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Profiling of peptides from wet- and dry-heated milk proteins after *in vitro* infant digestion and intestinal transport, and their effects on mucus production and innate immunity

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Milk proteins undergo both wet and dry heating steps during infant formula production, which can affect their gastro-intestinal digestion, subsequent peptide formation, intestinal transport, and immunoreactivity. Additionally, some milk peptides were previously shown to influence intestinal mucus production. However, the direct link between dry and wet heating of milk proteins, their digestion, and subsequent effects on mucus production, intestinal transport, and immunoreactivity remains unclear. To investigate this, milk proteins remained unheated, or were either wet or dry heated, followed by digestion using an *in vitro* infant digestion model. Peptides were identified with LC-MS/MS, and assessed for effects on intestinal mucus production with HT29-MTX-E12 cells, intestinal epithelial transport with Caco-2 monocultures and Caco-2/HT29-MTX-E12 cocultures, and immunoreactivity by predicting epitopes and by stimulating primary immature dendritic cells (iDCs) with transported peptides. Results showed that both wet and dry heating affected the HLA-II epitope survival during intestinal digestion, whereas linear IgE epitope survival was unaffected. The digestion-derived peptides did not alter mucus production. Wet heating reduced peptide transport in both cell models, whereas dry heating reduced peptide transport in the monoculture but did not affect peptide transport in the coculture. Moreover, dry heating increased transport of HLA-II epitopes from β-casein and transport of IgE epitopes in the coculture. Transported milk peptides did not affect cytokine production by iDCs. Together, this shows that wet and dry heating affect milk protein digestion, survival of immunoreactive peptides, and their intestinal transport. Further research is needed to clarify their effects on the immunoreactivity of intestinal and transported milk peptides.

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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.[1] In contrast, spray drying, which can be mimicked on lab scale by dry heating, leads to glycation.[1] 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.[2] Protein denaturation generally leads to higher hydrolysis of milk proteins and shorter peptides as a result of more easily accessible cleavage sites after unfolding, although a few studies have reported lower protein hydrolysis after denaturation.[1] 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.[3] This study showed that glycated milk peptides were transported across a Caco-2 monolayer to a greater extent 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

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heating.[4] 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.[5] Intestinal mucus provides an important defensive barrier and consists of water, lipids, salts, and proteins, of which mucin glycoproteins are the most abundant ones. Mucins can be either secretory or transmembrane glycoproteins. MUC 5AC is the main secreted mucin in the stomach, and MUC 2 is the main secreted mucin in the small intestine. From the transmembrane glycoproteins, MUC 1 is the most expressed, but MUC 3, MUC 4, MUC 13, and MUC 17 are also highly expressed in the small intestine.[6] The secreted mucins are mainly responsible for the gel-like structure of mucus and are usually negatively charged because of their carboxyl and sulfate groups.[7] 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.[8] 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.[9–11] 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.[12–14] 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.[15] 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.[15] For example, the formation of advanced glycation end products (AGEs) has been shown to influence the capacity of milk proteins to bind to IgE[16–18] and to several receptors on antigen-presenting cells.[12,13,19] In addition, digestion can change the allergenicity of milk proteins, although the effect seems to differ depending on the milk protein ingredient used and the heating conditions. The increased susceptibility of denatured β-Lg to enzymatic digestion was shown to result in reduced allergenicity by disrupting B-cell epitopes.[20] In contrast, digestion of glycated β-Lg led to a higher survival of structures that can bind receptors on antigen presenting cells.[13] 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.[3] 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,[21] 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 peptides transported across the intestinal barrier. Wet and dry heating were performed under conditions that induce either denaturation or glycation, respectively, which allows for distinction between the effects of these two protein modifications.

2. Materials and methods

2.1 Materials

Dulbecco's Modified Eagle's medium with 4.5 g L^{-1} 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, and IL-1β from Merck Millipore. 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 from the VECTASTAIN® Elite® ABC-HRP Kit (PK-6100) were obtained from Vector Laboratories, and sulfuric acid was obtained from Brunschwig. Porcine pepsin (P6887), porcine pancreatin (P7545), porcine bile extract (B8631), Dulbecco's Phosphate buffered saline (DPBS) with and without $CaCl_2^+$/$MgCl^{2+}$, prostaglandin E2 (PGE2), mucin from porcine stomach (M1778), bovine serum albumin (BSA; A3059), o-phenylenediamine dihydrochloride (P8787), Alcian Blue 8GX (A5268), PAS staining kit (1.1646.0001) and all other chemicals were obtained from Sigma Aldrich.

2.2 Infant formula model system

An infant formula (IF) model system was made from raw bovine milk as described previously.[2] In brief, fresh raw bovine milk was skimmed, and a whey protein concentrate (WPC) was created by acidifying the skim milk to pH 4.6, followed by centrifugation and removing the casein pellet. The resulting WPC was demineralized by dialysis. Thereafter, it was mixed with skim milk and lactose to adjust the casein : whey ratio 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. To enable distinguishing between the effect

of protein denaturation and glycation, wet heating was performed at conditions which result in denaturation but not glycation, and dry heating was performed at conditions which result in glycation but no denaturation. Dry heating was conducted in a desiccator at 60 °C for 72 h (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 per 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). Approximately 80 ml of each IF model system sample (UH, DH-72 and WH-40) was created at a protein concentration of 12 mg ml^{-1}. 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.[2] In brief, protein denaturation was assessed by the difference in band intensity of β-Lg on non-reducing SDS-PAGE between unheated and wet heated samples. Lysine and furosine contents were measured using LC-MS/MS, and these values were used to calculate the lysine blockage of the samples. These results showed that UH, WH-40 and DH-72 contained 1.34 ± 0.28%, 1.52 ± 0.32% and 68.3 ± 3.1% lysine blockage, respectively, and WH-40 contained 60% whey protein denaturation.[2]

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.[21] The main differences between this infant model and the INFOGEST adult model[22] are the higher gastric pH, shorter gastric phase and lower activities of digestive enzymes to resemble infant digestion conditions. Before the digestions were performed, enzyme activities were measured following the INFOGEST protocol.[22] Briefly, the IF model system at a concentration of 12 mg protein per 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^{-1} 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^{-1} 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 (filtration of digests to ensure compatibility with cell culture models; Kondrashina *et al.*, 2023[23]) 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.

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% CO2 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 5×10^5 cells per ml (500 µl; 2.5×10^5 cells per well) and were cultured for 7 days at 37 °C, 5% CO2 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. Three days post-confluency, which was 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 4 h with 500 µl of 1 : 1 filtered 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 4 h. After the 0.5–4 h exposure of the cells to the digests, the medium was harvested and split into two parts. One part was centrifuged at 2000*g* for 5 min for the LDH assay, and one part was centrifuged at 13 500*g* 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. In addition to the HT29-MTX-E12 cells stimulated by the filtered intestinal digestion samples as described above, a few wells of unstimulated HT29-MTX-E12 cells were harvested with 600 µl of TRIzol for RNA isolation, to be used in a pilot experiment to assess the general mucin gene expression profile of HT29-MTX-E12 cells.

2.5 LDH cytotoxicity assay

The LDH assay was performed by use of the 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 2000*g* 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, cells exposed to a medium control (non-stimulated cells), and cells exposed to a positive control (2% Triton X-100) as described in section 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. To calculate the cytotoxicity, the average absorbance value of the blank was first subtracted from all absorbance values, followed by applying the following formula: Cytotoxicity = (absorbance cells exposed to intestinal digest − absorbance cells exposed to medium control)/(absorbance cells exposed to positive control − absorbance cells exposed to medium control) × 100%.

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

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.[10] In brief, centrifuged samples (as described in section 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 per 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 1 h. Thereafter, the wells were washed four times, 100 µl biotinylated wheat germ agglutinin (5 µg ml^{-1}) in PBS-T with 2% BSA was added, and the plate was incubated at 37 °C for 1 h. The plate was washed four times with PBS-T. Then, 100 µl avidin-peroxidase conjugate in PBS (100 µl kit component A was added to 5 ml PBS, followed by adding 100 µl kit component B) was added, and the plate was incubated again at 37 °C for 1 h. The plate was washed four times with PBS-T, and 100 µl 0.4 mg ml^{-1} *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.

2.7 RNA isolation and mucin gene expression

The HT29-MTX-E12 cells in TRIzol were thawed, and total RNA of the cells was isolated by chloroform extraction (40 µl on 0.2 ml TRIzol sample). The upper aqueous phase was transferred to a new tube and mixed with 1.5× volume 70% ethanol. Total RNA was further purified, including on-column DNase I treatment using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. The quantity and purity of the RNA were checked with the NanoDrop, and the quality and integrity 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 A_{260}/A_{280} ratio of 2.0–2.1 and an A_{260}/A_{230} ratio of >1.5 as measured with the Nano-drop and those that showed two clear ribosomal RNA bands (18S and 28S rRNA) 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 total 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). 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 diluted cDNA, and the efficiencies ranged from 82 to 101%. First, the general mucin gene expression profile of unstimulated HT29-MTX-E12 cells was assessed (that were harvested separately from the main experiment) using forward and reverse primers for MUC1, MUC2, MUC3A and B, MUC4, MUC5A, MUC6, MUC12, MUC13, MUC15 and MUC17, as well as the two reference genes Cyclophilin A (hCyclo) and GAPDH.

Based on the Cq results from this pilot experiment, MUC5A, MUC13, MUC17 were selected for the main experiment. Primers for the reference genes SF3A1, RPS18, HPRT1, hCyclo and GAPDH were also included for normalization purposes. Primer details are provided in Table S1. Data were analyzed by use of qBASE software.[24] 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 (ΔCT method), relative mucin gene expression levels (ΔΔCT method) 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 4 h of exposure to the filtered intestinal digests). For time point 4 h, also a normalization towards medium exposed cells was performed per passage number of HT29-MTX-E12 cells.

2.8 Caco-2 and Caco-2/HT29-MTX cocultures on inserts

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% CO2 and 90% humidity. Medium was refreshed once 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 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 $2.25{\cdot}10^5$ cells per ml in Caco-2 culture medium. 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 and subsequently used for peptides transport studies.

For mucus staining, Caco-2 monocultures and Caco-2/HT29-MTX-E12 cocultures (90 : 10) were seeded into 24-well translucent 0.4 µm Transwell™ inserts (Greiner Bio-one) at a

concentration of 2.25×10^5 cells per ml in Caco-2 culture medium. Both apical (150 µl) and basolateral medium (700 µl) were changed three times a week till monolayers reached 21 days of development.

2.9 Transport of peptides, TEER, and cytotoxicity in Caco-2 and Caco-2/HT29-MTX-E12 cell monolayers

Transwell™ inserts seeded for peptide transport were first quality tested. At 21 days, transepithelial electrical resistance (TEER) was measured, and only wells with a TEER value >400 Ω cm^2 were used. After apical and basolateral medium was removed, 1500 µl medium without phenol red nor FBS with 1% pen/strep was added to the basolateral side and 500 µl 1 : 1 diluted intestinal digest (filtered, as described in section 2.3) in medium without phenol red nor FBS with 1% pen/strep was added to the apical side. This was performed in triplicate, each time using a different passage of cells. TEER values were measured again at 0, 1, 3, and 6 h after the addition of the samples. After 6 h, 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 6 h were included as medium control, and cells that were exposed to DMEM for 6 h 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. First, absorbance values were obtained from the apical and basolateral sides of each sample with the LDH assay as described in section 2.5. Then, the overall absorbance value of each sample was calculated by using the following formula: Total absorbance sample = (absorbance apical side × volume apical side) + (absorbance basolateral side × volume basolateral side).

Finally, cytotoxicity was calculated as described in section 2.5.

2.10 Peptidomics

Peptides were measured in the filtered intestinal digests and in the basolateral media after 6 h 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,[2] starting with 75 µl sample. The sample preparation of the basolateral media was performed with eight times as much volume (600 µl) of both the sample and trichloroacetic acid (TCA) to compensate for the dilution during the intestinal transport experiment compared to the intestinal digestion samples. TCA precipitation was performed as a standard step to eliminate large intact proteins, if present, from the analysis. 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.[3] Data were analyzed with MaxQuant version 2.2.0.0 with the Andromeda search engine.[25] The digestion model was set to unspecific, and the same bovine milk database was used as described previously[2] 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.[26] 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. In case a peptide was not identified in one of the three replicates, its value was considered absent, and the average peptide intensity was calculated using the peptide intensities of the other two replicates. A peptide alignment tool[27] 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[28] were considered bioactive peptides. Prediction of HLA-II binding epitopes and linear IgE epitopes was performed as described previously by Zenker *et al.*[3] In brief, the IEDB MHC-II Binding Predictions tool was used to predict HLA-II binding epitopes (https://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. The prediction tool only accepts peptides ≥11 AAs, so peptides meeting this length requirement were included in the analysis. 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 comparison with literature in which known linear IgE binding epitopes were reviewed.[29] 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; Fig. 2B, 3B, 7, 8B, 9B and Fig. S1, S2, S10, S11), and excluding their signal peptides for the identified bioactive peptides and the predicted HLA-II and linear IgE epitopes (Tables S2–S4 and S6–S8).

2.11 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).

2.12 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.[30] 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^{-1} IL-4 and 50 ng ml^{-1} GM-CSF (200 µl per well; 2.5 × 10^5 cells per ml). Cells were incubated at 37 °C with 5% CO_2 and humidified atmosphere (90%). After 3 days, half of the medium was refreshed by new IMDM with 10% FBS, 1% pen/strep, 60 ng ml^{-1} IL-4 (final concentration: 30 ng ml^{-1}) and 100 ng ml^{-1} GM-CSF (final concentration: 50 ng ml^{-1}), 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 6 h of intestinal transport. In addition, iDCs were stimulated with fresh medium (IMDM with 10% FBS, 1% pen/strep, 30 ng ml^{-1} IL-4, 50 ng ml^{-1} GM-CSF) as non-treated (NT) control, with fresh medium plus 100 ng ml^{-1} lipopolysaccharide (LPS) as dendritic cell type 1 (DC1) control, or with fresh medium plus 50 ng ml^{-1} TNF-α, 25 ng ml^{-1} IL-1β, 10 ng ml^{-1} IL-6 and 1 µg ml^{-1} 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 LPS content of the intestinal transport samples (basolateral samples) was measured as described previously with a Pyros Kinetix Flex tube reader.[31] The LPS content of the samples (<3 pg ml^{-1}) was considered too low to affect the iDC response.[32] Media of the iDCs 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).

2.13 Statistical analysis

Statistical analysis of the data was performed by use of GraphPad prism v8.0.2 (GraphPad Software, San Diego, California USA). One-way ANOVA and Tukey's multiple comparisons test were used for comparisons between heat treatments, and *t*-test were used for comparisons between cell models. Differences were considered significant if $p < 0.05$.

3. Results

3.1 Peptides after *in vitro* infant digestion

To investigate the impact of heating on milk protein digestion and subsequent intestinal transport, IF model system samples were heat treated, subjected to an infant *in vitro* digestion simulation and subsequently prepared for cell culture stimulations. Peptides in the filtered 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 different detected peptides (peptide count) was similar for both WH-40 and DH-72 compared to UH, and was higher for WH-40 compared to DH-72 (Fig. 1A). In all three digests, most peptides originated from α_{s1}-casein, β-casein and β-Lg. The intestinal digests also tended to differ with regard to summed peptide intensity (Fig. 1B). There was a trend towards lower peptide intensities in WH-40 (36%) compared to UH ($p = 0.051$). This lower 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 and phosphorylated peptides, whereas peptides in the DH-72 digest had relatively less unmodified peptides and relatively more lactosylation- and CML-modified peptides originating from glycation (Fig. 1C). Most identified peptides in all digests had a length between 6 and 13 amino acids, but the DH-72 digest contained a lower relative intensity of short peptides (6–9 AA) and a higher relative intensity of longer peptides (14–17 AA) compared to UH and WH-40 digests (Fig. 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 (Fig. S1), and per modification (Fig. 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 modifications over the proteins differed between the heat treatments, the distribution of peptides over the proteins was largely the same for all intestinal digests.

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 identified by matching with a database and by matching with literature. Their intensity and peptide alignment are visualized in Fig. 2 and 3, whereas all peptide sequences and their modifications are shown in Tables S2 and S3. No significant differences were found between in intensity of peptides containing HLA-II epitopes (Fig. 2A) or IgE epitopes (Fig. 3A) in the UH, WH-40 and DH-72. 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. For example, WH-40 contained higher intensities of an HLA-II epitope located in the 103–119 AA region of α_{S1}-casein (Fig. 2B), which was detected in phosphorylated form (Table S2). 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 (Fig. 2B), which were mainly detected in glycated forms (Table S2). The distribution of peptides over the proteins was more similar between the heat treatments for the linear IgE epitopes (Fig. 3B). WH-40 contained a higher intensity of peptides containing an IgE epitope in the 109–120 AA region of α_{S1}-casein, whereas it con-

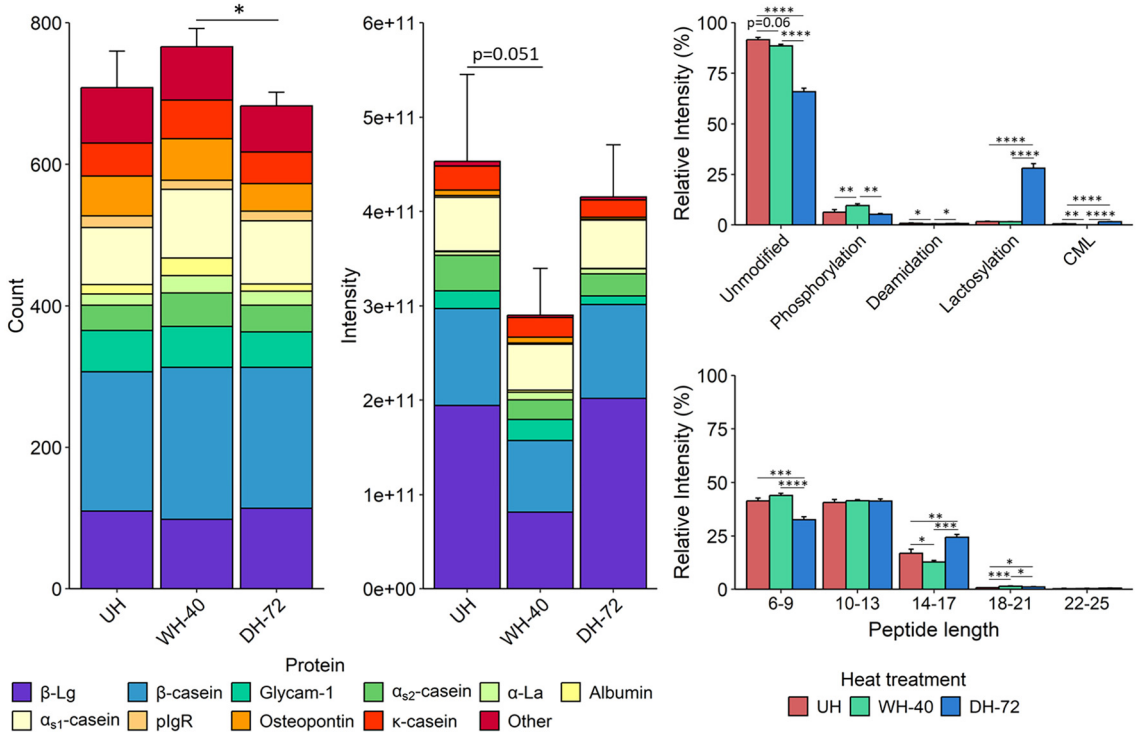

Fig. 1 Milk peptides detected with LC-MS/MS in the filtered intestinal digests (n = 3). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40) or dry heated for 72 h (DH-72) and all samples were digested by use of an *in vitro* infant digestion model. (a) Summed peptide count (b) Summed peptide intensity (c) Relative peptide intensity per modification (d) Relative peptide intensity per peptide length group. Error bars represent standard deviation. Statistical differences between samples are indicated with *, **, ***, **** for $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

tained 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 S4). 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,[33] and YPVEPF (neocasomorphin-6), which can increase MUC4 expression.[34]

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. Expression levels of 11 different mucin genes were tested within a pilot, which indicated that MUC2, MUC4, MUC6, MUC12 and MUC15 were lowly expressed. The three genes with the highest expression levels (*i.e.* lowest Cq values) were MUC5A, MUC13, and MUC17. These were selected for further analysis of the main experiment (Fig. S3). Cytotoxicity effects of exposures on the HT29-MTX-E12 cells remained below 4% during the 4 h incubation with the digests (Fig. S4). As the mucins were influenced on a genetic level by peptides formed through self-digestion of the digestive enzymes in the control digest, we decided to normalize the data to the control digest rather than to the medium control (Fig. S5A). The expression of MUC5A, MUC13 and MUC17 was not changed by the digests with milk peptides compared to the control digest (Fig. 4A–C). Over time, MUC5A, MUC13, and MUC17 expression appeared to vary after stimulation with the digestion samples, although no significant differences were observed (Fig. S5A). At the 4 h time point, we also normalized the data to the medium control as a reference (Fig. S5B). Also with this normalization, MUC 5A, MUC13 and MUC17 expression did not differ from each other, the control digest or the medium control. The mucus production increased during the 4 h of stimulation for all digests, including the control digest (Fig. 4D). Cells that were stimulated with the intestinal digests had a higher mucus production than the medium control, which was only measured at the 4 h time point. However, stimulating the cells with the digests containing

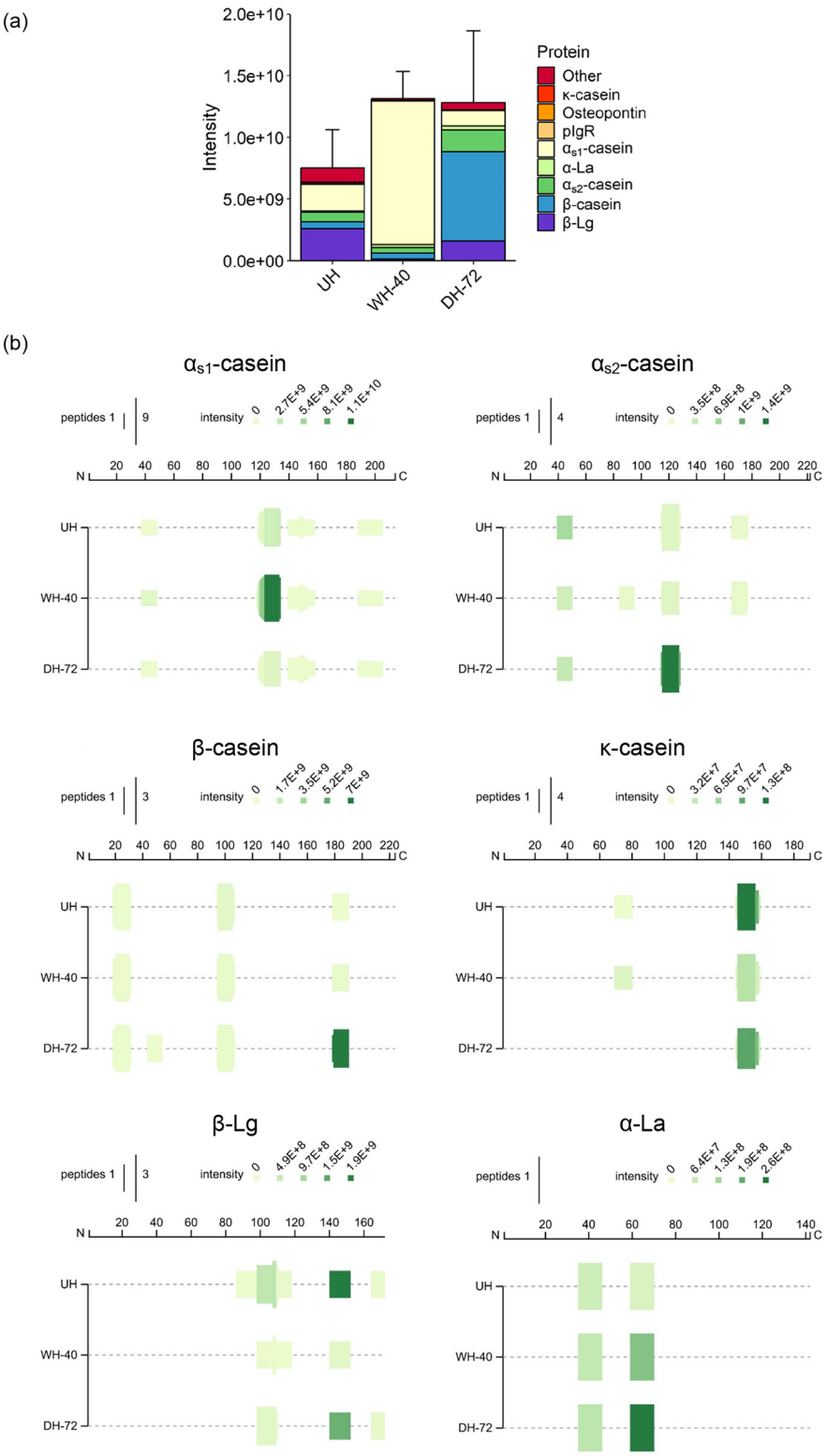

Fig. 2 HLA-II epitopes predicted in the filtered intestinal digests by use of the IEDB MHC Class II Binding Prediction tool (n = 3). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40) or dry heated for 72 h (DH-72) and all samples were digested by use of an *in vitro* infant digestion model. (a) Summed peptide intensity (b) Peptide alignment for 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, 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. Error bars represent standard deviation. No statistical differences were found between the samples in (a).

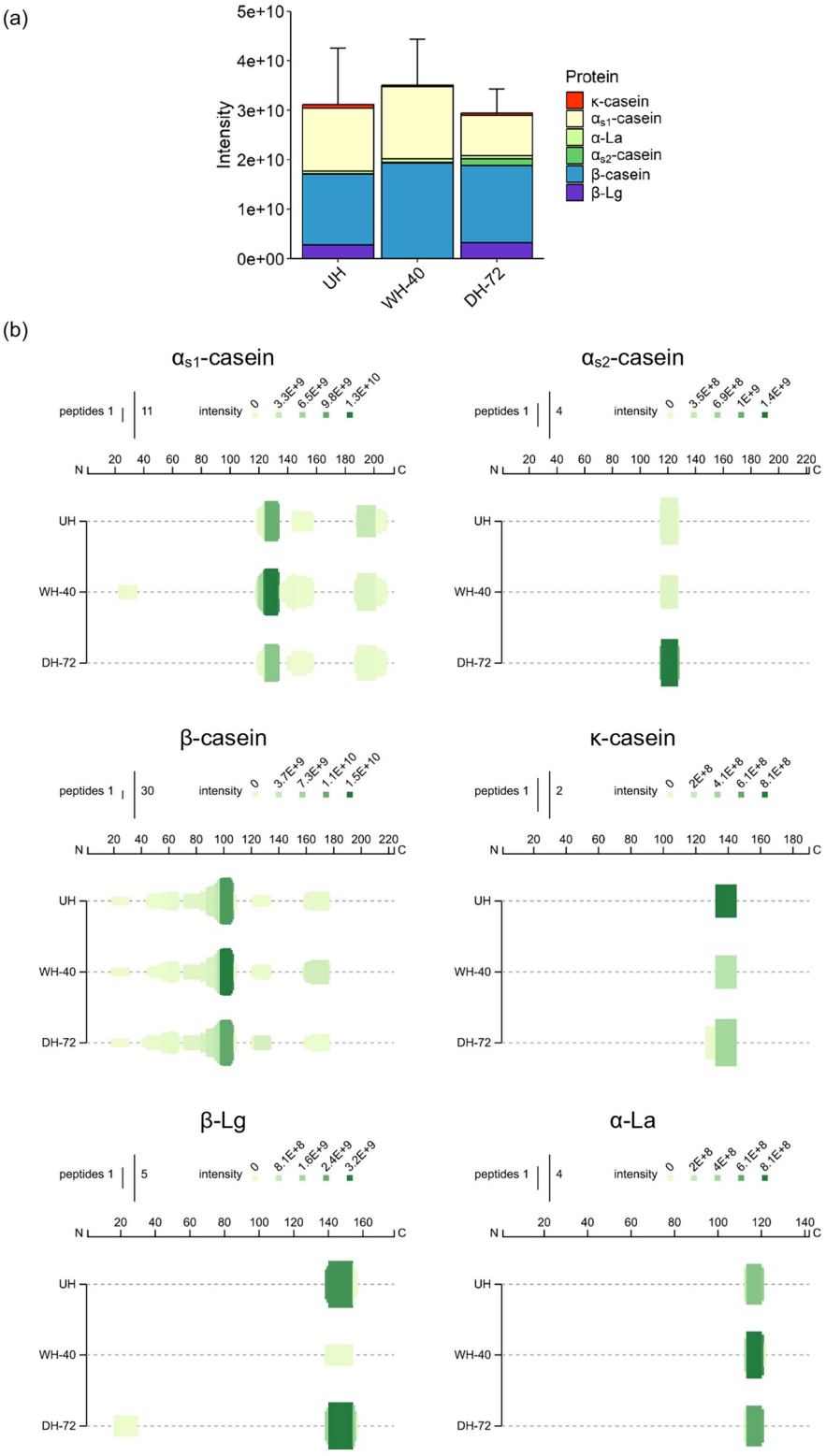

Fig. 3 Linear IgE epitopes predicted in the filtered intestinal digests (n = 3). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40) or dry heated for 72 h (DH-72) and all samples were digested by use of an *in vitro* infant digestion model. (a) Summed peptide intensity (b) Peptide alignment for 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, 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. Error bars represent standard deviation. No statistical differences were found between the samples in (a).

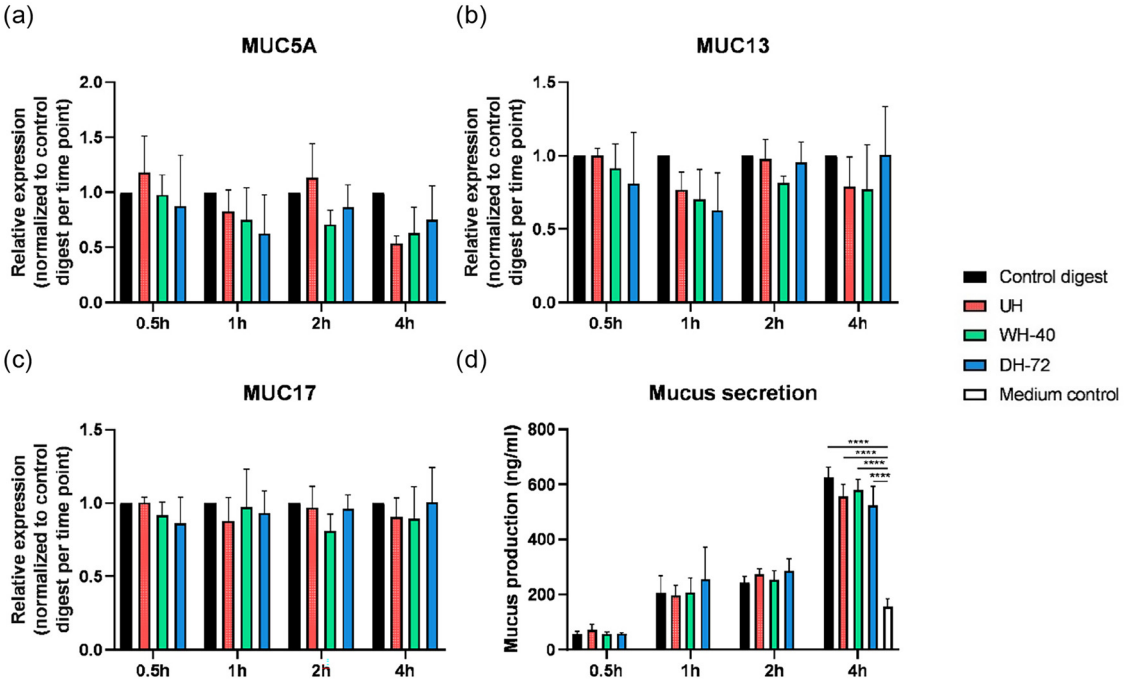

Fig. 4 Mucus secretion and mucin gene expression after application of the filtered intestinal digests to HT29-MTX-E12 cells for 0.5, 1, 2, or 4 h (n = 3). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40) or dry heated for 72 h (DH-72), and all samples were 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 exposed to Dulbecco's Modified Eagle's medium (DMEM) (a) MUC5A expression (b) MUC13 expression (c) MUC17 expression (d) Mucus secretion. MUC5A, MUC13 and MUC17 gene expressions of the digestion samples are presented relative to the expression of control digest at each corresponding time point. Statistical differences between samples at the same time point are indicated with *, **, ***, **** for $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively. Error bars represent standard deviation.

milk peptides did not result in a different mucus production compared to the control digest.

3.4 Peptides after intestinal transport

To measure the intestinal transport of milk peptides, 21 day differentiated Caco-2 monolayers were used, as well as a coculture of Caco-2 and HT29-MTX-E12 cells to analyze the influence of mucus on transport. A pilot experiment confirmed that HT29-MTX-E12 cells remained present and continued to produce mucus in coculture with Caco-2 cells, without being outcompeted after 21 days of culture (Fig. S6). Primarily neutral mucins were observed in the Caco-2 monoculture (magenta staining) with a few patches of cells producing neutral mucins (light blue staining), while both neutral and acidic mucins were observed in the Caco-2/HT29-MTX-E12 coculture (dark purple staining). Next, in a second pilot experiment, we observed that the unfiltered intestinal digests were toxic for differentiated Caco-2 cells as measured by TEER values (Fig. S7). Therefore, the digests were first filtered, and these filtered digests were used for peptide identification after intestinal digestion (sections 3.1–3.2) and for cell exposure experiments, to ensure comparability between peptides before and after intestinal transport and compatibility with the cell culture models. Intestinal digests before and after filtration were analyzed on protein composition and concentration (Fig. S8 and Table S5). The SDS-PAGE showed that digestive enzymes were removed after filtration, but also that a protein band around 14 kDa and a light smear just below that molecular weight were lost in the UH digest upon filtration. The recovery of milk proteins was 69%, indicating that a portion of the milk proteins were lost in this step. 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 6 h, 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 6 h of intestinal transport (Fig. S9). Moreover, TEER and cytotoxicity levels did not differ between the mono- and cocultures or between the different milk samples. A higher peptide count ($p = 0.002$ and $p < 0.0001$ for UH and DH-72, respectively) and a higher peptide intensity ($p = 0.04$ and $p = 0.008$ for UH and DH-72, respectively) were detected in the basolateral medium of the coculture compared to the monoculture for UH and DH-72 (Fig. 5). A relatively high intensity of transported peptides originated from β-Lg (Fig. 5B and F) compared to the peptide composition before intestinal transport for UH ($p < 0.0001$) and DH-72 ($p < 0.01$) (Fig. 1B). WH-40 contained a lower count and intensity of transported peptides compared to UH in the monoculture, and intensity in the coculture. However, the peptide transport for DH-72 differed compared

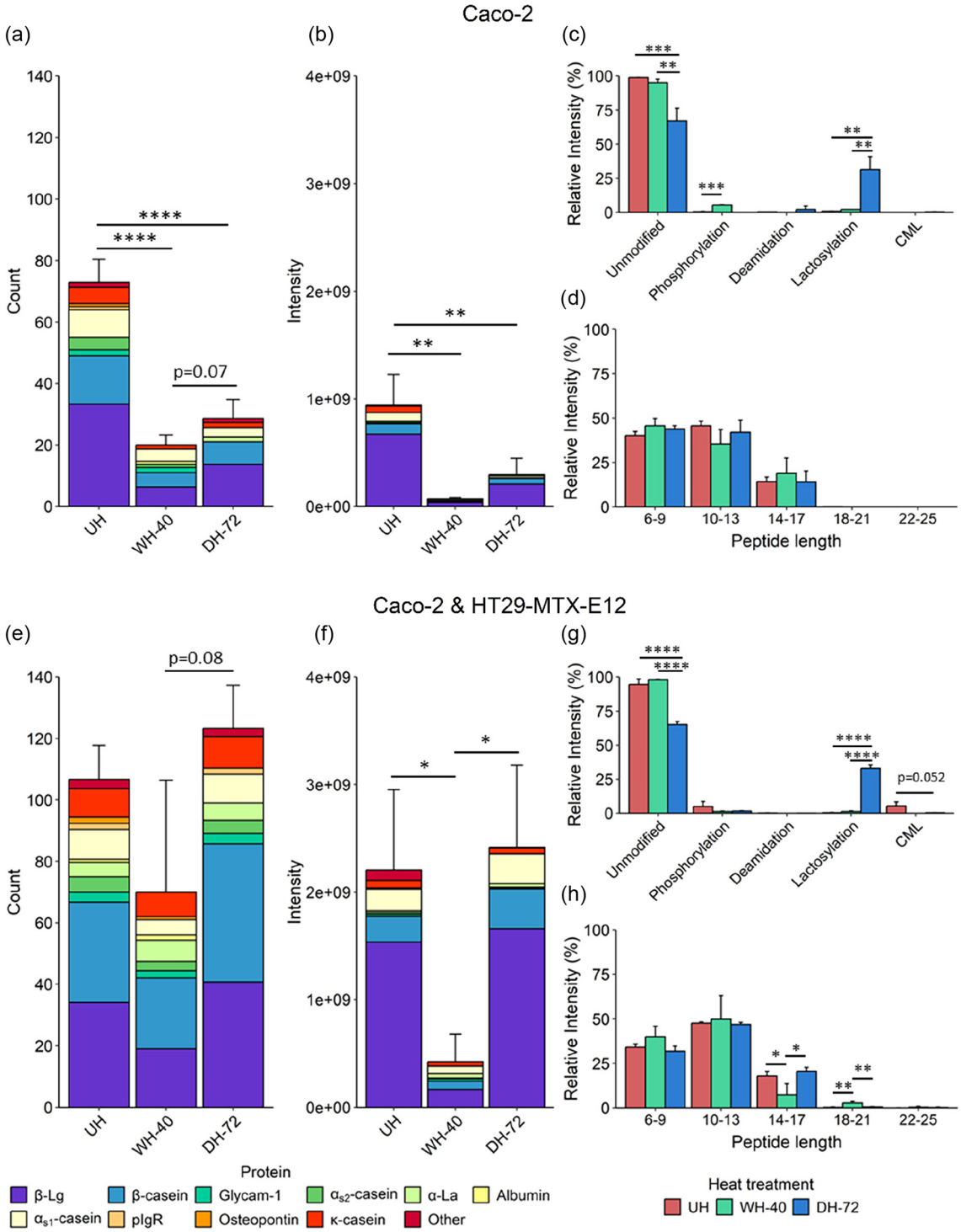

Fig. 5 Milk peptides detected with LC-MS/MS on the basolateral side after transport across a 21-days differentiated Caco-2 monolayer (a–d) or Caco-2/HT29-MTX-E12 (90/10) monolayer (e–h) (n = 3). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40) or dry heated for 72 h (DH-72) and all samples were digested by use of an *in vitro* infant digestion model and filtered before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6 h. (a and e) Summed peptide count. (b and f) Summed peptide intensity. (c and g) Relative peptide intensity per modification. (d and h) Relative peptide intensity per peptide length group. Error bars represent standard deviation. Statistical differences between samples are indicated with *, **, ***, **** for $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

to UH between the mono- and cocultures. DH-72 had a lower peptide count and intensity than UH in the monoculture but similar peptide count and intensity as UH in the coculture. The transported peptides consisted mainly of unmodified or lactosylated peptides, whereas transported peptides with other modifications were relatively low abundant in both cell models (Fig. 5C and G). Most transported peptides had a length between 6 and 13 AA, but also some longer peptides between 14 and 21 AA were transported (Fig. 5D and H). In the coculture, a lower relative intensity of shorter peptides between 6 and 9 AA for UH (p = 0.02) and DH-72 (p = 0.003) 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 (Fig. 6). 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 higher extent for DH-72 and to a lower 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 (Fig. 7). 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 modifications (Fig. S10). 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 modifications since one of these regions (164–175 AA in β-casein) mainly consisted of peptides with lactosylation or CML-modified AA, whereas the other regions consisted mainly of unmodified peptides (Fig. S11).

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

While DH-72 did not have a significantly higher total intensity of peptides with HLA-II epitopes than UH and WH-40 on the basolateral side of both the mono- and cocultures (Fig. 8A), it did have a higher intensity of the peptide SLSQSKVLPVPQ. This peptide originates from β-casein and is positioned at 164–175 AA (Fig. 8B), and was only detected on the basolateral side in either lactosylated or CML-modified form (Table S6). Whereas WH-40 seemed to contain the lowest intensity of peptides with HLA-II epitopes in both the mono- and the cocultures, no significant differences were found. 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 (Fig. 9A). In the coculture, DH-72 contained a higher intensity of transported peptides with potential linear IgE epitopes compared to WH-40 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 region in α_{S1}-casein and the 83–92 region in β-casein (Fig. 9B), which were either transported in unmodified or phosphorylated form (Table S7). Moreover, several bioactive peptides with different properties were detected in the basolateral media (Table S8). 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.

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 (Fig. 10). No large differences were observed in IL-18 and IL-8 levels between the medium control, control digest, and the digests with milk peptides. 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 (Fig. S12). The production of these cytokines was very low (<1 pg ml^{-1}) and did not significantly differ between the samples. In addition, the production of IL-10, IL-1β, IFN-α2, MCP-1, IL-12p70, IL-17A, IL-23, and IL-33 in the basolateral compartment was measured, but the concentrations of these cytokines were below the detection limit for the majority of the samples (data not shown).

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 (Fig. 11). The non-treated iDCs, medium controls and digestion samples did not reach the elevated levels of TNF-α, IL-6 and IL-10 production as observed in the DC1 and DC2 controls. Moreover, stimulating the iDCs with basolateral medium from the control digest or digests with milk peptides did not lead to significant changes in TNF-α, IL-6 or IL-10 compared to the non-treated cells. In addition, the production of IL-12p70, IL-1β, arginase, IL-12p40, IL-23, IFN-γ, IP-10, IL-1RA, and TARC was measured (Fig. S13 and S14). No differences were found for these additional cytokines between NT, medium control, control digest and the digests with milk peptides.

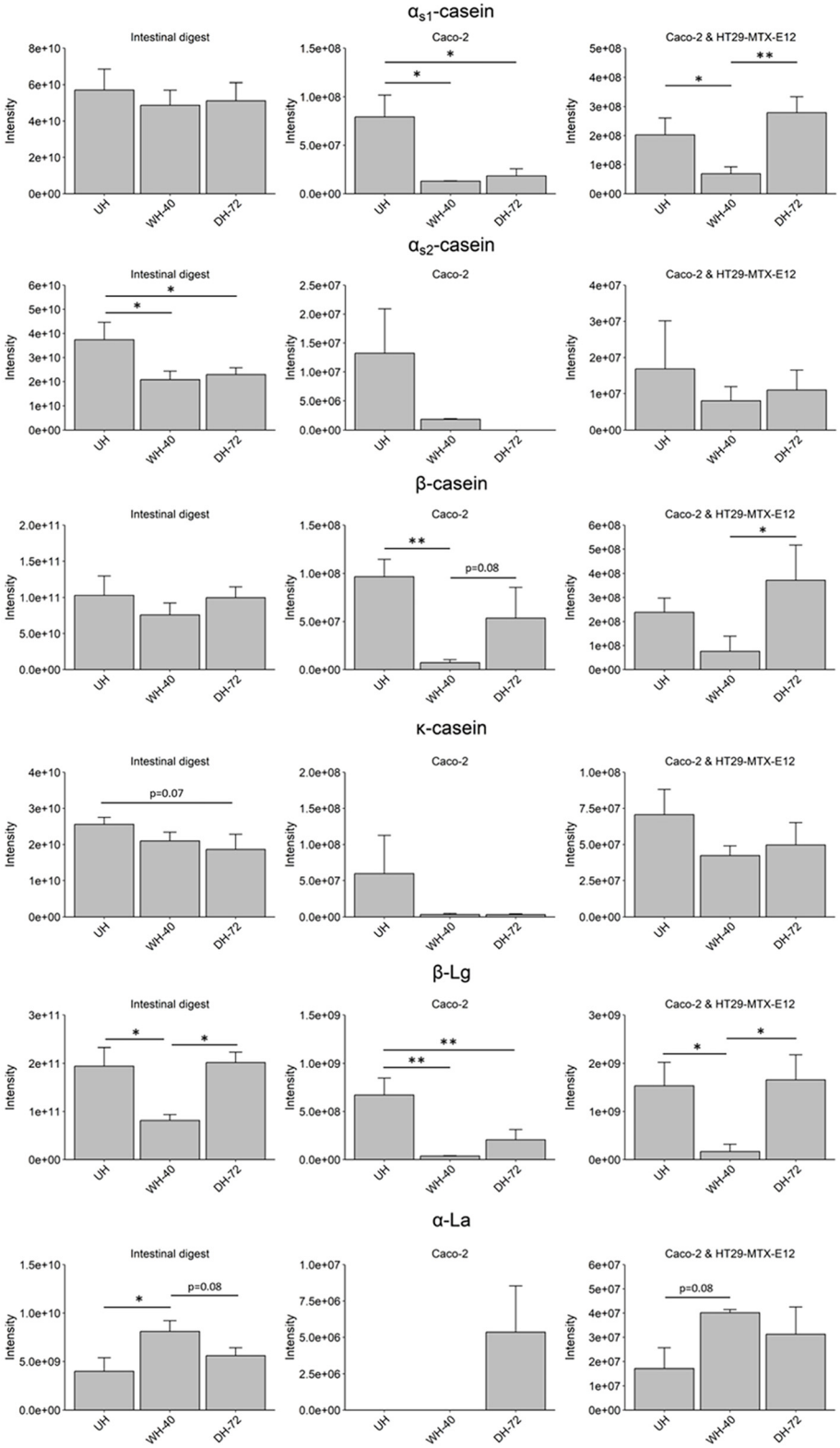

Fig. 6 Summed intensity of milk peptides originating from α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) detected with LC-MS/MS in the intestinal digests and on the basolateral side after transport across a 21 days differentiated Caco-2 monolayer or Caco-2/HT29-MTX-E12 (90/10) monolayer (n = 3). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40) or dry heated for 72 h (DH-72) and all samples were digested by use of an *in vitro* infant digestion model and filtered before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6 h. Error bars represent standard deviation.

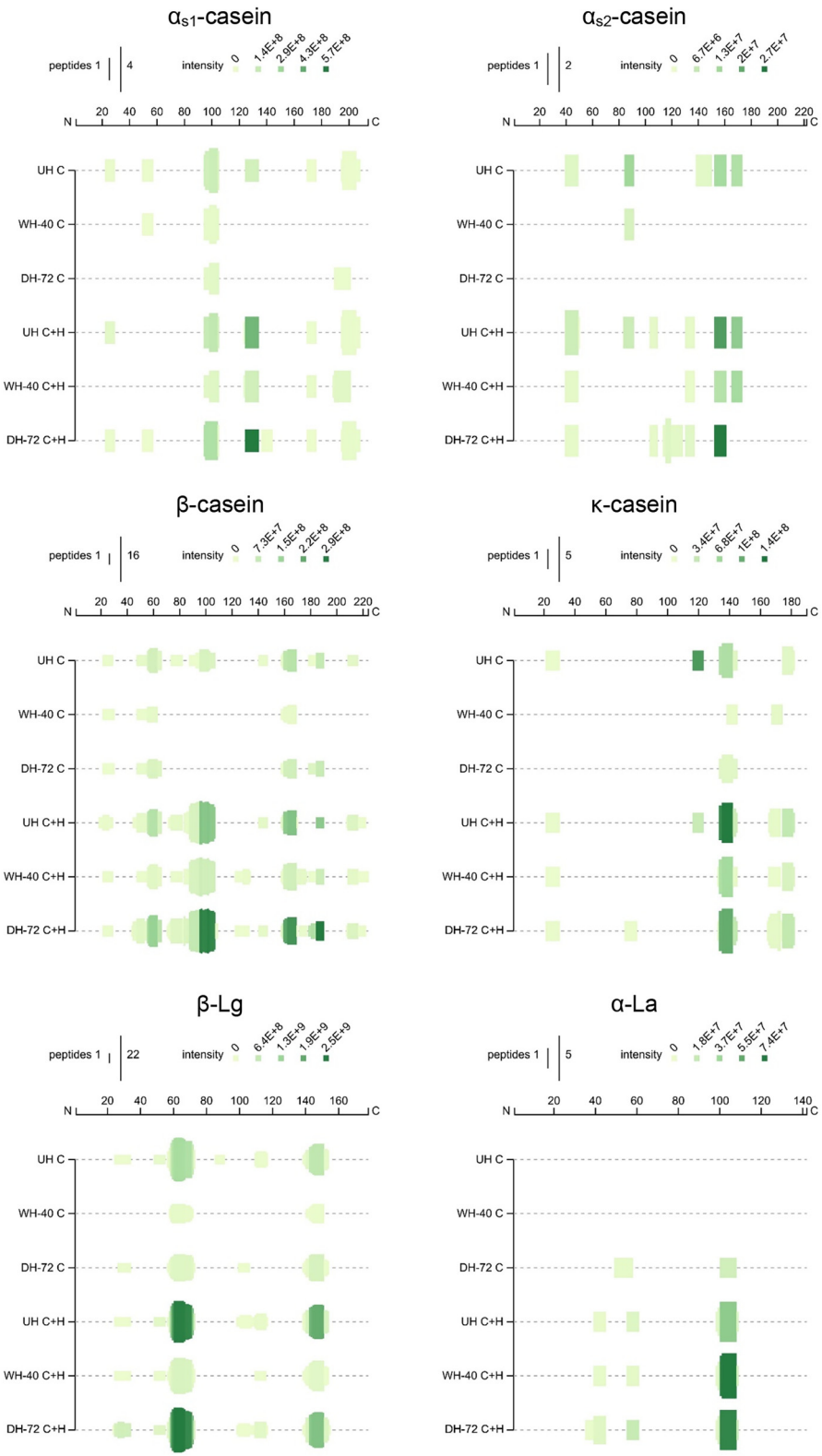

Fig. 7 Peptide alignment for α_{S1}-casein, α_{S2}-casein, β-casein, κ-casein, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) detected with LC-MS/MS on the basolateral side after transport across a 21-days differentiated Caco-2 monolayer (C) or Caco-2/HT29-MTX-E12 (90/10) monolayer (C + H) (n = 3). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40) or dry heated for 72 h (DH-72) and all samples were digested by use of an *in vitro* infant digestion model and filtered before the intestinal digests (1 : 1 in DMEM) were applied to the Caco-2 and HT29-MTX-E12 cells for 6 h. 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.

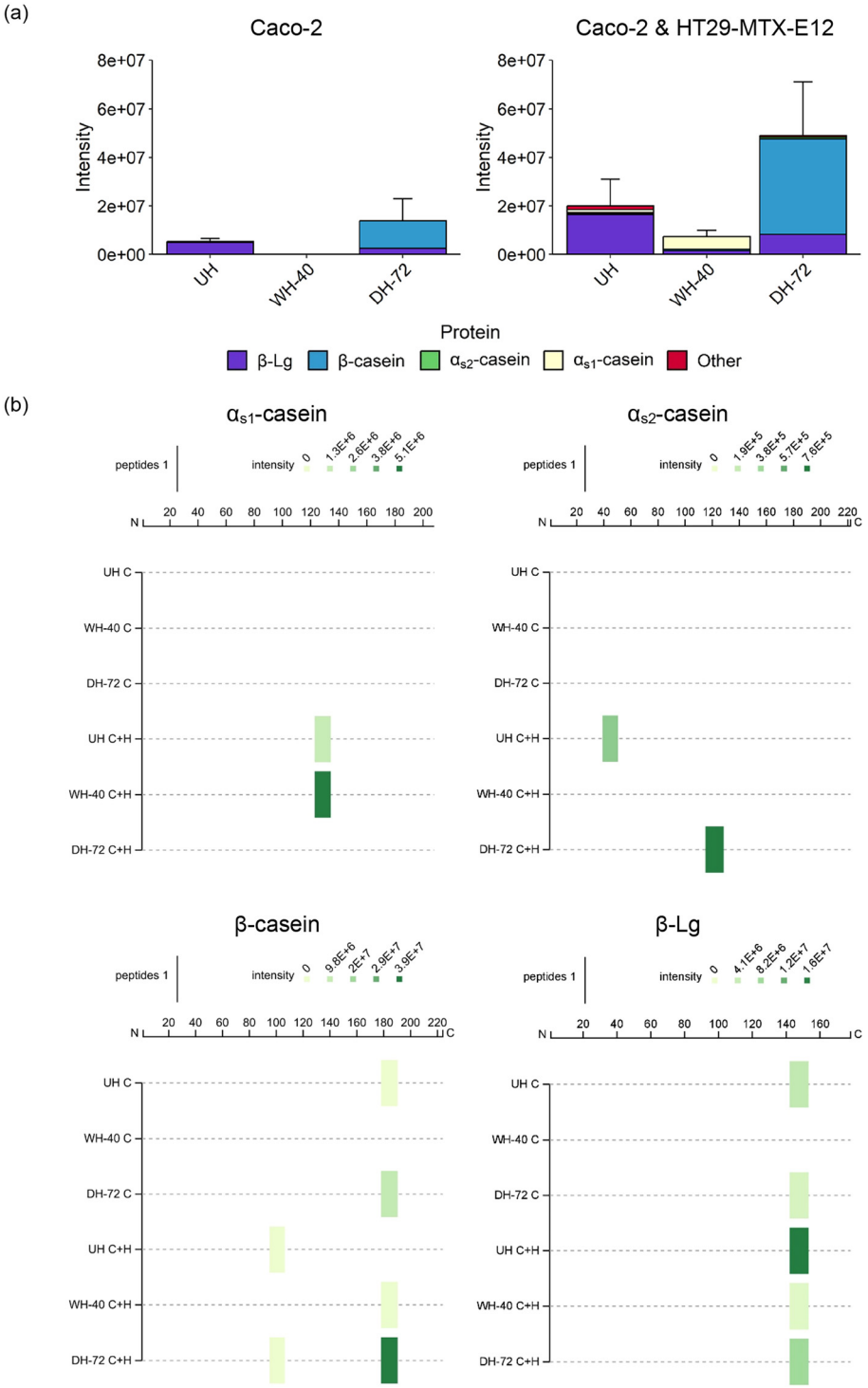

Fig. 8 HLA-II epitopes identified by the use of the IEDB MHC Class II Binding Prediction tool on the basolateral side after transport across a 21-days differentiated Caco-2 monolayer (C) or Caco-2/HT29-MTX-E12 (90/10) monolayer (C + H) (n = 3). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40) or dry heated for 72 h (DH-72) and all samples were digested by use of an *in vitro* infant digestion model and filtered before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6 h. (a) Summed peptide intensity. (b) Peptide alignment for α_{S1}-casein, α_{S2}-casein, β-casein, β-lactoglobulin (β-Lg). 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, and 16 amino acids for α_{S1}-casein, α_{S2}-casein, β-casein, and β-Lg, respectively. Error bars represent standard deviation. No statistical differences were found between the samples in (a).

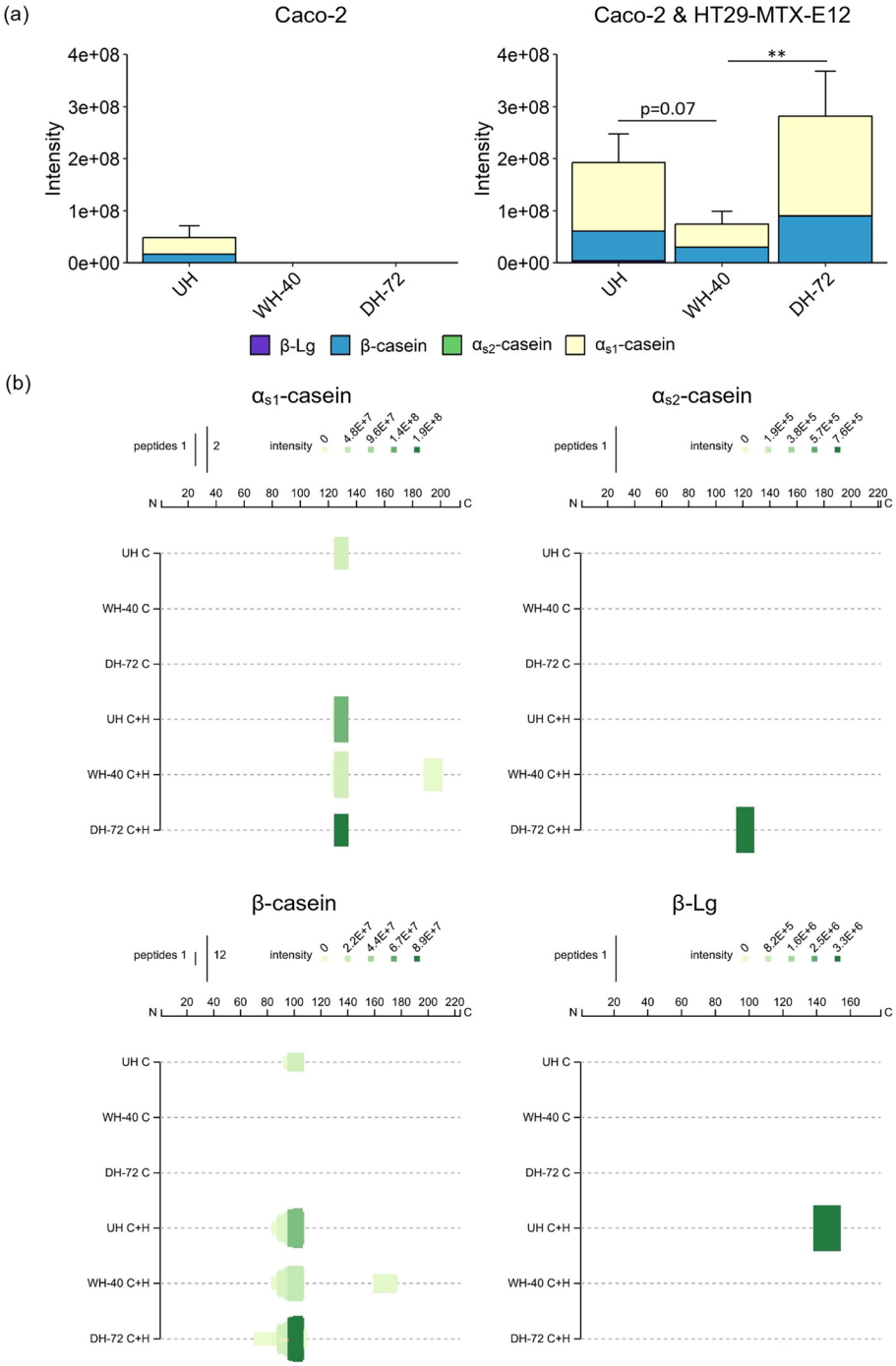

Fig. 9 Linear IgE epitopes identified on the basolateral side after transport across a 21-days differentiated Caco-2 monolayer (C) or Caco-2/HT29-MTX-E12 (90/10) monolayer (C + H). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40) or dry heated for 72 h (DH-72) and all samples were digested by use of an *in vitro* infant digestion model and filtered before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6 h (n = 3). (a) Summed peptide intensity. (b) Peptide alignment for α_{S1}-casein, α_{S2}-casein, β-casein, β-lactoglobulin (β-Lg). 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, and 16 amino acids for α_{S1}-casein, α_{S2}-casein, β-casein, and β-Lg, respectively. Error bars represent standard deviation. Statistical differences between samples are indicated with *, **, ***, **** for $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

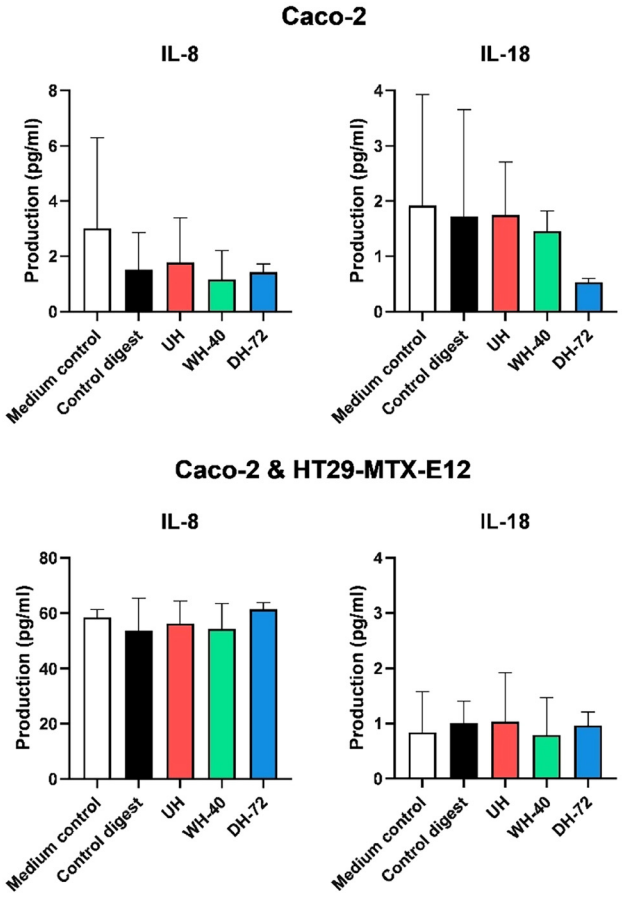

Fig. 10 IL-8 and IL-18 concentrations measured with a LEGENDplex assay on the basolateral side of a 21-days differentiated Caco-2 monolayer or a Caco-2/HT29-MTX-E12 (90/10) monolayer after stimulation with intestinal digests for 6 h (n = 3). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40) or dry heated for 72 h (DH-72) and all samples were digested by use of an *in vitro* infant digestion model and filtered before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6 h. 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 exposed to DMEM. The detection limits of IL-8 and IL-18 measured with the LEGENDplex assay were 1.14 and 0.95 pg ml^{-1}, respectively. Cytokine concentrations were measured in triplicate, and the average of the three replicates is shown. If one of the replicates was below the detection limit, a value of zero was assigned to that replicate, and the mean was calculated accordingly. Error bars represent standard deviation. No statistical differences were found between medium control, control digest, and milk digest samples.

4. Discussion

During the production of infant formula, milk proteins are heated at both wet and dry conditions, resulting in a combination of protein denaturation, aggregation, and glycation. To enable distinction between the effect of protein denaturation and glycation, we chose wet heating conditions that resulted in denaturation but no glycation, and dry heating conditions that resulted in glycation but no denaturation. The protein denaturation and glycation levels of the WH-40 and DH-72 samples were in line with the levels found in commercial infant formulas, although they were at the higher end of the range.[2] Protein aggregation may have differed from that in commercial infant formulas under the same heating conditions due to differences in mineral concentration and composition.[35] This study aimed to investigate the effect of wet and dry heating of milk proteins on (1) the composition and immunoreactivity of peptides released after *in vitro* infant digestion, (2) intestinal mucus production, and (3) the composition and immunoreactivity of peptides transported across the intestinal barrier.

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 first filtered with a 10 kDa filter and then identified with LC-MS/MS. Filtration resulted into loss of digestive enzymes, which was desired, but also into loss of larger peptides that were still present in the intestinal digests (Fig. S8). The recovery of milk proteins was 69%, indicating that part of the milk proteins were lost during filtration. Although losing milk proteins at this step was undesirable, the step was crucial to ensure the samples could be applied to the Caco-2 and HT29-MTX-E12 cells. Wet heating tended to result in a lower peptide intensity (p = 0.051), mainly due to the lower intensity of peptides originating from β-Lg (Fig. 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 AA) to be identified with the used LC-MS/MS measurement and identification parameters. The faster digestion of β-Lg after wet heating has been reported previously,[36,37] 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 of lactosylated peptides and longer peptides (Fig. 1C and 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[2] as well as with Zenker *et al.*[38] DH-72 contained highly glycated regions in all six major proteins, but especially in β-casein and β-Lg (Fig. S2), which were detected at higher intensities in DH-72 than in WH-40 (Fig. 1B and Fig. S1). This indicates that milk proteins are digested slower with higher glycation levels, which was in agreement with our previous study.[2] 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.

4.2 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 released after *in vitro* infant

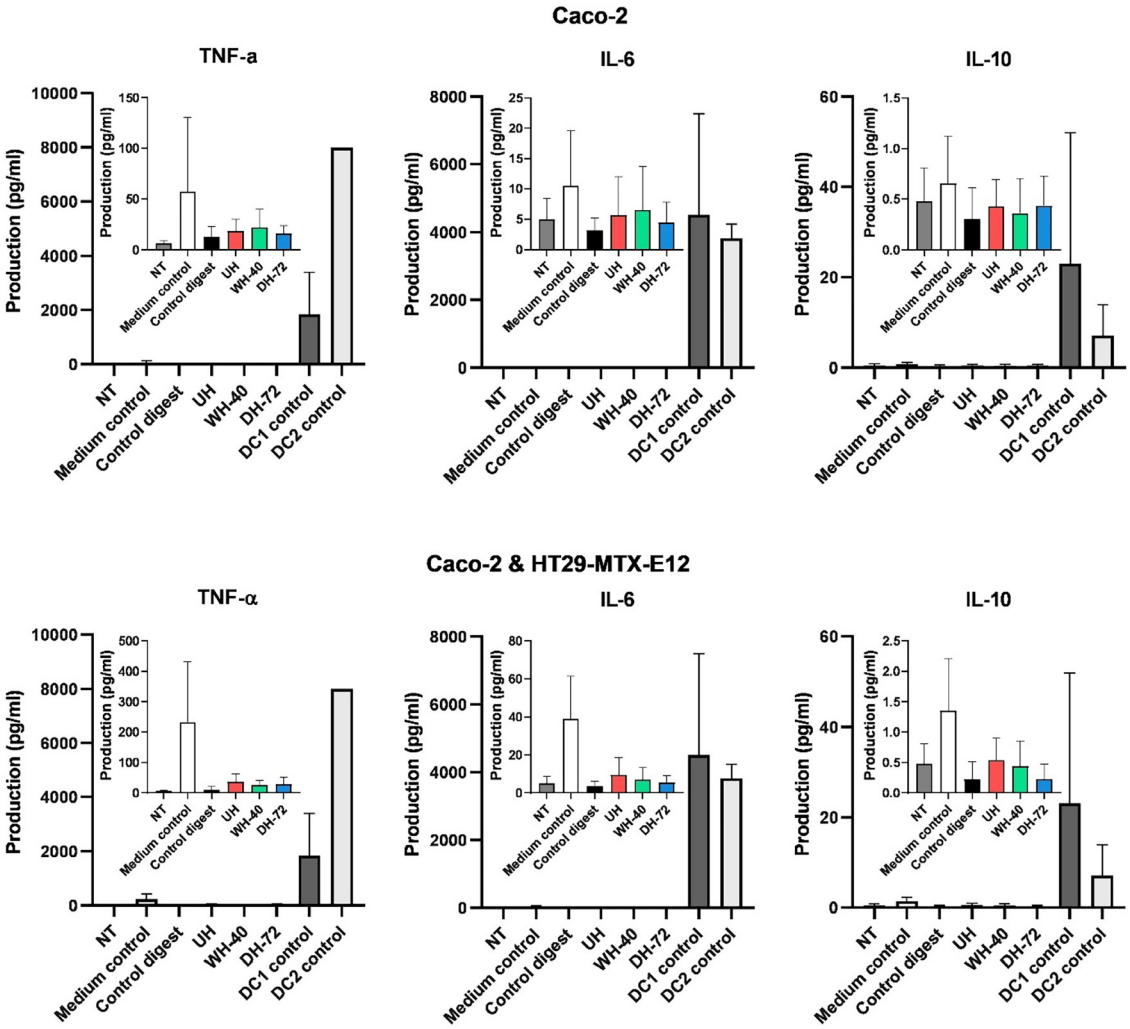

Fig. 11 TNF-α, IL-6 and IL-10 concentrations measured with a LEGENDplex assay after stimulating immature dendritic cells (iDCs) with medium from the basolateral side of a 21-days differentiated Caco-2 monolayer or a Caco-2/HT29-MTX-E12 (90/10) monolayer (n = 5). An infant formula model system remained either unheated (UH), was wet heated for 40 min (WH-40) or dry heated for 72 h (DH-72) and all samples were digested by use of an *in vitro* infant digestion model and filtered before the intestinal digests were applied to the Caco-2 and HT29-MTX-E12 cells for 6 h. 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 exposed to DMEM. Non-treated (NT) iDCs, DC1 controls and DC2 controls were grown and stimulated in IMDM. The detection ranges of the cytokines measured with the LEGENDplex assay were: TNF-a: 0.55–8000 pg ml^{-1}, IL-6: 0.46–10000 pg ml^{-1}, and IL-10: 0.54–11000 pg ml^{-1}. Cytokine concentrations were measured in five replicates, and the average of the five replicates is shown. If one of the replicates was below the detection limit, a value of zero was assigned to that replicate, and the mean was calculated accordingly. Error bars represent standard deviation. No statistical differences were found between NT, medium control, control digest, and milk digest samples.

digestion was evaluated by predicting potential HLA-II and IgE epitopes in the digests. No significant differences were found in survival of HLA-II epitopes during intestinal digestion between the heat treatments (Fig. 2). However, the composition of these HLA-II epitopes differed per heat treatment: WH-40 digests mainly contained HLA-II epitopes originating from one phosphorylated region in α_{s1}-casein (103–119 AA) (Fig. 2 and Fig. S2), while DH-72 digests mainly contained glycated HLA-II epitopes from α_{s2}-casein (100–113 AA) and β-casein (164–175 AA) (Fig. 2 and Fig. S2). Modifications have previously been shown to alter the binding capacity of several epitopes in milk proteins. 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,[39] whereas phosphorylation can decrease the IgE and IgG binding capacity of α-La,[40] also after *in vitro* digestion.[41] Glycation was also reported to modulate the immunoreactivity of whey proteins after digestion, with glycated β-Lg showing increased binding to sRAGE and galectin-3[13] and with glycated β-Lg and α-La showing reduced IgE binding,[16–18] both after *in vitro* digestion. Some of the HLA-II epitopes that were detected in the intestinal digests were also found by Zenker *et al.*,[3] although we detected three times as many, including epitopes from different regions in the pro-

teins, possibly due to the higher sensitivity of the LC-MS/MS in our study. In contrast, the variation in linear IgE epitopes was low between the heat treatments (Fig. 3 and Table S3). Nearly all IgE epitopes detected in our study were also reported by Zenker *et al.*[3] after *in vitro* digestion, demonstrating that mainly the same regions of the proteins survived digestion. Most of these IgE epitopes are derived from caseins, which aligns with the higher prevalence of casein-based compared to whey protein-based cow's milk allergy.[42] In summary, both wet and dry heating of milk proteins did not significantly increase HLA-II epitope survival, but did result in a different composition of HLA-II epitopes, which may potentially modulate immunoreactivity after *in vitro* digestion. However, the limited and contradicting literature on the composition of IgE epitope and modifications of these epitopes makes it difficult to determine the impact of wet and dry heating on immunoreactivity. Therefore, directly measuring the immunoreactivity of the intestinal digests (*e.g.* binding assays or immune cell experiments) instead of an *in silico* bioinformatic approach would be of interest for future research.

4.3 Mucus production

The influence of milk peptides on intestinal mucus, *via* mucus production and secretion as well as gene expression analysis, was analyzed in one set of cells to allow for direct comparison. Mucus production of HT29-MTX-E12 cells was not affected by the digests with milk peptides compared to the control digest (water digested instead of milk; Fig. 4). However, all digests, including the control digest, increased mucus secretion compared to the medium control, suggesting that the control digest stimulated mucus secretion, although the responsible component is unknown, which might be of interest to analyze in future research. In the intestinal digests, the peptide YPVEPF (neocasomorphin-6) had a lower intensity in WH-40 digest, and YPFPGPI (β-casomorphin 7) had a lower intensity in DH-72 digest (Table S4). These two peptides can increase mucus secretion and mucin gene expression: YPVEPF can increase MUC4 expression[34] and YPFPGPI can increase mucus production and the expression of MUC2, MUC3, and MUC5A.[33] However, no significant differences were found in total mucus production or in the gene expression of mucins that we measured, which were MUC5A, MUC13 and MUC17 (Fig. 4). MUC2, MUC3 and MUC4 expression were not analyzed due to low expression in HT29-MTX-E12 cells (Fig. S3) and would likely not provide meaningful data when measured. Interestingly, while mucus production increased more than 10-fold during the 4 h of stimulation with the digests (Fig. 4D), the expression of the mucin genes did not show a corresponding increase (Fig. S5A). This suggests that either the mucin gene expression was not directly related to mucus production in this model, or that mucin genes, other than the ones measured, might be involved in the observed increase in mucus production. Of note, since the ELLA assay only allows for the quantification of secretory mucins, we may have missed the regulatory effects of milk peptides on anchored mucins. In contrast to our findings, some previous studies showed increased mucus production and mucin gene expression (*e.g.* MUC5A, MUC2 and MUC3) with isolated milk peptides (*e.g.* β-Lg, α-La, α_{s1}-casein) both *in vitro* an *in vivo*.[9,10,43–45] However, our study used a milk peptide mixture instead of isolated milk peptides, which may have faded the effect of individual isolated milk peptides. One study showed that heated infant milk formula decreased gene expression of MUC2 and MUC5A compared to unheated formula,[46] but this was measured in Caco-2/HT29-MTX monolayers, whereas we measured mucin gene expression only in HT29-MTX cells. Moreover, the cell model used in our study produces a mucus more representative of the stomach, rather than the small intestine and the primary secretory mucin MUC2 is known to be very low expressed in HT29-MTX cells.[47] While commonly used in *in vitro* studies due to the lack of a simple intestinal mucus model, its use leads to differences between *in vitro* (stomach-like) and *in vivo* (intestinal) mucus environments. Furthermore, although the quantification of mucus production and gene expression is typically performed in a well-plate format,[9,10] it is questionable whether these analyses would not be better performed using cells cultured on permeable inserts, since the degree of cell differentiation and polarization can influence mucus production.[48]

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 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 (Fig. 5). Peptides were transported more proportionally to the peptide intensities in the intestinal digests in the coculture than in the monoculture (Fig. 6). 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 and goblet cells. Interestingly, more peptides from all major milk proteins were transported across the coculture compared to the monoculture (Fig. 5), whereas no differences in TEER values or cytotoxicity levels within the cells were observed (Fig. S9). However, directly measuring permeability, such as *via* 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 (Fig. 5C and G), and relatively fewer phosphorylated peptides were transported compared to the composition of the intestinal digests (Fig. 1). This suggests that differences in peptide modifications were not related to the observed differences in transport between the mono- and cocultures. Moreover, specific regions of proteins were not transferred across the monoculture after both wet and dry heating, whereas mainly the same regions of proteins were transferred for all heat treatments across the coculture (Fig. 7). However, no correlation could be found between these differences in the transported regions of milk proteins in the monoculture with certain protein modifi-

cations (Fig. S10 and S11) or with other properties of these regions such as hydrophobicity or charge. As peptide properties can influence transport,[49] 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.*,[3] and the lower peptide intensity of transported β-Lg across a Caco-2 monolayer after wet heating was in line with Deng *et al.*[4] 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. The presence of goblet cells in the coculture may contribute to the higher peptide transport, which can transport low molecular weight antigens across the intestinal barrier,[50] but this does not explain the larger differences in peptide transport between the cell models for the dry heated sample. We hypothesize that peptide-peptide interactions induced by wet and dry heating may hinder peptides from being transported across the monoculture. In dry heated digests, more peptide-peptide interactions may be formed that involved lactosylation, which occurred to a higher degree in the dry heated digests (Fig. 1C). Since lactosylation induces a negative charge, and these peptide-peptide interactions may be weakened by negatively charged mucins in the coculture,[7] resulting in a higher transport. However, this is only a hypothesis and should be further investigated. Furthermore, different routes of transport might be involved in the translocation of peptides in the mono- and cocultures. Whereas a higher permeability of cocultures with HT29 cells has been reported in literature,[51] possibly due to the lower expression of tight junction proteins by HT29 cells,[52,53] no differences in TEER values were observed, and thus no indication for a higher permeability, of the coculture (Fig. S9). However, still a higher peptide intensity and relatively more longer peptides (10–13 AA) were observed on the basolateral side of the coculture (Fig. 5), suggesting that different routes of transport (*e.g.* paracellular transport, passive diffusion, endocytosis, or carrier-mediated transport) were involved in the mono- and cocultures, which is of interest for future research. 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.

4.5 Immunoreactivity of transported peptides

The immunoreactivity of transported peptides was evaluated by predicting HLA-II and linear IgE epitopes and by measuring iDC response after stimulation with the basolateral media. Only some epitope-containing regions detected in the intestinal digests (Fig. 2 and 3) were able to cross the intestinal barrier (Fig. 8 and 9). DH-72 contained higher intensity of HLA-II epitopes from β-casein than UH and WH-40 in both cell models (Fig. 8), and were mainly from one region within β-casein (164–175 AA) (Fig. 8 and Table S6), which seemed to be transferred according to its intensity in the intestinal digests (Fig. 2 and Table S2). However, other regions with high intensities in the intestinal digests (*e.g.* 103–119 AA in α_{s1}-casein) were transported only to a low extent (Fig. 2 and 8). This suggests that other factors than peptide intensity are involved, such as hydrophobicity, charge, 3D-structure, and modifications, which may influence the transfer of peptides,[49] although no clear correlation was found in our study (section 4.4). The HLA-II epitope from the 164–175 AA region in β-casein in DH-72 was only detected in lactosylated and CML-modified form (Table S6). As discussed in section 4.2, glycation may change the immunoreactivity of epitopes, although literature is contradictory on whether it enhances or reduces recognition of epitopes.[13,16–18] Linear IgE epitopes intensities were similar in the intestinal digests (Fig. 3), but after transport, DH-72 had a higher IgE epitope intensity compared to WH-40 in the coculture (Fig. 9). Most transported IgE epitopes were unmodified (Table S7), suggesting that the higher IgE epitope transfer in DH-72 was not related to glycation modifications.

These differences detected in transferred HLA-II and linear IgE epitopes, however, did not lead to changes in cytokine response by iDCs compared to the control digest (Fig. 11). 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. IL-8, however, was significantly higher in the basolateral medium of the coculture than the monoculture, suggesting that this increase was driven by HT29-MTX-E12 cells, as also observed in an earlier study.[54] Previous studies showed increased binding to or uptake of wet and dry heated milk proteins by DCs or macrophages, including after intestinal digestion, but no differences in cytokine response.[12–14] This suggests that the peptides in the basolateral medium might have been able to bind to and taken up by surface receptors of iDCs, such as RAGE and galectin-3, but the peptide concentrations were probably too low to affect the cytokine production. Using a direct-contact model of Caco-2 and DCs[55] or HT29-MTX-E12 and DCs could avoid peptide dilution in the basolateral compartment, and allows DCs to sample through the intestinal barrier, where higher concentrations of peptides (Fig. 1) and HLA-II and IgE epitopes (Fig. 2 and 3) were detected, potentially inducing a different DC response. Additionally, a DC-T cell model could be explored to study how DCs present milk-derived HLA-II epitope presentation and resulting T cell responses. In summary, the intensities of peptides containing predicted HLA-II and IgE epitopes suggested that dry heating increased immunoreactivity after intestinal transport in the coculture. However, these differences in HLA-II and IgE epitopes did not change the cytokine response by iDCs.

5. Conclusion

This study showed that wet and dry heating of milk proteins affected the peptide composition after *in vitro* infant digestion. Wet heating tended to result in lower peptide intensities,

especially of β-Lg, and dry heating resulted in relatively longer and more lactosylated peptides. Peptides containing linear IgE epitopes survived intestinal digestion equally across heat treatments, but the composition of peptides containing HLA-II epitopes surviving intestinal digestion differed after wet and dry heating compared to the unheated IF model system. This suggests that wet and dry heating affect the immunoreactivity of digestion-derived milk peptides, 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 compared to wet heating. More HLA-II epitopes from β-casein were transported across the intestinal barrier after dry heating in both cell models, and more linear IgE epitopes crossed the intestinal barrier after dry heating compared to wet heating in the coculture. This indicates that dry heating may 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 DCs. All in all, wet and dry heating of milk proteins changed their digestion, survival of immunoreactive structures, and intestinal transport, but did not affect mucus production or DC response after intestinal transport. This shows the importance of studying heat treatment effects on milk proteins during IF production and subsequent implications on digestion, intestinal transport, and immunoreactivity, although the immunological consequences should be further investigated.

Author contributions

Julie Miltenburg: conceptualization, methodology, investigation, data Curation, formal Analysis, visualization, writing – original Draft. Tamara Hoppenbrouwers: conceptualization, supervision, methodology, investigation, writing – review & editing. Monic M. M. Tomassen: methodology, investigation, data curation, formal analysis. Dianne Somhorst: methodology, investigation, data curation, formal analysis. Anouk Boudewijn: investigation, data Curation. Els Oosterink: investigation, methodology. Harry Wichers: conceptualization, supervision, writing – review & editing. Kasper Hettinga: conceptualization, funding acquisition, supervision, writing – review & editing. Shanna Bastiaan-Net: conceptualization, supervision, methodology, investigation, data curation, formal analysis, writing – review & editing.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d5fo02883c.

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