found at I10 but heating affected the degree of hydrolysis at I60. A lower degree of hydrolysis was observed for DH-72 compared to UH, all WH samples and DH-4 but not compared to DH-24. Whereas all WH samples as well as DH-4 and DH-24 samples did not differ from UH, DH-24 had a significantly lower degree of hydrolysis than WH-15 and WH-40.

3.3.3 Peptide composition

Changes in peptide levels after intestinal digestion were investigated at I10 and I60 for UH, WH-40 and DH-72 samples by use of LC-MS/MS. In total, 198 different peptides were detected that originated from 34 different milk proteins. Dry heating resulted in a lower number of different peptides (peptide count) at both I10 and I60 compared to UH and WH-40 (Figure 3.4A). Moreover, larger differences between the heat treatments were found in peptide intensities (Figure 3.4B). UH and DH-72 digests had a higher peptide intensity than WH-40 digests, and the peptide intensity decreased during intestinal digestion for all samples. The peptide length distribution differed slightly in DH-72 compared to UH and WH-40 (Figure 3.4C+D). DH-72 had a relatively higher abundance of peptides between

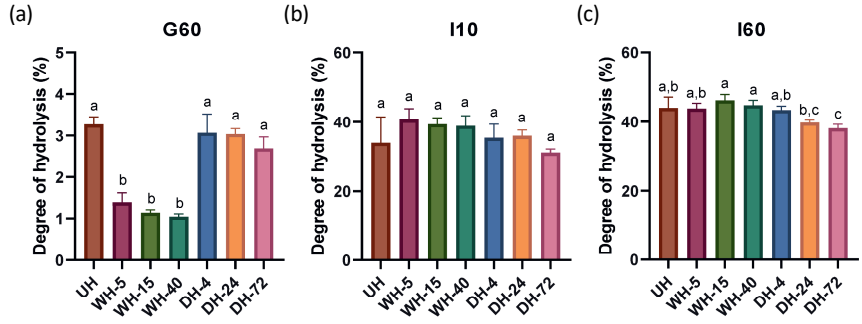

Figure 3.3: Degree of hydrolysis of unheated (UH), wet heated (WH) and dry heated (DH) infant formula (IF) model system after *in vitro* infant digestion. WH IF model system was heated at 80°C for 5 (WH-5), 15 (WH-15) and 40 min (WH-40), and DH IF model system was heated at 60°C for 4 (DH-4), 24 (DH-24) and 72h (DH-72). (a) After gastric digestion for 60 min (G60), (b) after intestinal digestion for 10 min (I10), (c) after intestinal digestion for 60 min (I60). Different letters indicate significant differences ($p<0.05$).

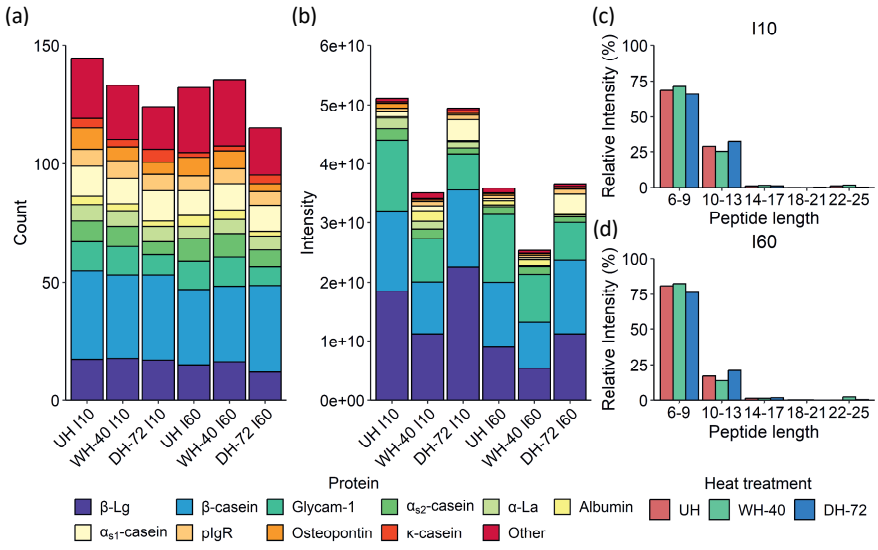

Figure 3.4: Identified peptides in unheated (UH), wet heated (WH-40) and dry heated (DH-72) infant formula (IF) model systems after 10 (I10) and 60 min (I60) intestinal digestion. (a) Count of peptides (number of different peptides) from all milk proteins, (b) intensity of peptides from all milk proteins, (c) peptide length distribution at I10, (d) peptide length distribution at I60. β-Lg: β-lactoglobulin, Glycam-1: Glycosylation-dependent cell adhesion molecule 1, α-La: α-lactalbumin, pIgR: Polymeric immunoglobulin receptor.

10-13 AAs and a relatively lower abundance of peptides between 6-9 AAs than UH and WH-40. All samples contained a high abundance of small peptides with a length between 6-13 AAs, and the peptide length decreased during intestinal digestion.

Focusing on the six major proteins (Figure 3.5), WH-40 digests had a lower peptide intensity compared to UH digests at I10 and/or I60 for all proteins except α_{s1}-casein, which had a similar intensity compared to UH digests. In contrast, DH-72 digests had higher intensities for most proteins, in particular for α_{s1}-casein, β-casein, κ-casein and β-Lg, compared to both UH and WH-40 digests. Lower intensities in DH-72 were measured for α_{s2}-casein at I10 and I60, and for α-La at I10. Whereas the intensities of both caseins and whey proteins decreased between I10 and I60 in UH and WH-40 digests, the intensity of caseins remained relatively stable during intestinal digestion in DH-72 digests.

Relatively more modified peptides were detected for α_{s1}-casein and α_{s2}-casein than for the other proteins in all digests, regardless of heat treatment. Whereas α_{s2}-casein consisted mainly of phosphorylated peptides in all digests, α_{s1}-casein consisted of peptides with many different modifications (i.e. phosphorylation, oxidation, deamidation, lactosylation, CML and pyrraline). DH-72 digests, which showed a higher intensity for peptides from α_{s1}-casein, β-casein, κ-casein and β-Lg, also showed relatively high intensities of peptides containing glycated AAs for these proteins. This was especially the case for α_{s1}-casein, κ-casein and β-Lg, which were highly abundant in peptides with lactosylated AAs.

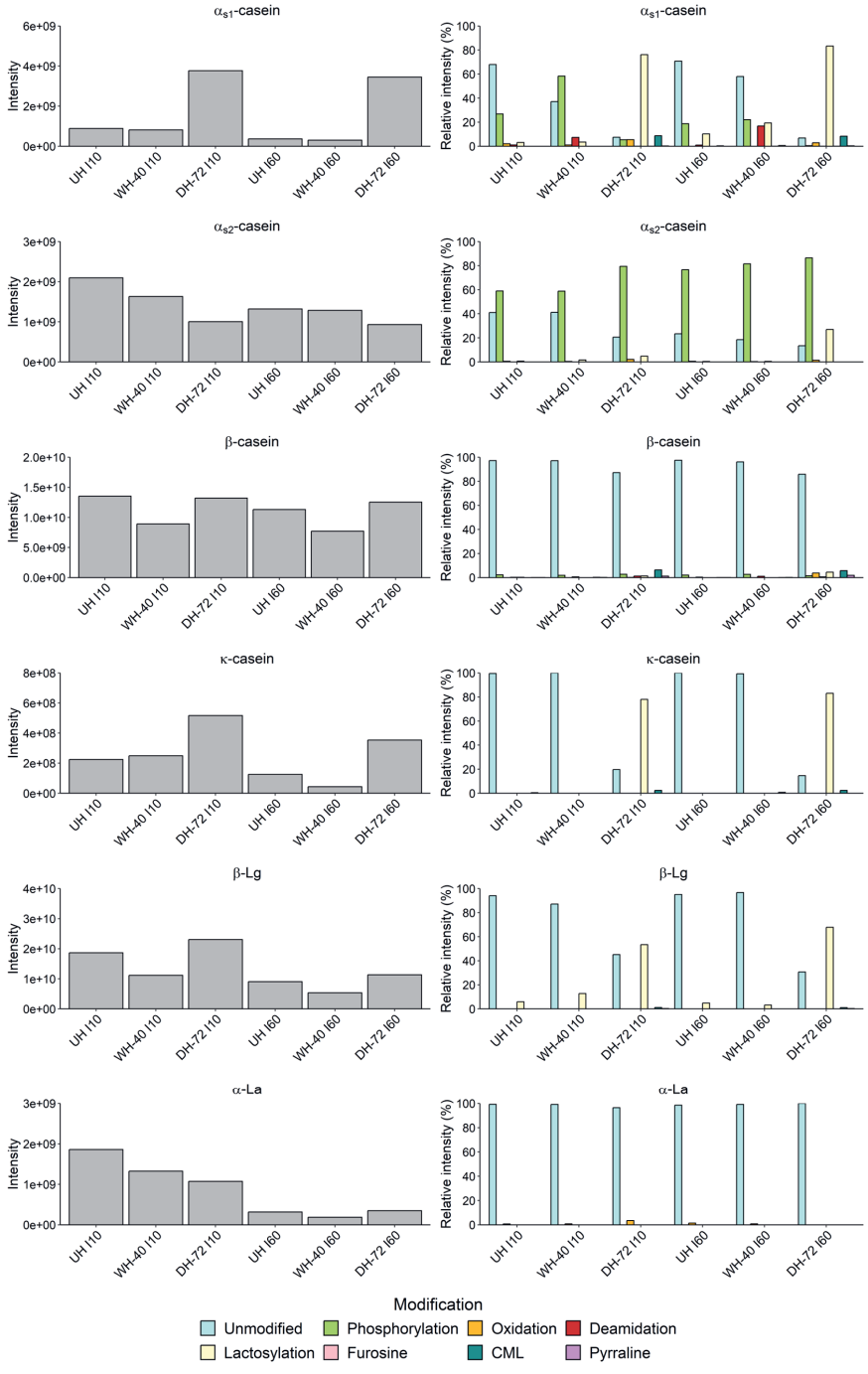

Figure 3.5: (Caption on next page)

Figure 3.5: Intensity (left) and relative intensity of modified peptides compared to total intensity (right) for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). Peptides were identified in unheated (UH), wet heated (WH-40) and dry heated (DH-72) infant formula (IF) model systems after 10 (I10) and 60 min (I60) intestinal digestion. CML: carboxymethyllysine.

Furthermore, the relative intensity of peptides with lactosylated AAs increased between I10 and I60 in DH-72 digests for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein and β-Lg, with an increase between I10 and I60 ranging from 3.1% for β-casein up to 22.1% for α_{s2}-casein.

The peptide distributions of the six major milk proteins were also visualized with a peptide alignment tool (Figure 3.S2). DH-72 digests contained higher intensities of a specific region in α_{s1}-casein, κ-casein and β-Lg, which were located between 80-89 AA, 112-121 AA and 41-45 AA, respectively, not including their signal peptides. These regions had a relatively high intensity of peptides containing glycated AAs, which were located close to cleavage sites for trypsin. UH digests contained a higher intensity of one region in α_{s2}-casein and α-La, which mainly consisted of non-glycated peptides. This region in α_{s2}-casein was located between 25-34 AA and in α-La between 80-90 AA, not including their signal peptides.

3.3.4 Bioactive peptides

The presence of bioactive peptides in the intestinal digests was determined by matching the identified peptides with the milk bioactive peptide database (MBPDB). Seventeen bioactive peptides from five different milk proteins were detected in the digests (Table 3.2). Digests at I10 consisted of approximately 20% bioactive peptides of the total peptide intensity, regardless of heat treatment. At I60, WH-40 digests had a slightly higher relative intensity of bioactive peptides compared to UH digest, and DH-72 digests had an even higher relative intensity of bioactive peptides. The composition of these bioactive peptides differed between the heat treatments at both I10 and I60. At I10, DH-72 digests contained a higher relative intensity for one bioactive peptide from β-Lg, TPEVDDEALEK. This peptide has DPP-IV inhibitory and antimicrobial properties, and was present in the digests in unmodified, lactosylated, CML, and pyrraline modified forms. The intensity of this peptide was decreased at I60, although the DH-72 digests still contained a higher intensity of this peptide compared to the other samples at I60. In addition, higher relative intensities of bioactive peptides from β-casein were detected at I60 for both WH-40 and DH-72. For β-casein, 13 different bioactive peptides were identified with varying bioactive functions. Most of these β-casein peptides were detected in all samples, and were present in similar relative intensities. One peptide from β-casein with prolyl endopeptidase (PEP)-inhibitory properties (VYPFPGPI) had a higher relative intensity in DH-72 at I60.

Table 3.2: Identified bioactive peptides in unheated (UH), wet heated (WH-40) and dry heated (DH-72) infant formula (IF) model system with the milk bioactive peptide database (MBPDB) after 10 (I10) and 60 min (I60) intestinal digestion and their relative intensity compared to total intensity detected with LC-MS/MS with ND meaning not detected. Amino acid (AA) position represents the position of the bioactive peptide within the protein not including its signal peptide. Modification represents which modifications the bioactive peptides contained. PEP-inhibitory: Prolyl endopeptidase inhibitory, ACE-inhibitory: Angiotensin-converting enzyme inhibitory, DPP-IV inhibitory: dipeptidylpeptidase-4 inhibitory, CML: carboxymethyllysine, β-Lg: β-lactoglobulin, α-La: α-lactalbumin.

Protein of origin	Peptide	AA position	Relative intensity (%) at I10			Relative intensity (%) at I60			Bioactive function	Modification
			UH	WH-40	DH-72	UH	WH-40	DH-72		
α_{s2}-casein	NMAINPSK	25-32	0.20	0.25	ND	0.19	0.17	0.09	ACE-inhibitory	Phosphorylation
β-casein	NVPGEIVESL	7-16	0.46	0.29	0.54	0.41	0.55	0.40	Antioxidant	Phosphorylation
	VYPFPGPI	59-66	7.66	8.14	6.75	12.15	14.06	16.35	PEP-inhibitory	Unmodified
	VYPFPGPIP	59-67	0.08	0.07	0.05	0.16	0.10	0.05	PEP-inhibitory	Unmodified
	VYPFPGPIPN	59-68	1.86	2.88	0.05	3.97	3.55	2.38	Antioxidant, ACE-inhibitory	Unmodified, Deamidation
	YPFPGPI	60-66	0.26	0.27	0.23	0.68	0.66	0.38	Increases MUC expression and mucus secretion, immunomodulatory, Opioid, ACE-inhibitory, Antioxidant	Unmodified
	YPFPGPIPN	60-68	ND	ND	0.02	ND	ND	0.03	DPP-IV inhibitory, ACE-inhibitory, Antioxidant	Unmodified
	PFPGPI	61-66	0.05	0.10	0.01	0.06	ND	0.04	Cathepsin B inhibitory	Unmodified
	PFPGPIPN	61-68	1.16	1.75	0.70	2.23	3.11	1.95	ACE-inhibitory	Unmodified, Deamidation
	SLPQNIPPL	69-77	ND	0.23	ND	ND	0.01	0.02	DPP-IV inhibitory	Unmodified
	NIPPLTQTPV	73-82	0.91	0.78	0.96	0.40	0.45	0.45	ACE-inhibitory	Unmodified
	HKEMPFPK	106-113	ND	ND	0.01	ND	ND	ND	Antimicrobial	Furosine
	MPFPKYPVEP	109-118	ND	ND	0.32	ND	ND	0.30	ACE-inhibitory	Lactosylation, CML

Table 3.2: (Continued)

Protein of origin	Peptide	AA position	Relative intensity (%) at I10			Relative intensity (%) at I60			Bioactive function	Modification
			UH	WH-40	DH-72	UH	WH-40	DH-72		
β-casein	HQPHQPLPPT	145-154	1.26	1.94	1.10	0.92	0.70	1.46	ACE-inhibitory	Unmodified
κ-casein	YYQQKPVA	42-49	0.01	ND	0.01	ND	ND	ND	Antimicrobial	Unmodified
β-Lg	TPEVDDEALEK	125-135	6.52	4.29	9.85	1.15	1.44	2.91	DPP-IV inhibitory, Antimicrobial	Unmodified, Lactosylation, CML, Pyrraline
α-La	ILDKVGINY	95-103	0.03	0.01	ND	0.004	0.003	ND	DPP-IV inhibitory	Unmodified
Total			**20.47**	**20.81**	**20.59**	**22.31**	**24.81**	**26.79**		

3.4 Discussion

3.4.1 Effect of wet and dry heating on physio-chemical properties of milk proteins

This study aimed to directly compare the effect of wet and dry heating on the digestion of milk proteins in infants. First, an IF model system was made and was either wet heated or dry heated for different durations to induce different levels of protein denaturation or glycation, respectively. Whey protein denaturation increased with increasing wet heating duration, resulting in samples with different whey protein denaturation levels (Table 3.1). The denaturation levels ranged from 26 to 60%, which is comparable to the levels found in a previous study in which an IF model system was pasteurized [27]. Dry heating led to increased glycation levels, which was both observed by the upward shift for all proteins in SDS-Page (Figure 3.1) and by the increase in lysine blockage, and furosine and CML formation (Table 3.1). In DH-4, an increased lysine blockage and furosine concentration was observed compared to UH, while the CML level was not significantly increased, indicating early MR [7]. In DH-24 and DH-72, higher lysine blockage levels and furosine concentrations were found, and CML levels were increased compared to UH and DH-4, indicating advanced MR. Furosine and CML concentrations in the DH samples were similar to those in commercial IFs [28]. They ranged from the lowest to the highest furosine and CML concentrations measured in commercial IFs, but the majority of commercial IFs contained furosine and CML concentrations similar to DH-24. No significant increase in MRPs was observed after wet heating, demonstrating that the used wet heating conditions did not induce glycation.

Surface hydrophobicity was significantly increased after wet heating but did not increase to a higher level with increasing wet heating duration (Table 3.1). During wet heating at 80°C, whey proteins unfold, which exposes hydrophobic regions that are hidden in the interior of native proteins, increasing the surface hydrophobicity. However, simultaneously with protein denaturation, aggregation can take place via hydrophobic or -SH/S-S interchange reactions. This can shield exposed hydrophobic regions after unfolding, thereby leading to a decreased surface hydrophobicity [29]. Since some large aggregates (>200 kDa) were detected at the top of the non-reducing SDS-PAGE (Figure 3.1B) in the WH samples, the combined effect of these two processes of denaturation and aggregation may explain why the surface hydrophobicity increased after wet heating but did not further increase with longer wet heating durations. A similar effect was seen in a study in which whey proteins were heated at 85°C for different durations [30]. These authors showed that the surface hydrophobicity steeply increased during the first 10 min of heat treatment and only slightly increased during longer heating durations. In contrast, dry heating did not affect the surface hydrophobicity regardless of heating duration. The surface hydrophobicity was expected to decrease after dry heating due to glycation, in which hydrophilic lactose binds to the milk proteins. The unchanged surface hydrophobicity after dry heating may be a combination of the binding of hydrophilic sugars, which decrease surface hydrophobicity, and glycation-induced aggregation, which shields these hydrophilic regions in the aggregates. Although no aggregates larger than 200 kDa were observed on top of the non-reducing SDS-PAGE, some smears were present (Figure 3.1B), indicating that smaller glycation-induced aggregates were probably formed during dry heating. The change in surface hydrophobicity after dry

heating seems to depend on the exact combination of heating temperature, water activity and the type of milk proteins, which may explain why decreases [31], no changes [16] as well as increases [32,33] in surface hydrophobicity have previously been reported after dry heating.

In addition, an increased fluorescence intensity in the ThT assay was measured with increasing wet but not dry heating duration (Table 3.1), which is in agreement with previous studies in which β-Lg alone was either wet or dry heated in the presence of lactose [16,34]. Although this increase in fluorescence intensity may suggest that wet heating resulted in increased fibril structure levels, ThT is known to bind to both fibril structures and hydrophobic regions [35]. The increased fluorescence intensities after wet heating were, therefore, most likely caused by the increase in surface hydrophobicity levels (Table 3.1) and not by an increase in fibril structure. Since dry heating did not result in changed surface hydrophobicity, the unchanged fluorescence intensities in the ThT assay indicate that dry heating did not cause the formation of fibril structures.

3.4.2 Effect of wet and dry heating on digestion of milk proteins

The milk proteins with different heat-induced physio-chemical changes were digested, and the effect of these changes on their digestion behavior were evaluated. At the end of gastric digestion, casein digestion was decreased after wet heating, with more pronounced decreases with increasing wet heating duration. In contrast, no intact caseins were present anymore in UH and all DH samples, and whey proteins remained largely intact in all samples during gastric digestion (Figure 3.2). The slower gastric casein digestion after wet heating was probably caused by denatured whey proteins that bound to the outside of casein micelles, thereby preventing pepsin from reaching the cleavage sites within the caseins, which was also reported in a previous study [36]. The survival of β-Lg and α-La during gastric infant digestion was in agreement with one study [37], whereas other studies showed an increase in gastric whey protein digestion after wet heating, which has been related to denaturation and subsequent increased accessibility of cleavage sites [8,38]. The difference between studies may be caused by the type of whey aggregates that were formed during heat treatment, as reported by Peram et al. [39]. They showed that high molecular weight aggregates of β-Lg were rapidly digested by pepsin, whereas lower molecular weight aggregates were either not digested or more slowly digested. The degree of hydrolysis at G60 was lower for WH samples compared to UH and DH samples (Figure 3.3), which was mainly caused by a decrease in soluble protein concentration in WH samples during gastric digestion (Figure 3.S1). During wet heating, whey proteins denature and aggregate with caseins, resulting in whey proteins ending up in the gastric clot [40]. This leads to a decreased soluble protein concentration compared to raw milk, whose gastric clot consists only of caseins. As the degree of hydrolysis was colorimetrically evaluated, insoluble protein could not be included in the measurement, resulting in a lower degree of hydrolysis for samples with less soluble protein. The unchanged degree of hydrolysis of DH-4 during gastric digestion is in line with literature [41]. However, DH-24 and DH-72 also showed no change in degree of hydrolysis during gastric digestion, whereas a lysine blockage level of 44.5%, which corresponds to the lysine blockage level of the DH-24 sample, was previously reported to result in a reduced degree of hydrolysis during gastric digestion [41].

During intestinal digestion, caseins and whey proteins were quickly digested into peptides of various sizes (Figure 3.2), and the degree of hydrolysis increased compared to gastric digestion for all samples (Figure 3.3). At the end of intestinal digestion, a lower degree of hydrolysis was measured for DH-72 compared to UH, DH-4 and all WH samples, and a lower degree of hydrolysis was measured for DH-24 compared to WH-15 and WH-40 samples. This indicates that dry heating for a long duration decreased milk protein digestibility, and that the difference in the digestibility between wet and dry heated milk proteins was larger than between unheated and heated milk proteins. The decreased degree of hydrolysis after dry heating is in line with previous studies [41,42], although they also reported a decreased protein digestion at lower glycation levels, whereas we only found a reduced protein digestibility at high glycation levels. The unchanged degree of hydrolysis of wet heated compared to unheated milk proteins was also in agreement with literature [9,10], but sometimes a slight increase in protein digestion was reported after wet heating [43].

3.4.3 Effect of wet and dry heating on peptides after intestinal digestion

Peptides in intestinal digests from UH, WH-40 and DH-72 were identified with LC-MS/MS. Digests from WH-40 had a lower peptide intensity than digests from UH and DH-72 (Figure 3.4), which was most likely caused by an increased protein digestion after wet heating. This was supported by the decrease in peptide intensity during intestinal digestion, indicating that peptides were broken down to a length that was too small (<6 AAs) to detect with the used LC-MS/MS method, and by the degree of hydrolysis data, which ranged from 30 to 45% during intestinal digestion (Figure 3.3), demonstrating that the average peptide length was 2-3 AAs. The increased milk protein digestion after wet heating was in line with literature [44,45]. DH-72 contained a lower number of different peptides (peptide count) than UH and WH-40 (Figure 3.4A), a similar peptide intensity as UH (Figure 3.4B), and a longer average peptide length compared to UH and WH-40 (Figure 3.4C+D). This indicates that dry heating decreased milk protein digestion, which was also observed in the lower degree of hydrolysis for DH-72 (Figure 3.3). The decreased protein digestion after dry heating is in line with literature in which dry heated milk peptides were detected with LC-MS/MS after *in vitro* digestion [20,41]. In these studies also longer peptide lengths and a lower number of different peptides were observed after dry heating.

The effect of wet and dry heating on peptide intensity differed per milk protein. WH-40 digests contained a lower α-La, β-Lg, and β-casein peptide intensity at both I10 and I60 and a lower α_{s1}-casein, α_{s2}-casein and k-casein peptide intensity at either I10 or I60 (Figure 3.5). The increased digestion of wet heated whey proteins has often been reported [44,45], and was caused by an increased accessibility to cleavage sites of whey proteins after unfolding. Literature on the effect of wet heating on casein digestion is, however, contradictory. A previous study also showed an increased digestion of κ-casein by trypsin after wet heating, which was suggested to be caused by a less defined structure of κ-casein after heat treatment, which made cleavage sites more available [44]. However, another study showed a decreased casein digestion after heat treatment [46]. The used heat treatments in both these studies probably resulted in protein denaturation and glycation, indicating that depending on the exact heating conditions and induced protein modifications,

wet heating can result in either an increased or decreased casein digestion. Dry heating resulted in a higher intensity for β-Lg, α_{s1}-casein, β-casein and κ-casein peptides at both I10 and I60, and a lower intensity for α-La peptides at I60 and for α_{s2}-casein peptides at both I10 and I60. These differences in intensity seemed to correlate with the relative intensity of peptides containing glycated AAs (Figure 3.5), indicating that peptides containing glycated AAs were digested slower than peptides without glycated AAs. This was in line with the increase in relative intensity of peptides with glycated AAs between I10 and I60 (Figure 3.5) and with the high intensities of peptides with glycated AAs that were located close to trypsin cleavage sites (Figure 3.S2). The position of highly glycated regions in the proteins were in agreement with literature [47], which could have blocked the cleavage sites for digestive enzymes, as shown previously [48]. In contrast, dry heating resulted in a decreased α-La intensity at I10 and a decreased α_{s2}-casein intensity during the whole intestinal digestion, indicating a faster digestion of these proteins after dry heating. This might be due to the low level of glycated peptides originating from these proteins (Figure 3.4), in which cleavage sites were not blocked. However, in a previous study both α-La and α_{s2}-casein were shown to contain highly glycated lysine residues after dry heating [47]. The higher detection of glycated peptides in that study may be caused by the use of several combinations of proteases in the LC-MS/MS sample preparation to maximize peptide identification, whereas always the same combination of proteases was used in our study.

3.4.4 Bioactive peptides after intestinal digestion

DH-72 digests contained a higher relative intensity compared to UH and WH-40 digests for one bioactive peptide from β-Lg at both I10 and I60 and for one bioactive peptide from β-casein at I60 (Table 3.2). This peptide from β-Lg, TPEVDDEALEK (AA position 125-135), was relatively more glycated in DH-72 and has DPP-IV inhibitory and antimicrobial properties. This peptide has previously been reported to be resistant to gastro-intestinal digestion [20,49], and was shown to be more resistant to digestion after glycation [50]. However, whether these bioactive properties of TPEVDDEALEK are retained after glycation is currently unknown. At I60, DH-72 digests contained the highest relative intensities of bioactive peptides, followed by WH-40 digests, whereas UH digests contained the lowest relative intensities of bioactive peptides. This difference was mainly caused by the abundance of one peptide from β-casein (VYPFPGPI), which has PEP inhibitory properties. Although this peptide had a higher relative intensity after dry heating, it was not detected in a glycated form. The survival of peptides from this region in β-casein (AA position 59-66) is in agreement with literature [20]. Further research is needed to determine whether these differences in bioactive peptides at I10 and I60 lead to an overall difference in bioactivity of the digests. In addition, their resistance to intestinal brush border enzymes as well as translocation rate over the intestinal epithelial lining is of interest for future research.

3.5 Conclusion

This study aimed to directly compare the effect of wet versus dry heating on the digestion of milk proteins in infants. Wet heating for longer durations resulted in samples with increased whey protein denaturation levels and a higher surface hydrophobicity, without inducing glycation. Dry heating for longer durations led to samples with increasing glycation levels, at different stages in the Maillard reaction, but no changes in surface hydrophobicity. During gastric digestion, whey protein digestion was not affected by either wet or dry heating, but caseins were hydrolyzed slower with increasing wet heating duration, which was probably due to the formation of casein-whey complexes during wet heating. During intestinal digestion, wet heating resulted in a faster digestion of the most abundant milk proteins, most likely because of unfolding and an increased surface hydrophobicity after wet heating, which increased the accessibility of cleavage sites in the proteins. In contrast, dry heating led to a slower digestion of milk proteins due to glycation, which can decrease the accessibility of cleavage sites for digestive enzymes. Dry heating also resulted in a relatively higher abundance of two bioactive peptides: one with DPP-IV inhibitory and antimicrobial properties and one with PEP-inhibitory properties. Moreover, digestion of milk proteins differed more between wet and dry heated proteins than between unheated and either wet or dry heated proteins.

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Supplementary information

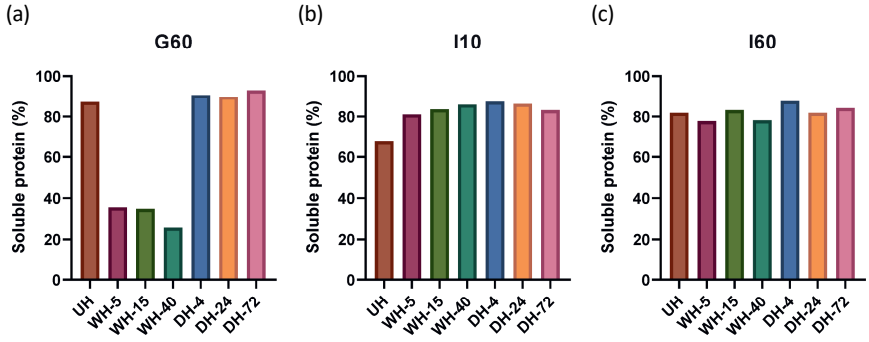

Figure 3.S1: Percentage soluble protein of unheated (UH), wet heated (WH) and dry heated (DH) infant formula (IF) model system after *in vitro* infant digestion (n=1). WH IF model system was heated at 80°C for 5 (WH-5), 15 (WH-15) and 40 min (WH-40), and DH IF model system was heated at 60°C for 4 (DH-4), 24 (DH-24) and 72h (DH-72). (a) After gastric digestion for 60 min (G60), (b) after intestinal digestion for 10 min (I10), (c) after intestinal digestion for 60 min (I60).

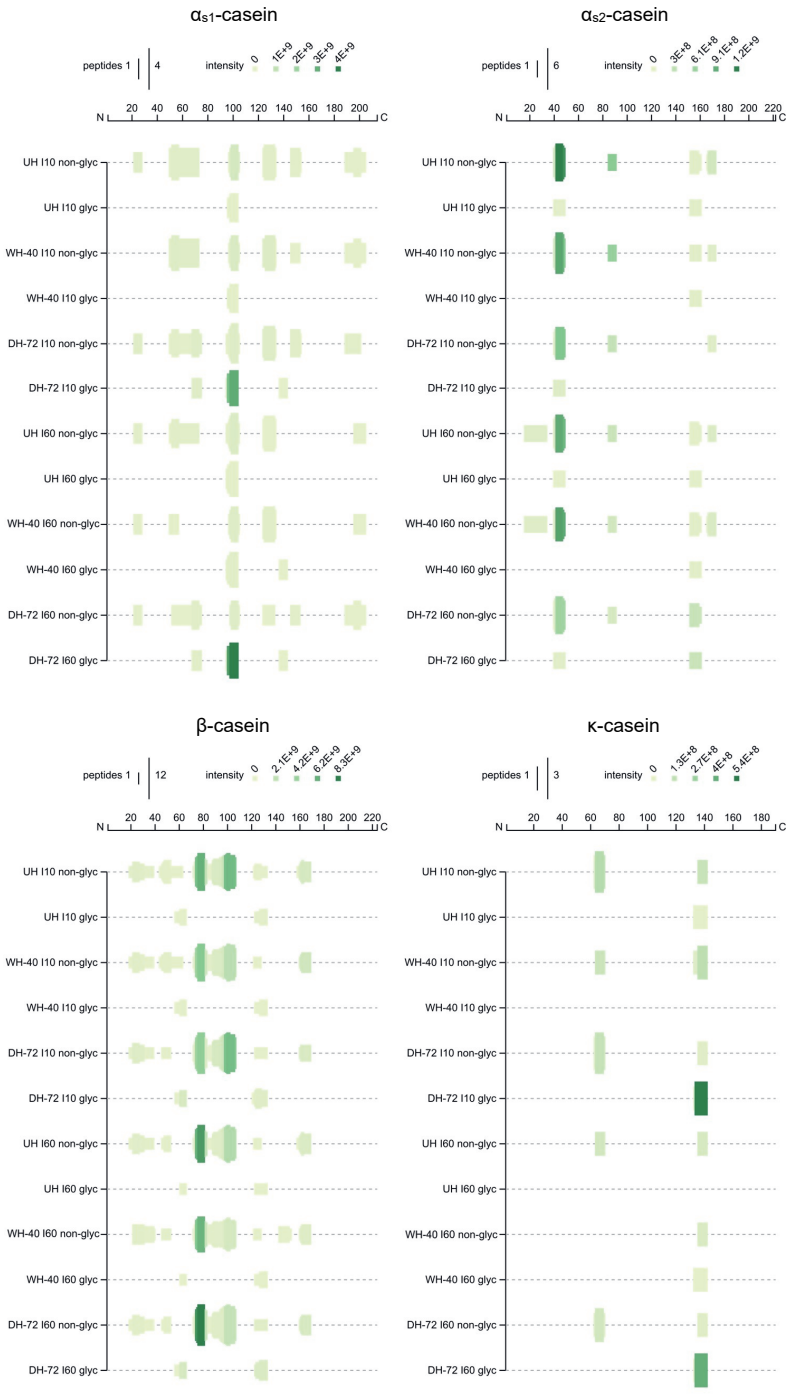

Figure 3.S2: (Continued on next page)

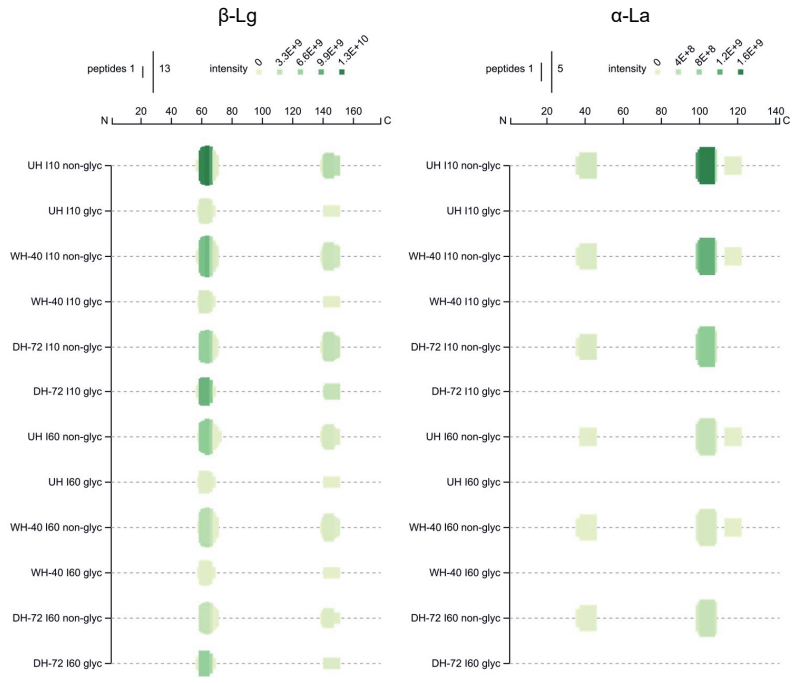

Figure 3.S2: Peptide alignment of α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). The color of the bars represents the sum of peptide intensities that overlap at this position from either peptides without glycated amino acids (non-glyc) or with glycated amino acids (glyc), and the height of the bars represents the number of peptides (peptide count) that overlap at this position. The amino acid position is given including the signal peptides of 15, 15, 15, 21, 16 and 19 amino acids for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-Lg and α-La, respectively.

Chapter 4

Differential effects in mucus production, intestinal transport and immunoreactivity of peptides released after *in vitro* infant digestion of wet and dry heated milk proteins

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Abstract

Milk proteins undergo both wet and dry heating steps during the production of infant formula, which can affect their gastro-intestinal digestion, subsequent peptide formation, and transport of these peptides across the intestinal barrier. Some milk peptides were previously reported to influence intestinal mucus production, and both wet and dry heating was shown to affect the intestinal transport of milk proteins as well as the immune response towards milk proteins. The direct link between dry and wet heating of milk proteins, their digestion, and subsequent mucus production, intestinal transport, and immunoreactivity is, however, still largely unclear. To investigate this, milk proteins remained unheated or were either wet heated to induce denaturation or dry heated to induce glycation. Subsequently, the samples were digested by use of an *in vitro* infant digestion model and peptides were identified with LC-MS/MS. The effect of these peptides on intestinal mucus production was investigated using HT29-MTX-E12 cells and intestinal epithelial transport was investigated with either a Caco-2 monoculture or a Caco-2/HT29-MTX-E12 coculture. Finally, immunoreactivity of the peptides was studied by predicting potential HLA-II and linear IgE epitopes before and after intestinal transport as well as by stimulating primary immature dendritic cells (iDCs) with transported peptides. Both wet and dry heating resulted in higher survival of HLA-II epitopes during intestinal digestion, whereas survival of linear IgE epitopes was similar regardless of heat treatment. Mucus production was not affected by the digestion-derived milk peptides. Wet heating resulted in lower peptide transport in both cell models, whereas dry heating resulted in lower peptide transport in the monoculture but a higher peptide transport in the coculture. In line with this, wet heating led to a lower intestinal transport of HLA-II and linear IgE epitopes in both cell models, whereas dry heating led to a higher intestinal transport of HLA-II in both cell models and of linear IgE epitopes in the coculture. Transported milk peptides did not affect the cytokine production by iDCs. Together, this shows that wet and dry heating affect the digestion of milk proteins, the survival of immunoreactive peptides, and the intestinal transport of milk peptides. However, the effect of wet and dry heating on the immunoreactivity of intestinal and transported milk peptides should be further investigated.

4.1 Introduction

During the production of infant formula (IF), different heat treatments including pasteurization and spray drying are applied to ensure microbiological safety, and to enable easy handling, transportation and storage of the product. Pasteurization is a form of wet heating and results in protein denaturation, whereas spray drying, which can be mimicked on lab scale by dry heating, leads to glycation. Both protein denaturation and glycation have been shown to influence the digestion of milk proteins and subsequent peptide formation during gastro-intestinal digestion [1], which was also observed in our previous study in which we studied the effect of wet versus dry heating of an IF model system on milk protein digestion (Chapter 3). Protein denaturation generally leads to a higher protein digestibility of milk proteins and shorter peptides as a result of more easily accessible cleavage sites after unfolding. In contrast, glycation decreases the digestibility and results in the formation of longer peptides due to the blockage of cleavage sites after the binding of a reducing sugar. Differences in peptide composition can also result in a changed composition of peptides that are transported across the intestinal barrier, which was shown for a digest from dry heated milk proteins [2]. This study showed that glycated milk peptides were transported relatively more across a Caco-2 monolayer compared to non-glycated peptides and were predicted to contain more bioactive and immunomodulatory properties. Another study showed that the intestinal transport of intact β-lactoglobulin (β-Lg) that survived *in vitro* gastric digestion was decreased after wet heating but not after dry heating [3]. This demonstrates that wet and dry heating can have a different effect on the intestinal transport of intact β-Lg, but the effect of wet and dry heating on the intestinal transport of other milk proteins after digestion has not been investigated. Moreover, peptide transport across the intestinal barrier was also shown to be affected by the presence of mucus [4]. Intestinal mucus provides an important defensive barrier and consists of water, lipids, salts, and proteins, of which mucin glycoproteins are the most abundant ones. These mucins are mainly responsible for the gel-like structure of mucus and are usually negatively charged because of their carboxyl and sulfate groups [5]. Due to this gel-like structure and negative charge of mucins, the combination of charge, hydrophobicity and spatial configuration of peptides seem to play an important role in the transport of peptides through a mucus layer [6]. In addition, some peptides can modulate the composition or quantity of the produced mucins. An increased mucin expression and production was observed for peptides derived from different milk proteins in both *in vitro* and *in vivo* studies and was thought to be caused by the activation of opioid receptors on mucus-producing cells [7–9]. Such changes in mucus production may impact the strength of the infant's intestinal defensive barrier, and might subsequently affect infant health. However, it remains unknown if mucus production is differently affected by peptides derived from heated compared to unheated milk proteins and how the presence of mucus affects the intestinal transport of milk peptides.

Peptides that are released during intestinal digestion can come into contact with dendritic cells (DCs) both before and after intestinal transport since DCs are able to sample through the intestinal barrier. Wet heating as well as dry heating have been shown to influence the immunoreactivity of milk proteins [10–12]. Heating can result in reduced immunoreactivity, which may be caused by the loss of conformational epitopes, or by decreased accessibility of epitopes that are hidden in aggregates [13]. On the other hand,

heating can also lead to increased immunoreactivity, probably due to exposure of hidden epitopes after unfolding or formation of neo-epitopes [13]. Especially glycation, resulting in the formation of advanced glycation end products (AGEs), has been shown to influence the capacity of milk proteins to bind to IgE [14– 16] and to several receptors on antigen-presenting cells [10,11,17]. In addition, digestion can change the allergenicity of milk proteins, although the effect seems to differ depending on the used milk proteins and the heating conditions. The increased susceptibility of denatured milk proteins to enzymatic digestion was shown to result in reduced allergenicity by disrupting B-cell epitopes [18]. In contrast. digestion of glycated β-Lg led to a higher survival of structures that can bind receptors on antigen presenting cells [11]. Another study showed that digestion of glycated milk proteins resulted in a lower survival but a similar intestinal transport of IgE epitopes compared to non-glycated milk proteins [2]. However, studies evaluating the immunoreactivity of wet and dry heated milk proteins after intestinal digestion and subsequent intestinal transport are limited, especially under infant digestion conditions. Milk proteins are less extensively hydrolyzed under the milder digestion conditions of infants compared to adults [19], resulting in a different composition of digestion-derived peptides, which may subsequently lead to changes in intestinal transport and immunoreactivity of those peptides compared to ones released under adult digestion conditions. This study, therefore, aimed to investigate the effect of wet and dry heating on the composition and immunoreactivity of peptides after *in vitro* infant digestion, on intestinal mucus production, and on the composition and immunoreactivity of peptide transported across the intestinal barrier.

4.2 Materials and Methods

4.2.1 Materials

Dulbecco's Modified Eagle Medium with 4.5 g/L D-glucose and L-glutamine and 25 mM HEPES (DMEM) with and without phenol red, MEM non-essential amino acids (NEAA) solution, Trypsin-EDTA (0.25%), Iscove's Modified Dulbecco's Medium (IMDM), and TRIzol Reagent were obtained from Thermo Fisher Scientific. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were purchased from R&D Systems, TNF-α and IL-6 from Miltenyi Biotec, IL-1β from Merck Millipore, and prostaglandin E2 (PGE_2) from Sigma Aldrich. HyClone Fetal Bovine serum was obtained from Cytiva. QPCR primers were purchased from Biolegio BV. Biotinylated wheat germ agglutinin (B-1025) and avidin-peroxidase conjugate (PK-6100) were obtained from Vector Laboratories, Brunschwig, and sulfuric acid was obtained from Brunschwig. Pepsin (P6887), pancreatin (P7545), porcine bile extract (B8631), Dulbecco's Phosphate buffered saline (DPBS) with and without $CaCl^{2+}$/$MgCl^{2+}$, mucin from porcine stomach (M1778), Bovine serum albumin (BSA; A3059), o-phenylenediamine dihydrochloride (P8787) and all other chemicals were obtained from Sigma Aldrich.

4.2.2 Infant formula model system

An infant formula (IF) model system was made from raw bovine milk as described previously (Chapter 3). In brief, the casein:whey ratio was adjusted to 40:60 and the protein:lactose

ratio to 12:70. After the IF model system was lyophilized, it remained unheated (UH), or it was either wet or dry heated to induce denaturation or glycation, respectively. Dry heating was conducted in a desiccator at 60°C for 72h (DH-72) with a saturated potassium iodide solution to obtain a water activity of 0.6. Before wet heating, the IF model system was dissolved in Milli-Q water at a concentration of 12 mg protein/ml. Wet heating was performed in a water bath at 80°C. Once the IF model system had reached 80°C, wet heating was continued for 40 min (WH-40). The IF model system samples were analyzed on protein composition, denaturation, lysine blockage, furosine concentration, carboxymethyllysine (CML) concentration, surface hydrophobicity, and fibril structure in our previous study (Chapter 3). These results showed that UH, WH-40 and DH-72 contained 1.34%, 1.52% and 68.3% lysine blockage, respectively, and WH-40 contained 60% whey protein denaturation.

4.2.3 *In vitro* infant digestion

UH, WH-40 and DH-72 IF model system samples were digested by use of an *in vitro* infant digestion model [19]. Briefly, the IF model system at a concentration of 12 mg protein/ml was mixed with simulated gastric fluid (SGF) in a 63:37 ratio (v:v). SGF was composed of 94 mM sodium chloride and 13 mM potassium chloride. The gastric digestion was started by setting the pH to 5.3 with 1 M hydrochloric acid and adding 268 U/ml pepsin. The samples were placed in an incubator at 37°C for 60 min under rotary movement (20 rpm). After gastric digestion, the pH was increased to 6.6 with 1 M sodium hydroxide, and simulated intestinal fluid (SIF), which was composed of 164 mM sodium chloride, 10 mM potassium chloride and 85 mM sodium bicarbonate, was added in a 62:38 gastric digest:SIF ratio (v:v). In addition, 3 mM calcium chloride, 3.1 mM bile salts, and pancreatin with a trypsin activity of 16 U/ml were added, and the samples were placed in an incubator at 37°C under rotary movement of 20 rpm. After 10 min of intestinal digestion, the digests were taken out of the incubator and were transferred to an Amicon 10 kDa centrifugal filter (Merck). They were centrifuged at 4°C for 30 min to remove digestive enzymes (detoxification of digests to ensure compatibility with cell culture models) and intact proteins, and the filtrate was snap-frozen in liquid nitrogen and stored at -20°C. Water instead of the IF model system was digested with the *in vitro* infant digestion model to obtain a control digest. Digestions were performed in triplicate, and these triplicate samples were pooled for the cell experiments.

4.2.4 HT29-MTX-E12 cell culture and stimulation with intestinal digests

HT29-MTX-E12 cells (ECACC 12040401) were purchased from Sigma Aldrich and were cultured in DMEM supplemented with 10% FBS and 1% NEAA at 37°C with 5% CO_2 and 90% humidity. Cells were split weekly when reaching 80% confluence by washing the cells with 10 ml DPBS, followed by trypsinization with 1 ml trypsin-EDTA (0.25%) for 5 min, and the medium was refreshed three times a week. For the mucin gene expression and mucus production experiments, HT29-MTX-E12 cells were used between cell passages 55 and 58. The cells were seeded in 24 well plates at a concentration of $5 \cdot 10^5$ cells/ml (500 µl; $2.5 \cdot 10^5$ cells/well) and were cultured for 7 days at 37°C, 5% CO_2 and 90% humidity. Culture medium

was refreshed on day 2 and day 4. On day 6, the medium was replaced by medium without FBS and NEAA. On day 7, cells were washed twice with PBS with $CaCl^{2+}$ and $MgCl^{2+}$ to remove excess mucus, and the cells were stimulated for 0.5, 1, 2, or 4h with the 500 µl of 1:1 detoxified intestinal digestion samples diluted in DMEM with 1% pen/strep. This was performed in triplicate, each time using a different passage of cells. In addition, cells that were only stimulated with medium were included as medium control and cells that were treated with 2% Triton X-100 in medium were included as positive control for the LDH cytotoxicity assay. These controls were incubated for 4h. After the 0.5-4h exposure of the cells to the digests, the medium was harvested and split into two parts. One part was centrifuged at 2000g for 5 min for the LDH assay, and one part was centrifuged at 13.500g at 4°C for 5 min for the enzyme linked lectin assay (ELLA) assay. The supernatants were transferred to new tubes. After the medium from the cells was collected, the cells were harvested by adding 600 µl TRIzol to each well. The plates were placed on a plate shaker at 500 rpm for 5 min, after which the cells were transferred to new Eppendorf tubes. All samples were stored at -80°C till further analysis.

4.2.5 LDH cytotoxicity assay

The LDH assay was performed by use of Cytotoxicity Detection Kit (LDH; Roche) according to the manufacturer's protocol to check the cytotoxicity level of the digests on the cells. After the medium from the cells was centrifuged at 2000g for 5 min, 50 µl sample was added in technical duplicate to a 96-well plate. The samples included medium from cells exposed to intestinal digests, a medium control, and a positive control as described in section 4.2.4. In addition, 50 µl DMEM was included on the 96 well plate as blank. Thereafter, 50 µl DMEM and 50 µl LDH reaction mix were added to all wells. The plate was incubated at 25°C for 30 min and absorbance was measured at 490 nm. Absorbance values of the DMEM wells were subtracted from all absorbance values. Cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity} = \frac{\text{Absorbance digest - Absorbance medium control}}{\text{Absorbance positive control - Absorbance medium control}} \cdot 100\%$$

A cytotoxicity level below 10% was considered non-cytotoxic.

4.2.6 Quantification of mucus secretion

The enzyme-linked lectin assay (ELLA) was performed to quantify the mucus production of the HT29-MTX-E12 cells as described previously [9]. In brief, centrifuged samples (as described in section 4.2.4) were diluted 10 or 20 times in 0.5 M sodium carbonate buffer pH 9.6. A 96-well plate was coated overnight at 4°C with 100 µl of the diluted samples and an 11-point calibration curve ranging from 0 to 500 ng mucin/ml. After coating the plate, each of the wells were washed four times by use of PBS with 0.1% Tween-20 (PBS-T) and were blocked with PBS-T with 2% BSA at 37°C for 1h. Thereafter, the wells were washed four times, 100 µl biotinylated wheat germ agglutinin in PBS-T with 2% BSA (1:1000) was added, and the plate was incubated at 37°C for 1h. Then, 100 µl avidin-peroxidase conjugate (1:50000) was added, and the plate was incubated again at 37°C for 1h. The plate was

washed four times with PBS-T, and 100 µl 0.04 mg/ml o-phenylenediamine dihydrochloride solution was added. After incubation in the dark for 10 min, the reaction was stopped by the addition of 25 µl 3 M sulfuric acid, and absorbance was measured at 492 nm.

4.2.7 mRNA isolation and mucin gene expression

The HT29-MTX-E12 cells in TRIzol were thawed, and total RNA of the cells was isolated by use of an RNeasy Mini kit (Qiagen) following the manufacturer's instructions. The quantity and purity of the RNA were checked with the NanoDrop, and the quality of the RNA was checked on a 1% agarose gel, which was stained with SYBR safe gel stain (Thermo Fisher Scientific). RNA samples that had an A260/A280 ratio of 2.0-2.1 and an A260/A230 ratio of >1.5 as measured with the Nano-drop and those that showed two clear bands on the agarose gel were used for cDNA synthesis. cDNA was synthesized by use of an iScript cDNA synthesis kit (BioRad) following the manufacturer's instructions, using 200 ng RNA per reaction. After cDNA synthesis, the reaction mixtures were diluted 2.5 times with MilliQ water to a final volume of 50 µl. For the qPCR reactions, 5 µl diluted cDNA was mixed with 10 µl iQ™ SYBR® Green Supermix (Bio-Rad), and 2.5 µl of the forward and reverse primers (3.2 µM for MUC5A, MUC13, MUC17, SF3A1, RPS18, and HPRT1; 1.6 µM for Cyclophilin A (PPIA) and GAPDH). The forward and reverse primers for the mucin genes were for MUC5A (PrimerBank ID 3334747a1): forward 5'-CAGCACAACCCCTGTTTCAAA-3', reverse 5'-GCGCACAGAGGATGA CAGT-3'; for MUC13 (PrimerBank ID 308736984c2): forward 5'-GATCCCTGTGCAGATAATTC GTT-3', reverse 5'-ACTATGCAAGTCTTGATAGGCCA-3'; for MUC17 (PrimerBank ID 91982771c1): forward 5'-TCTCAGCACGTTAGGACAGGT-3', reverse 5'-TCGAGGTCATCTCAG GGTTGG-3', and the forward and reverse primers for the reference genes were for GAPDH [20]: forward 5'-TGCACCACCAACTGCTTAGC-3', reverse 5'-GGCATGGACTGTGGTCATGAG-3'; Cyclophilin A: forward 5'-TCTTTGGGACCTTGTCTGCAA-3', reverse 5'-CCCACCGTGTTCTT CGACAT-3'; SF3A1 [21]: forward 5'-GGAGGATTCTGCACCTTCTAA-3', reverse 5'-GCGGTAGTAGGCATGGTAA-3'; RPS18 (PrimerBank ID 14165467c2): forward 5'-ATCACCATTATGCAGAATCCACG-3', reverse 5'-GACCTGGCTGTATTTTCCATCC-3'; HPRT1 (Primerbank 164518913c1): forward 5'-CCTGGCGTCGTGATTAGTGAT-3', reverse 5'-AGACGTTCAGTCCTGTCCATAA-3'. The PCR program started with initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s. Then, melt curve analysis was performed by heating at 95°C for 10 s, cooling to 65°C, and increasing to 95°C with increments of 0.5°C per 5 s. Primer amplification efficiencies were tested by performing qPCR reactions with a five-point calibration curve ranging from 1 to 10.000 times diluted cDNA, and the efficiencies ranged from 82 to 101%. Data were analyzed by use of qBASE software [22]. First, quantification cycle (Cq) values were obtained for all genes and samples. Then, geNorm analysis was performed to determine the most stable reference genes, which were HPRT1 and hCycloF. After normalization of the data to these reference genes, relative mucin gene expression levels were obtained by normalizing the samples to the control digest per passage number of the HT29-MTX-E12 cells and per time point (0.5, 1, 2, or 4h of exposure to the detoxified intestinal digests).

4.2.8 Transport of peptides across Caco-2 and Caco-2/ HT29-MTX-E12 cell monolayers

Caco-2 (line HTB-37) cells were purchased from the American Type Culture Collection (ATTC), and were cultured in DMEM with 10% FBS at 37°C with 5% CO_2 and 90% humidity. Medium was refreshed three times a week, and upon reaching 90% confluency, cells were subcultured by trypsinization. Caco-2 cells were used between passages 31 and 33. HT29-MTX-E12 cells were cultured as described in section 4.2.4. Caco-2 monocultures or Caco-2/ HT29-MTX-E12 cocultures (90:10) were seeded into 12-well translucent 0.4 µm Transwell™ inserts (Greiner Bio-one) at a concentration of $0.225 \cdot 10^6$ cells/ml. Both apical (500 µl) and basolateral medium (1500 µl) were changed three times a week. Cells were grown for 21 days to develop into small intestinal-like cells. After 21 days, trans epithelial electrical resistance (TEER) was measured, and only wells with a TEER value >400 $\Omega \cdot cm^2$ were used. After apical and basolateral medium was removed, 1500 µl medium without phenol red with 1% pen/strep was added to the basolateral side and 500 µl 1:1 diluted intestinal digest (detoxified, as described in section 4.2.3) in medium without phenol red with 1% pen/strep was added to the apical side. This was performed in triplicate, each time using a different passage of cells. After 0, 1, 3 and 6h, TEER values were measured. After 6h, samples from the apical and basolateral sides were collected and were stored at -80°C till further analysis. Cells that were exposed to DMEM for 6h were included as medium control, and cells that were exposed to DMEM for 6h and subsequently exposed to 2% Tritin X-100 in DMEM were included as positive control for the LDH cytotoxicity assay. The LDH assay was performed to evaluate the cytotoxicity of the exposure samples as described in section 4.2.5.

4.2.9 Peptidomics

Peptides were measured in the detoxified intestinal digests and in the basolateral media after 6h of intestinal transport by use of LC-MS/MS. Sample preparation of the intestinal digests for LC-MS/MS measurement was performed as described previously (Chapter 3), whereas sample preparation of the basolateral media was performed with eight times as much sample volume and trichloroacetic acid (TCA) to compensate for the dilution during the intestinal transport experiment compared to the intestinal digestion samples. The samples were run on a Thermo nLC 1000 system (Thermo Fisher Scientific) coupled to an Orbitrap Exploris 480 (Thermo Fisher Scientific) with settings as described previously [2]. Data were analyzed with MaxQuant version 2.2.0.0 with the Andromeda search engine [23]. The digestion model was set to unspecific, and the same bovine milk database was used as described previously (Chapter 3) as well as a common contaminant database. This bovine milk database included all proteins that were detected after the intestinal digests were matched to a larger bovine milk database, which is available on ProteomeXchange with the identification number PXD003011 [24]. Peptides ranging from 6 to 25 amino acids (AAs) in length were identified, and variable modifications were set for ST-phosphorylation, NQ-deamidation, K-lactosylation, and K-carboxymethyllysine. Peptides that were present in at least two out of three biological replicates of the intestinal digests or basolateral digests were included, and average peptide intensities (abundancy) and peptide count (number of different peptides) were reported as calculated by MaxQuant. A peptide alignment tool

[25] was used to visualize the distribution of the detected peptides over the milk proteins, and peptides that exactly matched with peptides in the milk bioactive peptide database [26] were considered bioactive peptides. Predication of HLA-II binding epitopes and linear IgE epitopes was performed as described previously by Zenker et al. [2]. In brief, the IEDB MHC-II Binding Predictions tool was used to predict HLA-II binding epitopes (http://tools.iedb.org/mhcii/). Predictions were performed by use of the recommended 'NetMHCIIpan 4.1 BA' prediction method, and by use of the full HLA reference set, which included 27 HLA alleles. Peptides >11 AAs in length were included in the prediction, and peptides were considered potential HLA-II binding epitopes if they had a percentile rank <10%, as recommended by the prediction tool. Linear IgE epitopes in the intestinal digests and basolateral media were determined by comparing with literature in which known linear IgE binding epitopes were reviewed [27]. Peptides that contained at least 80% of a known IgE epitope were considered potential IgE binding epitopes. The AA position of peptides within the proteins was presented including their signal peptides for their distribution over the proteins (peptide alignment), and excluding their signal peptides for the identified bioactive peptides and the predicted HLA-II and linear IgE epitopes.

4.2.10 Cytokine measurement in basolateral medium

Cytokine production by Caco-2 and HT29-MTX-E12 cells in the basolateral medium was measured by performing a LEGENDplex assay (BioLegend) according to the manufacturer's protocol. The Human Inflammation Panel 1 was used, which included IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1 (CCL2), IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33. The samples were measured by use of a CytoFLEX flow cytometer (Beckman Coulter), and data were analyzed with LEGENDplex software (BioLegend).

4.2.11 Dendritic cell culture and stimulation with basolateral medium

Buffy coats were obtained from healthy donors (Sanquin), and primary human monocytes were isolated from the buffy coats as described previously [28]. Monocytes were differentiated into immature dendritic cells (iDCs) by culturing them in 96 well plates in IMDM supplemented with 10% FBS, 1% pen/strep, 30 ng/ml IL-4, and 50 ng/ml GM-CSF. Cells were incubated at 37°C with 5% CO_2. After 3 days, half of the medium was refreshed by new IMDM with 10% FBS, 1% pen/strep, 60 ng/ml IL-4 (final concentration: 30 ng/ml) and 100 ng/ml GM-CSF (final concentration: 50 ng/ml), and the cells were cultured for another 3 days. Thereafter, the medium was removed and cells were stimulated with 200 µl basolateral medium from the Caco-2 monoculture or Caco-2/HT29-MTX-E12 coculture after 6h of intestinal transport. In addition, iDCs were stimulated with fresh medium (IMDM with 10% FBS, 1% pen/strep, 30 ng/ml IL-4, 50 ng/ml GM-CSF) as non-treated (NT) control, with fresh medium plus 100 ng/ml LPS as dendritic cell type 1 (DC1) control, or with fresh medium plus 50 ng/ml TNF-α, 25 ng/ml IL-1β, 10 ng/ml IL-6 and 1 µg/ml PGE_2 as dendritic cell type 2 (DC2) control. This was performed five times, using iDCs derived from monocytes from five different healthy donors. The NT control with IMDM did not differ from an NT control with DMEM, the medium used to culture Caco-2 and HT29-MTX-E12 cells, as determined in a pilot experiment. Before the samples were applied to the iDCs, the lipopolysaccharide

(LPS) content of the samples was measured as described previously with a Pyros Kinetix Flex tube reader [29]. The LPS content of the samples (<3 pg/ml) was considered too low to affect the iDC response. Samples were harvested after 2 days of stimulation and cytokine concentrations were measured with a LEGENDplex kit with a human macrophage/microglia panel (BioLegend) according to the manufacturer's instruction. This panel consists of IL-12p70, TNF-α, IL-6, IL-4, IL-10, IL-1β, Arginase, TARC, IL-1RA, IL-12p40, IL-23, IFN-γ, and IP-10. Samples were measured with a CytoFLEX flow cytometer (Beckman Coulter), and data were analyzed by use of LEGENDplex software (BioLegend).

4.2.12 Statistical analysis

Statistical analysis of the data was performed by use of GraphPad prism v8.0.2 (GraphPad Software). One-way ANOVA and Tukey's multiple comparisons test were used for comparisons between heat treatments. Differences were considered significant if $p<0.05$.

4.3 Results

4.3.1 Peptides after *in vitro* infant digestion

Peptides in the detoxified intestinal digests from the unheated (UH), wet heated (WH-40) and dry heated (DH-72) IF model system were measured by LC-MS/MS. Overall, 911 peptides were detected originating from 35 different milk proteins. The number of detected peptides (peptide count) was similar for UH and DH-72, and was slightly higher for WH-40 (Figure 4.1A). In all three digests, most peptides originated from α_{s1}-casein, β-casein and β-Lg. The intestinal digests differed more with regard to summed peptide intensity (Figure 4.1B). The peptide intensities of DH-72 and WH-40 were respectively 8% and 36% lower than UH. The low peptide intensity in the WH-40 digest was mainly caused by a decreased intensity of peptides from β-Lg and β-casein compared to UH and DH-72 digests. Peptides in the UH and WH-40 digests consisted mainly of unmodified peptides without post-translational modifications (PTMs) and phosphorylated peptides, whereas peptides in the DH-72 digest had relatively less unmodified peptides and relatively more lactosylation and CML-modified peptides (Figure 4.1C). Most peptides in all digests had a length between 6-13 AAs, but the DH-72 digest contained a lower relative intensity of short peptides (6-9 AAs) and a higher relative intensity of longer peptides (14-17 AAs) compared to UH and WH-40 digests (Figure 4.1D).

The distribution of peptides over the proteins was visualized for the major milk proteins, α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-Lg, and α-La (Figure 4.S1), and per PTM (Figure 4.S2). Especially α_{s1}-casein, α_{s2}-casein, β-casein, and β-Lg contained regions that were highly glycated in the DH-72 digests compared to the UH and WH-40 digests. Whereas the distribution of PTMs over the proteins differed between the heat treatments, the distribution of peptides over the proteins was largely the same for all intestinal digests.

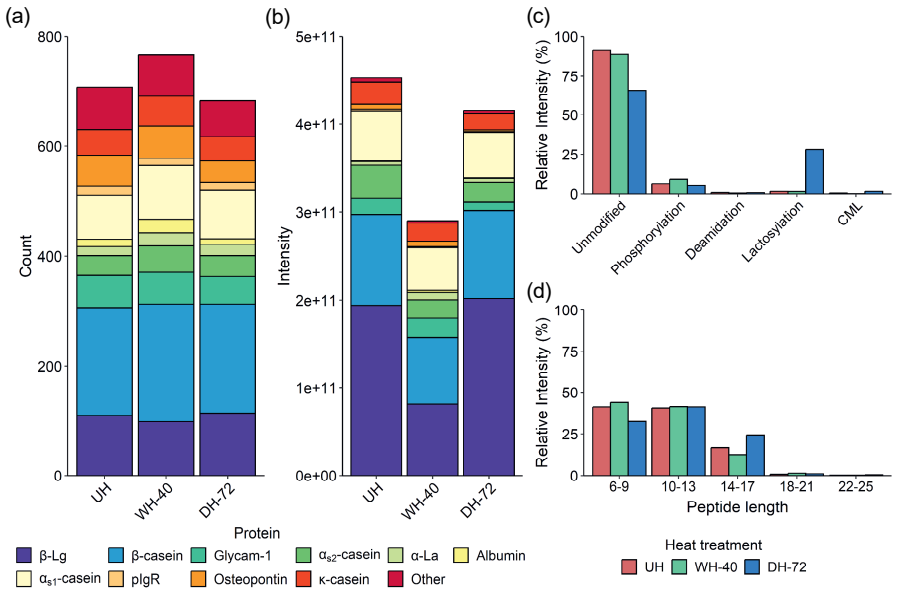


Figure 4.1: Milk peptides detected with LC-MS/MS in the detoxified intestinal digests. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model. (a) Summed peptide count, (b) summed peptide intensity, (c) relative peptide intensity per post-translational modification, (d) relative peptide intensity per peptide length group. β-Lg: β-lactoglobulin, Glycam-1: Glycosylation-dependent cell adhesion molecule-1, α-La: α-lactalbumin, pIgR: polymeric immunoglobulin receptor.

4.3.2 HLA-II epitopes, IgE epitopes and bioactive peptides in the intestinal digests

To evaluate the immunoreactivity of the intestinal digests, potential HLA-II epitopes and linear IgE epitopes were predicted by matching with a database and by matching with literature. Their intensity and peptide alignment are visualized in Figure 4.2, whereas all peptide sequences and their modifications are shown in Tables 4.S1 and 4.S2. WH-40 and DH-72 had a higher intensity of peptides containing HLA-II epitopes compared to UH (Figure 4.2A), and all digests contained a similar intensity of potential IgE epitopes (Figure 4.2C). Most peptides containing HLA-II epitopes were present in all three digests, but the intensities of specific regions containing epitopes highly differed between the heat treatments. WH-40 contained higher intensities of an HLA-II epitope located in the 103-119 AA region of α_{s1}-casein (Figure 4.2B), which was detected in phosphorylated form (Table 4.S1). In contrast, DH-72 contained higher intensities of an HLA-II epitope in the 100-113 AA region of α_{s2}-casein and one in the 164-175 AA region of β-casein (Figure 4.2B), which were mainly detected in glycated forms (Table 4.S1). The distribution of peptides over the proteins was more similar between the heat treatments for the linear IgE epitopes (Figure 4.2D). WH-40 contained a slightly higher intensity of peptides containing an IgE epitope in the 109-120

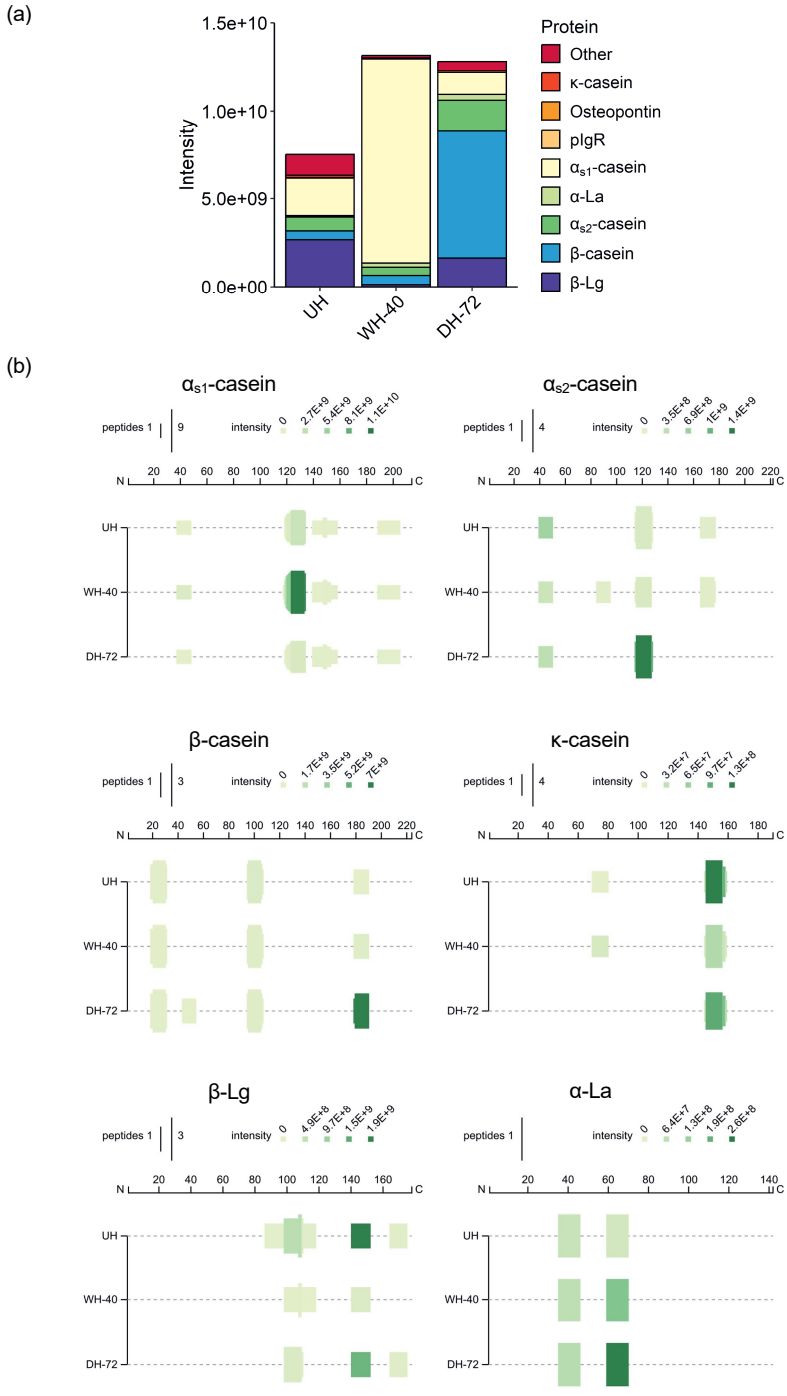


Figure 4.2: (Continued on next page)

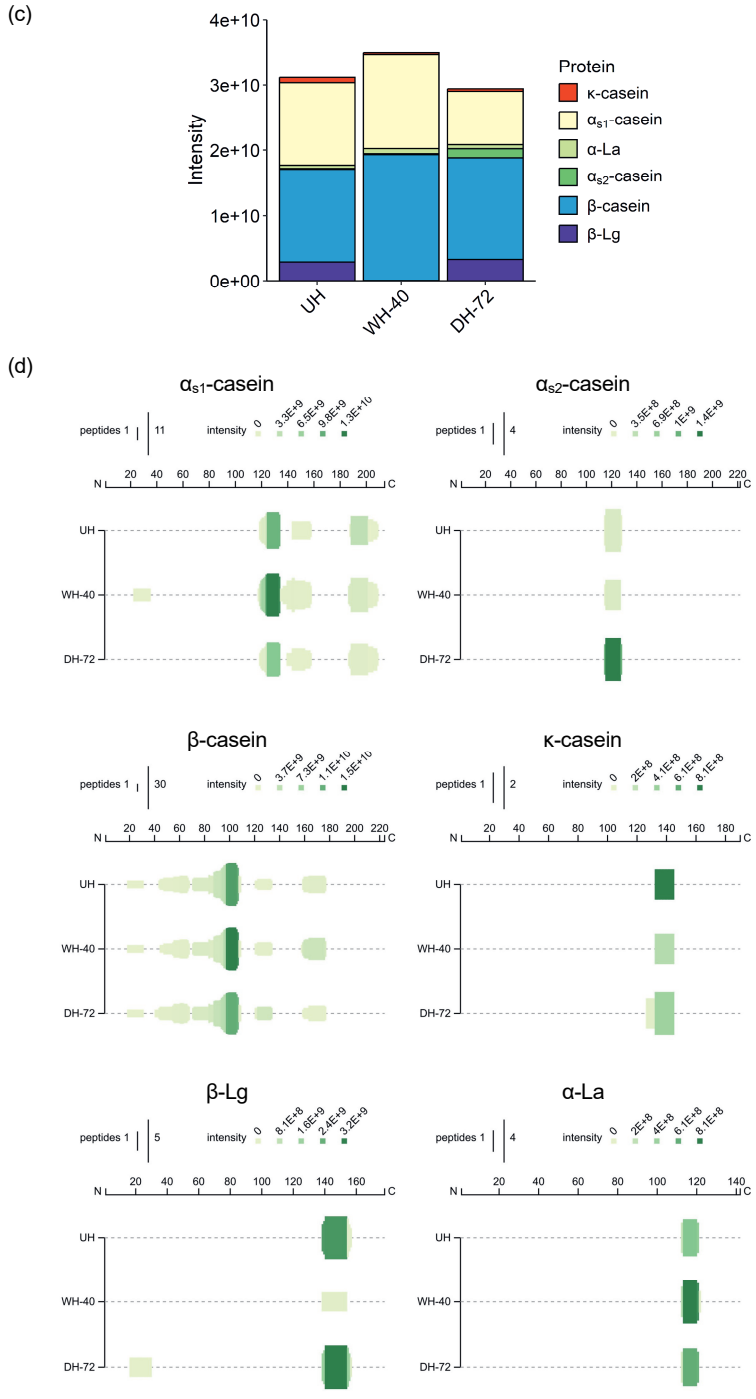


Figure 4.2: (Caption on next page)

Figure 4.2: HLA-II epitopes and linear IgE epitopes predicted in the detoxified intestinal digests. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model. (a) Summed peptide intensity of predicted HLA-II epitopes, (b) peptide alignment of predicted HLA-II epitopes for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin (β-Lg), and α-lactalbumin (α-La), (c) summed peptide intensity of predicted linear IgE epitopes, (d) peptide alignment of predicted linear IgE epitopes for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-Lg, and α-La. The color of the bars in the peptide alignments represents the sum of peptide intensities that overlap at this position, and the height of the bars represents the number of peptides (peptide count) that overlap at this position. The amino acid position is given including the signal peptides of 15, 15, 15, 21, 16, and 19 amino acids for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-Lg, and α-La, respectively.

AA region of α_{s1}-casein, whereas it contained a lower intensity of an IgE epitope in the 121-140 AA region of β-Lg.

Peptides were also matched with the milk bioactive peptide database to determine the presence of additional bioactive peptides in the digests (Table 4.S3). In total, 57 bioactive peptides with various functions were detected in the intestinal digests. UH and DH-72 digests contained a similar intensity of bioactive peptides, whereas that of the WH-40 digest was lower. Approximately half of the summed intensity of bioactive peptides in the UH, WH-40 and DH-72 digests originated from TPEVDDEALEK, a peptide from β-Lg with DPP-IV inhibitory and antimicrobial properties. In addition, two peptides that can increase mucin gene expression and mucus production were found in all digests and had a lower intensity in the DH-72 digest compared to the UH and WH-40 digests. These two peptides were YPFPGPI (β-casomorphin 7), which can increase mucus production and the expression of MUC2, MUC3, and MUC5A [30], and YPVEPF (neocasomorphin-6), which can increase MUC4 expression [31].

4.3.3 Mucin gene expression and mucus production

The effect of the intestinal digests on mucin gene expression and mucus production was investigated with HT29-MTX-E12 cells. Cytotoxicity effects of exposures on the cells remained below 4% during the 4h incubation with the detoxified digests (Figure 4.S3). The expression of MUC5A, MUC13 and MUC17 was not changed by the digests with milk peptides compared to the control digest (Figure 4.3A-C). The mucus production increased during the 4h of stimulation for all digests, including the control digest (Figure 4.3D). Cells that were stimulated with the intestinal digests had a higher mucus production than the medium control, which was only measured at the 4h timepoint. However, stimulating the cells with the digests containing milk peptides did not result in a different mucus production compared to the control digest.

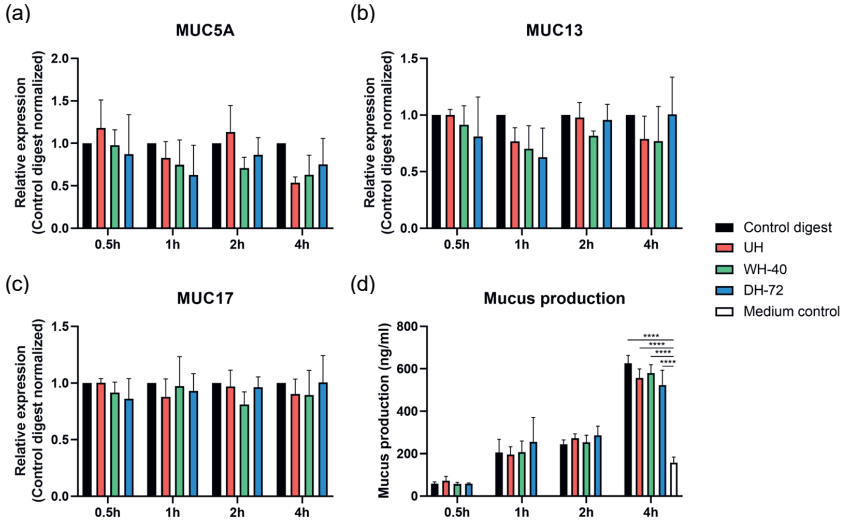


Figure 4.3: Mucus production and mucin gene expression after application of the detoxified intestinal digests to HT29-MTX-E12 cells for 0.5, 1, 2, or 4h. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model. Water instead of the infant formula model system was digested with the *in vitro* digestion model to obtain a control digest. Medium control were HT29-MTX-E12 cells grown in DMEM. (a) MUC5A expression, (b) MUC13 expression, (c) MUC17 expression, (d) mucus production. Statistical differences between samples at the same time point are indicated with *, **, ***, and **** for $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.0001$, respectively.

4.3.4 Peptides after intestinal transport

In a pilot experiment, we observed that the unfiltered intestinal digests were toxic for differentiated Caco-2 cells as measured by TEER values (data not shown). Therefore, the digests were first detoxified before cell exposures. Peptides smaller than 10 kDa from UH, WH-40 and DH-72 intestinal digests were applied to Caco-2 monocultures or to Caco-2/HT29-MTX-E12 cocultures for 6h, and transported peptides were identified with LC-MS/MS. TEER values remained above 90% and cytotoxicity of exposures on the cells remained below 1% during the 6h of intestinal transport (Figure 4.S4). Moreover, TEER and cytotoxicity levels did not differ between the mono- and cocultures or between the different milk samples. A higher peptide count and a higher peptide intensity were detected in the basolateral medium of the coculture compared to the monoculture for all heat treatments (Figure 4.4). A relatively high intensity of transported peptides originated from β-Lg (Figure 4.4B+F) compared to the peptide composition before intestinal transport (Figure 4.1B). WH-40 contained a lower count and intensity of transported peptides compared to UH and DH-72 in both the mono- and cocultures. However, the peptide transport for DH-72 differed compared to UH between the mono- and cocultures. DH-72 had a lower peptide count and intensity than UH in the monoculture but a higher peptide count and intensity than UH in the coculture. The transported peptides consisted mainly of unmodified or lactosylated

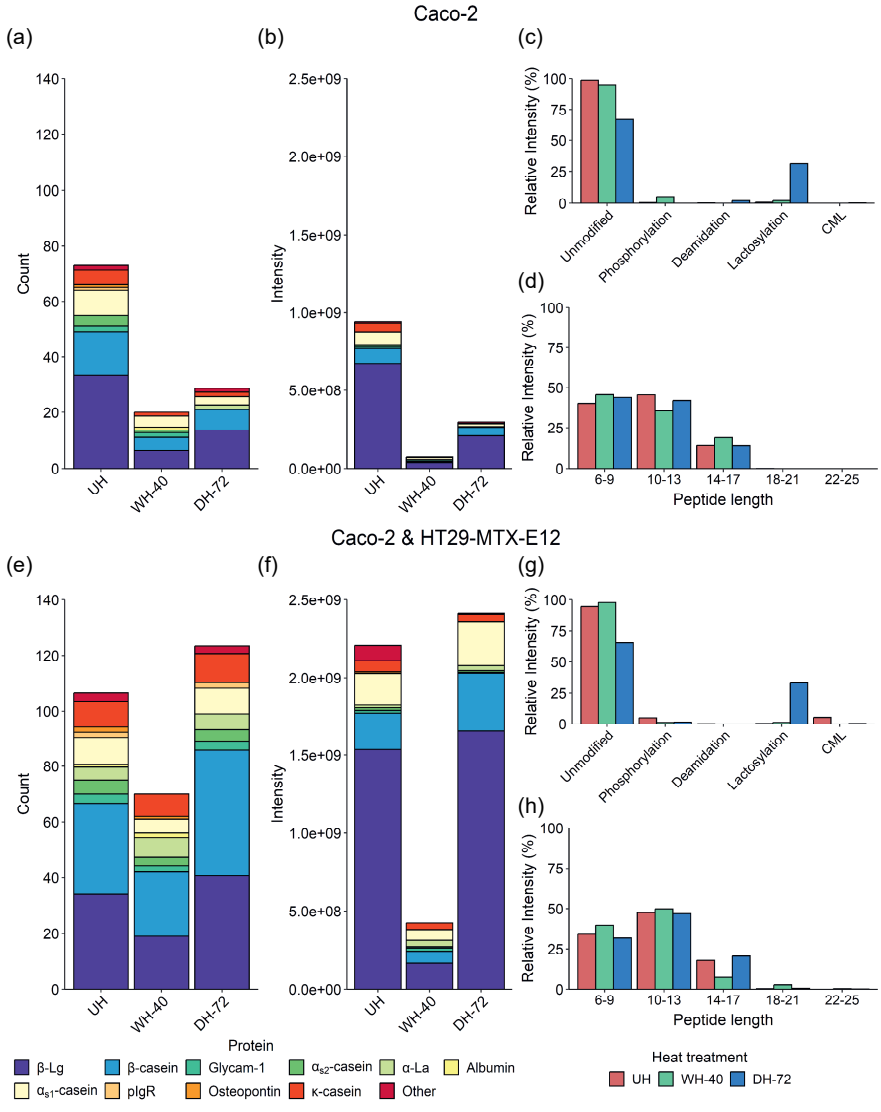


Figure 4.4: Milk peptides detected with LC-MS/MS on the basolateral side after transport across a 21-days differentiated Caco-2 monolayer (a-d), or Caco-2/HT29-MTX-E12 (90/10) monolayer (e-h). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6h. (a+e) Summed peptide count, (b+f) summed peptide intensity, (c+g) relative peptide intensity per modification, (d+h) relative peptide intensity per peptide length group.

peptides, whereas transported peptides with other PTMs were relatively low in both cell models (Figure 4.4C+G). Most transported peptides had a length between 6-13 AAs, but also some longer peptides between 14-21 AAs were transported (Figure 4.4D+H). In the coculture, a higher relative intensity of longer peptides between 10-13 AAs for all heat treatments and between 14-17 AAs for UH and DH-72 were detected compared to the monoculture.

Peptides from some major milk proteins seemed to be transported across the coculture proportionally to their abundance after intestinal digestion (Figure 4.5). For instance, the intensity of peptides from α_{s2}-casein and α-La showed a similar ratio between the heat treatments before and after transport across the coculture. In contrast, peptides from α_{s1}-casein and β-casein were transported across the coculture to a high extent for DH-72 and to a low extent for WH-40 compared to the ratio of peptide intensity between the heat treatments in the intestinal digests. In the monoculture, peptides from all major proteins except α-La were transferred to a lower extent in the WH-40 and DH-72 digests compared to the UH digest. Peptides from the most abundant proteins, β-casein and β-Lg, were transferred to an even lower extent in WH-40 than in DH-72, whereas peptides from other proteins, such as κ-casein and α_{s2}-casein, were transferred to the same or even to a higher extent in WH-40 than in DH-72.

Peptides detected after transfer over the monoculture were differently distributed over the proteins in the UH, WH-40 and DH-72 samples (Figure 4.6). For both WH-40 and DH-72 samples, fewer regions of all major milk proteins except α-La were transferred compared to UH, and these regions consisted of both unmodified peptides as well as peptides with different PTMs (Figure 4.S5). In contrast, approximately the same regions of the major milk proteins were transferred across the coculture independent of heat treatment, although the intensity of these regions differed between heat treatments. For instance, specific regions in α_{s1}-casein (110-119 AA) and β-casein (81-92 AA, 143-154 AA, 164-175 AA) seemed to be transferred to a higher extent across the coculture in the DH-72 compared to the UH sample. These regions did not seem to correlate with peptides containing particular PTMs since one of these regions (164-175 AA in β-casein) mainly consisted of peptides with lactosylation or CML-modified AAs, whereas the other regions consisted mainly of unmodified peptides (Figure 4.S6).

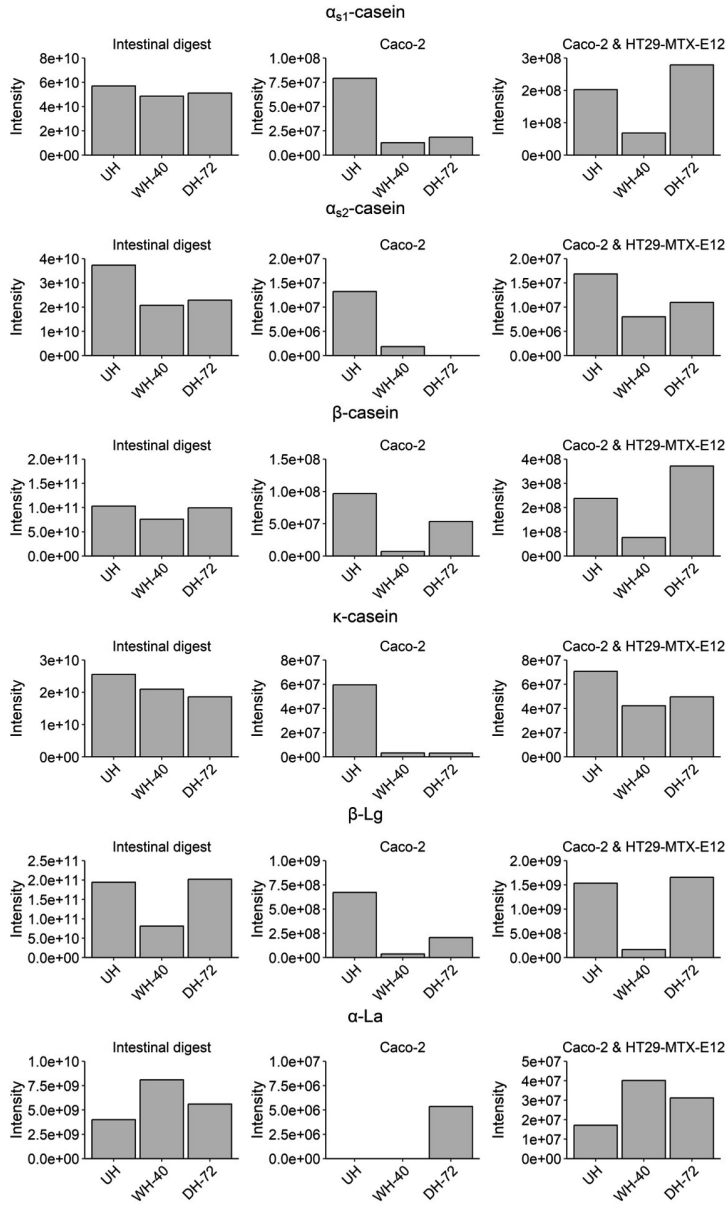


Figure 4.5: Summed intensity of milk peptides originating from α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin (β-Lg), and α-lactalbumin (α-La) detected with LC-MS/MS in the detoxified intestinal digests and on the basolateral side after transport across a 21-days differentiated Caco-2 monolayer or Caco-2/HT29-MTX-E12 (90/10) monolayer. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6h.

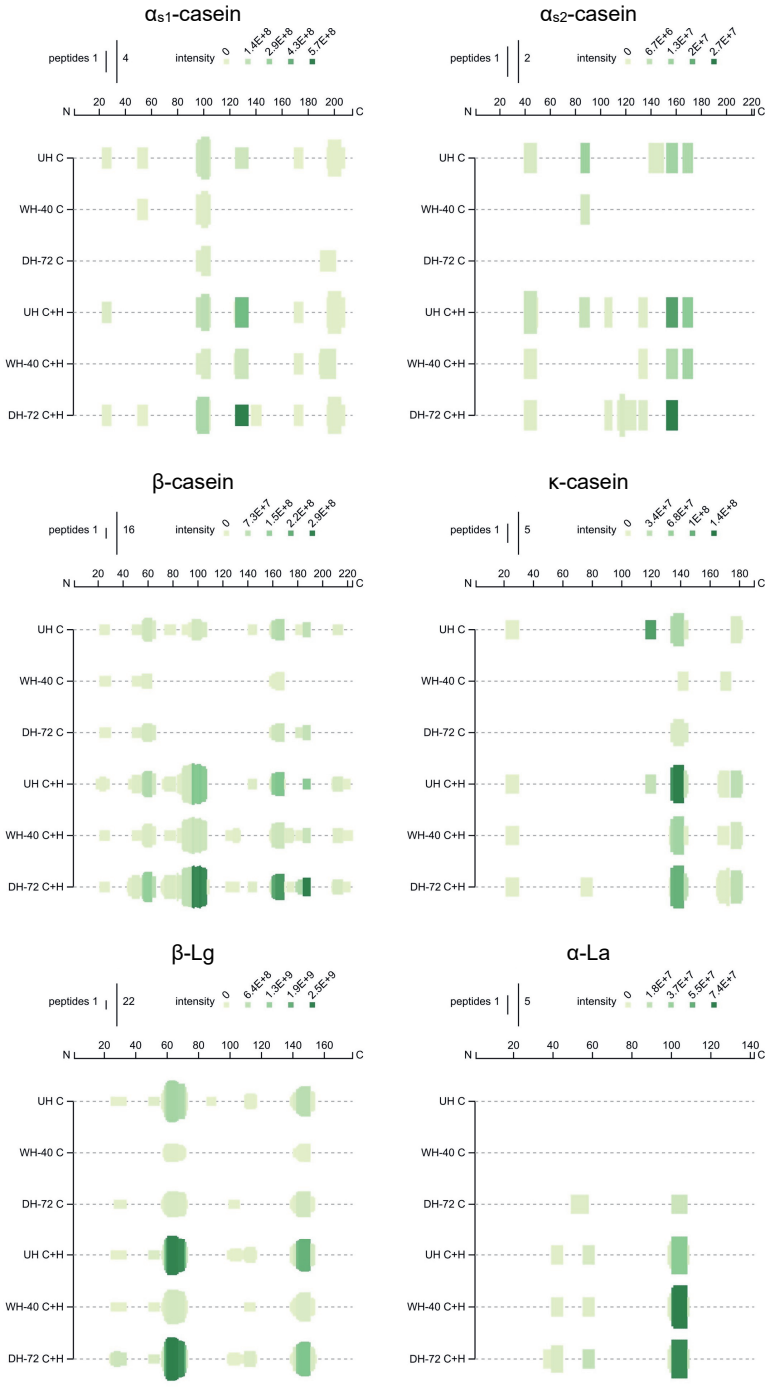
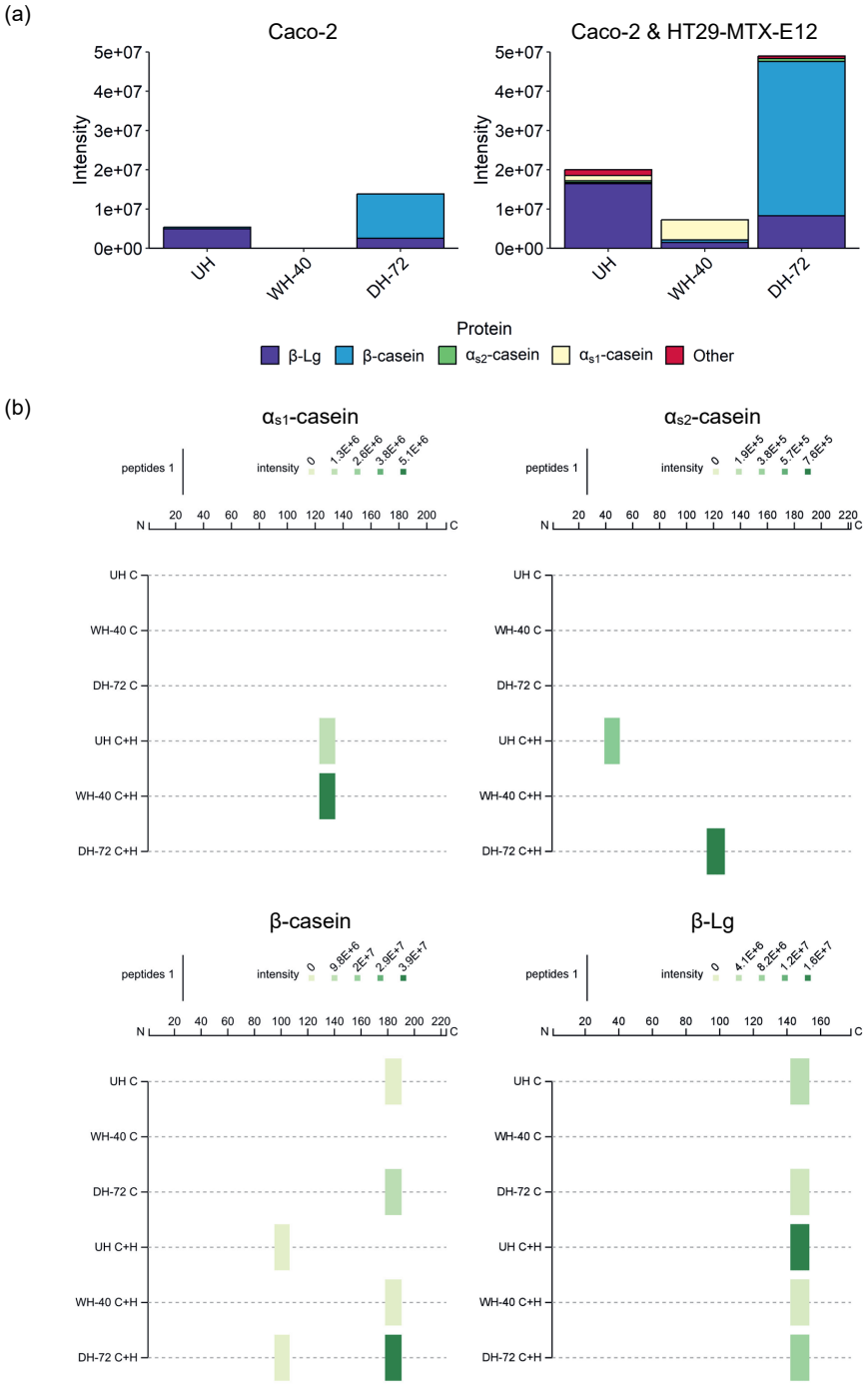


Figure 4.6: (Caption on next page)

Figure 4.6: Peptide alignment for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin (β-Lg), and α-lactalbumin (α-La) detected with LC-MS/MS on the basolateral side after transport across a 21-days differentiated Caco-2 monolayer (C) or Caco-2/HT29-MTX-E12 (90/10) monolayer (C+H). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6h. The color of the bars represents the sum of peptide intensities that overlap at this position, and the height of the bars represents the number of peptides (peptide count) that overlap at this position. The amino acid position is given including the signal peptides of 15, 15, 15, 21, 16, and 19 amino acids for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-Lg, and α-La, respectively.

4.3.5 HLA-II epitopes, IgE epitopes and bioactive peptides after intestinal transport

DH-72 contained the highest intensity of peptides with predicted HLA-II epitopes on the basolateral side of both the mono- and cocultures (Figure 4.7A). This was mainly caused by the presence of SLSQSKVLPVPQ, a peptide originating from β-casein positioned at 164-175 AA (Figure 4.7B), which was only detected on the basolateral side in either lactosylation or CML-modified form (Table 4.S4). WH-40 contained the lowest intensity of peptides with HLA-II epitopes in both the mono- and the cocultures. Potential linear IgE epitopes were only detected in UH on the basolateral side of the monoculture, whereas they were detected in UH, WH-40 and DH-72 on the basolateral side of the coculture (Figure 4.7C). In the coculture, DH-72 contained the highest intensity and WH-40 the lowest intensity of transported peptides with potential linear IgE epitopes on the basolateral side. Largely the same IgE epitopes were detected in UH, WH-40 and DH-72 samples, with the highest intensities for the peptides containing potential epitopes from the 109-120 AA region in α_{s1}-casein and the 83-92 AA region in β-casein (Figure 4.7D), which were either transported in unmodified or phosphorylated form (Table 4.S5). Moreover, several bioactive peptides with different properties were detected in the basolateral media (Table 4.S6). The most abundant bioactive peptides contained either ACE-inhibitory, inhibition of cholesterol solubility, DPP-IV inhibitory, or antimicrobial properties. Whereas the intensity of transported bioactive peptides was the highest in UH in the monoculture, it was the highest in DH-72 in the coculture.



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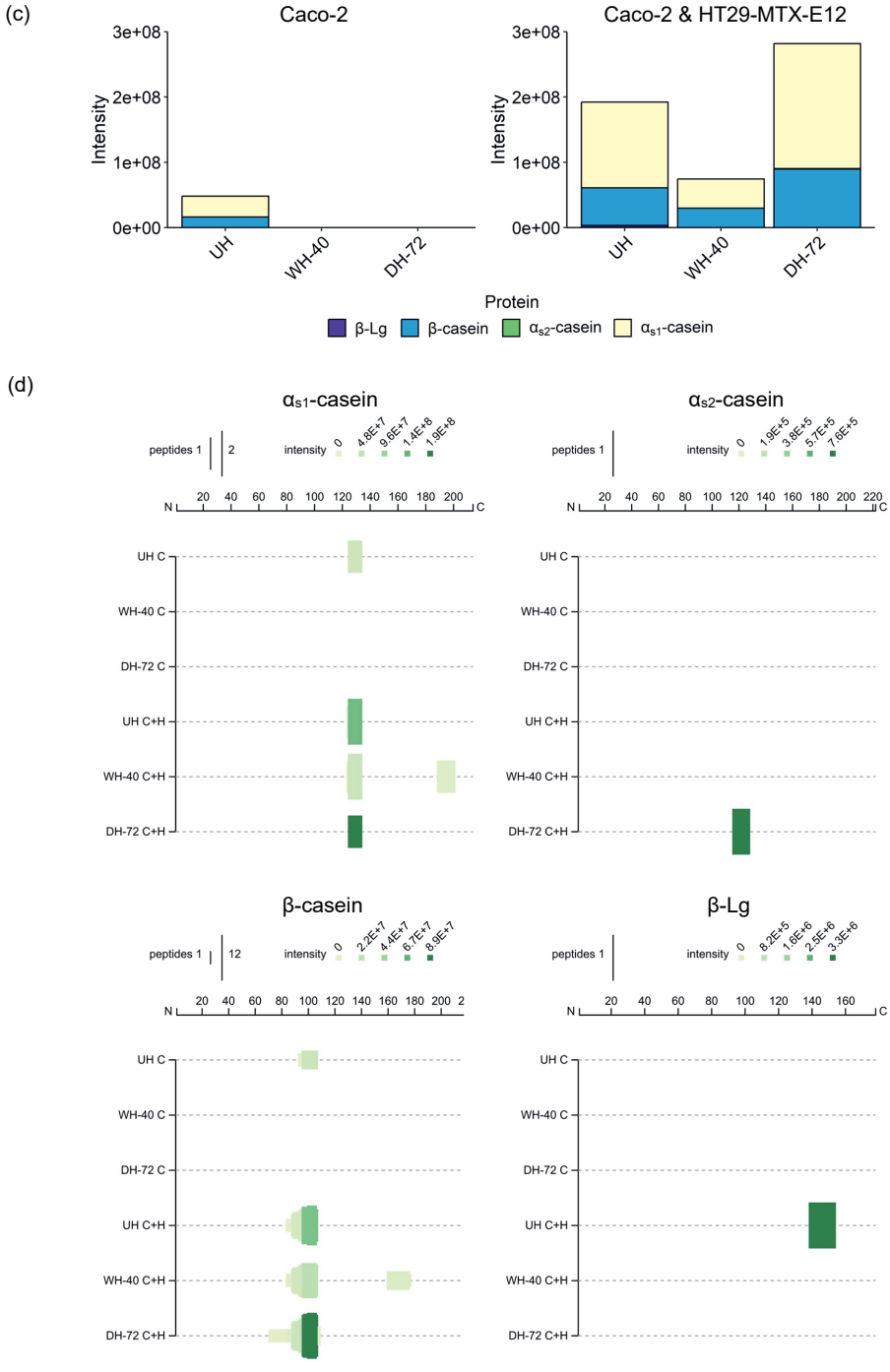

Figure 4.7: (Caption on next page)

Figure 4.7: HLA-II epitopes and linear IgE epitopes predicted on the basolateral side after transport across a 21-days differentiated Caco-2 monolayer (C) or Caco-2/HT29-MTX-E12 (90/10) monolayer (C+H). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6h. (a) Summed peptide intensity of predicted HLA-II epitopes, (b) peptide alignment of predicted HLA-II epitopes for α_{s1}-casein, α_{s2}-casein, β-casein, and β-lactoglobulin (β-Lg), (c) summed peptide intensity of predicted linear IgE epitopes, (d) peptide alignment of predicted linear IgE epitopes for α_{s1}-casein, α_{s2}-casein, β-casein, and β-Lg. The color of the bars in the peptide alignments represents the sum of peptide intensities that overlap at this position, and the height of the bars represents the number of peptides (peptide count) that overlap at this position. The amino acid position is given including the signal peptides of 15, 15, 15, and 16 amino acids for α_{s1}-casein, α_{s2}-casein, β-casein and β-Lg, respectively.

4.3.6 Cytokine production by Caco-2 and HT29-MTX-E12 cells

Cytokines in the basolateral compartment of the Caco-2 and HT29-MTX-E12 mono- and cocultures were measured to determine the effect of the intestinal digests on the cells (Figure 4.8). No large differences were observed between the medium control, control digest, and the digests with milk peptides. IL-18 production tended to be lower for the DH-72 sample in the monoculture, although this was not significant. Remarkably, the coculture produced higher IL-8 levels for all samples compared to the monoculture. Besides IL-8 and IL-18, other cytokines were measured including IL-6, TNF-α, and IFN-γ (Figure 4.S7). The production of these cytokines was very low (<1 pg/ml) and did not significantly differ between the samples. In addition, IL-10 production in the basolateral compartment was measured, but the IL-10 concentration was below the detection limit of 0.27 pg/ml for the majority of the samples (data not shown).

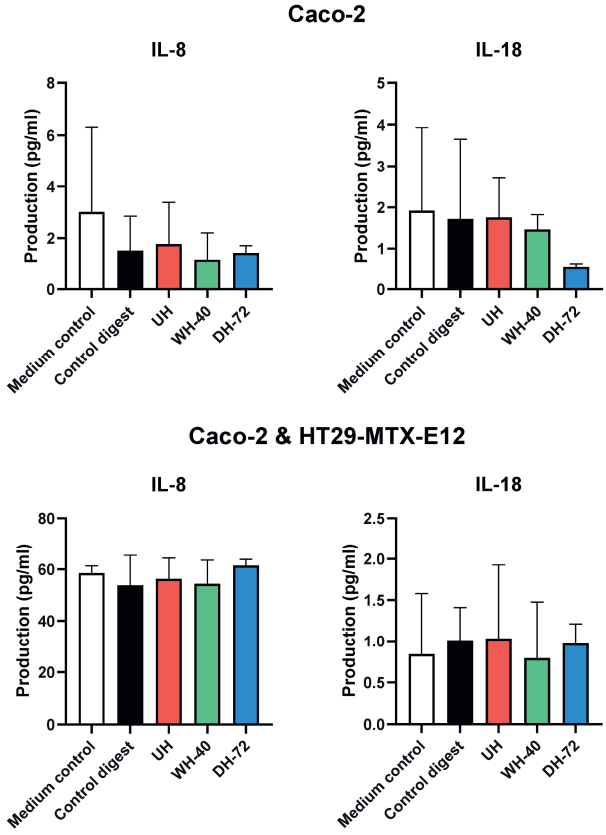

Figure 4.8: IL-8 and IL-18 concentrations measured with a LEGENDplex assay on the basolateral side of a 21-days differentiated Caco-2 or Caco-2/HT29-MTX-E12 (90/10) monolayer after stimulation with intestinal digests for 6h (n=3). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6h. Water instead of the infant formula model system was digested with the *in vitro* digestion model to obtain a control digest. Medium control were Caco-2 and HT29-MTX-E12 cells grown in DMEM.

4.3.7 Dendritic cell response to basolateral medium

The immunoreactivity of the transported peptides was studied by stimulating primary iDCs with the basolateral media and measuring changes in cytokine production (Figure 4.9). The medium control from the mono- and cocultures resulted in an increased production of TNF-α, IL-6 and IL-10 compared to the non-treated iDCs. However, stimulating the iDCs with basolateral medium from the control digest or digests with milk peptides did not lead to changes in TNF-α, IL-6 or IL-10 compared to the non-treated cells.

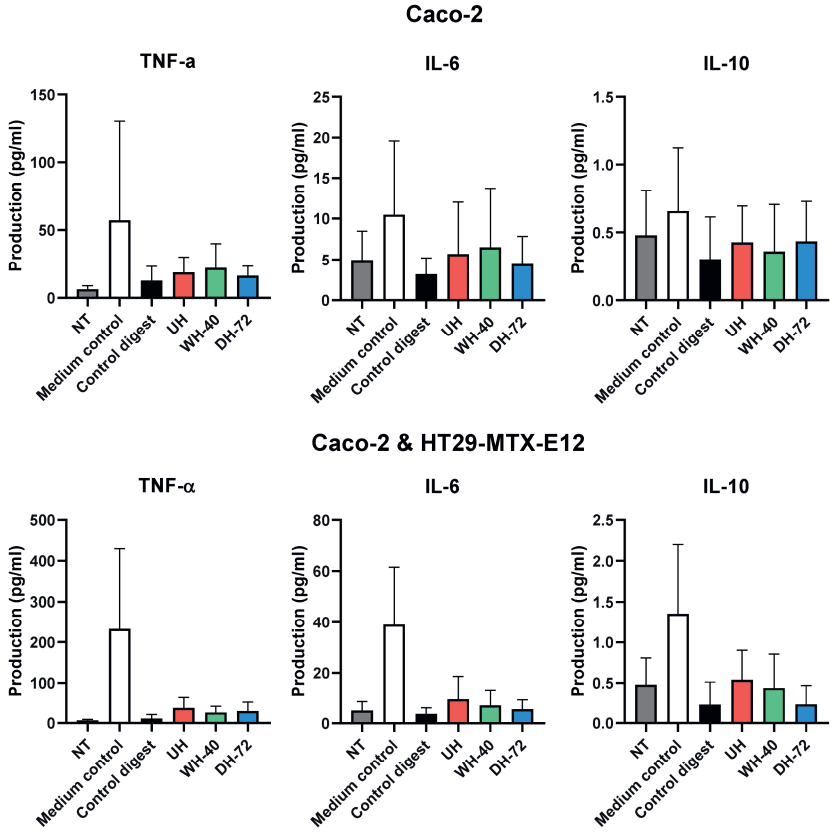

Figure 4.9: TNF-α, IL-6 and IL-10 concentrations measured with a LEGENDplex assay after stimulating immature dendritic cells (iDCs) with medium from the basolateral side of a 21-days differentiated Caco-2 or Caco-2/HT29-MTX-E12 (90/10) monolayer (n=5). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6h. Water instead of the infant formula model system was digested with the *in vitro* digestion model to obtain a control digest. Medium controls were Caco-2 and HT29-MTX-E12 cells grown in DMEM. Non-treated (NT) iDCs were grown in IMDM.

4.4 Discussion

During the production of infant formula, milk proteins are heated at both wet and dry conditions, resulting in protein denaturation and glycation. This study aimed to investigate the effect of wet and dry heating of milk proteins (1) on the composition and immunoreactivity of peptides released after *in vitro* infant digestion, (2) on intestinal mucus production, and (3) on the composition and immunoreactivity of peptides transported across the intestinal barrier.

4.4.1 Peptides released after *in vitro* infant digestion

After unheated, wet heated, and dry heated milk proteins were digested with an *in vitro* infant digestion model, the released peptides were identified with LC-MS/MS. Wet heating resulted in a lower peptide intensity, mainly due to the lower intensity of peptides originating from β-Lg (Figure 4.1). This was probably caused by the more extensive digestion of β-Lg after wet heating, resulting in more peptides that were too small (<6 AAs) to be detected with the used LC-MS/MS method. The faster digestion of β-Lg after wet heating has been reported previously [32,33], which was most likely due to the unfolding of β-Lg upon wet heating, leading to a higher accessibility of cleavage sites for digestive enzymes. The DH-72 intestinal digest contained a higher relative intensity lactosylated peptides and longer peptides (Figure 4.1C+D). At the used dry heating conditions (60°C, a_w = 0.6), the Maillard reaction takes place, in which lactose binds to the free amino group of lysine residues, resulting in lactosylated peptides. Since trypsin cleaves next to lysine residues, lactosylation can hinder the accessibility of those cleavage sites, leading to relatively longer peptides. The presence of longer peptides after *in vitro* digestion of dry heated milk proteins was in agreement with our previous study (Chapter 3) as well as with Zenker et al. [34]. DH-72 contained highly glycated regions in all six major proteins, but especially in β-casein and β-Lg (Figure 4.S2), which were detected at higher intensities in DH-72 than in WH-40 (Figures 4.1B and 4.S1). This indicates that milk proteins are digested slower with increasing glycation levels, which was in agreement with our previous study (Chapter 3). Together, this showed that wet heating increased and dry heating decreased the digestion of milk proteins under *in vitro* infant digestion conditions, resulting in a different composition of the digestion-derived peptides from wet and dry heated milk proteins.

4.4.2 Immunoreactivity of the peptides after *in vitro* infant digestion

The effect of wet and dry heating of milk proteins on the immunoreactivity of the peptides after *in vitro* infant digestion was evaluated by predicting potential HLA-II and IgE epitopes in the digests. Since DCs are able to sample through the intestinal barrier, changes in the peptide composition in the intestinal digests due to heat treatment, even before transport across the intestinal barrier, may modulate the immune response towards milk proteins [35]. Both wet and dry heating resulted in increased survival of HLA-II epitopes during intestinal digestion compared to unheated milk proteins (Figure 4.2), even though wet heating resulted in a lower overall peptide intensity (Figure 4.1B). In addition, the composition of these HLA-II epitopes was very different for the different heat treatments. In the digest from WH-40, most HLA-II epitopes originated from one region in α_{s1}-casein (103-119 AA) (Figure 4.2), which was highly phosphorylated (Figure 4.S2). In the digest from DH-72, mainly HLA-II epitopes from α_{s2}-casein (100-113 AA) and β-casein (164-175 AA) survived intestinal digestion, which were mostly glycated (Figures 4.2 and 4.S2). PTMs have previously been shown to alter the binding capacity of several epitopes in milk proteins, probably due to the destruction or neo-formation of either structural or linear epitopes. This has, however, mainly been shown for IgE and IgG epitopes and not for HLA-II epitopes. For instance, dephosphorylation can decrease the IgE binding capacity of caseins [36], whereas phosphorylation can decrease the IgE and IgG binding capacity of α-La [37], also after *in*

vitro digestion [38], showing that phosphorylation can affect the binding capacity of milk protein epitopes. Glycation was also reported to modulate the immunoreactivity of whey proteins after digestion. Both increased binding to sRAGE and galectin-3 for glycated β-Lg [11] as well as reduced IgE binding for glycated β-Lg and α-La were reported [14–16], both after *in vitro* digestion. Some of the HLA-II epitopes that were detected in the intestinal digests were also detected previously by Zenker et al. [2], although we detected three times as many epitopes and also some epitopes from different regions in the proteins, possibly due to the longer incubation of the intestinal digests in the intestinal barrier models in our study. In contrast to the variation in HLA-II epitopes between the heat treatments, the variation in linear IgE epitopes was low between the heat treatments (Figure 4.2, Table 4.S2). WH-40 had higher intensities of one phosphorylated region in α_{s1}-casein (109-120 AA), and it contained lower intensities of IgE epitopes from β-Lg, whereas DH-72 had higher intensities of one glycated region in α_{s2}-casein (105–114 AA). Nearly all IgE epitopes detected in our study were also reported by Zenker et al. [2] after *in vitro* digestion, demonstrating that mainly the same regions of the proteins survived digestion. In summary, both wet and dry heating resulted in increased survival of HLA-II epitopes, which may result in a higher immunoreactivity of milk protein digests after either wet or dry heating. However, the limited and contradicting literature on the effect of PTMs on linear epitopes derived from caseins and whey proteins makes it difficult to determine the impact of wet and dry heating on the immunoreactivity of milk proteins after intestinal digestion. Therefore, directly measuring the immunoreactivity of the intestinal digests instead of a bioinformatic approach would be of interest for future research.

4.4.3 Mucus production

Mucus production was not affected by the digests with milk peptides compared to the control digest (Figure 4.3). However, the mucus production of all digests, including the control digest, was increased compared to the medium control. This suggests that the control digest stimulated the mucus production, although it is unclear what component in the digest may be responsible for this effect. In the intestinal digests, two peptides were detected that can increase mucus production and mucin gene expression (Table 4.S3): YPVEPF (neocasomorphin-6) and YPFPGPI (β-casomorphin 7). YPVEPF can increase MUC4 expression [31] and was present in lower intensities in the DH-72 digest. However, we did not measure MUC4 expression, which may be of interest for future research. YPFPGPI can increase mucus production and the expression of MUC2, MUC3, and MUC5A [30], and was present in higher intensities in UH and WH-40 than in DH-72. However, no significant differences were found in mucus production or in the gene expression of mucins that we measured, which were MUC5A, MUC13 and MUC17. In contrast to our findings, multiple milk peptides were shown to increase mucus production or mucin gene expression in previous studies. For instance, a peptide from α_{s1}-casein significantly increased mucus production and MUC5A expression *in vitro* [39,40], and significantly increased MUC2 and MUC3 expression *in vivo* [41]. In addition, peptides from β-Lg and α-La were also shown to increase mucus production and MUC5A expression [8,9]. These studies were, however, conducted with isolated milk peptides instead of with a mixture of all digestion-derived milk peptides as in our study, in which the complex peptide composition may have faded the effect of individual isolated milk peptides.

In summary, the mucus production and mucin gene expression by HT29-MTX-E12 cells were not affected by the digestion-derived peptides from the differently heated milk proteins.

4.4.4 Transported peptides across the Caco-2 and Caco-2/HT29-MTX-E12 monolayers

The effect of wet and dry heating on the intestinal transport of milk peptides was evaluated by the use of Caco-2 monocultures and Caco-2/HT29-MTX-E12 cocultures. Wet heating resulted in a lower peptide transport in both cell models, whereas dry heating resulted in a lower peptide transport only in the monoculture (Figure 4.4). Peptides were transported more proportionally to the peptide intensities in the intestinal digests in the coculture than in the monoculture (Figure 4.5). Together, this indicates that the transport of milk peptides depends on both heat treatment and the used cell model, which may be related to the presence or absence of a mucus layer. Interestingly, more peptides from all major milk proteins were transported across the coculture compared to the monoculture (Figure 4.4), whereas no differences in TEER values or cytotoxicity of the cells were observed (Figure 4.S4). However, directly measuring permeability, such as through an FITC-dextran assay, would help to confirm whether the mono- and cocultures have similar permeabilities. Most of the transported peptides were either unmodified or lactosylated in both cell models (Figure 4.4C+G), and relatively less phosphorylated peptides were transported compared to the composition of the intestinal digests (Figure 4.1). This suggests that differences in modifications of the peptides were not related to the higher transport of milk peptides across the coculture than the monoculture or to the difference in the effect of dry heating in the coculture compared to the monoculture. Moreover, compared to the unheated digest, specific regions of proteins in the wet and dry heated digests were not transferred across the monoculture, whereas mainly the same regions of proteins were transferred for all heat treatments across the coculture (Figure 4.6). However, no correlation could be found between these differences in the transported regions of milk proteins in the monoculture with certain protein modifications (Figures 4.S5 and 4.S6) or with other properties of these regions such as hydrophobicity or charge. Since different properties of peptides are known to affect the transfer of peptides [42], it may be of interest to investigate why certain regions are more easily transported in the coculture than in the monoculture. The lower peptide transfer across a Caco-2 monolayer after dry heating and the efficient transfer of glycated milk peptides were in agreement with Zenker et al. [2]. In addition, the lower peptide intensity of transported β-Lg across a Caco-2 monolayer after wet heating was in line with Deng et al. [3]. However, to our knowledge no studies have previously been performed that investigated the effect of dry heating on the transport of peptides with both a Caco-2 monoculture and a Caco-2/HT29-MTX-E12 coculture. We hypothesize that peptide-peptide interactions induced by wet and dry heating may hinder peptides from being transported across the monoculture. The difference in these peptide-peptide interactions formed by wet versus dry heating may explain why the wet heated digest is transported to a low extent in both cell models, while dry heated digest is only transported to a low extent in the monoculture. Lactosylation, which occurred to a higher degree in the dry heated digests (Figure 4.1C), induces a negative charge, which may be involved in peptide-peptide interactions in the dry heated digest. Since mucins that are produced by HT29-MTX-E12 cells

are also negatively charged [5], they might weaken these peptide-peptide interactions in the dry heated digest, resulting in a higher transport of the dry heated digest across the coculture than the monoculture. However, this hypothesis on the role of peptide-peptide interactions on the transport of milk proteins, and how this is impacted by mucins, should be further investigated. Furthermore, different routes of transport might be involved in the translocation of peptides in the mono- and cocultures, which may affect which peptides are transported to the basolateral side. Since a higher permeability of cocultures with HT29 cells has been reported in literature [43], which was described to be caused by the lower expression of tight junction proteins by HT29 cells [44,45], the coculture may show more paracellular transport compared to the monoculture. Whereas no differences in TEER values were observed that indicate a higher permeability of the coculture (Figure 4.S4), a higher peptide intensity and relatively more longer peptides (10-13 AAs) were observed on the basolateral side of the coculture (Figure 4.4). Hence, investigating the routes of transport in both the mono- and cocultures may aid in understanding the different effects of wet and dry heating observed in the monoculture compared to the coculture. Together, we showed that wet heating resulted in a decreased transport of milk peptides, whereas the effect of the dry heating differed depending on the intestinal barrier model.

4.4.5 Immunoreactivity of transported peptides

The immunoreactivity of peptides that were transported across the intestinal barrier was evaluated by predicting HLA-II and linear IgE epitopes and by measuring iDC response after stimulation with the basolateral media. Only some of the regions that contained HLA-II and IgE epitopes as detected in the intestinal digests (Figure 4.2) were able to cross the intestinal barrier (Figure 4.7). The highest intensity of peptides containing an HLA-II epitope was detected in DH-72 and the lowest intensity in WH-40 in both cell models (Figure 4.7). Especially one region from β-casein (164-175 AA) and one region from β-Lg (127-137 AA) were able to cross the intestinal barrier (Figure 4.7, Table 4.S4). These regions seemed to be transferred according to their intensities in the intestinal digests, which showed higher intensities in the UH and DH-72 samples (Figure 4.2, Table 4.S1). However, other regions with high intensities in the intestinal digests, such as the 103-119 AA region of α_{s1}-casein, were transferred only to a low extent across the intestinal barrier (Figures 4.2 and 4.7). This suggests that the transport of peptides containing HLA-II epitopes is affected not only by their intensity in the intestinal digests but also by other properties of the peptides. Properties such as hydrophobicity, charge and PTMs are often thought to influence the transfer of peptides [42], although a correlation between peptide properties and their transfer across the barrier was not found in our study, as described in section 4.4.4. The HLA-II epitopes in DH-72 consisted mainly of one region from β-casein (164-175 AA), which was only detected in lactosylation and CML-modified form (Table 4.S4). As discussed in section 4.4.2, glycation may change the immunoreactivity of epitopes, although literature is contradictory on whether glycation results in an enhanced or reduced recognition of epitopes [11,14–16]. Whereas the intensity of peptides containing linear IgE epitopes was very similar between the heat treatments in the intestinal digests (Figure 4.2), UH had the highest intensity IgE epitopes on the basolateral side of the monoculture and DH-72 had the highest intensity IgE epitopes on the basolateral side of the coculture (Figure 4.7). Most transported peptides

containing IgE epitopes were unmodified (Table 4.S5), suggesting that the higher peptide transfer with IgE epitopes in the DH-72 sample was not related to glycation modifications.

These differences detected in the transfer of HLA-II and linear IgE epitopes across the intestinal barrier, however, did not lead to changes in cytokine response by iDCs compared to the control digest (Figure 4.9). This could indicate that the differences between the heat treatments in transferred peptides were too small to induce a change in cytokine response by the iDCs. Interestingly, iDCs stimulated with basolateral medium from the medium control caused an enhanced TNF-α, IL-6 and IL-10 response, especially for the basolateral medium from the coculture (Figure 4.8). These cytokines were either not or only at very low concentrations produced by Caco-2 and HT29-MTX-E12 cells (Figure 4.S7), indicating that the cytokines were produced by the iDCs. Although Caco-2 and HT29-MTX-E12 cells did produce IL-8 and IL-18 in the basolateral medium (Figure 4.8), the concentrations of these cytokines did not significantly differ between the medium control and the digests, and thereby could not explain the higher cytokine production by iDCs stimulated with the medium control. More insight into the different effect of the medium control and control digest on iDCs may be obtained by the of use cocultures of iDCs with Caco-2 or HT29-MTX cells, in which their direct interaction can be investigated. Both wet and dry heating of milk proteins were previously shown to increase the binding to or uptake by DCs or macrophages, although no differences in cytokine response were observed [10–12]. Dry heating was also reported to result in increased binding to THP-1 macrophages after intestinal digestion [11]. This suggests that the peptides in the basolateral medium might have been able to bind to receptors on the surface of iDCs, such as RAGE and galectin-3, and might have been taken up and processed by the iDCs. However, since no changes in the cytokine response were detected between the digests with milk peptides and the control digest, the peptide concentrations in the basolateral medium were probably too low to affect the cytokine production by iDCs. Investigating the DC response to intestinal digests containing peptides from unheated, wet heated and dry heated milk proteins would be of interest for future research since DCs are able to sample through the intestinal barrier, and the intestinal digests contained a higher concentration of peptides (Figure 4.1) and predicted HLA-II and IgE epitopes (Figure 4.2). Therefore, these intestinal digests may induce a different DC response than the peptides that were transported across the intestinal barrier, and this would provide a more direct measurement of the effect of wet and dry heating on the immunoreactivity of milk proteins after intestinal digestion. In summary, the intensities of transported peptides containing predicted HLA-II and linear IgE epitopes suggested that wet and dry heating affect the immunoreactivity of transported peptides. However, these differences in HLA-II and linear IgE epitopes did not result in a changed cytokine response by iDCs.

4.5 Conclusion

This study showed that wet and dry heating of milk proteins affected the peptide composition after *in vitro* infant digestion. Wet heating resulted in lower peptide intensities, especially of β-Lg, and dry heating resulted in relatively longer and more lactosylated peptides. Whereas peptides containing linear IgE epitopes survived intestinal digestion to an equal extent regardless of heat treatment, more peptides containing HLA-II epitopes survived intestinal digestion after wet and dry heating compared to the unheated IF model system. This suggests that wet and dry heating affect the immunoreactivity of milk peptides that are formed during digestion, although this should be further investigated. Mucin gene expression and mucus production by HT29-MTX-E12 cells were not affected by the milk peptides derived from the differently heated IF model systems. In both the mono- and cocultures, wet heating resulted in a lower intestinal transport of milk peptides. In contrast, dry heating led to a lower intestinal transport in the monoculture, but a higher intestinal transport in the coculture. More HLA-II epitopes were transported across the intestinal barrier after dry heating and less after wet heating in both cell models, and more linear IgE epitopes were transported across the intestinal barrier after dry heating and less after wet heating in the coculture. This indicates that both wet and dry heating modulate the immunoreactivity of transported milk peptides. However, these differences in peptide composition after intestinal transport did not result in a changed cytokine production by iDCs. All in all, wet and dry heating of milk proteins changed their digestion, survival of immunoreactive structures, and intestinal transport, but did not lead to a difference in mucus production or to a difference in iDC response after intestinal transport. This shows the importance of studying the effect of heat treatment on milk proteins during the production of IF and subsequent implications on digestion, intestinal transport, and immunoreactivity, although the immunological consequences should be further investigated.

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Supplementary information

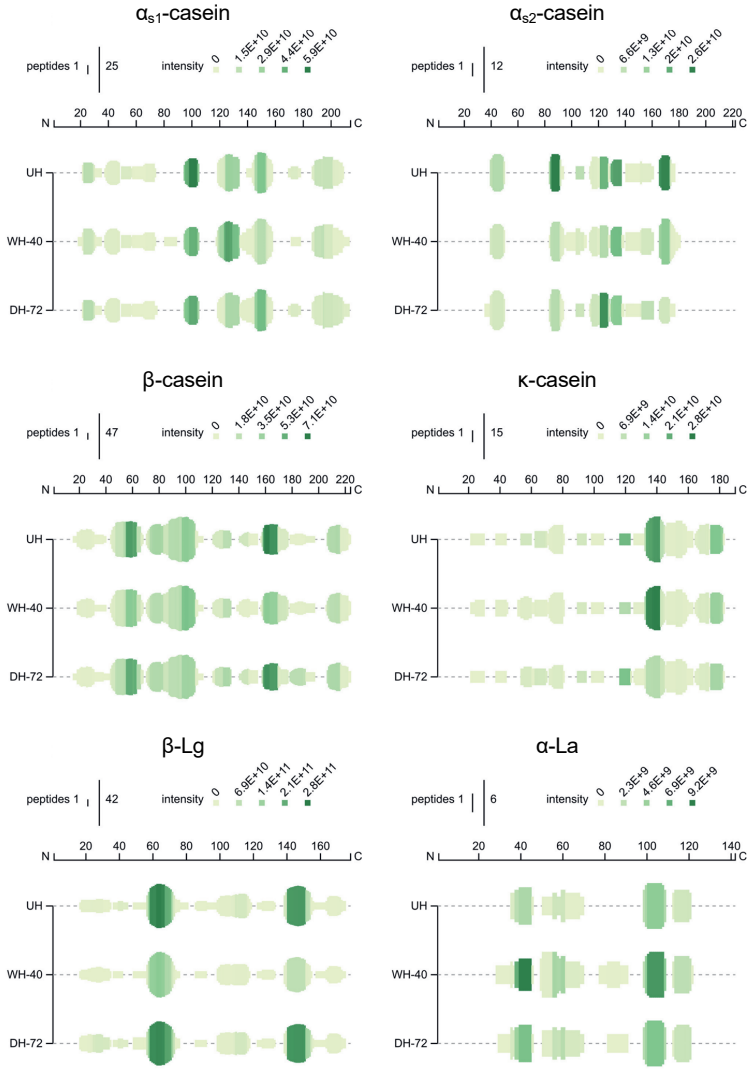

Figure 4.S1: Peptide alignment for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin (β-Lg), and α-lactalbumin (α-La) detected with LC-MS/MS in the detoxified intestinal digests. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model. The color of the bars represents the sum of peptide intensities that overlap at this position, and the height of the bars represents the number of peptides (peptide count) that overlap at this position. The amino acid position is given including the signal peptides of 15, 15, 15, 21, 16, and 19 amino acids for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-Lg, and α-La, respectively.

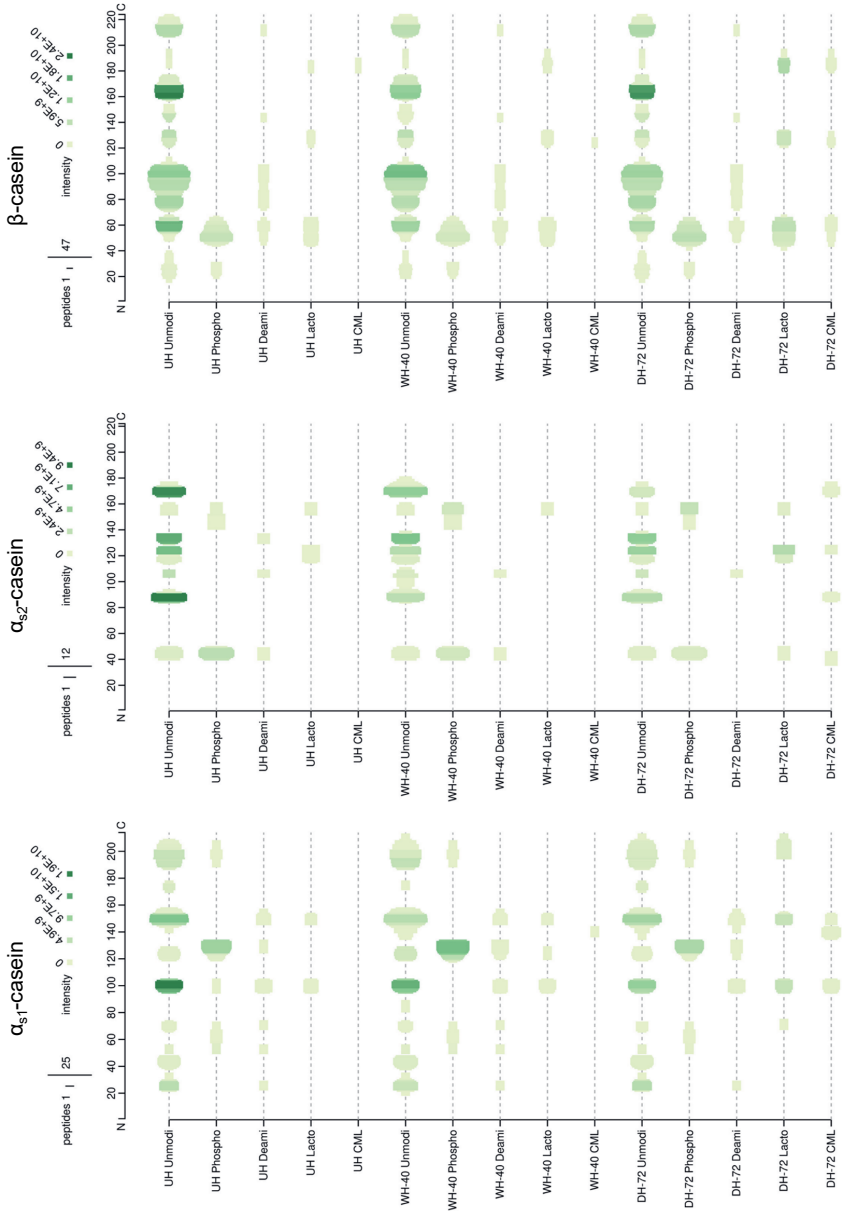

Figure 4.S2: (Continued on next page)

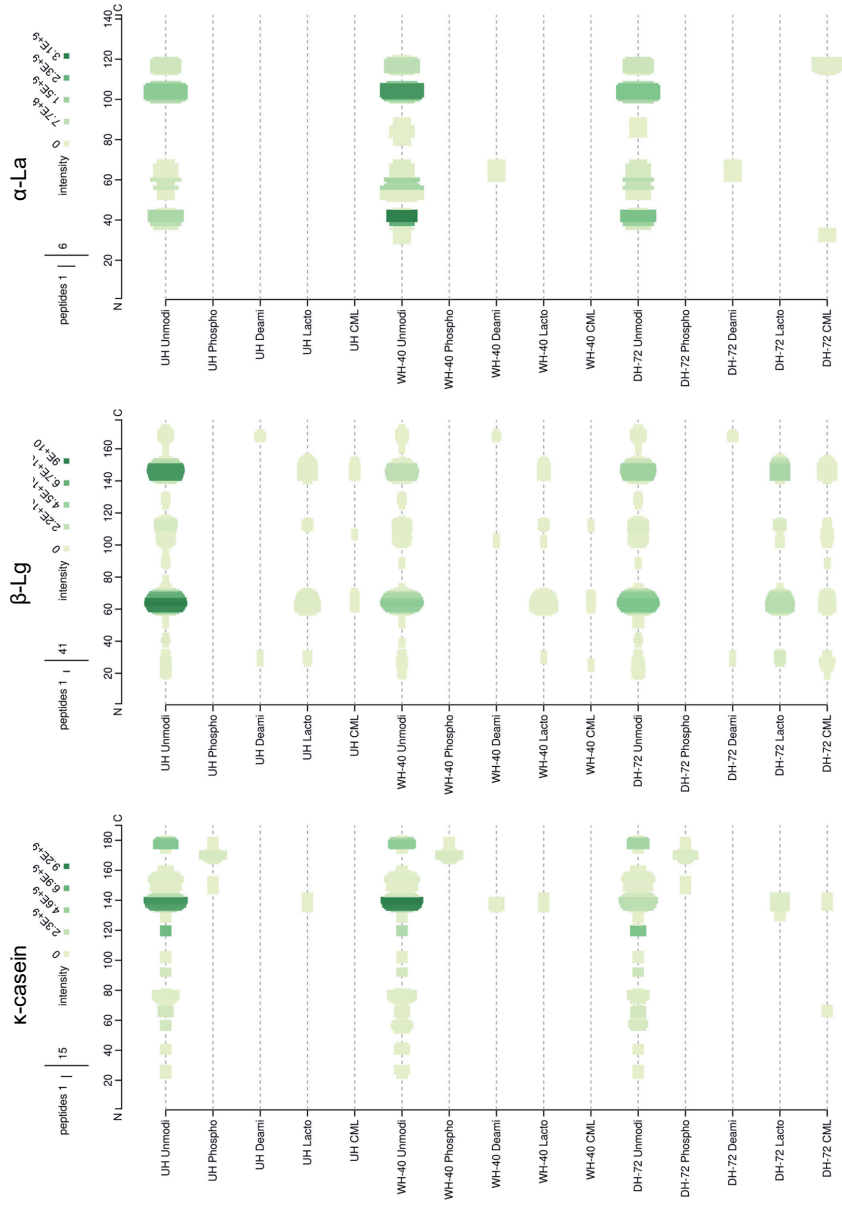

Figure 4.S2: (Caption on next page)

Figure 4.S2: Peptide alignment for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin (β-Lg), and α-lactalbumin (α-La) detected with LC-MS/MS in the detoxified intestinal digests. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model. Peptide alignments are shown separately for unmodified (Unmodi), phosphorylated (Phospho), deamidation (Deami) modified, lactosylated (Lacto) and carboxymethyllysine (CML) modified peptides. The color of the bars represents the sum of peptide intensities that overlap at this position, and the height of the bars represents the number of peptides (peptide count) that overlap at this position. The amino acid position is given including the signal peptides of 15, 15, 15, 21, 16, and 19 amino acids for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-Lg, and α-La, respectively.

Table 4.S1: HLA-II epitopes predicited in the detoxified intestinal digests by use of the IEDB MHC Class II Binding Prediction tool. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model. Amino acid (AA) position provides the position of the peptide containing an HLA-II epitope in the protein without the signal peptide. Modification indicates if the peptide was detected in the digest in unmodified, phosphorylated, deamidation modified, lactosylated, or carboxymethyllysine (CML) modified form. All missing values were not detected. β-Lg: β-lactoglobulin, α-La: α-lactalbumin.

Protein of origin	Peptide	AA position	HLA gene	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
α_{s1}-casein	FFVAPFPEVFG	23-33	HLA-DP, HLA-DQ, HLA-DR	9.14E+06	6.57E+08	1.08E+07	Unmodified
	KYKVPQLEIVPNSAEER	103-119	HLA-DR		1.13E+08		Phosphorylation
	YKVPQLEIVPNSAEE	104-118	HLA-DR	1.24E+07	1.96E+08		Phosphorylation
	YKVPQLEIVPNSAEER	104-119	HLA-DR	1.02E+08	1.35E+09	2.78E+07	Phosphorylation
	KVPQLEIVPNSAEE	105-118	HLA-DR		2.91E+08		Phosphorylation
	KVPQLEIVPNSAEER	105-119	HLA-DR	3.48E+08	2.78E+09	7.26E+07	Phosphorylation, Deamidation
	VPQLEIVPNSAEER	106-119	HLA-DR	2.97E+08	1.55E+09	2.11E+08	Phosphorylation, Deamidation
	PQLEIVPNSAEER	107-119	HLA-DR		1.81E+06		Phosphorylation
	QLEIVPNSAEER	108-119	HLA-DR	1.59E+07	1.44E+07	1.24E+07	Phosphorylation
	LEIVPNSAEER	109-119	HLA-DR	1.29E+09	4.50E+09	7.07E+08	Phosphorylation, Deamidation
	EGIHAQQKEPM	125-135	HLA-DR	2.67E+07	2.48E+07	5.37E+07	Unmodified, Deamidation

Table 4.S1: (Continued)

Protein of origin	Peptide	AA position	HLA gene	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
α_{s1}-casein	EGIHAQQKEPMIGV	125-138	HLA-DR		7.44E+06	7.51E+06	Unmodified
	EPMIGVNQELA	133-143	HLA-DQ	4.10E+07	1.10E+08	8.49E+07	Unmodified
	TDAPSFSDIPNPIGSEN	174-190	HLA-DR	1.52E+07	4.29E+07	4.11E+07	Unmodified
α_{s2}-casein	NMAINPSKENL	25-35	HLA-DR	6.37E+08	2.68E+08	3.67E+08	Unmodified, Phosphorylation, Lactosylation
	KITVDDKHYQK	70-80	HLA-DR		3.53E+07		Unmodified
	YQGPIVLNPWDQV	100-112	HLA-DR	4.30E+07	1.10E+07	2.60E+08	Unmodified
	YQGPIVLNPWDQVK	100-113	HLA-DR	5.29E+06		7.59E+08	Lactosylation
	QGPIVLNPWDQV	101-112	HLA-DR, HLA-DP	6.50E+07	1.09E+08	1.31E+08	Unmodified
	QGPIVLNPWDQVK	101-113	HLA-DR, HLA-DP	2.93E+06		2.31E+08	Lactosylation
	TKLTEEEKNRL	151-161	HLA-DR		1.21E+07		Unmodified
	TKLTEEEKNRLN	151-162	HLA-DR	3.32E+07	2.56E+07		Unmodified
β-casein	EELNVPGEIVES	4-15	HLA-DQ	6.18E+07	3.06E+07	2.11E+07	Unmodified
	EELNVPGEIVESL	4-16	HLA-DQ	2.21E+07	2.46E+07	8.93E+06	Phosphorylation
	LNVPGEIVESL	6-16	HLA-DQ	5.54E+06	5.87E+06	5.69E+06	Phosphorylation
	KIEKFQSEEQQ	29-39	HLA-DR, HLA-DQ			1.21E+07	Phosphorylation, CML
	TPVVVPPFLQP	80-90	HLA-DP	5.35E+07	2.01E+07	9.39E+06	Unmodified
	TPVVVPPFLQPEV	80-92	HLA-DP	1.14E+08	1.04E+08	6.29E+07	Unmodified
	PVVVPPFLQPE	81-91	HLA-DP	2.82E+08	9.53E+07	1.48E+08	Unmodified
	SLSQSKVLPVPQ	164-175	HLA-DR, HLA-DQ	3.20E+06	2.30E+08	6.89E+09	Lactosylation, CML
	LSQSKVLPVPQ	165-175	HLA-DQ			9.71E+07	Lactosylation
κ-casein	ALINNQFLPYP	49-59	HLA-DP, HLA-DR, HLA-DQ	2.55E+05	1.32E+07		Unmodified

Table 4.S1: (Continued)

Protein of origin	Peptide	AA position	HLA gene	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
κ-casein	TIASGEPTSTPT	124-135	HLA-DQ	2.16E+07	1.34E+07	1.69E+07	Unmodified, Phosphorylation
	IASGEPTSTPT	125-135	HLA-DQ	1.48E+07	8.21E+06		Unmodified
	IASGEPTSTPTTE	125-137	HLA-DQ	5.18E+07	1.21E+07	4.64E+07	Unmodified
	IASGEPTSTPTTEA	125-138	HLA-DQ	4.12E+07	1.23E+07	3.74E+07	Unmodified
β-Lg	IIAEKTKIPAVF	71-82	HLA-DR	9.09E+06			Unmodified
	KIDALNENKVL	83-93	HLA-DR	4.85E+08	1.58E+07	1.03E+08	Unmodified, CML
	KIDALNENKVLV	83-94	HLA-DR	8.74E+07		3.48E+07	Unmodified, CML
	VLVLDTDYKKY	92-102	HLA-DR	4.73E+07	3.80E+06		Unmodified
	TPEVDDEALEKF	125-136	HLA-DQ	1.95E+09	1.11E+08	1.39E+09	Unmodified, Lactosylation, CML
	LSFNPTQLEEQ	149-159	HLA-DQ	4.47E+07		7.06E+07	Unmodified
α-La	GYGGVSLPEWV	17-27	HLA-DQ	5.18E+07	6.86E+07	7.95E+07	Unmodified
	IVQNNDSTEYG	41-51	HLA-DR	3.21E+07	1.58E+08	2.55E+08	Unmodified, Deamidation
Osteopontin	DKNKHSNLIESQ	207-218	HLA-DQ	8.50E+06	6.65E+06		Phosphorylation
Desmoplakin	LQKYQAECSQFK	1072-1083	HLA-DR	3.34E+08	1.30E+07	5.26E+07	Phosphorylation, CML
GRIP and coiled-coil domain containing 2	IDQLKLKLQDTQNS	1358-1371	HLA-DR	5.98E+08	6.92E+07	4.32E+08	Deamidation, CML
Inositol 1,4,5-trisphosphate receptor	IQKSFYNLMTS	1761-1771	HLA-DP, HLA-DQ, HLA-DR	1.71E+08	2.91E+07	4.26E+07	Phosphorylation
Nucleobindin-1	ILHDINSDGVLDEQ	247-260	HLA-DQ, HLA-DR		1.38E+07	1.99E+07	Unmodified
Polymeric immunoglobulin receptor	KVVQGEPSLKVPK	455-467	HLA-DP, HLA-DR	3.25E+07			Unmodified

Table 4.S1: (Continued)

Protein of origin	Peptide	AA position	HLA gene	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
TSPO associated protein 1	WQSWTPGRGGDA	24-35	HLA-DQ	6.66E+07	1.61E+07		Deamidation, Phosphorylation
Count				**42**	**43**	**37**	
Total intensity				**7.54E+09**	**1.32E+10**	**1.28E+10**	

Table 4.S2: Linear IgE epitopes predicted in the detoxified intestinal digests. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model. Amino acid (AA) position provides the position of the peptide containing a linear IgE epitope in the protein without the signal peptide. Modification indicates if the peptide was detected in the digest in unmodified, phosphorylated, deamidation modified, lactosylated, or carboxymethyllysine (CML) modified form. All missing values were not detected. β-Lg: β-lactoglobulin, α-La: α-lactalbumin.

Protein of origin	Peptide	AA position	IgE epitope position	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
α_{s1}-casein	HQGLPQEVLNENL	8-20	6-20		6.39E+06		Unmodified
	KYKVPQLEIVPNSAEER	103-119	109-120		1.13E+08		Phosphorylation
	YKVPQLEIVPNSAEE	104-118	109-120	1.24E+07	1.96E+08		Phosphorylation
	YKVPQLEIVPNSAEER	104-119	109-120	1.02E+08	1.35E+09	2.78E+07	Phosphorylation
	KVPQLEIVPNSAEE	105-118	109-120		2.91E+08		Phosphorylation
	KVPQLEIVPNSAEER	105-119	109-120	3.48E+08	2.78E+09	7.26E+07	Phosphorylation, Deamidation
	VPQLEIVPNSAEER	106-119	109-120	2.97E+08	1.55E+09	2.11E+08	Phosphorylation, Deamidation
	PQLEIVPNSAEER	107-119	109-120		1.81E+06		Phosphorylation
	QLEIVPNSAEER	108-119	109-120	1.59E+07	1.44E+07	1.24E+07	Phosphorylation
	LEIVPNSAEE	109-118	109-120	1.05E+08	5.87E+08	5.71E+07	Phosphorylation
	LEIVPNSAEER	109-119	109-120	1.29E+09	4.50E+09	7.07E+08	Phosphorylation, Deamidation
	EIVPNSAEER	110-119	109-120	7.23E+09	1.65E+09	6.66E+09	Phosphorylation, Deamidation

Table 4.S2: (Continued)

Protein of origin	Peptide	AA position	IgE epitope position	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
α_{s1}-casein	LHSMKEGIHAQ	120-130	122-132		5.89E+07		Unmodified
	SMKEGIHAQ	122-130	122-132		1.26E+07		Unmodified
	EGIHAQQKEPMIGV	125-138	126-140		7.44E+06	7.51E+06	Unmodified
	EGIHAQQKEPMIGVNQEL	125-142	126-140		1.09E+07		Unmodified
	EGIHAQQKEPMIGVNQELA	125-143	126-140		9.74E+07		Unmodified
	AQQKEPMIGVNQEL	129-142	126-140	7.58E+06	5.82E+06	1.16E+07	Unmodified
	AQQKEPMIGVNQELA	129-143	126-140	1.34E+07	1.86E+07	1.59E+07	Unmodified
	QYTDAPSFSDIPNPI	172-186	171-185		1.07E+09		Unmodified
	QYTDAPSFSDIPNPIGSENSEK	172-193	171-185; 173-194		1.59E+07	7.01E+06	Unmodified
	YTDAPSFSDIPNPI	173-186	171-185	2.95E+08	4.47E+07	2.99E+08	Unmodified
	TDAPSFSDIPNPI	174-186	171-185	2.95E+09	7.23E+06	1.27E+07	Unmodified, Phosphorylation
	TDAPSFSDIPNPIGSEN	174-190	171-185	1.52E+07	4.29E+07	4.11E+07	Unmodified
	TDAPSFSDIPNPIGSENSEK	174-193	171-185; 173-194	1.76E+07	1.15E+08	5.57E+07	Unmodified
	TDAPSFSDIPNPIGSENSEKT	174-194	171-185; 173-194	5.19E+06	3.18E+07	1.02E+07	Unmodified
α_{s2}-casein	YQGPIVLNPWDQV	100-112	105-114	4.30E+07	1.10E+07	2.60E+08	Unmodified
	YQGPIVLNPWDQVK	100-113	105-114	5.29E+06		7.59E+08	Lactosylation
	QGPIVLNPWDQV	101-112	105-114	6.50E+07	1.09E+08	1.31E+08	Unmodified
	QGPIVLNPWDQVK	101-113	105-114	2.93E+06		2.31E+08	Lactosylation
β-casein	EELNVPGEIVESL	4-16	1-16	2.21E+07	2.46E+07	8.93E+06	Phosphorylation
	INKKIEKFQSEEQQQTEDELQDK	26-48	25-50			4.31E+07	Phosphorylation, Lactosylation
	KIEKFQSEEQQQTEDELQDKIHP	29-51	25-50	5.51E+06		9.03E+07	Phosphorylation, Lactosylation
	IEKFQSEEQQQTEDELQDKIHP	30-51	25-50	5.99E+07	3.18E+07	2.03E+08	Phosphorylation, Lactosylation

Table 4.S2: (Continued)

Protein of origin	Peptide	AA position	IgE epitope position	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
β-casein	FQSEEQQQTEDELQDKIHPF	33-52	45-54	6.00E+06	3.83E+07		Phosphorylation
	QTEDELQDKIHPF	40-52	45-54		8.28E+06		Unmodified
	QTEDELQDKIHPFA	40-53	45-54			6.53E+07	Lactosylation
	TEDELQDKIHPF	41-52	45-54	2.22E+07	5.85E+07	1.94E+07	Unmodified
	TEDELQDKIHPFA	41-53	45-54		1.65E+07	4.61E+07	Unmodified, Lactosylation
	QDKIHPFA	46-53	45-54	2.91E+08	1.48E+08	1.85E+08	Unmodified
	QSLVYPFPGPIPN	56-68	55-70	9.38E+08	1.04E+09	1.23E+09	Unmodified
	QSLVYPFPGPIPNS	56-69	55-70	2.50E+07	2.61E+07	4.70E+07	Unmodified
	SLVYPFPGPIPNS	57-69	55-70	5.53E+07	1.01E+08	6.95E+07	Unmodified
	NSLPQNIPPLTQTPVVVPPFLQPEV	68-92	83-92	1.73E+07	8.40E+06	6.44E+07	Unmodified
	SLPQNIPPLTQTPVVVPPFLQP	69-90	83-92	9.03E+06	5.56E+06	1.61E+07	Unmodified
	SLPQNIPPLTQTPVVVPPFLQPE	69-91	83-92	4.14E+07	4.43E+07	3.83E+07	Unmodified
	SLPQNIPPLTQTPVVVPPFLQPEV	69-92	83-92	1.05E+09	7.36E+08	1.71E+09	Unmodified, Deamidation
	LPQNIPPLTQTPVVVPPFLQPEV	70-92	83-92	3.67E+07	2.71E+07	6.88E+07	Unmodified
	QNIPPLTQTPVVVPPFLQPEV	72-92	83-92	7.60E+06	4.16E+06	1.21E+07	Unmodified
	NIPPLTQTPVVVPPFLQP	73-90	83-92	2.10E+07	1.63E+07	2.41E+07	Unmodified
	NIPPLTQTPVVVPPFLQPE	73-91	83-92	8.77E+07	7.92E+07	6.09E+07	Unmodified
	NIPPLTQTPVVVPPFLQPEV	73-92	83-92	1.45E+09	7.79E+08	1.22E+09	Unmodified
	NIPPLTQTPVVVPPFLQPEVMG	73-94	83-92	1.71E+07	1.31E+07	2.76E+07	Unmodified
	IPPLTQTPVVVPPFLQPEV	74-92	83-92	5.80E+07	5.18E+07	1.16E+08	Unmodified
	PPLTQTPVVVPPFLQPEV	75-92	83-92	8.31E+06	5.52E+06		Unmodified
	TQTPVVVPPFLQPEV	78-92	83-92	2.34E+08	2.73E+08	1.47E+08	Unmodified
	QTPVVVPPFLQP	79-90	83-92	7.30E+07	4.23E+07	2.50E+07	Unmodified
	QTPVVVPPFLQPE	79-91	83-92	2.06E+07	1.23E+07	2.03E+07	Unmodified
	QTPVVVPPFLQPEV	79-92	83-92	4.17E+08	2.45E+08	6.43E+08	Unmodified, Deamidation
	QTPVVVPPFLQPEVMG	79-94	83-92	7.39E+06	6.53E+06	1.61E+07	Unmodified

Table 4.S2: (Continued)

Protein of origin	Peptide	AA position	IgE epitope position	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
β-casein	TPVVVPPFLQP	80-90	83-92	5.35E+07	2.01E+07	9.39E+06	Unmodified
	TPVVVPPFLQPEV	80-92	83-92	1.14E+08	1.04E+08	6.29E+07	Unmodified
	PVVVPPFLQP	81-90	83-92	4.06E+07	1.39E+07	3.60E+07	Unmodified
	PVVVPPFLQPE	81-91	83-92	2.82E+08	9.53E+07	1.48E+08	Unmodified
	PVVVPPFLQPEV	81-92	83-92	3.35E+07	2.00E+09	5.17E+07	Unmodified, Deamidation
	PVVVPPFLQPEVM	81-93	83-92	3.98E+07	3.26E+06	1.26E+07	Unmodified
	PVVVPPFLQPEVMG	81-94	83-92	1.55E+08	3.72E+07	1.52E+08	Unmodified
	VVVPPFLQPEV	82-92	83-92	6.41E+07	3.72E+07	5.65E+07	Unmodified
	VVPPFLQPE	83-91	83-92	3.83E+08	4.72E+08	1.68E+08	Unmodified
	VVPPFLQPEV	83-92	83-92	7.23E+09	9.13E+09	5.90E+09	Unmodified, Deamidation
	VVPPFLQPEVMG	83-94	83-92	1.28E+08	1.57E+08	1.36E+08	Unmodified
	VPPFLQPEV	84-92	83-92	1.30E+07	1.01E+07	6.93E+06	Unmodified
	PPFLQPEV	85-92	83-92	2.55E+08	2.49E+08	2.19E+08	Unmodified
	HKEMPFPKYPVEP	106-118	107-120	3.07E+06	3.50E+06	3.28E+07	Lactosylation
	HKEMPFPKYPVEPF	106-119	107-120		2.40E+07	3.33E+07	Unmodified, Lactosylation
	EMPFPKYPVEPF	108-119	107-120	2.89E+06	1.90E+08	2.28E+09	Unmodified, Lactosylation
	WMHQPHQPLPPTVMFPPQS	143-161	149-164		6.31E+06		Unmodified
	MHQPHQPLPPTVMFPPQS	144-161	149-164	1.00E+08	7.57E+08		Unmodified
	MHQPHQPLPPTVMFPPQSV	144-162	149-164	1.96E+08	1.09E+09	3.68E+07	Unmodified
	HQPHQPLPPTVMFPPQS	145-161	149-164	2.29E+07	2.61E+08		Unmodified
	HQPHQPLPPTVMFPPQSV	145-162	149-164	1.03E+08	5.74E+08	8.22E+06	Unmodified
	QPHQPLPPTVMFPPQSV	146-162	149-164		3.52E+06		Unmodified
	QPLPPTVMFPPQS	149-161	149-164		5.99E+07	1.28E+06	Unmodified
	QPLPPTVMFPPQSV	149-162	149-164	1.85E+07	1.22E+08	2.83E+06	Unmodified
κ-casein	MAIPPKKNQDKTEIPTINT	106-124	111-126			1.72E+07	Lactosylation

Table 4.S2: (Continued)

Protein of origin	Peptide	AA position	IgE epitope position	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
κ-casein	KNQDKTEIPTINT	112-124	111-126	8.11E+08	2.74E+08	3.78E+08	Unmodified, Lactosylation
β-Lg	LIVTQTMKGLDIQK	1-14	1-16			3.06E+06	CML
	VRTPEVDDEALEKFDK	123-138	121-140	2.72E+09	5.12E+07	1.46E+09	Unmodified, Lactosylation, CML
	VRTPEVDDEALEKFDKA	123-139	121-140	5.50E+07		2.24E+08	Unmodified, Lactosylation
	RTPEVDDEALEKFDKA	124-139	121-140	9.64E+06		4.21E+06	Unmodified
	TPEVDDEALEKFDKAL	125-140	121-140	2.34E+07		1.43E+09	Unmodified, Lactosylation, CML
	TPEVDDEALEKFDKALK	125-141	121-140	4.28E+07		1.28E+08	Unmodified, Lactosylation
α-La	KILDKVGI	94-101	93-102	4.55E+07	7.83E+07	2.37E+07	Unmodified
	KILDKVGIN	94-102	93-102	1.27E+08	2.05E+08	7.91E+07	Unmodified, CML
	ILDKVGIN	95-102	93-102	3.20E+08	4.92E+08	5.14E+08	Unmodified, CML
	ILDKVGINY	95-103	93-102		3.24E+07		Unmodified
Count				**73**	**84**	**77**	
Total intensity				**3.04E+10**	**3.48E+10**	**2.91E+10**	

Table 4.S3: Bioactive peptides identified in the detoxified intestinal digests by use of the milk bioactive peptide database (MBPDB). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model. Amino acid (AA) position provides the position of the peptide in the protein that was identical to a peptide in the MBPDB without the signal peptide. Modification indicates if the peptide was detected in the digest in unmodified, phosphorylated, deamidation modified, lactosylated, or carboxymethyllysine (CML) modified form. All missing values were not detected. β-Lg: β-lactoglobulin, α-La: α-lactalbumin.

Protein of origin	Peptide	AA position	Bioactive function	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
α_{s1}-casein	FVAPFPEVFG	24-33	ACE-inhibitory	5.47E+08	3.49E+08	8.81E+08	Unmodified
	DIGSESTEDQAMEDIK	43-58	Promotes calcium uptake	3.31E+07	7.22E+06	8.13E+06	Phosphorylation
	YKVPQLEIVPNSAEER	104-119	Promotes calcium uptake	1.02E+08	1.35E+09	2.78E+07	Phosphorylation
	SDIPNPIGSENSEK	180-193	Antimicrobial	3.90E+08	1.11E+07	1.02E+09	Unmodified, Phosphorylation, Lactosylation
α_{s2}-casein	NMAINPSK	25-32	ACE-inhibitory	6.02E+07	3.82E+07	3.18E+07	Unmodified, Phosphorylation
	ALNEINQFYQK	81-91	ACE-inhibitory		6.99E+06		Unmodified
	VPITPT	117-122	DPP-IV Inhibitory	3.34E+08	1.35E+08	3.28E+08	Unmodified
β-casein	LNVPGEIVE	6-14	ACE-inhibitory	3.58E+07		3.05E+07	Unmodified
	NVPGEIVESL	7-16	Antioxidant	7.39E+07	9.53E+07	1.01E+08	Phosphorylation
	FQSEEQQQTEDELQDK	33-48	Promotes calcium uptake	9.68E+08	8.37E+08	1.16E+09	Unmodified, Phosphorylation, Deamidation
	FQSEEQQQTEDELQDKIHPF	33-52	Promotes calcium uptake	6.00E+06	3.83E+07		Phosphorylation
	VYPFPGPI	59-66	PEP-inhibitory	6.92E+07	4.91E+07	3.63E+07	Unmodified
	VYPFPGPIPN	59-68	Antioxidant, ACE-inhibitory	4.31E+09	3.28E+09	3.22E+09	Unmodified, Deamidation

Table 4.S3: (Continued)

Protein of origin	Peptide	AA position	Bioactive function	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
β-casein	YPFPGPI	60-66	Satiety, Reduces pancreas MDA level, Opioid, Increases MUC5A expression, Increases MUC3 expression, Increases MUC2 expression, Increases jejunal mucus secretion, immunomodulatory, Anxiolytic, Anticancer, ACE-inhibitory, Antioxidant	9.30E+07	1.36E+08	4.74E+07	Unmodified
	YPFPGPIPN	60-68	DPP-IV Inhibitory, ACE-inhibitory, Antioxidant	3.25E+08	3.76E+08	1.68E+08	Unmodified
	YPFPGPIPNS	60-69	Anti-anxiety	2.61E+07	1.13E+07	5.94E+06	Unmodified
	PFPGPIPN	61-68	ACE-inhibitory	9.53E+08	7.58E+08	5.08E+08	Unmodified
	FPGPIPN	62-68	DPP-IV Inhibitory	1.74E+06	5.30E+06		Unmodified
	PGPIPN	63-68	immunomodulatory, Anticancer, Anti-inflammatory, ACE-inhibitory	6.20E+07	4.44E+07	5.71E+07	Unmodified
	SLPQNIPPL	69-77	DPP-IV Inhibitory	2.07E+08	1.36E+08	1.82E+08	Unmodified
	SLPQNIPPLTQTPVVVPPF	69-87	Anticancer	1.24E+08	1.08E+08	1.12E+08	Unmodified
	LPQNIPPLT	70-78	DPP-IV Inhibitory	6.06E+06	3.61E+06	5.85E+06	Unmodified
	NIPPLTQTPV	73-82	ACE-inhibitory	6.66E+08	1.58E+09	4.72E+08	Unmodified
	IPPLTQT	74-80	DPP-IV Inhibitory	3.11E+07	1.10E+07	4.03E+07	Unmodified
	TPVVVPPFLQP	80-90	ACE-inhibitory	5.35E+07	2.01E+07	9.39E+06	Unmodified
	HKEMPFPK	106-113	Antimicrobial	2.67E+09	1.41E+09	5.44E+08	Unmodified, CML
	FPKYPVEPF	111-119	Antioxidant	2.49E+07	2.85E+07	1.94E+07	Unmodified

Table 4.S3: (Continued)

Protein of origin	Peptide	AA position	Bioactive function	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
β-casein	YPVEPF	114-119	Opioid, Increases MUC4 expression, DPP-IV Inhibitory, Antioxidant, Antimicrobial	7.02E+09	6.48E+09	4.93E+09	Unmodified
	DVENLHLPLPL	129-139	Antimicrobial	2.98E+06	1.82E+07	1.04E+07	Unmodified
	WMHQPHQPLPPT	143-154	Anti-inflammatory, ACE-inhibitory	3.26E+09	8.51E+08	3.19E+09	Unmodified
	HQPHQPLPPT	145-154	ACE-inhibitory	3.78E+09	1.52E+09	2.11E+09	Unmodified
	HQPHQPLPPTVMFPPQ	145-160	Anti-inflammatory, ACE-inhibitory	5.74E+06	1.12E+08		Unmodified
	SQSKVLPVPQ	166-175	ACE-inhibitory			1.78E+07	CML
	SKVLPVPQ	168-175	ACE-inhibitory	2.10E+07	8.81E+06	1.32E+07	Unmodified
	LYQEPVLGPVR	192-202	Anti-inflammatory, ACE-inhibitory	3.40E+07	9.52E+07	3.80E+07	Unmodified
	YQEPVLGPVR	193-202	ACE-inhibitory, Immunomodulatory, Antithrombotic, Antioxidant, Anti-inflammatory	8.83E+07	2.57E+07	1.19E+08	Unmodified
	YQEPVLGPVRGPFPIIV	193-209	ACE-inhibitory, Immunomodulatory, Antithrombotic, Antioxidant, Anticancer	3.35E+06	3.76E+06	1.22E+07	Unmodified
	QEPVLGPVRGPFPIIV	194-209	ACE-inhibitory		8.40E+07		Unmodified
κ-casein	YPSYGLN	35-41	Opioid		4.48E+07	2.51E+07	Unmodified
	YYQQKPVA	42-49	Antimicrobial	7.25E+08	1.22E+08	8.25E+08	Unmodified, CML
	MAIPPKKNQDKTEIPTINT	106-124	Antimicrobial			1.72E+07	Lactosylation
β-Lg	LIVTQTMK	1-8	Cytotoxic	3.43E+08	3.71E+07	1.25E+08	Unmodified

Table 4.S3: (Continued)

Protein of origin	Peptide	AA position	Bioactive function	Intensity UH	Intensity WH-40	Intensity DH-72	Modification
β-Lg	GLDIQKVAGT	9-18	Antimicrobial	2.08E+08	5.91E+06	8.86E+09	Unmodified, Deamidation, Lactosylation, CML
	DAQSAPLRVY	33-42	ACE-inhibitory			4.08E+07	Unmodified
	VYVEELKPTPEGDLEILLQK	41-60	Hypocholesterolemic	5.65E+06	8.71E+05	7.24E+06	Unmodified
	LKPTPEGDL	46-54	DPP-IV Inhibitory	2.33E+07	4.24E+08	1.84E+07	Unmodified
	LKPTPEGDLE	46-55	DPP-IV Inhibitory	2.67E+07	3.18E+08	1.65E+07	Unmodified
	IDALNENK	84-91	Stimulates proliferation, Antimicrobial	1.13E+08	1.37E+08	1.41E+08	Unmodified
	VLVLDTDYK	92-100	DPP-IV Inhibitory, Antimicrobial	8.06E+08	1.47E+08	5.46E+08	Unmodified, Lactosylation
	VLDTDYK	94-100	ACE-inhibitory	6.66E+09	1.30E+09	8.25E+09	Unmodified, Lactosylation
	TPEVDDEALEK	125-135	DPP-IV Inhibitory, Antimicrobial	3.74E+10	1.92E+10	3.69E+10	Unmodified, Lactosylation, CML
	ALPMHIR	142-148	Stimulates proliferation, Reduces vasoconstrictor endothelin-1 release, ACE-inhibitory	1.74E+08	1.63E+07	6.88E+07	Unmodified
	LSFNPTQ	149-155	ACE-inhibitory	1.70E+07		2.11E+07	Deamidation
α-La	GYGGVSLPEW	17-26	ACE-inhibitory	1.89E+08		2.52E+08	Unmodified
	VSLPEW	21-26	ACE-inhibitory	3.68E+08	7.46E+08	4.59E+08	Unmodified
	IWCKDDQNPH	59-68	Antioxidant		2.51E+06		Unmodified
	ILDKVGINY	95-103	DPP-IV Inhibitory		3.24E+07		Unmodified
Count				**49**	**51**	**50**	
Total intensity				**7.35E+10**	**4.25E+10**	**7.60E+10**	

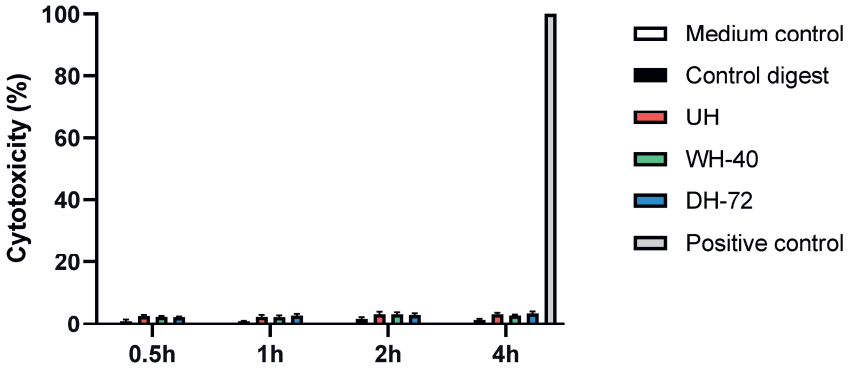

Figure 4.S3: Cytotoxicity effects on HT29-MTX-E12 cells after exposure to the detoxified intestinal digests for the mucin gene expression and mucus production experiments as measured with the LDH assay. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the HT29-MTX-E12 cells for 0.5-4h. Water instead of the infant formula model system was digested with the *in vitro* digestion model to obtain a control digest. Medium control were HT29-MTX-E12 cells grown in DMEM. The LDH positive control Triton X100 releases the maximum amount of LDH present within the HT29-MTX-E12 cells, and was set at 100%.

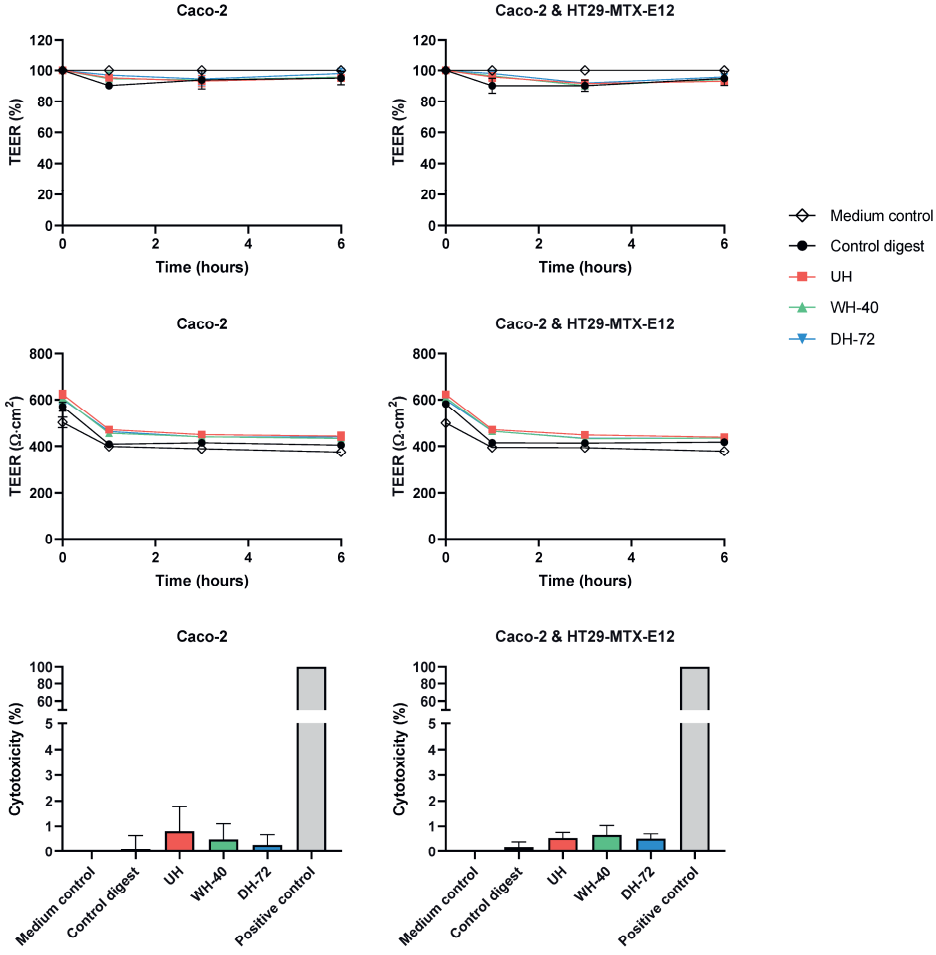

Figure 4.S4: Relative and absolute transepithelial electrical resistance (TEER) values 0-6h and cytotoxicity effects 6h after detoxified intestinal digests were applied to the apical side of the 21-days differentiated Caco-2 and Caco-2/HT29-MTX-E12 monolayers. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 0-6h. Water instead of the infant formula model system was digested with the *in vitro* digestion model to obtain a control digest. Medium control were Caco-2 and HT29-MTX-E12 cells grown in DMEM. Cytotoxicity was measured with the LDH assay. The LDH positive control Triton X100 releases the maximum amount of LDH present within the cell monolayers, and was set at 100%.

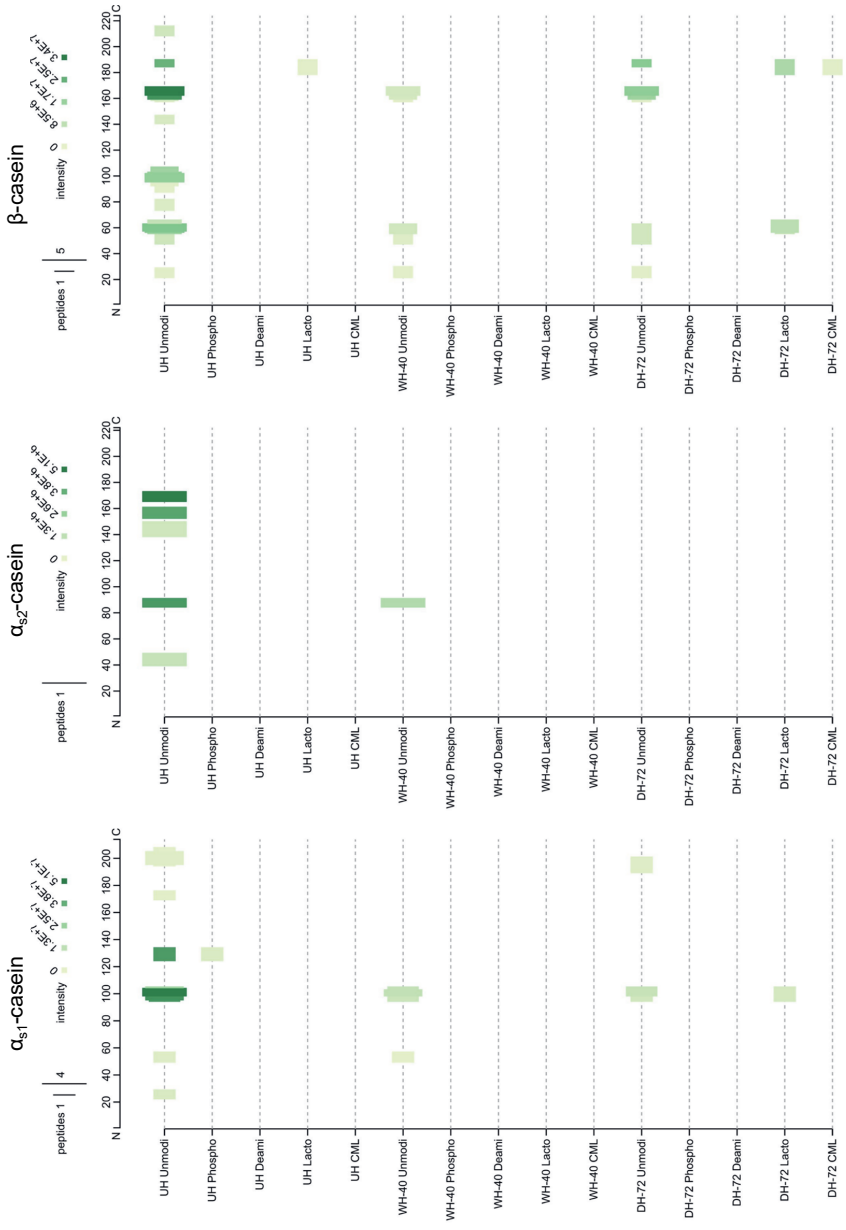

Figure 4.S5: (Continued on next page)

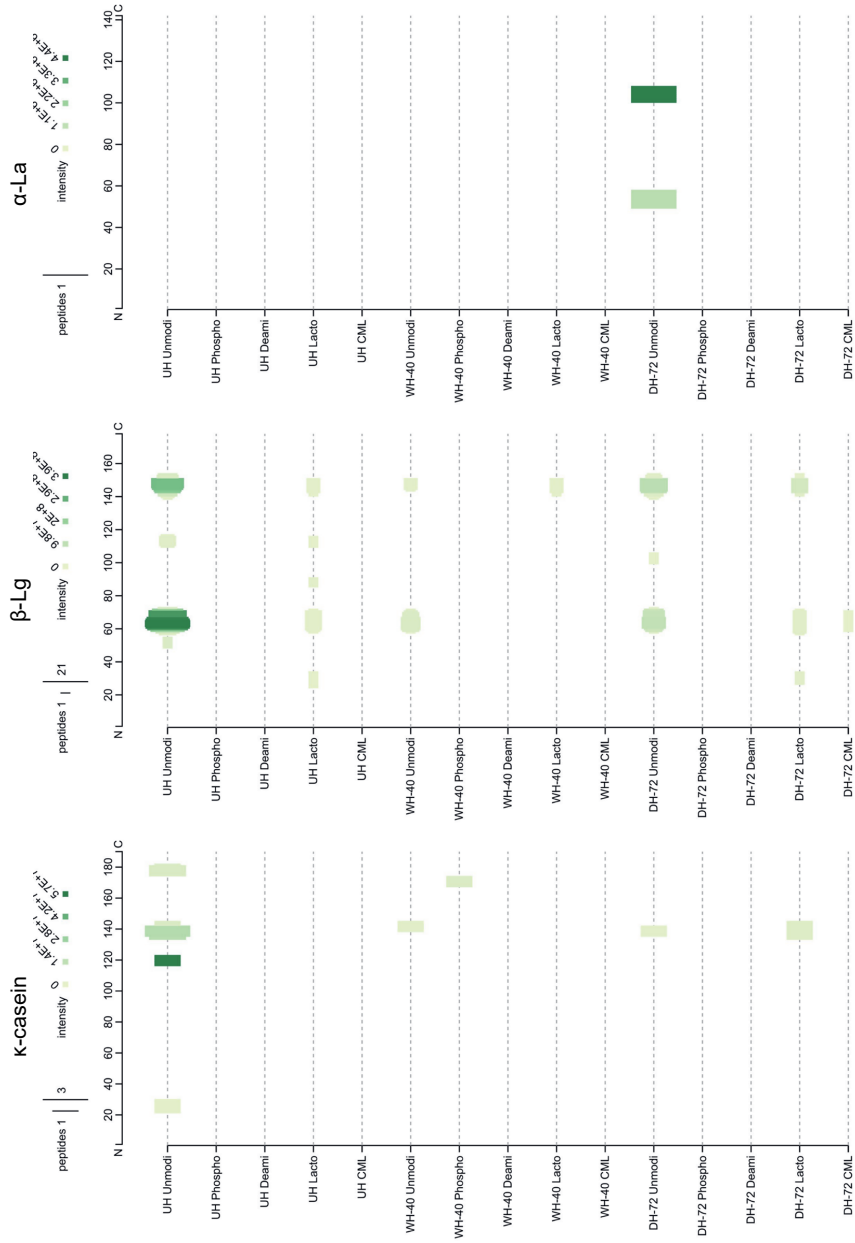

Figure 4.S5: (Caption on next page)

Figure 4.S5: Peptide alignment for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin (β-Lg), and α-lactalbumin (α-La) detected with LC-MS/MS at the basolateral side after transport across a 21-days differentiated Caco-2 monolayer. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 cells for 6h. Peptide alignments are shown separately for unmodified (Unmodi), phosphorylated (Phospho), deamidation (Deami) modified, lactosylated (Lacto) and carboxymethyllysine (CML) modified peptides. The color of the bars represents the sum of peptide intensities that overlap at this position, and the height of the bars represents the number of peptides (peptide count) that overlap at this position. The amino acid position is given including the signal peptides of 15, 15, 15, 21, 16, and 19 amino acids for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-Lg, and α-La, respectively.

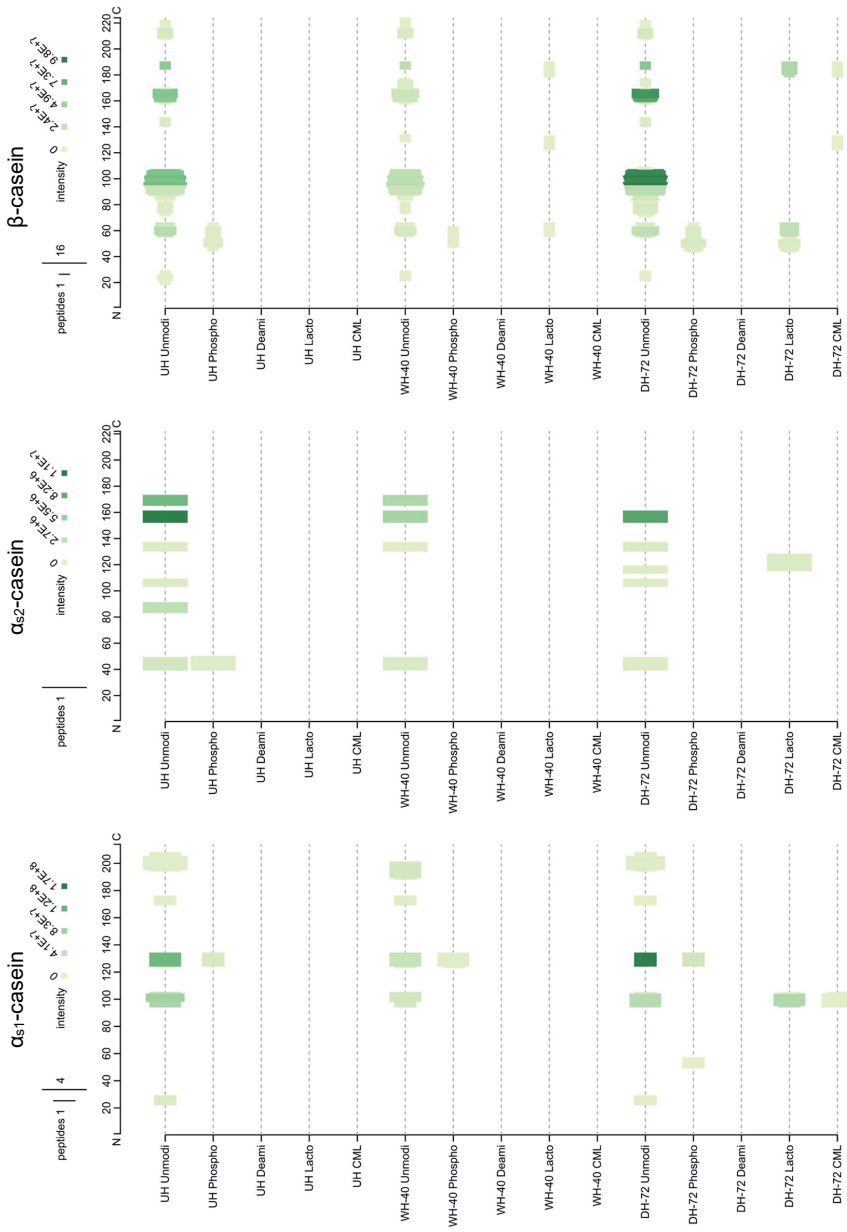

Figure 4.S6: (Continued on next page)

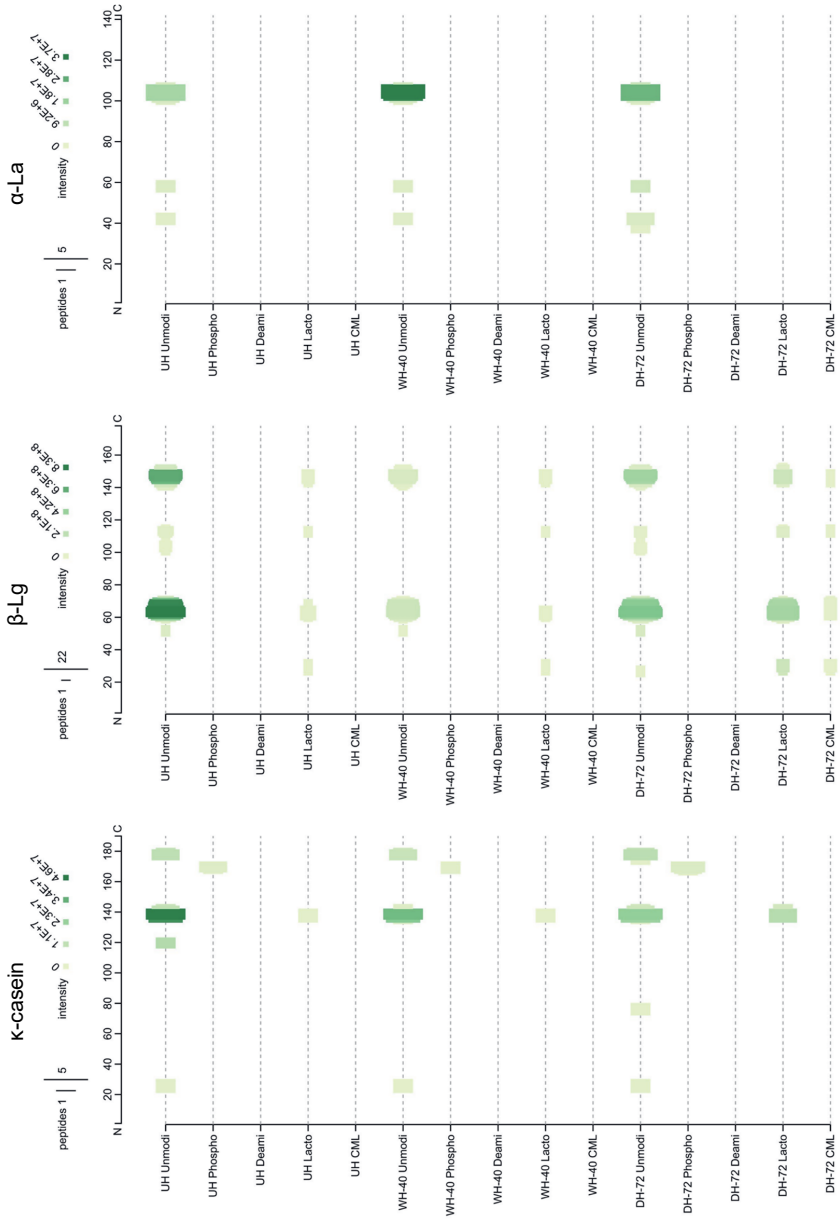

Figure 4.S6: (Caption on next page)

Figure 4.S6: Peptide alignment for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin (β-Lg), and α-lactalbumin (α-La) detected with LC-MS/MS at the basolateral side after transport across a 21-days differentiated Caco-2/HT29-MTX-E12 (90/10) monolayer. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6h. Peptide alignments are shown separately for unmodified (Unmodi), phosphorylated (Phospho), deamidation (Deami) modified, lactosylated (Lacto), and carboxymethyllysine (CML) modified peptides. The color of the bars represents the sum of peptide intensities that overlap at this position, and the height of the bars represents the number of peptides (peptide count) that overlap at this position. The amino acid position is given including the signal peptides of 15, 15, 15, 21, 16, and 19 amino acids for α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-Lg, and α-La, respectively.

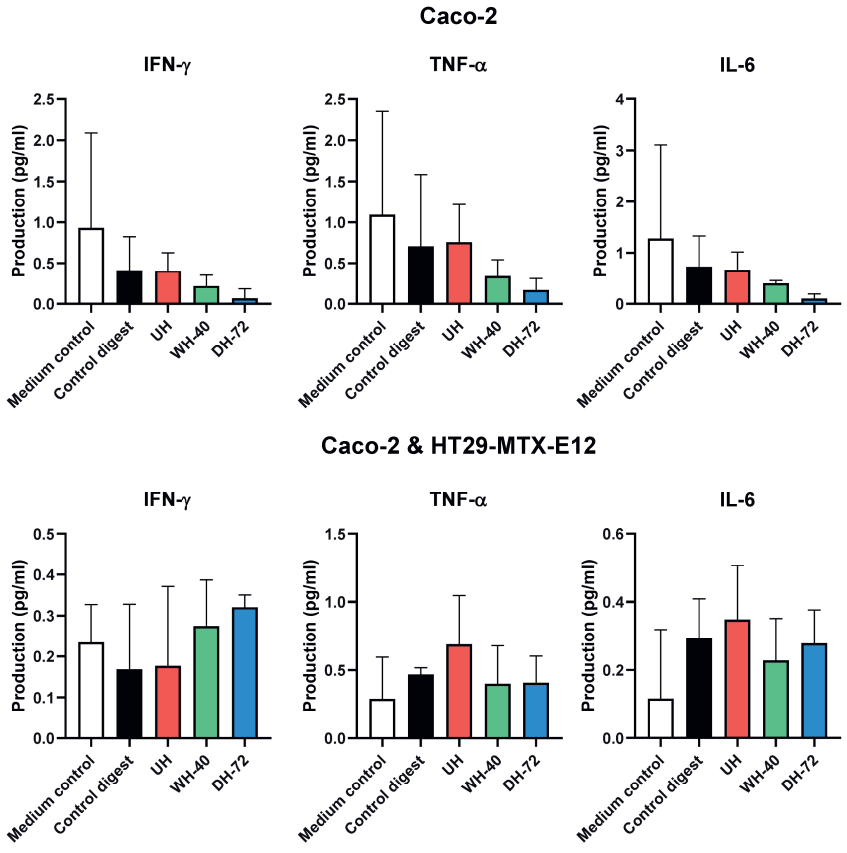

Figure 4.S7: IFN-γ, TNF-α and IL-6 concentrations measured with a LEGENDplex assay at the basolateral side of a 21-days differentiated Caco-2 or Caco-2/HT29-MTX-E12 (90/10) monolayer after stimulation with the detoxified intestinal digests for 6h. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6h. Water instead of the infant formula model system was digested with the *in vitro* digestion model to obtain a control digest. Medium controls were Caco-2 and HT29-MTX-E12 cells grown in DMEM.

Table 4.S4: HLA-II epitopes predicted by the use of the IEDB MHC Class II Binding Prediction tool at the basolateral side after transport across a 21-days differentiated Caco-2 or Caco-2/HT29-MTX-E12 (90/10) monolayer. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6h. Amino acid (AA) position provides the position of the peptide containing an HLA-II epitope in the protein without the signal peptide. Modification indicates if the peptide was detected in the digest in unmodified, phosphorylated, deamidation modified, lactosylated, or carboxymethyllysine (CML) modified form. All missing values were not detected. β-Lg: β-lactoglobulin, ND: not detected.

Protein of origin	**Peptide**	**AA position**	**HLA gene**	**Intensity Caco-2**			**Intensity Caco-2 & HT29-MTX-E12**			**Modification**
				UH	**WH-40**	**DH-72**	**UH**	**WH-40**	**DH-72**	
α_{s1}-casein	LEIVPNSAEER	109-119	HLA-DR				1.33E+06	5.13E+06		Unmodified, Phosphorylation
α_{s2}-casein	NMAINPSKENL	25-35	HLA-DR				4.42E+05			Phosphorylation
	QGPIVLNPWDQVK	101-113	HLA-DR, HLA-DP						7.55E+05	Lactosylation
β-casein	PVVVPPFLQPE	81-91	HLA-DP				2.92E+05		2.79E+05	Unmodified
	SLSQSKVLPVPQ	164-175	HLA-DR, HLA-DQ	3.83E+05		1.13E+07		6.34E+05	3.90E+07	Lactosylation, CML
β-Lg	EVDDEALEKFD	127-137	HLA-DQ	4.96E+06		2.55E+06	1.65E+07	1.49E+06	8.28E+06	Unmodified
GRIP and coiled-coil domain containing 2	IDQLKLKLQDTQNS	1358-1371	HLA-DR				1.47E+06		6.29E+05	Deamidation, CML
Count				**2**	**ND**	**2**	**5**	**3**	**5**	
Total intensity				**5.35E+06**	**ND**	**1.38E+07**	**2.00E+07**	**7.25E+06**	**4.90E+07**	

Table 4.S5: Linear IgE epitopes predicted at the basolateral side after transport across a 21-days differentiated Caco-2 or Caco-2/HT29-MTX-E12 (90/10) monolayer. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72) and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6h. Amino acid (AA) position provides the position of the peptide containing a linear IgE epitope in the protein without the signal peptide. Modification indicates if the peptide was detected in the digest in unmodified, phosphorylated, deamidation modified, lactosylated, or carboxymethyllysine (CML) modified form. All missing values were not detected. β-Lg: β-lactoglobulin, ND: not detected.

Protein of origin	Peptide	AA position	IgE epitope position	Intensity Caco-2			Intensity Caco-2 & HT29-MTX-E12			Modification
				UH	WH-40	DH-72	UH	WH-40	DH-72	
α_{s1}-casein	LEIVPNSAEER	109-119	109-120				1.33E+06	5.13E+06		Unmodified, Phosphorylation
	EIVPNSAEER	110-119	109-120	3.19E+07			1.30E+08	3.22E+07	1.91E+08	Unmodified, Phosphorylation
	TDAPSFSDIPNPI	174-186	171-185					7.51E+06		Unmodified
α_{s2}-casein	QGPIVLNPWDQVK	101-113	105-114						7.55E+05	Lactosylation
β-casein	QSLVYPFPGPIPN	56-68	55-70						8.13E+05	Unmodified
	SLPQNIPPLTQTPVVVPPFLQPE	69-91	83-92				2.78E+05			Unmodified
	SLPQNIPPLTQTPVVVPPFLQPEV	69-92	83-92					2.08E+06	4.11E+06	Unmodified
	NIPPLTQTPVVVPPFLQPE	73-91	83-92				3.17E+05	4.46E+05	5.06E+05	Unmodified
	NIPPLTQTPVVVPPFLQPEV	73-92	83-92				8.16E+06	9.57E+06	1.06E+07	Unmodified
	NIPPLTQTPVVVPPFLQPEVMG	73-94	83-92						2.11E+05	Unmodified
	IPPLTQTPVVVPPFLQPEV	74-92	83-92				4.04E+05	4.16E+05	7.85E+05	Unmodified
	TQTPVVVPPFLQPEV	78-92	83-92	4.64E+05			9.99E+05	8.54E+05	9.66E+05	Unmodified
	QTPVVVPPFLQPEV	79-92	83-92				8.33E+05	8.46E+05	1.52E+06	Unmodified
	PVVVPPFLQPE	81-91	83-92				2.92E+05		2.79E+05	Unmodified
	PVVVPPFLQPEV	81-92	83-92	1.58E+07			4.58E+07	1.00E+07	6.74E+07	Unmodified

Table 4.S5: (Continued)

Protein of origin	Peptide	AA position	IgE epitope position	Intensity Caco-2			Intensity Caco-2 & HT29-MTX-E12			Modification
				UH	WH-40	DH-72	UH	WH-40	DH-72	
β-casein	PVVVPPFLQPEVMG	81-94	83-92						5.54E+05	Unmodified
	VVVPPFLQPEV	82-92	83-92						1.27E+06	Unmodified
	PPFLQPEV	85-92	83-92				4.76E+05		6.78E+05	Unmodified
	HQPHQPLPPTVMFPPQS	145-161	149-164					2.92E+05		Unmodified
	HQPHQPLPPTVMFPPQSV	145-162	149-164					5.15E+06		Unmodified
β-Lg	VRTPEVDDEALEKFDK	123-138	121-140				3.29E+06			Unmodified
Count				**3**	**ND**	**ND**	**12**	**12**	**15**	
Total Intensity				**4.82E+07**	**ND**	**ND**	**1.92E+08**	**7.44E+07**	**2.82E+08**	

Table 4.S6: Bioactive peptides identified by use of the milk bioactive peptide database (MBPDB) at the basolateral side after transport across a 21-days differentiated Caco-2 or Caco-2/HT29-MTX-E12 (90/10) monolayer. An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40), or dry heated for 72h (DH-72), and all samples were digested by use of an *in vitro* infant digestion model and detoxified before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6h. Amino acid (AA) position provides the position of the peptide in the protein that was identical to a peptide in the MBPDB without the signal peptide. Modification indicates if the peptide was detected in the digest in unmodified, phosphorylated, deamidation modified, lactosylated, or carboxymethyllysine (CML) modified form. All missing values were not detected. β-Lg: β-lactoglobulin, α-La: α-lactalbumin, ND: not detected.

Protein of origin	Peptide	AA position	Bioactive function	Intensity Caco-2			Intensity Caco-2 & HT29-MTX-E12			Modification
				UH	WH-40	DH-72	UH	WH-40	DH-72	
α_{s1}-casein	SDIPNPIGSENSEK	180-193	Antimicrobial				7.30E+05			Unmodified
β-casein	FQSEEQQQTEDELQDK	33-48	Promote calcium uptake	5.73E+06	1.41E+06	4.65E+06	3.71E+06	1.03E+06	3.57E+06	Unmodified, Phosphorylation
	VYPFPGPIP	59-67	PEP-inhibitory	1.96E+06			4.98E+06	2.72E+06	6.99E+06	Unmodified
	SLPQNIPPLTQTPVVVPPF	69-87	Anticancer					6.37E+05	1.00E+06	Unmodified
	NIPPLTQTPV	73-82	ACE-inhibitory				2.34E+06	3.09E+06	2.07E+06	Unmodified
	IPPLTQT	74-80	DPP-IV Inhibitory				4.61E+05		6.92E+05	Unmodified
	YPVEPF	114-119	Opioid, Increases MUC4 expression, DPP-IV Inhibitory, Antioxidant, Antimicrobial					1.56E+06		Unmodified
	WMHQPHQPLPPT	143-154	Anti-inflammatory, ACE-inhibitory	8.16E+05	2.43E+05	6.73E+05	1.71E+06	4.47E+05	3.90E+06	Unmodified
	HQPHQPLPPT	145-154	ACE-inhibitory	2.50E+07	3.57E+06	1.58E+07	4.42E+07	8.64E+06	5.85E+07	Unmodified
	SQSKVLPVPQ	166-175	ACE-inhibitory						5.29E+06	Lactosylation

Table 4.S6: (Continued)

Protein of origin	Peptide	AA position	Bioactive function	Intensity Caco-2			Intensity Caco-2 & HT29-MTX-E12			Modification
				UH	WH-40	DH-72	UH	WH-40	DH-72	
β-casein	VLPVPQ	170-175	Inhibition of cholesterol solubility	2.47E+07		1.96E+07	5.64E+07	2.85E+07	5.84E+07	Unmodified
	QEPVLGPVRGPFPIIV	194-209	ACE-inhibitory					3.00E+05		Unmodified
β-Lg	GLDIQKVAGT	9-18	Antimicrobial	8.30E+05			2.12E+06	4.45E+05	1.24E+08	Lactosylation, CML
	VLVLDTDYK	92-100	DPP-IV Inhibitory, Antimicrobial				1.19E+06		4.49E+05	Unmodified
	VLDTDYK	94-100	ACE-inhibitory	1.08E+07			2.28E+07	9.60E+05	5.16E+07	Unmodified, Lactosylation
	TPEVDDEALEK	125-135	DPP-IV Inhibitory, Antimicrobial	4.89E+07	8.63E+05	2.15E+07	8.45E+07	1.33E+07	7.43E+07	Unmodified, Lactosylation, CML
α-La	GYGGVSLPEW	17-26	ACE-inhibitory						1.94E+05	Unmodified
	VSLPEW	21-26	ACE-inhibitory				9.37E+05	1.24E+06	2.78E+06	Unmodified
Count				**8**	**4**	**5**	**13**	**13**	**15**	
Total Intensity				**1.19E+08**	**6.08E+06**	**6.23E+07**	**2.26E+08**	**6.30E+07**	**3.94E+08**	

Chapter 5

Milk and plant lipids influence the *in vitro* digestion of milk proteins

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Manuscript in preparation to be submitted

Abstract

Infant formula is usually made from bovine milk but the milk lipids are partly or fully replaced by plant lipids to more closely mimic the fatty acid composition of human milk. Whereas the digestion of either milk proteins or lipids has been extensively studied, little is known about how interactions between these components affect their digestion. Therefore, this study aimed to investigate the effect of milk lipids and plant lipids, respectively, on the digestion of milk proteins during *in vitro* infant digestion. Emulsions were made from raw bovine skim milk diluted to 1.2% protein (SM), containing either no lipids, 4% bovine milk lipids (SM-ML), or 4% plant lipids (SM-PL; 80% palm, 10% sunflower, 10% coconut oil), and all samples were digested with an *in vitro* infant digestion model. Results showed that SM-ML and SM-PL had a very similar particle size distribution and average particle size, which were comparable to commercial infant formulas. During gastric digestion, caseins were digested faster in SM-ML compared to SM-PL and SM. Intestinal milk protein digestion was slower in the presence of lipids and was slower in SM-PL than in SM-ML. These effects were probably caused by the difference in free fatty acid (FFA) composition between SM-PL and SM-ML, and in particular by the higher concentrations of the LC-PUFA linoleic acid in SM-PL. Moreover, we showed that the addition of linoleic acid as FFA to milk proteins decreased the intestinal digestion of milk proteins in a concentration-dependent manner. Taken together, we showed that the digestion of milk proteins was influenced by milk and plant lipids as well as by FFAs and was differently affected by different types of lipids.

5.1 Introduction

Human milk is a complex mixture of protein, lipids, carbohydrates, minerals, and other minor components, and optimally supports the healthy growth and development of infants. An alternative to human milk is infant formula (IF), which is usually made from bovine milk. Since bovine milk differs in composition from human milk, its composition is adjusted during the production of IF to better resemble the composition of human milk. This includes partly or fully replacing the bovine milk lipids with different plant oils because the fatty acid (FA) composition of human milk lipids is more similar to a mixture of plant oils than to bovine milk lipids. Human and bovine milk both contain lipids that consist for 98% of triacylglycerols (TAGs), which are esters with three FAs at three stereospecific positions (sn-1, sn-2, and sn-3). The composition of these FAs, however, differs greatly between human and bovine milk. Human milk contains less short-chain FAs (C4:0–C8:0), more oleic acid (C18:1), less saturated FAs, and more long-chain polyunsaturated FAs (LC-PUFAs; ≥C18:2) compared to bovine milk [1]. The FA composition of human milk is associated with several health benefits for infants, including the promotion of intestinal development and inflammation regulation [2]. Therefore, bovine milk lipids are replaced by a blend of different plant lipids during the production of IF, which contain less saturated FAs and more LC-PUFAs than bovine milk, such as palm oil, (high oleic) sunflower oil, coconut oil, rapeseed oil, and safflower oil. However, the placement of FAs within TAGs can differ in plant oils compared to human milk. For instance, palmitic acid, the most abundant FA in human milk, is present in higher abundancies at the sn-2 position of TAGs in human milk than in plant oils. The high concentration of palmitic acid as well as its sn-2 position in TAGs are thought to be important for the development of the gut microbiome, calcium absorption, and bone health of infants [3]. Hence, bovine milk lipids, whose TAG structure is more similar to human milk, are sometimes included in IF together with plant oils.

The presence of lipids was shown to influence the structure of the clot during gastric digestion [4,5]. The formation of this clot starts with pepsin cleaving κ-casein on the outside of casein micelles. This leads to destabilization and coagulation of casein micelles, and lipid globules are entrapped in this network, together forming the gastric clot. A softer gastric clot is formed from whole milk compared to skim milk because lipid droplets in whole milk weaken the protein-protein network, resulting in less tight coagulation of casein micelles in the presence of lipids. The softer, more open clot from whole milk allows pepsin to enter the clot more easily, leading to higher casein hydrolysis during gastric digestion [4,5].

The digestion of proteins may also be affected by free fatty acids (FFAs), which are released during gastric and intestinal digestion. For instance, β-lactoglobulin (β-Lg) was digested slower in whole than in skim milk, which might be due to FFAs stabilizing β-Lg during digestion [6]. The release of FFAs during gastric and intestinal digestion depends on both the composition and stereospecific positioning of FFAs. Gastric lipase can cleave at all TAG positions but has a preference for the sn-3 position. Since human and bovine milk mainly contain short and medium-chain FAs (≤C12) at the sn-3 position, these FAs are released during gastric digestion. During intestinal digestion, pancreatic lipase further hydrolyzes the lipids and has a preference for the sn-1 and sn-3 positions, leading to the release of mainly unsaturated FAs from TAGs in human milk, and a monoacylglycerol (MAG) with an FA, often palmitic acid, at the sn-2 position. The difference in FA composition and placement in TAGs

in human milk as well as in IFs with bovine and plant lipids is known to result in a different release of FFAs during *in vitro* digestion [7–9]. However, whether this variation in FFA release also leads to differences in milk protein digestion has not been investigated. This study, therefore, aimed to determine how milk lipids and plant lipids influence the digestion of milk proteins in infants.

5.2 Materials & Methods

5.2.1 Materials

Fresh whole bovine milk was provided by CARUS Farm from Wageningen University & Research. Sunflower and coconut oil were purchased from a local supermarket, and palm oil was purchased from Aman Prana. NuPAGE 4 to 12% Bis-Tris protein gel, NuPAGE LDS sample buffer (4x), NuPAGE sample reducing agent (10X), NuPAGE MES SDS running buffer (20X), and HyperSep™ aminopropyl cartridges aminopropyl cartridges (60108-425) were obtained from Thermo Fisher Scientific. BlueRay prestained protein marker was purchased from Jena Bioscience, and F.A.M.E. Mix (CRM18918) was obtained from Supelco. Pepsin (P6887), lipase from rhizopus oryzae (80612), pancreatin (P7545), porcine bile extract (B8631), and all other chemicals were obtained from Sigma Aldrich.

5.2.2 Processing of milk

Raw bovine was centrifuged at 6000g at 4°C for 20 min, and the cream layer was removed. The skim milk was diluted with simulated milk ultrafiltrate [10] to a protein concentration of 1.2%. The removed cream layer was placed in a sonication bath at 40 kHz for 10 min, followed by centrifugation at 4500g, 4°C for 20 min. Thereafter, the milk lipid layer was transferred to a new tube. The skim milk and milk lipids were stored at -20°C till further use.

5.2.3 Preparation of emulsions

Skim milk with 1.2% protein (SM) was mixed with either no lipids as control, 4% (w/w) milk lipids (ML), or 4% (w/w) plant lipids (PL). PL was a mixture of 80% palm oil, 10% sunflower oil, and 10% coconut oil, which was based on the FA composition of commercial infant formulas [11,12]. The mixtures were dispersed using an Ultra-Turrax (IKA T 25 digital Ultra-Turrax, S25N 18G; IKA) with a starting speed of 3000 rpm, which was slowly increased to 7000 rpm, and the dispersing was continued at 7000 rpm for 2 min. Thereafter, the mixtures were passed through a homogenizer (PandaPLUS 2000, GEA) for 3 cycles at a pressure of 1000 bar. Samples were stored in at 4°C until they were digested the following day.

5.2.4 Particle size

The particle size distribution and average particle size were measured by laser light scattering using a Mastersizer 3000 laser diffraction particle size analyzer (Malvern Instruments). Refractive indexes of 1.458 and 1.333 were used for milk and water, respectively. Several drops of each sample were added to reach a laser obscuration range

of 4-8%. The average particle size was recorded as the volume-weighted mean diameter ($D_{4,3}$). The $D_{4,3}$ and the volumetric size distribution were calculated by the Mastersizer software (Malvern Instruments).

5.2.5 *In vitro* infant digestion

Digestions were performed with an *in vitro* infant digestion model [13]. Simulated gastric fluid (SGF), which was composed of 94 mM sodium chloride and 13 mM potassium chloride, was added to the SM, SM-ML, or SM-PL samples in a 63:37 meal:SGF ratio, and the pH was set to 5.3 with 1 M hydrochloric acid. Pepsin (268 U/ml) and lipase from rhizopus oryzae (19 U/ml) were added, and gastric digestion was conducted at 37°C for 60 min under rotary movement of 20 rpm. Gastric digestion was stopped by increasing the pH to 6.6 with 1 M sodium hydroxide. Thereafter, simulated intestinal fluid (SIF), which consisted of 164 mM of sodium chloride, 10 mM of potassium chloride, and 85 mM of sodium bicarbonate, was added in a 62:38 gastric phase:SIF ratio. Moreover, 3 mM calcium chloride, 3.1 mM bile salts, and pancreatin with a trypsin activity of 16 U/ml were added. Intestinal digestion was conducted at 37°C for 60 min under rotary movement of 20 rpm. Gastric digestion was stopped after 60 min (G60) by increasing the pH to 6.6, and intestinal digestion was stopped after 10 and 60 min (I10, I60) by adding 50 µL of 0.1 M Pefabloc per ml digest. In addition, gastric digests without pepsin were prepared as a reference (G0). Gastric digests were separated into a soluble and an insoluble part by centrifuging them at 4500g for 10 min, and the supernatant (soluble digest) and the pellet (insoluble digest) were stored separately at -20°C. Digestions were performed in triplicate.

5.2.6 SDS-Page

Reducing SDS-PAGE was performed to follow the disappearance of intact proteins during *in vitro* digestion. After the insoluble digests were freeze-dried, 0.22 mg was mixed with 25 µl 4x LDS sample buffer, 10 µl 10x reducing agent, and 65 µl Milli-Q water. To prepare the soluble gastric digests and the intestinal digests, 4 µl digest was mixed with 5 µl 4x LDS sample buffer, 2 µl 10x reducing agent, and 9 µl Milli-Q water. After heating all samples at 70°C for 10 min, 10 µl of each sample and 4 µl marker were loaded on a 4-12% Bis-Tris polyacrylamide precast gel. Gels were run at 120V for 75 min with MES running buffer. Thereafter, they were stained with Coomassie brilliant blue solution for 2h, and destained with 10% ethanol and 7.5% acetic acid in demi water. The gels were scanned with a GS-900 Calibrated Densitometer (Bio-Rad) with Image Lab software (Bio-Rad).

5.2.7 Soluble protein concentration

The total nitrogen contents of the soluble digests were determined as a measure of soluble protein. A DUMAS Flash EA 1112 Protein analyzer (Thermo Fisher Scientific) was used to measure the total nitrogen content of the samples, and nitrogen content was converted to protein content with a conversion factor of 6.38.

5.2.8 OPA

The o-phthaldialdehyde (OPA) assay was conducted to determine the degree of hydrolysis as described previously [14]. First, 500 µl of SM per ml 6 M hydrochloric acid was heated at 110°C for 22h to create an acidic protein hydrolysate, which was used to determine the total content of free amino groups in the sample. In addition, an L-leucine standard curve was prepared ranging from 5 to 50 µM. Undigested (G0) and digested samples (G60, I10, I60) were centrifuged at 2000g, 4°C for 5 min. Thereafter, 200 µl OPA reagent was added to 10 µl sample, acid hydrolysate, or L-leucine standard in a 96-well plate, and the plate was incubated in the dark at RT for 15 min. The absorbance was measured at 340 nm using a Spectramax M2 microplate reader (Molecular Devices). The degree of hydrolysis was calculated using the following formula:

$$\text{Degree of hydrolysis} = \frac{(NH_2 \text{ final} - NH_2 \text{ initial})}{(NH_2 \text{ acid} - NH_2 \text{ initial})} \cdot 100\%$$

in which NH_2 final, NH_2 initial and NH_2 acid are the amount of free amino groups in the digested sample, undigested sample, and acidic hydrolysate, respectively.

5.2.9 Free fatty acids

Short-chain FFAs (≤C5:0) in the digests were determined as described previously [15]. In brief, standard solutions of acetic acid (C2:0), propionic acid (C3:0), butyric acid (C4:0), isobutyric acid (C4:0), valeric acid (C5:0), and isovaleric acid (C5:0) in water were prepared ranging from 10 to 450 ppm. The digestion samples were centrifuged at 9000g, 4°C for 5 min. Thereafter, 500 µl supernatant of the digestion samples and 500 µl of the standard solutions were mixed with 250 µl of 450 ppm 2-ethylbutyric acid in 0.3 M hydrochloric acid and 0.45 M oxalic acid, which served as internal standard. FFAs were measured by use of a gas chromatograph coupled to a flame ionization detector (GC-FID, GC-2014AFSC, Shimatdzu) with a capillary FA-free Stabil wax-DA column (30 m × 0.25 µm × 0.25 µm, Restek). Nitrogen was used as carrier gas at a flow rate of 10 ml/min. The initial oven temperature was 100°C, increased to 180°C at 8°C/min, held at this temperature for 1 min, increased to 200°C at 20°C/min and held at this temperature for 5 min.

To measure longer FFAs (≥C8:0), first lipids were extracted from the digests as described previously [16] with some modifications. Gastric and intestinal digests (10 ml) were thoroughly mixed in a 1:2 (v:v) ratio with a solution of chloroform, methanol, and demi water (2:1:1, v:v:v). After the samples were centrifuged at 2000g for 15 min, the upper layer was discarded and the bottom layer was completely dried under a nitrogen flow. Thereafter, 3 ml of a diethyl ether and n-hexane solution (1:1, v:v) was added, and the samples were thoroughly mixed. Then, they were centrifuged at 400g for 3 min, and the upper organic phase was transferred to a new tube. To extract all lipids from the samples, this process was repeated twice and the three upper organic phases were combined. FFAs were separated from the bound FAs (in TAGs) by the use of solid phase extraction with an aminopropyl cartridge as described previously [17]. After the aminopropyl cartridges were conditioned with 10 ml n-hexane, the samples were loaded on the cartridges. First, 10 ml of chloroform and 2-propanol (2:1, v:v) was added to wash away the neutral lipids, followed by the addition of 5 ml 2% acetic acid in diethyl ether (v:v) to elute the FFA. After the FFAs

were completely dried under a nitrogen flow, they were dissolved in 2 ml methanol. Thereafter, 2 ml hydrochloric acid in methanol (1:3, v:v) was added, and 1 ml of 1 mg/ml nonadecanoic acid (C19:0) in n-hexane was added as a control to check the methylation efficiency. Then, the samples were heated at 100°C for 60 min to convert the FFA into fatty acid methyl esters (FAMEs). After the samples were cooled down to RT, 2 ml water was added, followed by centrifuging at 400g for 3 min. The upper organic phase was collected and filtered through a 0.2 µm PTFE filter, and 200 µl filtrate was mixed with 100 µl of 3 mg/ml methyl heptadecanoate (C17:0) in n-hexane, which served as internal standard. In addition, a calibration curve of the 14-component F.A.M.E Mix and methyl nonadecanoate was made in n-hexane, ranging from 20 to 2000 ppm. The FAMEs were analyzed with a gas chromatograph coupled to a flame ionization detector (GC-FID; Thermo Scientific Trace GC Ultra Interscience DSQ II, Thermo Fisher Scientific) with a capillary column (FAMEWAX GC Capillary Column, 30 m, 0.25 mm ID, 0.25 µm, Restek). Nitrogen was used as the carrier gas with a flow rate of 15 ml/min. The injector and detector temperatures were both 250°C. The program of the column oven temperature was as follows: 180°C for 2 min, 180–240°C at 10°C/min, and finally held at 240°C for 10 min. The FAMEs were identified and quantified by comparing their retention times and peak areas to those known FAME standards. Data were analyzed with Chromeleon software v7.2 SR5 (Thermo Fisher Scientific).

5.2.10 *In vitro* digestions with palmitic and linoleic acid

Milk proteins were digested in the presence of different concentrations of palmitic and linoleic acid to investigate the effect of FFAs on the digestion of milk proteins. For this aim, 1.2% β-Lg was mixed with 0.04, 0.4, or 4% palmitic acid, and SM (1.2% protein) was mixed with 5, 50, or 500 µM linoleic acid. The samples were digested with an *in vitro* infant digestion model as described in section 5.2.5. The digestions of β-Lg and palmitic acid were stopped at G0, G60, I10, and I60, and were performed once per digestion time point (n=1). The digestions with skim milk and linoleic acid were stopped at G0 and I60, and they were performed in triplicate. SDS-PAGE was performed on the digests of β-Lg and palmitic acid as described in section 5.2.6, and the OPA assay was performed on all digests as described in section 5.2.8.

5.2.11 Statistical analysis

Statistical analysis was conducted with GraphPad Prism version v8.0.2 (GraphPad Software) with one-way ANOVA, followed by Tukey's multiple comparisons test. Differences were considered significant if $p<0.05$.

5.3 Results

5.3.1 Particle size of emulsions before digestion

Emulsions were made from raw bovine skim milk diluted to 1.2% protein (SM) with either 4% bovine milk lipids (SM-ML) or 4% plant lipids (SM-PL). Thereafter, particle size distributions and average particle sizes of the emulsions were measured (Figure 5.1). SM-ML and SM-PL had a similar particle size distribution with most particles between the size of

0.1 and 1 µm, whereas SM contained smaller particles with most particles between 0.01 and 0.3 µm in size. The average particle sizes of the samples with lipids were both around 1.0 µm, whereas the average particle size of skim milk was only 0.1 µm.

5.3.2 Gastric clot

The SM, SM-ML, and SM-PL samples were digested with an *in vitro* infant digestion model to investigate whether lipids and different types of lipids affect the digestion of milk proteins. At the start of digestion (G0), the samples with lipids seemed to have a lower soluble protein content compared to SM, although this difference was not significant (Figure 5.2A). Moreover, gastric clots had a different structure in the absence than in the presence of lipids, which was visually observed. A firm gastric clot was present at the end of gastric digestion in SM, whereas very soft clots were formed in SM-ML and SM-PL. These clots from SM-ML and SM-PL rapidly disintegrated upon taking samples from the test tubes, which made it unfortunately impossible to further investigate their structures. The firmer clot of SM after gastric digestion was in line with the lower soluble protein concentration at this time point compared to SM-ML and SM-PL (Figure 5.2B). SM-PL had a slightly higher soluble protein concentration compared to SM-ML at the end of gastric digestion.

The protein composition of the clots during gastric digestion was visualized by reducing SDS-PAGE (Figure 5.3). At G0, the clots from all samples contained intact caseins and whey proteins, and more intact casein was present in the clot from SM compared to the clots from SM-ML and SM-PL. At the end of gastric digestion, less intact casein was detected in the clot from SM-ML than from SM and SM-PL. All clots still contained intact whey proteins at G60 as well as peptide bands with varying sizes (<8–24 kDa).

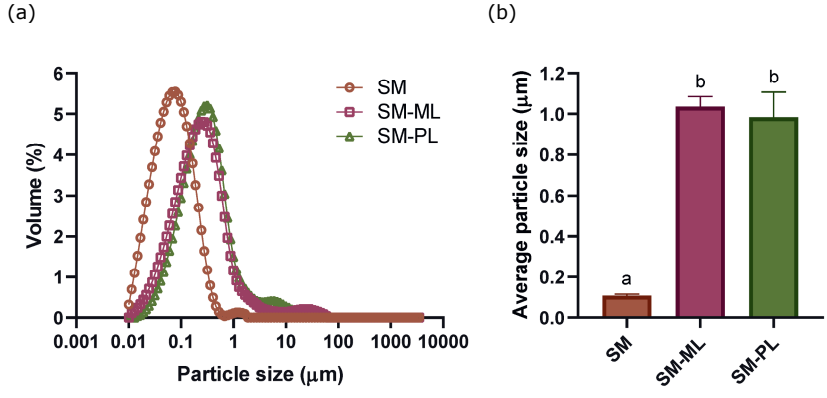

Figure 5.1: (a) Particle size distributions of skim milk (SM), SM with milk lipids (SM-ML), and SM with plant lipids (SM-PL), (b) Average particle size. Different letters indicate significant differences ($p<0.05$).

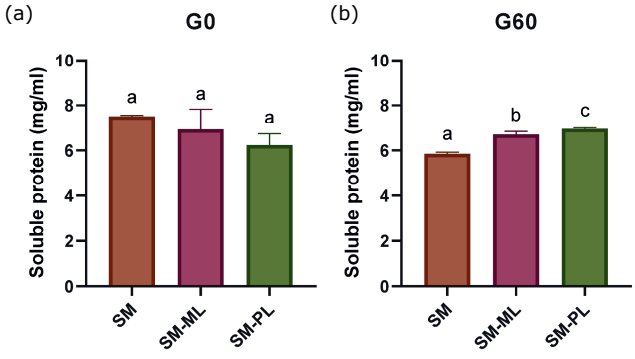

Figure 5.2: Protein concentration in soluble digests from skim milk (SM), SM with milk lipids (SM-ML), and SM with plant lipids (SM-PL) after (a) 0 min of gastric digestion (G0), (b) 60 min of gastric digestion (G60) with an *in vitro* infant digestion model. Different letters indicate significant differences ($p<0.05$).

5.3.3 Digestion of soluble protein

Intact caseins were observed in the soluble digests at G0, but no intact caseins were present anymore at the end of gastric digestion in all samples (Figure 5.4). All soluble digests also contained intact whey proteins at G0, which remained largely intact during gastric digestion. In addition, two distinct peptide bands with a size smaller than 8 kDa were present in all samples at G60. During intestinal digestion, insoluble protein from the gastric phase quickly dissolved, resulting in minimal differences in soluble protein concentration between the different samples (Figure 5.S1). At I10, a faint band in SM-ML at the height of intact β-Lg was present, contrary to SM and SM-PL. Moreover, SM-ML seemed to contain more intact α-La than the other samples and contained two distinct peptide bands around 8 kDa, which were

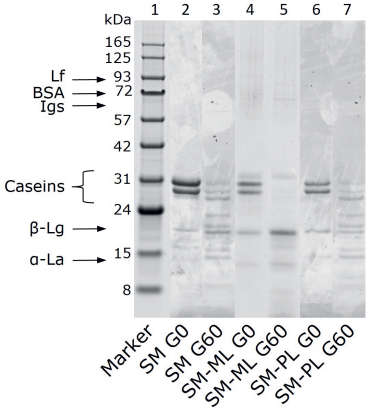

Figure 5.3: Reducing SDS-PAGE of the gastric clots from skim milk (SM), SM with milk lipids (SM-ML), and SM with plant lipids (SM-PL) after 0 min of gastric digestion (G0) and 60 min of gastric digestion (G60) with an *in vitro* infant digestion model. Lf: lactoferrin, BSA: bovine serum albumin, Igs: immunoglobulins, β-Lg: β-lactoglobulin, α-La: α-lactalbumin.

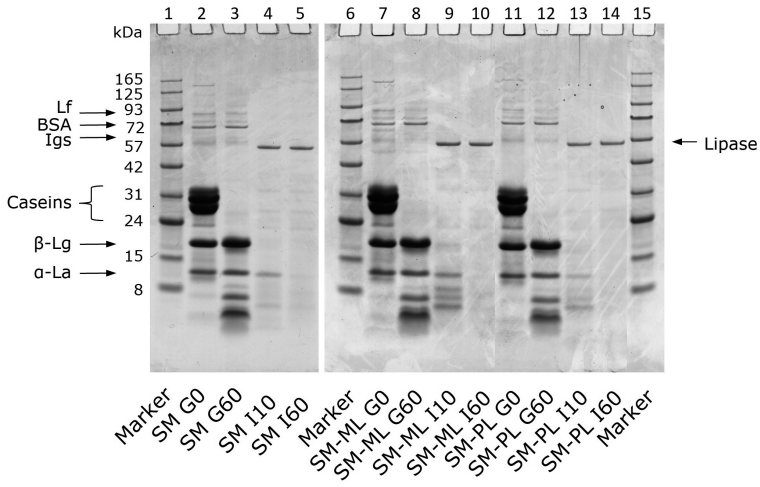

Figure 5.4: Reducing SDS-PAGE of the soluble digests from skim milk (SM), SM with milk lipids (SM-ML), and SM with plant lipids (SM-PL) after 0 min of gastric digestion (G0), 60 min of gastric digestion (G60), 10 min of intestinal digestion (I10), and 60 min of intestinal digestion (I60) with an *in vitro* infant digestion model. Lf: lactoferrin, BSA: bovine serum albumin, Igs: immunoglobulins, β-Lg: β-lactoglobulin, α-La: α-lactalbumin.

very faint in SM-PL and were not present in SM. At the end of intestinal digestion, no intact proteins were present anymore in all samples and only some faint peptide bands were observed.

Protein digestion was also monitored by measuring the degree of hydrolysis (Figure 5.5). The degree of hydrolysis slowly increased for all samples during gastric digestion, followed by a large increase at the beginning of the intestinal phase (Figure 5.5). At the end of gastric digestion, SM-ML had a higher degree of hydrolysis than SM and SM-PL, whereas SM and SM-PL had a similar degree of hydrolysis. After 10 min of intestinal digestion, both samples with lipids had a significantly lower degree of hydrolysis compared to SM, and SM-PL had an even lower degree of hydrolysis than SM-ML. The degree of hydrolysis remained the lowest for SM-PL till the end of intestinal digestion, followed by SM-ML and SM. Whereas the difference between SM and SM-ML was not significant at I60, the degree of hydrolysis of SM-PL was significantly lower than SM and SM-ML.

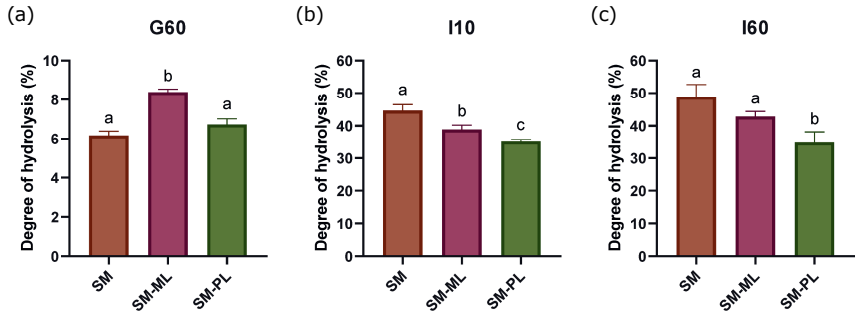

Figure 5.5: Degree of hydrolysis of the soluble digests from skim milk (SM), SM with milk lipids (SM-ML), and SM with plant lipids (SM-PL) after (a) 60 min of gastric digestion (G60), (b) 10 min of intestinal digestion (I10), (c) 60 min of intestinal digestion (I60) with an *in vitro* infant digestion model. Different letters indicate significant differences ($p<0.05$).

5.3.4 Free fatty acids

The composition and concentration of FFAs were determined at the end of gastric digestion and during intestinal digestion (Table 5.1). At the end of gastric digestion, SM-ML contained higher concentrations of the short- and medium-chain FFAs C4:0 and C16:1, whereas SM-PL contained higher concentrations of the LC-PUFA C18:2 (linoleic acid). During intestinal digestion, the concentration of total FFAs increased and larger differences in the FFA composition of SM-ML and SM-PL were observed. SM-ML contained higher concentrations of nearly all short- and medium-chain FFAs (C2-C16) at I10, and SM-PL contained a sixfold higher linoleic acid concentration. Whereas C16:0 (palmitic acid) was significantly higher in SM-ML, high concentrations of this FFA were also detected in SM-PL. In addition, C18:1 (oleic acid) concentrations were high in both samples but did not significantly differ between SM-ML and SM-PL. At the end of intestinal digestion, SM-ML still contained a higher concentration of most medium-chain FFAs, and SM-PL still contained a higher concentration of the LC-PUFA linoleic acid.

Table 5.1: Free fatty acid (FFA) composition in digests from skim milk with milk lipids (SM-ML) and skim milk with plant lipids (SM-PL) after 60 min of gastric digestion (G60), 10 min of intestinal digestion (I10), and 60 min of intestinal digestion (I60) with an *in vitro* infant digestion model. Measured FFA concentrations were converted to their concentrations in the meal before *in vitro* digestion. Significant differences between SM-ML and SM-PL at the corresponding digestion time point are indicated with * ($p<0.05$). SC-FFA: short-chain FFA (C2–C10), MC-FFA: medium-chain FFA (C12–C16), LC-FFA: long-chain FFA (C18–C22), S-FFA: saturated FFA, MU-FFA: mono-unsaturated FFA, PU-FFA: poly-unsaturated FFA.

	FFA (µg/ml meal)					
	at G60		at I10		at I60	
	SM-ML	**SM-PL**	**SM-ML**	**SM-PL**	**SM-ML**	**SM-PL**
C2:0	295 ± 36	242 ± 4.9	470 ± 4.9	429 ± 23 *	444 ± 34	432 ± 2.6
C3:0	11 ± 1.3	9.1 ± 0.2	18 ± 0.2	16 ± 0.8 *	17 ± 1.2	16 ± 0.1
C4:0	1770 ± 298	1076 ± 87 *	1607 ± 202	1037 ± 177 *	1429 ± 333	928 ± 127
C5:0	1356 ± 374	1361 ± 193	2446 ± 493	1955 ± 542	2561 ± 387	2111 ± 147
C8:0	75 ± 82	66 ± 10	138 ± 49	40 ± 1.8 *	325 ± 38	312 ± 21
C10:0	197 ± 159	76 ± 14	361 ± 12	67 ± 2.5 *	494 ± 75	326 ± 29 *
C12:0	209 ± 131	74 ± 13	431 ± 58	66 ± 2.5 *	541 ± 118	319 ± 28 *
C14:0	498 ± 258	261 ± 71	1029 ± 277	192 ± 29 *	1055 ± 405	345 ± 93 *
C16:0	1110 ± 678	2631 ± 769	2186 ± 184	1409 ± 322 *	2284 ± 875	1852 ± 592
C16:1	153 ± 0.3	24 ± 4.3 *	458 ± 106	43 ± 3.2 *	458 ± 108	76 ± 38 *
C18:0	578 ± 409	419 ± 110	1064 ± 46	372 ± 63 *	1170 ± 403	661 ± 181
C18:1	2127 ± 490	2926 ± 759	5891 ± 1890	8761 ± 669	6284 ± 1610	11118 ± 4260
C18:2	174 ± 55	964 ± 243 *	515 ± 214	3422 ± 74 *	600 ± 177	3796 ± 1075 *
C18:3	40 ± 17	15 ± 2.6	137 ± 48	57 ± 1.7 *	141 ± 42	67 ± 34
C20:0	7.9 ± 3.8	25 ± 6.7 *	19 ± 1.7	26 ± 4.5	35 ± 2.3	52 ± 12.1
C22:0	2.7 ± 0.2	5.6 ± 1.4 *	3.6 ± 0.6	6.7 ± 1.6 *	9.4 ± 0.8	14 ± 5.6
C22:1	11 ± 5.4	6.8 ± 3	15 ± 10	5.3 ± 1.4	69 ± 16	23 ± 2 *
SC-FFA	3704 ± 796	2830 ± 288	5040 ± 741	3545 ± 728	5270 ± 791	4124 ± 217
MC-FFA	1970 ± 1067	2990 ± 852	4104 ± 577	1710 ± 351 *	4337 ± 1505	2592 ± 680
LC-FFA	2941 ± 188	4361 ± 1116	7644 ± 2114	12649 ± 541 *	8308 ± 2250	15730 ± 5550
S-FFA	6109 ± 2039	6247 ± 1232	9773 ± 316	5616 ± 1095 *	10364 ± 2518	7366 ± 690
MU-FFA	2291 ± 495	2956 ± 763	6363 ± 1985	8810 ± 666	6810 ± 1734	11217 ± 4226
PU-FFA	215 ± 72	978 ± 246 *	652 ± 262	3479 ± 74 *	741 ± 219	3862 ± 1107 *
Total FFA	8615 ± 1574	10181 ± 2192	16788 ± 2044	17904 ± 1136	17915 ± 4451	22446 ± 5992

5.3.5 Protein digestion in the presence of palmitic or linoleic acid

Since SM-PL had the lowest degree of hydrolysis and the highest concentration of polyunsaturated FFAs (PU-FFAs), and in particular of the LC-PUFA linoleic acid, the effect of different concentrations of linoleic acid on the digestion of milk proteins was investigated. A lower degree of hydrolysis was observed with increasing linoleic acid concentration (Figure 5.6). Whereas low linoleic acid concentrations (5 µM; 1.40 µg/ml) did not affect the degree of hydrolysis, a significantly lower degree of hydrolysis was found in the presence of 50 µM (14.0 µg/ml) and 500 µM (140 µg/ml) linoleic acid. Furthermore, the effect of different palmitic acid concentrations on the digestion of β-Lg was studied because SM-ML contained a higher palmitic acid concentration and more intact β-Lg at I10 than SM and SM-PL. However, no difference in the digestion of β-Lg was observed in the presence of different palmitic acid concentrations, as observed on SDS-PAGE (Figure 5.S2) and in the degree of hydrolysis (Figure 5.S3).

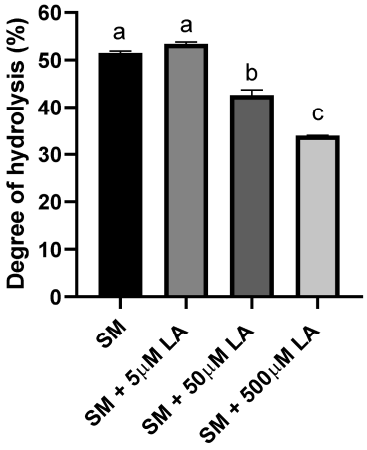

Figure 5.6: Degree of hydrolysis of digests from skim milk (SM) without linoleic acid (LA) or with different LA concentrations after 60 min of intestinal digestion (I60) with an *in vitro* infant digestion model. Different letters indicate significant differences ($p<0.05$).

5.4 Discussion

During the production of IF, bovine milk lipids are partly or fully replaced by plant lipids to better resemble the FA composition of human milk. Therefore, this study investigated how milk lipids and plant lipids affect the digestion of milk proteins in infants.

The emulsions of skim milk with milk lipids (SM-ML) and plant lipids (SM-PL) had a similar average particle size and particle size distribution with most particles between 0.1 and 1 µm in size (Figure 5.1), which is comparable to commercial IFs [18,19]. The similar particle distributions of SM-ML and SM-PL indicate that they had similar interfacial areas prior to digestion, which allows for similar accessibility for digestive enzymes. However, the lipid composition, which affects the interphase composition, is also thought to influence the digestion of lipids [20,21]. Mainly polyunsaturated FAs and long-chain FAs

were shown to increase the stability of emulsions by enhancing the repulsion between particles in emulsions and increasing the attraction strength, whereas medium-chain FAs, saturated FAs, and monounsaturated FAs were shown to decrease the stability of emulsions and lower the coverage of the surface with proteins [22]. Since plant lipids contain more LC-PUFAs than milk lipids, SM-PL may have had higher emulsion stability and higher surface coverage with milk proteins than SM-ML prior to digestion.

To investigate the effect of the presence and type of lipids, SM, SM-ML, and SM-PL were digested with an *in vitro* infant digestion model. At the end of gastric digestion, SM-ML and SM-PL had a higher soluble protein concentration compared to SM (Figure 5.2), indicating that less protein was involved in the gastric clot in the presence of lipids. SM-ML also contained less intact casein in the gastric clot (Figure 5.3) and no intact casein was present in the soluble digests of all samples at the end of gastric digestion (Figure 5.4), suggesting that casein was broken down more easily in the presence of milk lipids. This was supported by the higher degree of hydrolysis in SM-ML at this time point (Figure 5.5), indicating that milk proteins were hydrolyzed to a further extent during gastric digestion in the presence of milk lipids. Since, visibly, a softer gastric clot was formed in the presence of milk lipids than in SM, pepsin probably entered the gastric clot more easily, resulting in a faster cleavage of caseins. This was in line with Ye, who reported the formation of a looser gastric clot from whole milk than from skim milk, resulting in faster gastric digestion of caseins [5]. However, a softer gastric clot structure was also observed in SM-PL but did not result in a faster digestion of intact caseins (Figure 5.3) or a higher degree of hydrolysis (Figure 5.5) during gastric digestion compared to SM. This indicates that the breakdown of the gastric clot and the digestion of proteins during gastric digestion was differently affected by plant lipids than by milk lipids and that other factors than only the structure of the gastric clot play a role in the gastric digestion of milk proteins. For instance, the stability of the emulsion before and during gastric digestion may affect the digestion of milk proteins. SM-PL contained more LC-PUFAs, which are thought to stabilize the lipid droplets better and facilitate more protein on the surface [22]. Therefore, SM-ML may have been less stable in comparison, resulting in a faster destabilization of the lipid droplets during gastric digestion, thereby increasing the accessibility for pepsin to hydrolyze the proteins. In addition, the composition of FFAs released during gastric digestion differed between SM-ML and SM-PL (Table 5.1), which might affect the digestion of milk proteins. SM-ML contained a higher concentration of C4:0 and C16:1, whereas SM-PL contained a higher concentration of the LC-PUFA linoleic acid (C18:2). Pre-incubation of pepsin with linoleic acid was previously shown to decrease casein cleavage by pepsin [23], indicating that the concentration of linoleic acid may directly affect the gastric digestion of milk proteins. Moreover, the presence of FFAs was shown to decrease the digestion of serum albumin by inhibiting the formation of the enzyme-substrate complex [24]. However, it is not clear if FFAs inhibit the digestion of milk proteins due to binding to the milk proteins or pepsin, and whether different FFAs inhibit the digestion of milk proteins by pepsin to a different extent.

After 10 min of intestinal digestion, slightly lower digestion of intact whey proteins was found in the presence of milk lipids but not in SM-PL or SM (Figure 5.4). This difference between SM-ML and SM-PL may be caused by the composition of released FFAs at I10 (Table 5.1). The digestion of β-Lg by trypsin was previously shown to be influenced by the

presence of lipids and FFAs [6,25] and may be caused by FFAs binding to β-Lg as shown for palmitic acid [26], resulting in the stabilization of the protein, by FFAs binding to bile salts [25], resulting in less free bile salt that can destabilize β-Lg, or by binding directly to trypsin [27]. Since palmitic acid can bind to β-Lg [26] and was present at a higher concentration in SM-ML at I10 (Table 5.1), we investigated the effect of different palmitic acid concentrations on the digestion of β-Lg. However, no effect of palmitic acid on the digestion of β-Lg was found (Figures 5.S2 and 5.S3). Investigating the effect of other FFAs on the digestion of β-Lg and α-La may be of interest for future research to explain the lower digestion of these whey proteins in the presence of milk lipids. Moreover, less extensive protein hydrolysis was observed during intestinal digestion in the presence of lipids and was lower in SM-PL than in SM-ML (Figure 5.5). This was in contrast to de Figueiredo Furtado et al. [22] who found that proteolysis of milk proteins was not influenced by the lipid composition. However, they only evaluated proteolysis by SDS-PAGE, whereas the largest effect of lipid composition on the digestion of milk proteins was found in the degree of hydrolysis in our study. Several FFAs were reported to inhibit trypsin activity in a previous study, with longer FFAs resulting in stronger trypsin inhibition [27]. Since SM-PL contained a higher concentration of the LC-PUFA linoleic acid than SM-ML (Table 5.1), this difference in FFA composition may explain the lower degree of hydrolysis in SM-PL. We further investigated this by digesting milk proteins in the presence of different linoleic acid concentrations and found that milk proteins were digested to a lesser extent with increasing linoleic acid concentration (Figure 5.6). This is in agreement with Matsushita et al. who found a lower digestion of casein by trypsin in the presence of linoleic acid [23]. It is tempting to investigate the effect of other FFAs on the intestinal digestion of milk proteins as well as whether the digestion of some milk proteins is more affected by FFAs than other milk proteins. In summary, we showed that the presence and type of lipids in the form of TAGs and FFAs can affect the digestion of milk proteins, resulting in a different milk protein digestion in the presence of bovine milk lipids than in the presence of plant lipids. As commercial IFs contain different lipid sources and compositions [29], the digestion of milk proteins may be differently affected in different commercial IFs. Therefore, more detailed knowledge of the effect of interactions between different milk proteins and lipids on the digestion of both proteins and lipids may aid in developing improved IFs, which better support infant growth and health.

5.5 Conclusion

This study aimed to investigate the effect of milk and plant lipids on the digestion of milk proteins during *in vitro* infant digestion. We demonstrated that milk protein digestion was altered by the presence of lipids in TAGs as well as by FFAs and was differently affected by different types of lipids. During gastric digestion, caseins were digested faster in the presence of milk lipids but not in the presence of plant lipids. Intestinal digestion of milk proteins was lower in the presence of milk lipids and even lower in the presence of plant lipids. These effects were probably caused by the difference in FFA composition, and in particular by the higher concentrations of the LC-PUFA linoleic acid in the sample with plant lipids. We showed that adding linoleic acid to milk proteins decreased their hydrolysis during intestinal digestion, with higher linoleic acid concentrations resulting in lower proteolysis. However, a more comprehensive understanding is needed of the interactions between milk proteins and different types of FAs during digestion, and the consequences of these interactions for both protein and lipid digestion.

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Supplementary Information

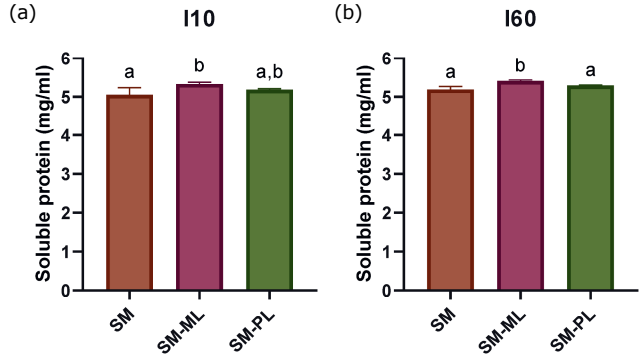

Figure 5.S1: Protein concentration in the intestinal digests from skim milk (SM), SM with milk lipids (SM-ML), and SM with plant lipids (SM-PL) after (a) 10 min of intestinal digestion (I10), (b) 60 min of intestinal digestion (I60) with an *in vitro* infant digestion model. Different letters indicate significant differences ($p<0.05$).

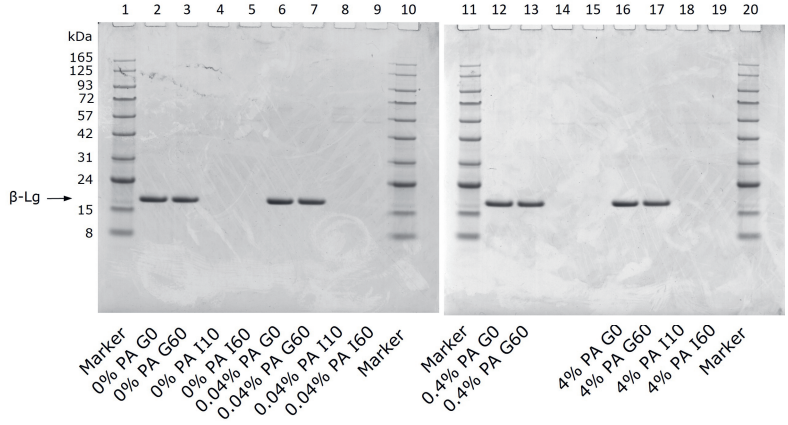

Figure 5.S2: Reducing SDS-PAGE of digests from β-lactoglobulin (β-Lg) without palmitic acid (PA) and β-Lg with different PA concentrations after 0 min of gastric digestion (G0), 60 min of gastric digestion (G60), 10 min of intestinal digestion (I10), and 60 min of intestinal digestion (I60) with an *in vitro* infant digestion model.

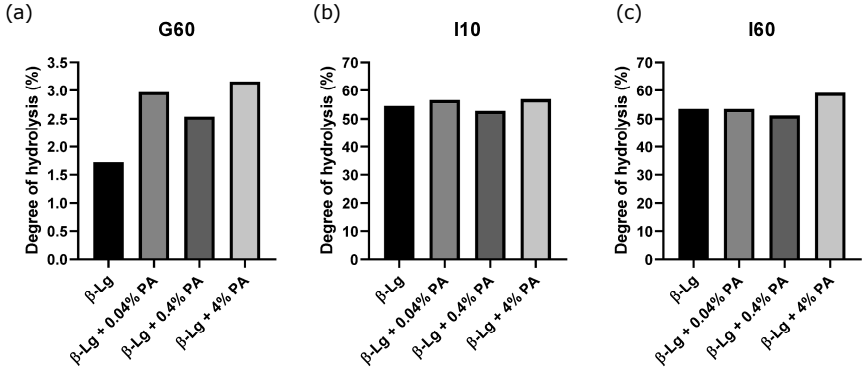

Figure 5.S3: Degree of hydrolysis of digests from β-lactoglobulin (β-Lg) without palmitic acid (PA) and β-Lg with different PA concentrations after (a) 60 min of gastric digestion (G60), (b) 10 min of intestinal digestion (I10), (c) 60 min of intestinal digestion (I60) with an *in vitro* infant digestion model (n=1).

Chapter 6

General discussion

6.1 Introduction

Providing infants with adequate nutrition is important to support healthy growth and development during infancy. Moreover, infant nutrition is also associated with health effects later in life, during childhood as well as adulthood. Proper digestion and absorption of the nutrients in infant nutrition is essential for infants as well as a healthy immune response towards these nutrients. Whereas human milk is the best nutrition for infants, many infants are partly or exclusively fed with infant formula (IF) during the first six months of life. To provide infants with the best alternative for human milk, manufacturers attempt to closely resemble its composition during the production of IF. However, achieving this is complicated since human milk has a complex composition that differs in several ways from bovine milk, which is usually the starting material for IF. To better mimic the lipids in human milk, the bovine milk lipids are partly or completely replaced by plant lipids during the production of IF. Moreover, IF undergoes several wet and dry heating steps, which are necessary to ensure microbiological safety and to facilitate easy packaging, storage, and transport. These processing steps can affect the digestion of milk proteins and can alter the subsequent intestinal transport and immunoreactivity of the digested milk proteins. However, the link between the processing of milk proteins, their digestion, intestinal absorption, and immunoreactivity is not completely understood. In addition, the digestive system of infants quickly develops during the first months of life, which can influence the digestion of milk proteins. Therefore, we studied the effect of processing as well as the changing digestion conditions of the developing infant on the digestion, intestinal uptake, and immunoreactivity of milk proteins. We accomplished this by:

- Examining the influence of age-related gastric digestion conditions on the digestion of unheated and wet heated milk proteins (Chapter 2)
- Investigating the effect of wet versus dry heating on the digestion of milk proteins (Chapter 3)
- Exploring the intestinal transport and immunoreactivity of peptides released after the digestion of wet and dry heated milk proteins (Chapter 4)
- Studying the effect of the presence and type of lipids on the digestion of milk proteins (Chapter 5)

In this chapter, the findings from Chapters 2 to 5 and some additional experiments are combined and discussed to unravel links between the processing, digestion, intestinal uptake, and immunoreactivity of milk proteins. Finally, the key findings are summarized and recommendations are made for future research.

6.2 Methodological considerations

6.2.1 Infant formula model system

Protein denaturation as well as glycation are known to affect the digestion of milk proteins. However, since IF and other milk products are usually heated at conditions that result in both denaturation and glycation, their effects are difficult to disentangle. To disentangle these

effects, we made an IF model system in Chapter 3 and heated it at wet conditions that induced protein denaturation but no glycation or at dry conditions that induced glycation but no denaturation. Unlike previous studies that used processed milk, our IF model system has the advantage of using raw bovine milk as starting material, whose composition was adjusted to the protein concentration (1.2%), casein:whey ratio (40:60), and protein:lactose ratio (12:70) of commercial IF. This enabled studying a mixture of different native milk proteins at similar protein and lactose concentrations as commercial IF, which is important because different milk proteins interact with each other and with lactose upon heat treatment. Therefore, heating one type of milk protein or heating in the absence of lactose results in the formation of different molecular interactions than heating different milk proteins and lactose as a mixture.

Wet and dry heating was conducted for different durations to reach protein denaturation or glycation levels in the range of those in commercial IFs. Wet heating was conducted at 80°C, which is above the denaturation temperature of whey proteins (~65°C), resulting in the unfolding of the globular whey proteins. Regions that were hidden in the interior of native whey proteins are exposed, which can interact with other whey proteins or with caseins via thiol-disulfide bond interactions or hydrophobic interactions. Therefore, whey protein denaturation usually also leads to the formation of denaturation-induced aggregates. Wet heating of our IF model system at 80°C for 5 min (WH-5), 15 min (WH-15), or 40 min (WH-40) resulted in a gradual increase in whey protein denaturation, from 0 to 60%, and in aggregation (Table 3.1). The surface hydrophobicity largely increased during the first 5 min and only slightly with longer wet heating durations (>15 min, Table 3.1), indicating that prolonged heating resulted in the formation of larger aggregates in which the exposed hydrophobic regions after unfolding were hidden. Whereas no protein denaturation and aggregation levels of commercial infant formula could be found in literature, the denaturation levels of our model system after wet heating were in agreement with a study in which an IF model system was heated according to industrial processing conditions [1]. The level of protein denaturation probably varies largely between commercial IFs because of differences in the processing history of the starting materials and differences in the used processing conditions during the production of IF. Therefore, studying the effect of different protein denaturation levels is relevant for industrially produced IFs, although which of our model system samples is most relevant for comparison may differ for differently processed IFs.

Dry heating of the IF model system was performed at 60°C, which is below the denaturation temperature of whey proteins, and at a water activity of 0.6. At these heating conditions, the Maillard reaction takes place in the presence of the reducing sugar lactose. Dry heating of our IF model system for 4h (DH-4) led only to increased lysine blockage, indicating early Maillard reaction, whereas dry heating for 24h (DH-24) and 72h (DH-72) led to increased lysine blockage, and furosine and CML concentrations, indicating advanced Maillard reaction and the formation of advanced glycation end products (AGEs). Glycation-induced aggregates were also formed since the surface hydrophobicity did not decrease upon the binding of hydrophilic lactose (Table 3.1) and some smears were observed on non-reducing SDS-PAGE (Figure 3.1). The lysine blockage levels of the dry heated samples ranged from 18 to 68%, while furosine and CML contents ranged from 5 to 22 mg/g and

from 29 to 265 µg/g, respectively (Table 3.1). These glycation levels were in line with the glycation levels in commercial IFs [2], and ranged from the lowest to the highest furosine and CML concentrations measured in commercial IFs. Whereas the DH-24 sample had furosine and CML levels that were comparable to the highest number of commercial IFs, including the DH-4 and DH-72 samples enabled studying the full range of industrially relevant glycation levels in IFs.

6.2.2 *In vitro* infant digestion models

We made use of a static *in vitro* infant digestion model to evaluate differences in the digestion of milk proteins in Chapters 2-5. This infant digestion model of Ménard et al. [3] is currently one of the most used infant digestion models and is based on *in vivo* data on the digestion system of one-month-old infants, as reviewed by Bourlieu et al. [4]. The use of a static *in vitro* digestion model has many advantages: it allows handling many samples simultaneously, is cost-efficient, does not require a high sample volume, and enables easy comparison of data with literature. However, static digestion models cannot simulate the complexity and dynamics of the physiological digestion conditions. Semi-dynamic or dynamic digestion models better resemble these physiological conditions since they include a gradual drop in gastric pH, secretion of digestive fluids and enzymes, gastric emptying, and peristaltic contractions. The use of such semi-dynamic or dynamic models is, however, time-consuming, does not allow for testing many samples, and induces more variation between replicates. To enable studying multiple samples simultaneously, the low sample volumes of our IF model system, and to facilitate easy comparison of data with literature, we chose to use the static *in vitro* infant digestion model.

One of the major limitations of *in vitro* studies on infant digestion is the lack of validated static and (semi-)dynamic *in vitro* infant digestion models with *in vivo* data, as has been done for adult digestion models [5]. Validation may be achieved by obtaining aspirates from the stomach and duodenum of infants via a nasogastric or nasoduodenum tube after they have received IF or human milk, which has been done previously in infants [6,7]. The same IF or human milk can be digested with the currently available *in vitro* infant digestion models to determine differences and similarities in the digestion patterns between the *in vivo* and *in vitro* obtained samples. In case obtaining *in vivo* data in infants is not possible, validation of *in vitro* infant digestion models with *in vivo* data obtained in piglets may be an alternative. Piglets are more often used as an *in vivo* model to study infant digestion since their digestion system closely resembles that of human infants [8,9]. *In vivo* data from piglets was used to validate a dynamic *in vitro* infant digestion model representing one-month-old infants [10]. However, static and semi-dynamic infant digestion models representing one-month-old infants have yet to be validated. Moreover, infant digestion models representing other infant ages, such as the static digestion models representing three- and six-month-old infants that were set up in Chapter 2, need to be validated to enable studying the age-dependent change in digestion of milk or IF. Hence, validating and optimizing *in vitro* infant digestion models with *in vivo* data raises ethical dilemmas since this requires conducting studies with either animal or human infants, but remains crucial for studying the digestion of IF or human milk under physiologically relevant conditions.

6.2.3 Intestinal barrier models

Caco-2 cells cultured on transwells for 21 days is a commonly used cell model to simulate small intestinal enterocytes and has previously been used to study the intestinal transfer of milk proteins and peptides [11–14]. These cells are frequently used to study intestinal absorption *in vitro* and show a good correlation with intestinal absorption in adults *in* vivo, especially for passive transport [15,16]. The small intestine of infants is, however, more permeable than that of adults [17], probably resulting in the transport of fewer peptides by the use of an *in vitro* Caco-2 model compared to the *in vivo* conditions. The use of intestinal barrier models that are more permeable to resemble infant conditions may be valuable to study the transport of milk peptides derived from IF or human milk at more physiologically relevant conditions. A more permeable infant Caco-2 model has recently been developed and validated by the use of isolated milk peptides [18], but cocultures of Caco-2 cells with other small intestinal cell types have not yet been developed for infant conditions. Since we aimed to study the effect of peptide transfer in the presence and absence of a mucus layer produced by goblet-like cells, we made use of the standard Caco-2 monoculture and a Caco-2/HT29-MTX-E12 (90/10) coculture in Chapter 4. Milk peptides have previously been shown to affect mucin gene expression and mucus production by goblet-like cells [19–22], and some milk peptides were transported through Caco-2/HT29(-MTX) cocultures [23,24]. However, more cell types are present in the small intestinal barrier besides enterocytes and goblet cells, which can affect the intestinal transport of and the immune response towards milk peptides. For instance, the intestinal barrier models we used did not include dendritic cells, which can sample through the small intestinal barrier, or M-cells, which are thought to play an important role in transporting antigens through the intestinal barrier. Whereas Caco-2 cells can differentiate into M-cells in the presence of lymphocytes [25], this is not known to occur in Caco-2 monocultures or Caco-2/HT29-MTX cocultures. Including such cell types would result in an intestinal barrier model that better resembles the physiological conditions, but also increases the complexity of the model. Therefore, different intestinal barrier models may be most appropriate for different studies depending on the specific research focus.

6.2.4 Analysis of digestion-derived peptides

Different methods were used to measure the breakdown of intact proteins and the formation of peptides, including SDS-PAGE, the OPA assay, and LC-MS/MS. Whereas SDS-PAGE and the OPA assay are simple methods to assess the disappearance of intact protein and the digestibility of proteins, which allow for easy comparison with other studies, they cannot provide information on the extent of digestion of individual proteins. Measuring changes in peptide patterns with LC-MS/MS gives useful insights into the effect of heat treatment on the formed peptides, which enables investigating differential effects on individual milk proteins and specific regions within those proteins. Furthermore, peptide patterns can reveal potential differences in the bioactivity or immunoreactivity of the digests. However, the used LC-MS/MS method was limited to measuring peptides with a length between 6-25 amino acids (AAs), meaning that information on peptides outside this size range was not obtained. Although digestion-derived peptides that contain immunoreactive relevant structures

generally fall in this size range, the detection of smaller peptides as well may provide more information on the extent of digestion of individual proteins and could be obtained using *de novo* sequencing. Next to the limitation in peptide identification due to length, peptide identification is also limited by the content of the database and the inclusion of post-translational modifications (PTMs). To maximize peptide identification, it is important to use a database that is as complete as possible and to include all relevant PTMs. However, the processing time increases significantly by increasing the database size and increases exponentially by adding more PTMs. Therefore, a compromise needs to be made between maximizing the number of identified peptides and maintaining an acceptable processing time. Such compromise was made in Chapter 3, in which a first run was performed with a larger milk protein database, but without the inclusion of PTMs, followed by a second run with a smaller database, which only included the milk proteins identified in the first run, and the inclusion of seven different PTMs. However, this still resulted in a long processing time (>3 weeks on a high-performance computer). To further reduce the processing time while minimizing a decrease in peptide identifications, the identification method in Chapter 4 included only the four most abundant PTMs. Studies on the effect of glycation require the inclusion of additional, glycation-related PTMs compared to studies on the digestion of milk proteins focusing on other effects. Therefore, making acceptable compromises is especially important for peptide identification in studies on the glycation of milk proteins.

6.2.5 Assessment of immunoreactivity of digested milk proteins

The immunoreactivity of milk proteins after intestinal digestion and after intestinal transport was predicted in Chapter 4 by predicting potential HLA-II and IgE binding epitopes. Evaluating the differences in potential HLA-II binding epitopes, which may be recognized by T-cells, and IgE binding epitopes, which may induce basophil and mast cell degranulation, is a fast method to screen for differences in immunoreactivity of the milk peptides that were identified with LC-MS/MS. While a commonly used HLA-II binding prediction tool (IEDB MHC-II Binding Predictions tool) was used to identify potential HLA-II epitopes in our samples, it remains a prediction and is not a direct measurement. Predicting of IgE epitopes was done by comparison with literature on known regions within milk proteins containing IgE epitopes but is only as complete as the ones that are described in literature. Moreover, literature did not always distinguish between linear and structural epitopes, whereas most structural epitopes present in intact proteins are destructed upon gastrointestinal digestion. Including a more direct measurement is important to link the differences in IgE and HLA-II epitopes to relevant changes in immune responses.

For this reason, we stimulated primary immature dendritic cells (iDCs) with milk peptides and measured changes in cytokine production. One challenge while performing this experiment was the presence of lipopolysaccharide (LPS) in the digestion samples, which stimulates iDCs to mature into dendritic cell type 1 (DC1), and makes it difficult to assess the effect of the milk peptides on iDCs. Since Caco-2 cells prevent the transfer of LPS to the basolateral side of the monolayer, we were able to use the basolateral media after intestinal transport to stimulate iDCs without performing LPS removal steps. Assessing the potency of milk proteins after gastric or intestinal digestion to affect the DC response is also physiologically relevant because DCs can sample through the intestinal barrier. However,

milk samples as well as the chemicals and enzymes added during *in vitro* digestion contained quantities of LPS that could not be completely blocked with the LPS inhibitor polymyxin B (PMB). These LPS quantities would thus lead to the maturation of iDCs, making it impossible to study the effect of the milk proteins without performing an LPS removal step beforehand. Therefore, we tried multiple methods to remove LPS from digestion samples without removing milk proteins and peptides. Since the EndoTrap HD column (Lionex) removed the highest concentration of LPS without changing the protein composition, we used this method to remove LPS from gastric digests of wet and dry heated milk proteins before stimulating iDCs with these gastric digests. The results of this experiment are presented and discussed later in this chapter (section 6.4.4). To measure changes in the immunoreactivity of milk proteins and peptides even more directly, DC-T-cell cocultures may be used to determine if differently heated milk proteins can affect the T-cell response and induce allergic sensitization. In addition, serum from cow's milk allergic patients could be used to measure the immunoreactivity of differently heated milk proteins in a direct manner [13,26].

6.3 Influence of age-related gastric digestion conditions on the digestion of milk proteins

The digestive system of infants greatly changes during the first six months of life [4], but the effect of these age-dependent changes on the digestion of milk proteins had not yet been investigated. Chapter 2 shows that the gastric digestion of milk proteins increased with infant age: a looser gastric clot was formed that was broken down more easily (Figures 2.1 and 2.2) and both caseins and whey proteins were hydrolyzed to a further extent (Figures 2.3-2.5). The higher pepsin concentration and the lower gastric pH resulted in a higher pepsin activity with increasing infant age, which is mainly responsible for the formation and breakdown of the gastric clot and the extent of protein hydrolysis during gastric digestion. Whereas whey proteins were resistant to pepsin activity at the digestion conditions of one-month-old infants due to their globular structure, they were partly digested with increasing infant age when gastric digestion develops towards more adult conditions (Figures 2.3 and 2.4). Moreover, the looser clot structure may facilitate higher accessibility of cleavage sites in caseins for pepsin. The structure and breakdown of the gastric clot were previously shown to be important to ensure a gradual release of AAs, which minimized nitrogen loss [27]. Moreover, changes in the gastric digestion of milk protein may affect the whole gastro-intestinal digestion kinetics. Therefore, a proper digestion of milk proteins that suits the changing needs of the developing infant is important. Whereas the composition of human milk changes to fulfill these changing needs [28], no different types of IF are available for different ages within the first six months of life. To further support healthy growth and development, IFs for different ages within the first six months of life could be developed, which mimic the composition of human milk and its digestion behavior at the corresponding lactation stage and infant age. To enable the development of such IFs, research is needed on the changing composition and digestion of proteins in human milk from different lactation stages.

6.4 Effect of wet and dry heating on the digestion, intestinal transport, and immunoreactivity of milk proteins

6.4.1 Gastro-intestinal digestion of wet heated milk proteins

Wet heating is considered to increase the digestion of whey proteins since cleavage sites in these globular proteins are more easily accessible after unfolding. Since caseins lack secondary and tertiary structure, they cannot unfold upon heat treatment, and therefore their digestion is considered to be less affected by wet heating compared to whey proteins. However, denatured whey proteins can bind to the casein micelles, which can influence the digestion of caseins. Chapter 2 showed that wet heating decreased gastric casein digestion in skim milk (Figure 2.3), and Chapter 3 showed the same effect in the IF model system (Figure 3.2). This was probably caused by the binding of denatured whey proteins to caseins that reduced the accessibility of cleavage sites in caseins for pepsin, which was also found by Sánchez-Rivera et al. [29]. Interestingly, wet heating resulted in increased gastric digestion of whey proteins in skim milk (Figure 2.4), but not in the IF model system (Figure 3.2). Most studies reported increased gastric digestion of whey proteins after wet heating [30,31], but a previous study that used an IF model system also found that the gastric digestion of β-Lg and α-La was unchanged after heat treatment [32]. This suggests that different molecular interactions were formed in our IF model system than in skim milk, which may explain the difference in the effect of whey protein digestion upon wet heating. The lower casein:whey ratio in the IF model system (40:60) compared to skim milk (80:20) might have resulted in relatively more covalent disulfide interactions and less hydrophobic interactions, leading to aggregates with stronger interactions that might hinder the digestion of whey proteins to a greater extent. Further investigation of the molecular interactions formed upon wet heating at different casein:whey ratios may aid in obtaining a more comprehensive understanding of the difference in the effect of wet heating on the gastric digestion of milk proteins in the IF model system compared to skim milk. Wet heating increased the intestinal digestion of milk proteins as observed by the lower intensity of peptides measured with LC-MS/MS in the WH-40 sample (Figures 3.4 and 4.1), indicating that more peptides were hydrolyzed till a size below the detection limit (<6 AAs). In particular, lower intensities for peptides from β-casein and β-Lg were found after wet heating (Figures 3.4 and 4.1). The more extensive digestion of whey proteins after wet heating is in line with literature [33], and was probably caused by the increased accessibility of cleavage sites after unfolding. The increased digestion of casein upon wet heating is in agreement with a study from Singh and Creamer [34], although slower digestion of caseins after wet heating was reported by Dupont et al. [35]. These previously conducted studies on the digestion of caseins, however, used heating conditions that most likely induced both denaturation and glycation, making it difficult to disentangle the effect of denaturation from glycation. Since the used wet heating conditions in Chapters 3 and 4 only induced denaturation and no glycation, our findings suggest that whey protein denaturation without the occurrence of glycation increases the intestinal digestion of both whey proteins and caseins.

6.4.2 Gastro-intestinal digestion of dry heated milk proteins

Dry heating in the presence of a reducing sugar results in glycation of caseins and whey proteins via the Maillard reaction. Glycation itself as well as glycation-induced aggregation reduce the digestion of milk proteins due to the blocking of cleavage sites. Since glycation occurs at lysine residues in both casein and whey proteins, dry heating affects the digestion of both caseins and whey proteins, especially during intestinal digestion in which trypsin cleaves next to lysine residues. Dry heating did not significantly change the gastric digestion of milk proteins in our IF model system (Figures 3.2 and 3.3). In contrast, lysine blockage levels that were similar to our DH-24 sample (42%) have previously been shown to result in a decreased degree of hydrolysis after gastric digestion of infant formula, although this decrease was small [36]. Dry heating had a larger effect on the digestion of milk proteins during intestinal digestion. The digestion of milk proteins was decreased to a further extent with increasing dry heating durations (Figure 3.3) and relatively longer digestion-derived peptides were found in the DH-72 sample (Figure 3.4). This was most likely caused by a decreased accessibility of cleavage sites in proteins upon glycation, which was supported by the correlation we found between the peptide intensities from milk proteins and their degree of glycation (Figure 3.5). In particular, dry heating resulted in a decreased digestion of specific regions in α_{s1}-casein, κ-casein, and β-Lg containing lactosylated lysine residues that were located close to cleavage sites for trypsin (Figure 3.S2). The decrease in milk protein digestion with increasing levels of glycation, resulting in longer peptide lengths, was also found by Zenker et al. [36]. Moreover, a correlation between a reduction in digestion and the degree of glycation due to the blocking of cleavage sites was reported previously for isolated α-La [37]. The reported correlation between highly glycated regions and reduced digestion in Chapter 3 was, however, observed in a different manner in Chapter 4. Whereas the same regions in the proteins contained higher intensities of glycated AAs, this did not result in a higher peptide intensity of these regions (Figures S4.1 and S4.2). The peptides originating from these highly glycated regions were, however, relatively long, which indicates that these regions were digested to a lower extent. These differences between Chapters 3 and 4 may be caused by the used batch of digestive enzymes. The proteins seemed to be hydrolyzed to a further extent in Chapter 3 (Figure 3.4) because relatively more small peptides and lower peptide intensities were detected than in Chapter 4 (Figure 4.1), suggesting that more peptides were too small (<6 AAs) to be detected in Chapter 3. In summary, the degree of glycation seems to be an important determinant in predicting the digestion of milk proteins, especially for cleavage by trypsin during intestinal digestion.

6.4.3 Intestinal transport of digestion-derived peptides

The transport of digestion-derived peptides across the intestinal barrier is important for infants as it enables them to use these peptides for their growth and development. In addition, several different immune cells are present in the *lamina propria* which can induce an immunological response upon encountering transported milk peptides. In Chapter 4, we showed that wet heating led to a lower intestinal transport of peptides between 6-25 AAs, whereas the effect of dry heating differed depending on the used intestinal barrier model: dry heating resulted in a lower peptide transport in the Caco-2 monoculture but a higher

peptide transport in the Caco-2/HT29-MTX-E12 coculture (Figure 4.4). This suggests that the intestinal transport of peptides is affected by the applied heat treatment prior to digestion as well as the used cell model for intestinal transport, in which the mucus layer may play a role. The lower peptide transfer across a Caco-2 monolayer has previously been reported after wet heating [12] and dry heating [11] of milk proteins. However, further research is needed to investigate why the effect of dry heating differs between the two intestinal barrier models, and which model better resembles the effect of dry heating compared to the physiological situation. Moreover, we showed that mainly unmodified and lactosylated peptides were transported across the intestinal barrier (Figure 4.4), as also shown by Zenker et al. [11]. This suggests that glycated peptides can come in contact with immune cells in the *lamina propria*. However, the effect of glycation on the immunoreactivity of milk proteins is not completely understood, which is further discussed in section 6.4.4.

6.4.4 Immunoreactivity of digested and transported milk proteins

Milk proteins come into contact with antigen-presenting cells (APCs) in the small intestine or after intestinal transport, and can induce an immunological response. Heat treatments and the digestion conditions affect the digestion of milk proteins and subsequently influence in what form milk proteins are presented to APCs. Therefore, it is important to consider heat treatment and digestion in evaluating the immunoreactivity of milk proteins. Chapter 4 showed that both wet and dry heating resulted in increased survival of peptides with predicted HLA-II epitopes (Figure 4.2), indicating that wet and dry heating increased the immunoreactivity of milk proteins. However, many peptides containing predicted HLA-II epitopes were phosphorylated or glycated (Table 4.S1) and it is unclear whether these modifications change their immunoreactivity. Peptides from specific regions in proteins were more efficiently transported across the intestinal barrier models, which resulted in a higher intensity of HLA-II epitopes after dry heating and a lower intensity of HLA-II epitopes after wet heating compared to unheated milk proteins in both the monoculture and coculture intestinal barrier models (Figure 4.7). Moreover, the intensity of peptides containing potential IgE epitopes was the highest for unheated milk proteins compared to wet and dry heated milk proteins in the monoculture and was the highest for dry heated milk proteins compared to unheated and wet heated milk proteins in the coculture (Figure 4.7). Together, this suggests that wet heating resulted in a decreased immunoreactivity after intestinal transport in both intestinal barrier models and dry heating in an increased immunoreactivity after intestinal transport in the coculture model. However, these transported peptides did not affect the cytokine production by iDCs (Figure 4.9). This indicates that the differences in immunoreactivity were too small to induce a change in response by the iDCs, or that the differences in immunoreactivity were insufficiently detected with the used method. This emphasizes that changes in HLA-II and IgE epitopes only provide a first indication of changes in immunoreactivity and that including more direct measurements is important to evaluate the immunoreactivity of milk proteins.

Dendritic cells can already encounter milk peptides directly after gastric digestion because they can sample through the intestinal barrier. At this stage of digestion, more immunoreactive structures are probably still intact and higher peptide concentrations can be expected than after intestinal transport. Therefore, we investigated differences in cytokine

production by iDCs after stimulation with the soluble part of unheated, wet heated, or dry heated IF model system after 60 min of gastric *in vitro* infant digestion. Moreover, the properties of the soluble part of the gastric digests were measured to enable linking these properties to the differences in cytokine production by the iDCs (Figure 6.1). Wet and dry heating seemed to change the TNF-α, IL-6, IL-10, and IP-10 production by iDCs after gastric digestion, although the effects were not significant due to the large variation between the different donors. Particularly the gastric digests from WH-5, DH-4, and DH-72 resulted in a high cytokine production, indicating that the cytokine production was not directly related to the wet or dry heating duration prior to digestion. The change in cytokine production did not resemble the cytokine production by either mature type 1 or type 2 DCs, which makes it difficult to interpret what the change in cytokine production by the iDCs means in terms of the potency of the gastric digests to induce a pro- or anti-inflammatory immune response. The principal component analysis (PCA) plot suggested that higher cytokine production by iDCs was related to fewer free NH_2 groups, higher surface hydrophobicity, and more fibril structures in the gastric digests (Figure 6.1C). These properties have also been linked to changes in the immunoreactivity of milk proteins in previous studies, including changed binding to receptors for AGEs [26,38,39] or to THP-1 macrophages and dendritic cells [39–41]. Furthermore, the PCA plot showed that the primary iDCs from donor 2 reacted more strongly, especially to the dry heated samples, than the ones from donors 1 and 3. This suggests that wet and dry heating may affect the immunoreactivity of milk proteins after gastric digestion, but the magnitude of this effect differs between individuals. Repeating this experiment with additional donors would aid in determining if more donors respond more extremely to digests from dry heated milk proteins than other donors, and in evaluating if the differently heated milk proteins induce a changed cytokine response by iDCs on average. In addition, since the infant immune system is Th2 skewed, it may be interesting to repeat this experiment with maturated type 2 DCs, possibly together with T-cells to measure potential differences in the DC-T-cell interaction. This may provide an indication of the potency of the digests to develop milk protein allergy.

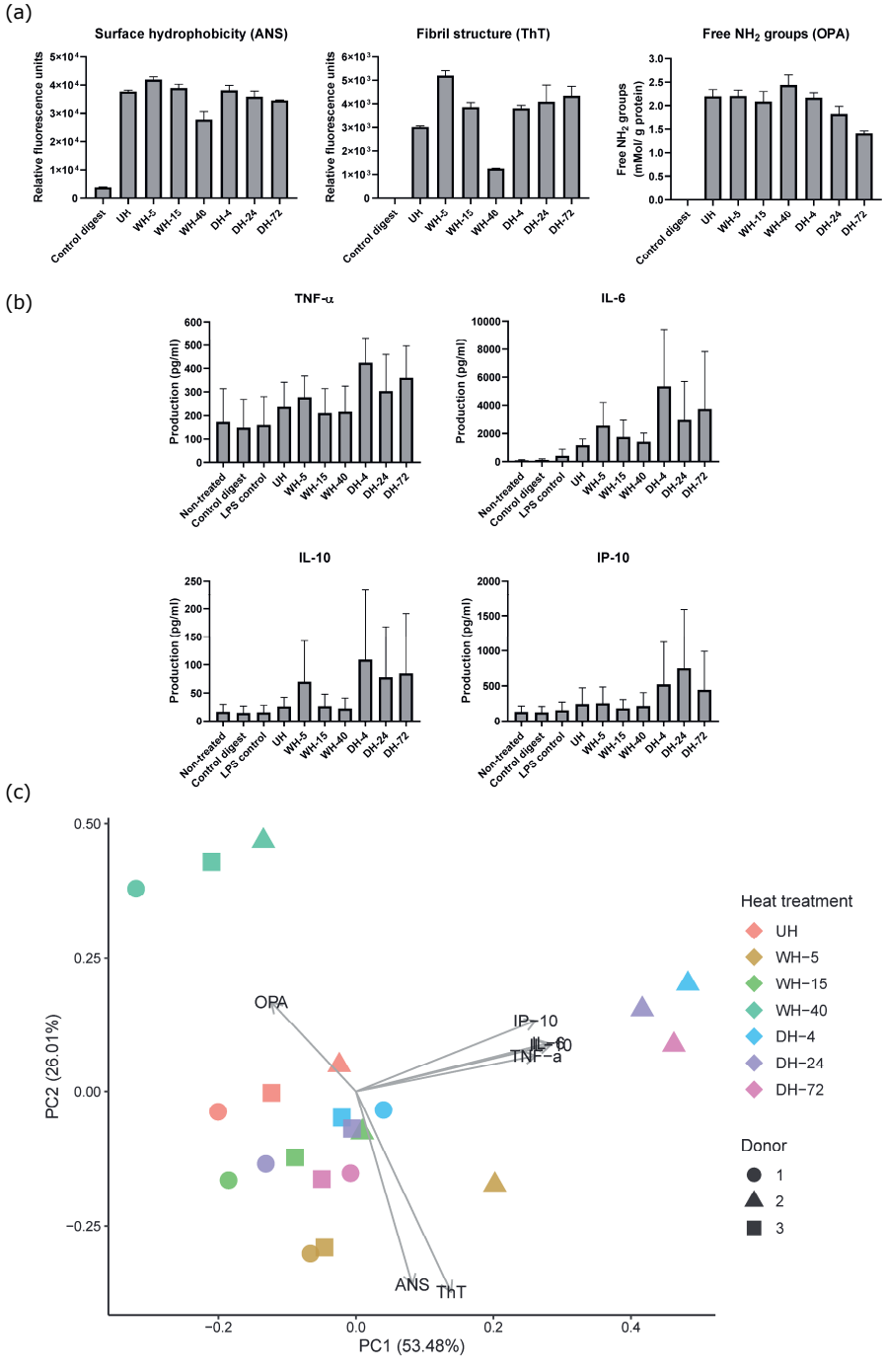

Figure 6.1: (Caption on next page)

Figure 6.1: Properties of soluble milk proteins after *in vitro* infant gastric digestion for 60 min and cytokine response of primary immature dendritic cells (iDCs) after stimulation with the gastric digests for 48h. (a) Surface hydrophobicity, fibril structure, and free amino (NH_2) group content in the soluble part of the gastric digests of unheated (UH) wet heated (WH) for 5 min (WH-5), 15 min (WH-15) or 40 min (WH-40), or dry heated (DH) for 4h (DH-4), 24h (DH-24) or 72h (DH-72) IF model system. Water instead of the IF model system was digested to obtain a control digest. Surface hydrophobicity was measured with the 8-anilino-1-naphthalenesulfonic acid (ANS) assay, fibril structure was measured with the thioflavin T (ThT) assay, and free NH_2 group content was measured with the o-phthaldialdehyde (OPA) assay. Values were corrected for protein content in the soluble part of the gastric digests. (b) TNF-α, IL-6, IL-10, and IP-10 production by iDCs after stimulation with the soluble part of the gastric digests after lipopolysaccharide (LPS) removal (n=3). LPS was removed from the samples by use of an EndoTrap HD column (Lionex). Thereafter, iDCs were stimulated with the samples at a concentration of 100 µg protein/ml (final concentration, as measured with the BCA assay). Non-treated were iDCs grown in Iscove's Modified Dulbecco's Medium (IMDM), and LPS control were iDCs stimulated with 14.8 ng/ml LPS, which was the highest LPS concentration measured in the gastric digests by use of the ENDONEXT™ EndoZyme® II Endotoxin Detection Assay (bioMérieux). Polymyxin B (3 µg/ml) was added to all iDCs to block the effect of LPS. (c) Principal component analysis (PCA) of the properties of the gastric digests and the production of TNF-α, IL-6, IL-10, and IP-10 by iDCs. ANS, ThT and OPA refer to the surface hydrophobicity, fibril structure and free NH_2 group content of the soluble part of the gastric digests as measured with the ANS assay, ThT assay and OPA assay, respectively.

6.5 Influence of lipids on the digestion of milk proteins

Whereas we studied the digestion of milk proteins in the absence of lipids in Chapters 2-4, infant formula also contains lipids, which may change the digestion of milk proteins. Therefore, we included milk lipids or plant lipids in Chapter 5 and studied their effect on the digestion of milk proteins. We found that lipids can decrease the digestion of milk proteins and that the extent of this decrease depends on the lipid composition and concentration. The composition of plant lipids, which contained more long chain and unsaturated fatty acids, decreased milk protein digestion to a greater extent than the composition of milk lipids (Figure 5.5). Moreover, we demonstrated that the presence of lipids in triacylglycerides (TAGs) as well as free fatty acids (FFAs) prior to digestion were able to decrease the digestion of milk proteins (Figures 5.5 and 5.6). The less extensive digestion of milk proteins depending on the lipid composition may result in the survival of larger peptides, which might be transported differently through the intestinal barrier and might contain more intact immunoreactive structures. Since the lipid sources and lipid profiles vary between different commercial IFs [42], the extent of milk protein digestion may differ between different IFs. Further investigating the effect of lipid composition on the digestion and uptake of milk proteins may aid in improving the lipid composition of IFs that better support infant growth and health. To investigate this, it is important to use an *in vitro* digestion model that resembles the physiological conditions for the digestion of proteins and lipids simultaneously because the digestion of milk proteins can be affected by FFAs, which are released from TAGs during gastric and intestinal digestion, and the digestion of lipids may be affected by proteins or digestion-derived peptides.

6.6 Conclusions

The research presented in this thesis has provided more insights into how processing as well as the changing digestion conditions of the developing infant impact the digestion of milk proteins, and how this subsequently leads to changes in intestinal transport and immunoreactivity of the digested milk proteins. This has resulted in the following conclusions:

- The changing gastric digestion conditions of infants in the first six months of life influenced the formation and breakdown of the gastric clot and the gastric digestion of milk proteins.
- Wet heating of the IF model system at 80°C decreased the gastric digestion of casein and increased the intestinal digestion of caseins and whey proteins, resulting in a different composition of released peptides with more potential immunoreactive structures.
- Dry heating at 60°C induced glycation and decreased the intestinal digestion of caseins and whey proteins by blocking cleavage sites for trypsin. This resulted in the release of longer glycated peptides with more potential immunoreactive structures.
- Heat treatment and the intestinal barrier model affected the intestinal transport of milk peptides and potential immunoreactive structures. These differences in transported potential immunoreactive structures did, however, not affect the cytokine response by iDCs.
- Wet and dry heating may increase the immunoreactivity of milk proteins after gastric digestion, which was more likely to be related to the properties of the digests rather than to wet or dry heating duration.
- Lipids affect the intestinal digestion of milk proteins differently depending on the lipid composition and concentration.

6.7 Future perspectives

This thesis shows that wet and dry heating as well as the digestion conditions influence the digestion of milk proteins in infants. However, future research is needed to determine how closely digesting milk proteins with *in vitro* infant digestion models resembles the physiological situation because these digestion models are not validated yet with *in vivo* data. Validation may be done by comparison of digestion patterns of milk proteins during *in vitro* digestion and *in vivo* digestion. Ideally, *in vivo* data would be obtained from the stomach and duodenum of human infants via a nasogastric or nasoduodenum tube, but piglets could be used as an alternative for obtaining *in vivo* data.

Moreover, Chapter 4 showed that wet and dry heating can affect potential immunoreactive structures in the intestinal digests and after intestinal transport. Many of the identified peptides with HLA-II and IgE epitopes contained phosphorylated or glycated AAs, whose effect on the immunoreactivity of the peptides is not known. Studying the binding capacity of peptides with and without modified AAs may aid in understanding how different

modifications affect the immunoreactivity of peptides. Whereas the change in immunoreactive structures after intestinal transport did not affect the iDC response, DCs can sample through the intestinal barrier, where they can encounter different and higher concentrations of milk peptides directly after gastric digestion. We made a start on investigating changes in DC response after gastric digestion of wet and dry heated milk proteins as shown in Figure 6.1. Linking properties of the digests to the DC response of more independent donors may help in predicting the immunoreactivity of differently modified milk proteins. In addition, testing this in DC-T-cell cocultures may provide more information on the effect of heating milk proteins on the induction of a pro- or anti-inflammatory response.

Lastly, IF is a complex system in which different components interact with each other upon heat treatment but also during digestion. We used a simplified IF model system in Chapters 3 and 4 and heated it at conditions to induce only protein denaturation or glycation, and we added different types of lipids to skim milk in Chapter 5, followed by studying the effects on the digestion of milk proteins. However, IF is heat treated at conditions that induce both denaturation and glycation simultaneously and contains lipids as well as other minor components. Since all these components can interact during the production of IF and during digestion, it would be valuable to slowly increase the complexity of the IF model system in which the effect of several interactions can be studied separately and simultaneously.

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Summary

Summary

Human milk is the best nutrition for infants during the first six months of life for healthy growth and development. Moreover, breastfeeding during infancy has been linked to several health benefits later in life. However, many infants are partly or completely fed with infant formula. Whereas human milk is usually drunk raw by infants, the production of infant formula from bovine milk includes multiple processing steps, including heating steps, to more closely mimic the composition of human milk and to ensure microbiological safety. These processing steps can alter the digestion of milk proteins and can result in subsequent changes in intestinal transport, and immunoreactivity of the digested milk proteins. However, the relationship between processing of milk proteins, and subsequent changes in digestion, intestinal transport, and immunoreactivity is not well understood. Moreover, the digestive system of infants quickly develops during the first months of life, which can also affect the digestion of milk proteins. Since milk proteins are the only source of protein that infants receive during the first six months of life, proper digestion and absorption of these proteins are highly important for infants as well as a healthy immune response towards these proteins. Therefore, this thesis aimed to investigate how processing as well as the changing digestion conditions of the developing infant impact the digestion of milk proteins and how this affects subsequent intestinal transport and immunoreactivity of the digested milk proteins.

The digestive system of infants changes tremendously during the first six months of life. In **Chapter 2**, we investigated how these age-related changes in digestion conditions influence the gastric digestion of milk proteins. For this aim, parameters of an existing *in vitro* digestion model representing one-month-old infants were changed to obtain gastric digestion models representing three- and six-month-old infants. Unheated and heated skim milk were digested by use of these models. Results show that, with increasing age, a looser gastric clot was formed, which was broken down more easily, and that caseins as well as whey proteins were digested to a further extent. Moreover, the difference in protein digestion with increasing age was larger for heated than unheated skim milk. Together, these findings show that milk protein digestion in infants increased with infant age and that heat treatment influenced milk protein digestion differently depending on age-related gastric digestion conditions.

Milk proteins are heated under both wet and dry conditions during the production of infant formula, resulting in the denaturation and glycation of these proteins. To disentangle the effects of protein denaturation and glycation during infant formula production, a model system for infant formula was made in **Chapter 3**, which was heated either under wet conditions that only induced protein denaturation or under dry conditions that only induced glycation. The wet and dry heating conditions resulted in protein denaturation levels and glycation levels that were comparable with infant formula model systems and commercial infant formulas. Wet heating decreased the digestion of caseins during gastric digestion, possibly due to casein-whey protein interactions after whey protein denaturation, whereas dry heating did not affect the gastric digestion of milk proteins. During intestinal digestion, wet heated milk proteins were digested faster, probably because of increased accessibility of cleavage sites for digestive enzymes after unfolding. In contrast, dry heating led to a decrease in intestinal protein digestion. The high intensities of peptides containing glycated

amino acids close to cleavage sites for trypsin indicate that the decreased digestion after dry heating was most likely due to glycation blocking the accessibility of cleavage sites.

The differential effects of peptides released after digestion of wet and dry heated milk proteins on intestinal mucus production, intestinal transport, and immunoreactivity were investigated in **Chapter 4**. Wet as well as dry heating led to higher survival of potential HLA-II epitopes during intestinal digestion, whereas no effect was seen for survival of potential IgE epitopes. This suggests that wet and dry heating increased the immunoreactivity of digestion-derived milk peptides. Mucus production by HT29-MTX cells was not affected by the released milk peptides. Intestinal transport of milk peptides was lower across both the Caco-2 monoculture and the Caco-2/HT29-MTX coculture after wet heating. In contrast, the effect of dry heating differed between the monoculture and the coculture: peptide transport across the monoculture was lower and across the coculture was higher after dry heating. The transport of potential HLA-II and IgE epitopes was lower after wet heating in the mono- and cocultures. Dry heating resulted in a higher transport of potential HLA-II epitopes in both the monoculture and the coculture and a higher transport of potential IgE epitopes in the coculture. This suggests that wet heating resulted in a decreased immunoreactivity after intestinal transport in the mono- and cocultures and that dry heating resulted in an increased immunoreactivity after intestinal transport in the coculture model. However, transported milk peptides did not affect the cytokine production by primary immature dendritic cells (iDCs). Together, wet and dry heating changed the survival of immunoreactive structures and intestinal transport of milk proteins, but did not affect mucus production after intestinal digestion or cytokine response by iDCs after intestinal transport.

During the production of infant formula, the bovine milk lipids are partly or fully replaced by plant lipids to better resemble the fatty acid composition of human milk. In **Chapter 5**, the effects of milk lipids as well as plant lipids on the digestion of milk proteins were investigated. During gastric digestion, caseins were digested faster in the presence of milk lipids. Both milk lipids and plant lipids decreased the digestion of milk proteins during intestinal digestion, and plant lipids decreased it to a further extent than milk lipids. This effect was probably attributed to the difference in free fatty acids that were released during digestion, and especially to the higher concentration of linoleic acid released from plant lipids than from milk lipids. We showed that adding linoleic acid as free fatty acid to milk proteins decreased the intestinal digestion of milk proteins in a concentration-dependent manner. All in all, we demonstrated that the digestion of milk proteins was affected by milk lipids and plant lipids as well as by free fatty acids and was differently affected by different types of lipids.

In **Chapter 6**, additional data was shown on the effect of wet and dry heating of milk proteins on their immunoreactivity after gastric digestion. Wet and dry heating of milk proteins seemed to change the TNF-α, IL-6, IL-10, and IP-10 production by primary iDCs after gastric digestion, although the effects were not significant due to the large variation between the different donors. Whereas no relation was found between wet or dry heating duration and cytokine response by iDCs, our results suggest that fewer free NH_2 groups, higher surface hydrophobicity, and more fibril structures in the gastric digests were related to higher productions of TNF-α, IL-6, IL-10, and IP-10 by iDCs. Together, we showed that

wet and dry heating may increase the immunoreactivity of milk proteins after gastric digestion, and this change in immunoreactivity was more likely to be related to the properties of the digests than to wet or dry heating duration.

To conclude, we have demonstrated that processing (i.e. heat treatment and addition of lipids) as well as the digestion conditions affect the digestion of milk proteins. The changes in the digestion of milk proteins after heat treatment were shown to subsequently alter the survival of immunoreactive structures during digestion and the intestinal transport of milk peptides and immunoreactive structures. Moreover, we have shown the importance of studying milk protein processing along with subsequent changes in their digestion, intestinal transport, and immunoreactivity.

Addenda

Acknowledgments

About the author

List of publications

Overview of completed training activities

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About the author

Julie Linde Miltenburg was born on September 14th, 1995 in Sassenheim, the Netherlands. She started a BSc in Molecular Life Sciences at Radboud University in Nijmegen in 2013. Thereafter, she continued with an MSc in Pharmaceutical Sciences at Utrecht University. During this MSc, she did an internship on the effect of raw and heated milk on the response of mast cells at the pharmacology department of Utrecht University. Her second internship was performed at the Karolinska Institute in Stockholm and focused on the organization of DNA in the cell nucleus. After completing her MSc studies, she started with a PhD in the Food Quality and Design group at Wageningen University & Research. Her PhD project focused on the effect of processing on the digestion and immunoreactivity of proteins in infant nutrition, and the results of her research are presented in this thesis.

List of publications

This thesis

Miltenburg, J., Bastiaan-Net, S., Hoppenbrouwers, T., Wichers, H., & Hettinga, K. (2024). Gastric clot formation and digestion of milk proteins in static in vitro infant gastric digestion models representing different ages. *Food Chemistry*, 432, 137209.

Others

Mayar, M., **Miltenburg, J. L.**, Hettinga, K., Smeets, P. A., van Duynhoven, J. P., & Terenzi, C. (2022). Non-invasive monitoring of in vitro gastric milk protein digestion kinetics by 1H NMR magnetization transfer. *Food Chemistry*, 383, 132545.

Abbring, S., Blokhuis, B. R., **Miltenburg, J. L.**, Olmedo, K. G. R., Garssen, J., Redegeld, F. A., & van Esch, B. C. (2020). Direct Inhibition of the Allergic Effector Response by Raw Cow's Milk—An Extensive In Vitro Assessment. *Cells*, 9(5), 1258.

Overview of completed training activities

Discipline-specific activities

Dairy protein biochemistry, VLAG & Aarhus University, 2019
International Milk Genomics Conference, IMGC, 2020
INFOGEST webinars, INFOGEST, 2020-2021
International Milk Genomics Conference, IMGC, 2021
Virtual International Conference on Food Digestion, INFOGEST, 2021
Maxquant Summer School on computational mass spectrometry-based proteomics, Max-Planck Institute of Biochemistry, 2021
Reactions and kinetics in food sciences, VLAG, 2021
Advanced food analysis, VLAG, 2022
International Conference on Food Digestion, Teagasc, 2022
Workshop Ghent University & those vegan cowboys, Ghent University, 2022
Advanced proteomics, VLAG, 2023
International Milk Genomics Conference, IMGC, 2023
NIZO Dairy Conference, NIZO, 2023

General courses

PhD week, VLAG, 2019
Introduction to R, VLAG, 2020
The essentials of scientific writing and presenting, WGS, 2020
Research data management, WGS, 2020
Applied statistics, VLAG, 2020
Supervising thesis students, Education Support Centre, 2021
Scientific writing, WGS, 2021

Assisting in teaching and supervision activities

Dairy science and technology, 2020-2022
Thesis supervision MSc students, 2021

Other activities

Preparation of research proposal, FQD, 2019
Group meetings, FQD, 2019-2023
Reviewing scientific article, FQD, 2021
PhD study trip, FQD, 2022

Colophon

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