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## **Cow's milk allergy prevention and treatment by heat-treated whey – a study in Brown Norway rats**

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Accepted Article

## ABSTRACT

Background: Food processing, including heat-treatment, can affect protein structure and stability, and consequently affect protein immunogenicity and allergenicity. A few studies have shown that structural changes induced by heat-treatment impact the intestinal protein uptake and suggest this as a contributing factor for altered allergenicity.

Objective: To investigate the impact of heat-treatment of a whey-based protein product on allergenicity and tolerogenicity as well as on intestinal uptake in various animal models.

Methods: Immunogenicity and sensitising capacity of the heat-treated whey product was compared to that of the unmodified product by intraperitoneal and oral exposure studies, while tolerogenic properties were assessed by oral primary prevention and desensitisation studies in high-IgE responder Brown Norway rats.

Results: Heat-treatment of whey induced partial protein denaturation and aggregation, which reduced the intraperitoneal sensitising capacity but not immunogenicity. In contrast, heat-treatment did not influence the oral sensitising capacity, but the heat-treated whey showed a significantly reduced eliciting capacity compared to unmodified whey upon oral challenge. Heat-treatment did not reduce the tolerogenic properties of whey, as both products were equally good at preventing sensitisation in naïve rats as well as desensitising already sensitised rats. Results from inhibitory ELISA and immunoblots with sera from sensitised rats demonstrated that heat-treatment caused an altered protein and epitope reactivity. Protein uptake studies showed that heat-treatment changed the route of uptake with less whey being absorbed through the epithelium but more into the Peyer's patches.

Conclusion & Clinical Relevance: These results support the notion that the physicochemical features of proteins affect their route of uptake and that the route of uptake may affect the protein allergenicity. Furthermore, the study highlights the potential for heat-treatment in the production of efficient and safe cow's milk protein-based products for prevention and treatment of cow's milk allergy.

## INTRODUCTION

Cow's milk allergy (CMA) is the most common food allergy among small children, with a prevalence of approximately 0.5-3%, varying from country to country[1–3]. The optimal way to prevent sensitisation to cow's milk is to exclusively breastfeed infants in their first months of life until introduction of solid foods. However, in many cases, breast milk must be supplemented with or replaced by infant formulas. Infants suffering from CMA or at high risk of developing an allergy, are recommended to use hypoallergenic

infant formulas, which are most often based on hydrolysed cow's milk proteins[4]. The risk of eliciting an allergic response is reduced because epitopes are degraded by hydrolysis, but this may also compromise the tolerogenic effects of these products[5,6]. Currently, there is no accepted treatment for CMA or any other food allergy, yet immunotherapeutic strategies for desensitising food allergic patients have shown promising results. The only accepted strategy to manage CMA is strict avoidance of foods containing cow's milk allergens[7]. Different types of processing, including heat-treatment of cow's milk proteins, are being investigated with the aim of reducing allergenicity but retaining immunogenicity, with the ultimate goal of producing safe and efficient products for prevention and treatment of CMA[8–11].

It is the general perception that heat-treatment reduces cow's milk allergenicity since many milk allergic children can tolerate baked milk[12], yet tolerogenic properties of heat-treated milk are not well characterised. It has been reported that the introduction of baked milk into the diet of cow's milk allergic patients can accelerate the development of tolerance to fresh milk[13–15], and baked milk has been used for oral immunotherapy in human clinical trials[16,17]. However, it has been questioned whether heat-treated milk in fact has a true tolerogenic effect, or whether tolerance to baked milk is just a biomarker for spontaneous resolution of milk allergy[18] since adverse reactions to baked milk is associated with severe CMA[16].

A direct comparison of allergenic and tolerogenic properties of unmodified and heat-treated milk can be addressed in controlled animal studies. A few animal studies have been performed to assess the sensitising and eliciting capacity of heat-treated milk, but results from these studies are conflicting[19–21]. These studies showed that effects on allergenicity are not only related to masking or exposure of antibody-binding epitopes as have previously been described for cow's milk allergic patients[22–24], but that heat-treatment also impacts on allergenicity by influencing the route of intestinal protein uptake[19]. Evidence of the tolerogenic properties of heat-treated milk proteins from controlled animal studies is very limited[25], but development of oral tolerance will likely also be affected by intestinal uptake.

The present study investigated the impact of heat-treatment on the immunogenicity, sensitising capacity, eliciting capacity, and on the ability to induce tolerance as well as on intestinal uptake of a whey product. This was done with the ultimate goal to guide the future design of modified cow's milk proteins with low allergenicity but retained tolerogenicity. The immunogenicity and sensitising capacity of unmodified and heat-treated whey were assessed by intraperitoneal (i.p.) and oral exposure studies, while the tolerogenic properties were assessed by oral primary prevention and desensitisation studies with recently established models[6]. The intestinal uptake was evaluated in naïve rats by quantifying protein levels in

different intestinal tissues at different time points after oral administration of the whey products by gavage. In addition, protein uptake was evaluated by two different *in vitro* assays. In all animal studies, high-IgE responder Brown Norway (BN) rats, resembling atopic individuals, were used.

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## METHODS

### *Product characterisation*

Whey protein isolate (WPI) with approx. 60%  $\beta$ -lactoglobulin (BLG) and a heat-treated (HT) (90°C for 10 min) version of the same WPI were kindly provided by Arla Foods Ingredients (Videbæk, Denmark).

### *Quantification of whey proteins*

To examine native protein composition, TSK gel permeation chromatography (GPC) high-performance liquid chromatography (HPLC) was performed. Whey products were dissolved in solvent (20 mM  $\text{NaH}_2\text{PO}_4$ , Merck KGaG, Darmstadt, Germany, pH 7.5) to a protein concentration of 0.1 % (w:v), left overnight at 4°C and subsequently filtered with a 0.22  $\mu\text{m}$  filter. A total of 25  $\mu\text{L}$  was loaded on two connected TSKgel3000PWxl (7.8mm, 30mm, TOSOH Bioscience GmbH, Stuttgart, Germany) columns equipped with a PWxl precolumn (6 mm, 4 cm, TOSOH Bioscience GmbH) at 25°C. Whey products were run at a flow rate of 0.4 mL/min with 47% (v:v) acetonitrile (CHEM-LAB NV, Zedelgem, Belgium) in Milli Q water, and proteins were detected at a wavelength of 210 nm. Peak areas were normalised with the peak area of the casein macropeptide (CMP).

### *Analysis of protein aggregation*

To examine protein aggregation status, ethylene bridged hybrid (BEH) GPC was performed. Whey products were dissolved in solvent (50 mM  $\text{NaH}_2\text{PO}_4$ , 0.15 M NaCl, Merck KGaG, pH 7.0) to a protein concentration of 0.25% (w:v), left overnight at 4°C and subsequently filtered with a 0.22  $\mu\text{m}$  filter. Five  $\mu\text{L}$  was loaded on an ACQUITY UPLC protein BEH SEC column (200Å, 1.7 $\mu\text{m}$ , 4.6 x 150mm, Waters Corporation, Massachusetts, US) with an ACQUITY UPLC protein BEH SEC Guard column (200Å, 1.7 $\mu\text{m}$ , 4.6 x 30mm, 10K-500K, Waters Corporation) at 25°C. The columns were connected on a Waters ACQUITY UPLC equipped with a Waters 2487 Dual  $\lambda$  Absorbance Detector. The whey products were run at a flow rate of 0.2 mL/min in solvent and detected at a wavelength of 214 nm.

### *Native and SDS-PAGE*

For analysis of proteins under non-reducing conditions by native polyacrylamide gel electrophoresis (PAGE), 40  $\mu\text{g}$  of whey product was dissolved in native sample buffer (62.5 mM Tris-HCL pH 6.8, 25% glycerol, 0.01% bromophenol blue; BioRad, Hercules CA, US) and loaded onto a 4-20% TGX polyacrylamide gel (BioRad). Gel electrophoresis was conducted in Tris/Glycin running buffer (25 mM Tris, 192 mM Glycin; BioRad) with 200 V constant current.

For analysis of proteins under reducing conditions, sodium dodecyl sulphate (SDS)-PAGE was conducted with 20 µg of whey product as previously described[26]. Native and SDS-PAGE gels were washed three times in deionised water, and proteins were stained by Bio Safe Coomassie stain (BioRad) for 1 h at RT and washed extensively (>10 times) in deionised water. Stained gels were photographed using Imager ChemiDoc XRS+ (BioRad).

### **Animals**

BN rats from the in-house breeding colony (National Food Institute, Technical University of Denmark, Denmark) were weaned at an age of three weeks. They were housed in macrolon cages (2-3/cage), with a 12 h light:dark cycle, at a temperature of  $22 \pm 1^\circ\text{C}$  and a relative humidity of  $55 \pm 5\%$ . The rats were observed twice daily and clinical signs were recorded. The rats were fed a milk-free diet for  $\geq 10$  generations to avoid tolerance to the studied allergens. The diet was produced in-house and based on rice flour, potato protein and fish meal as protein sources, as previously described[27], with the exception of maize flakes being substituted with rice flour. Diet and acidified water (pH 3.5) was given *ad libitum*. Animal experiments were carried out at the National Food Institute facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate and authorisation given (2015-15-0201-00553-C1). The experiments were overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use.

### **Intraperitoneal sensitisation experiment**

To investigate the inherent sensitising capacity of the two whey products, BN rats (6-8 weeks of age, both sex) were immunised i.p. with 200 µg of either WPI or HT-WPI in 0.5 mL phosphate buffered saline (PBS, 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L  $\text{Na}_2\text{HPO}_4$ , 1.8 mmol/L  $\text{KH}_2\text{PO}_4$ , pH 7.2) (n=8/group). Rats were immunised three times, at day 0, 14 and 28, without any use of adjuvant. They were sacrificed at day 35 by exsanguination using carbon dioxide inhalation as anaesthesia and blood was collected, converted to serum and stored at  $-20^\circ\text{C}$  until analysis (overview of animal experimental design in Fig. 2A).

### **Oral sensitisation experiment**

To investigate the oral sensitising capacity of the two whey products, BN rats (4-8 weeks of age, only females) were dosed by gavage with 10 mg of either WPI or HT-WPI together with 20 µg cholera toxin (CT, List Biological Laboratories Inc., Campbell, CA, US) in 0.5 mL PBS (n=12/group). Rats were dosed three times per week (Monday, Wednesday and Friday) for five consecutive weeks. Ten days after the last

dosing, blood samples were collected from the sublingual vein, converted to serum and stored at -20°C until analysis

(overview of animal experimental design in Fig. 3A).

#### ***In vivo tests***

Three days after the last dosing, rats were subjected to an oral challenge: The body temperature of each rat was measured by a rectal probe before and 30 min after gavage with 100 mg of the same product that was used for dosing, but without adjuvant, in 1 mL water. Six days after the last dosing, rats were subjected to an ear swelling test: Rats were anaesthetised with hypnorm-dormicum and the thickness of both ears was measured at the same spot before and one hour after intradermal injection of 10 µg of WPI or HT-WPI in 20 µL PBS (one product per ear).

#### ***Primary prevention experiment***

To investigate *de novo* tolerance inducing capacity, also designated primary preventive capacity, of the two whey products, naïve BN rats (4-6 weeks of age, both sex) were given either water, WPI or HT-WPI *ad libitum* in their drinking bottles (12.5 g protein per litre water) for 21 consecutive days (n=8/group). After one week of rest, rats were post-immunised i.p. with 100 µg WPI in 0.5 mL PBS once a week for eight weeks. Blood samples from the sublingual vein were collected after tolerance induction and after the fourth post-immunisation. Rats were sacrificed one week after the last post-immunisation by exsanguination using carbon dioxide inhalation as anaesthesia and blood was collected, converted to serum and stored at -20°C until analysis (overview of animal experimental design in Fig. 4A).

#### ***Desensitisation experiment***

To investigate the desensitising capacity of the two whey products, three groups of BN rats (4-8 weeks of age, only females, n=12) were orally sensitised to WPI with CT (as described above) and subsequently given either water, WPI or HT-WPI *ad libitum* in their drinking bottles (12.5 g protein per litre water) for 21 consecutive days. After one week of rest, desensitised rats together with naïve control rats were post-immunised i.p. with 50 µg WPI in 0.5 mL PBS once per week for three weeks. The day after the last post-immunisation, rats were subjected to an ear swelling test with WPI, as described above. Blood samples were collected from the sublingual vein the day after *ad libitum* administration and on the day of second and third post-immunisation. Rats were sacrificed by exsanguination using carbon dioxide inhalation as anaesthesia and blood was collected, converted to serum and stored at -20°C until analysis (overview of animal experimental design in Fig. 5A).

### ***In vivo uptake experiment***

To investigate the intestinal uptake of the two whey products, naïve BN rats (9-16 weeks of age, only females) were gavaged with 100 mg of either WPI or HT-WPI in 1 mL water and sacrificed at different time points (n=4/ group/time point). Rats were sacrificed 15, 30, 60 or 90 min after gavage by exsanguination using carbon dioxide inhalation as anaesthesia. The small intestine was excised and a 20 cm section (7 cm distal from the stomach) was rinsed with 0.9% (w:v) NaCl and divided into three fractions; Peyer's patches, lamina propria and epithelium; which were individually snap frozen in liquid nitrogen and stored at -80°C until analysis. Total proteins from Peyer's patches and epithelium samples were extracted by homogenisation by rotor stator in 10 µL/mg tissue lysis buffer (150 mM NaCl, 20 mM Tris, 1mM EGTA, 1% Triton X-100, 1 mM EDTA) with 2% (v:v) Halt protease inhibitor cocktail (Thermo Fisher, Hampton, VA, US) on ice. Lamina propria samples were homogenised in lysis buffer, and 2% (v:v) Halt protease inhibitor cocktail (Thermo Fisher) was immediately added to 1 mL of the homogenised tissue. Samples were incubated on ice for 20 min and mixed by vortexing every 5 min, and cleared by centrifugation at 15,000g for 20 min at 4°C. The supernatants were stored at -80°C until analysis. BLG concentrations of supernatants were quantified by a commercial bovine BLG ELISA kit (Bethyl Laboratories, Montgomery, AL, US) in 96-wells MaxiSorp plates (NUNC, Roskilde, Denmark) according to the manufacture's protocol. The ELISA kit was shown to detect WPI and HT-WPI equally well (data not shown).

### ***Analysis of product-specific IgG1 by various ELISAs***

For detection of product-specific IgG1 antibodies, indirect ELISA was performed as previously described[28]. For evaluation of the antigen-antibody binding strength, avidity IgG1 ELISA was performed as described elsewhere[28]. For evaluation of the specificity of the antigen-antibody binding, inhibitory IgG1 ELISA was performed as previously described[29] with the exception that sera were pre-incubated with WPI and HT-WPI inhibitor solutions.

### ***Antibody-capture ELISA for detection of product-specific IgE***

For detection of product-specific IgE antibodies, antibody-capture ELISA was performed as previously described[28] with two exceptions: That plates were blocked with 3% (v:v) horse serum (Biowest-bw, Nuaillé, France) in PBS with 0.01% (w:v) Tween 20, and product-specific IgE was detected by 50 µL/well of 0.1 µg/mL of 10:1 digoxigenin (DIG)-coupled WPI or HT-WPI in blocking solution.

### ***Immunoblot***

To investigate reactivity of IgG1 antibodies raised against WPI and HT-WPI after i.p. or oral administration, immunoblots were performed with serum pools as previously described[26] with SDS-PAGE loaded with 5 µg whey product. Serum pools were diluted 1:500 (i.p. study both groups), 1:800 (oral study, WPI group) or 1:1000 (oral study, HT-WPI group) for optimal visualisation.

### ***In vitro epithelium transcytosis***

To evaluate the *in vitro* epithelium permeability, transport of the whey products through a Caco-2 cell layer was examined as previously described[30]. In short, culture media were replaced with fetal bovine serum (FBS) free media on day 20 after seeding. The day after, whey products dissolved in FBS-free culture media were added to make a final concentration of 500 µg/mL in the apical compartment. After 1, 3, 6, and 24 h, 250 µL were sampled from the basolateral compartment and stored at -20°C. Only data from 24 h are shown. Supernatant BLG concentrations were quantified by a commercial bovine BLG ELISA kit (Bethyl Laboratories) in 96-wells MaxiSorp plates (NUNC) according to the manufacture's protocol. Tight junction integrity was confirmed by TEER (EVOM2, World Precision Instruments, Sarasota, FL, US) before and after the uptake assay. Each condition was performed in triplicate and repeated three times.

### ***In vitro dendritic cell uptake***

To evaluate the *in vitro* product uptake by dendritic cells, murine bone marrow-derived dendritic cells (BMDC) were generated as described elsewhere[31] and co-incubated with fluorescein isothiocyanate (FITC)-labelled whey products as previously described[32]. In short, bone marrow cells from femur bones of BALB/c mice were isolated and cultured six days in RPMI medium with 10% (v:v) FBS and 10 ng/mL GM-CSF (R&D, Oxon, UK). To assess protein uptake and endocytosis, 10 µg/mL of FITC-labelled products were incubated with  $1 \times 10^6$  BMDC/mL in 2.4 mL of cell culture medium in a 37°C water bath. After 0, 5, 15, 30, 45, 60 and 120 min, 300 µL of this cell culture was harvested and immediately washed twice in 1 mL cold PBS and twice in cold FACS buffer (PBS with 2 % (v:v) FBS and 0,05 % (w:v) NaN<sub>3</sub>) and centrifuged for 5 min at 250g at 4°C. The percentage of FITC positive cells and mean fluorescence intensity (MFI) was measured using FACS BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, US). Flow cytometry data were analysed in Cyflogic software (CyFlo Ltd, Turku, Finland). Each condition was performed twice in duplicates.

### ***Statistical analysis***

Curve and statistical analyses were made using GraphPad Prism (version 8.1.1 for Windows, GraphPad Software, San Diego, CA, US). For inhibitory ELISA curves, best fit slope estimates (HillSlope) with standard errors were compared by one-way ANOVA. IC50 values were only compared for graphs with slope estimates that were not significantly different.

ELISA results were expressed as log2 antibody titres. Normal distribution of data was tested by D'Agostino-Pearson normality test. For data that passed the normality test, group means are indicated on graphs and differences between groups were analysed by t-test (two groups) or one-way ANOVA followed by Tukey's post-test for multiple comparison (>2 groups). For data that did not pass the normality test, group medians are indicated on graphs, and group differences were analysed by non-parametric Mann-Whitney test (two groups) or Kruskal-Wallis test followed by Dunn's post-test for multiple comparison (>2 groups). MFI of BMDC was compared between the two groups at each time point by a two-way ANOVA of squarer root transformed data with Sidak's post-test. Asterisks indicate statistically significant difference between two given groups: \*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ , and \*\*\*:  $p \leq 0.001$ , ns: no statistically significant difference.

## RESULTS

### *Characterisation of products*

WPI and HT-WPI were analysed for native versus denatured protein composition and aggregation status, which showed that heat-treatment caused changes in the physicochemical features of the WPI. Results from TSK-HPLC showed that some but not all of the proteins in the HT-WPI were denatured; about 35% of the BLG, 50% of the  $\alpha$ -lactalbumin and 100% of glycomacropeptides (cGMP) remained in native form after the heat-treatment (Fig. 1A). Results from GPC showed that heat-treatment of WPI resulted in partial protein aggregation (Fig. 1B). SDS-PAGE confirmed that the protein distribution was the same in the two products (Fig. 1C). However, results from native PAGE revealed that protein conformation differed between the two products; while some distinctive protein bands were detectable for WPI, proteins in HT-WPI were retained in the top of the gel and formed a smear under non-reducing conditions (Fig. 1D).

### *Intraperitoneal sensitisation*

To compare the inherent sensitising capacity of the two whey products, serum from rats immunised i.p. with either WPI or HT-WPI (Fig. 2A) were analysed for specific antibody responses. A statistically significant reduction in the serum level of product-specific IgE was observed in the group of rats i.p. immunised with HT-WPI compared with those immunised with WPI (Fig. 2B). The reduction in sensitising capacity was not caused by a reduction in immunogenicity, as the serum level of product-specific IgG1 was similar in the two groups (Fig. 2C). The binding strength of the raised IgG1 antibodies were similar in the two groups (Fig. 2D). Results from inhibitory ELISAs demonstrated that the two products differed in competitive capacity; while HT-WPI fully inhibited the binding between WPI and IgG1 antibodies raised against WPI, WPI was not able to fully inhibit the binding between HT-WPI and antibodies raised against HT-WPI, indicating that new epitopes had developed as result of heat-treatment (Fig. 2E). In fact, for antibodies raised against HT-WPI, the IC50 values for HT-WPI were 70 times less than that of WPI, indicating that 70 times more WPI was needed to inhibit 50% of the binding between HT-WPI and antibodies raised against HT-WPI. Immunoblots of SDS-PAGE showed that serum from HT-WPI sensitised rats recognised more protein bands compared with that of WPI sensitised rats (Fig. 2F). Those bands were detectable in both products.

### **Oral sensitisation**

To compare the oral sensitising capacity of the two whey products, serum from rats orally gavaged with either WPI or HT-WPI together with the adjuvant CT (Fig. 2A) were analysed for specific antibody responses.

In contrast to the i.p. sensitisation study, oral dosing with WPI or HT-WPI resulted in similar serum levels of product-specific IgE (Fig. 3B) and IgG1 antibodies (Fig. 3C). However, the antigen-IgG1 binding strength was significantly increased in the group of rats that were dosed with the HT-WPI compared with WPI (Fig. 3D). This difference between the binding strength of IgG1 antibodies raised against the two products was observed consistently in both animal experiments in which the products were administered by the oral route (data not shown). Results from inhibitory ELISA confirmed results from the i.p. sensitisation study; while HT-WPI was able to fully inhibit the binding between WPI and IgG1 antibodies raised against WPI, WPI was not able to fully inhibit the binding between HT-WPI and antibodies raised against HT-WPI (Fig. 3E). However, for antibodies raised orally against HT-WPI, the IC<sub>50</sub> values for HT-WPI were only approximately 30 times less than that of WPI; hence, the difference between competitive capacities of the two products was much less for antibodies raised by oral administration compared with antibodies raised by i.p. immunisations.

To further evaluate the allergenicity, rats were subjected to two elicitation tests: An oral challenge and an ear swelling test. For the oral challenge, the body temperature of the rats was monitored before and 30 min after rats were administered with the same product as they were sensitised to, where a reduction of core body temperature is a sign of anaphylaxis. Despite a similar serum level of specific IgE in the two groups, the relative reduction in body temperature was significantly smaller in the group of rats dosed and challenged with HT-WPI compared with the group dosed and challenged with WPI (Fig. 3F). No statistically significant difference between acute allergic skin responses in the two groups of rats could be observed in the ear swelling test (Fig. 3G). Immunoblots of SDS-PAGE showed that serum from orally WPI sensitised rats recognised more protein bands compared with that of the i.p. WPI sensitised rats (Fig. 2H). The immunoblots with serum from WPI and HT-WPI orally sensitised rats were overall very similar, but did show different binding pattern in the region between 25-37 kDa, which are likely due to binding of different caseins present in small amounts. This showed that intestinal digestion diminished the difference between the products, but that some of the unique epitopes were still present on HT-WPI after ingestion.

### **Primary prevention**

To compare the *de novo* tolerance inducing properties of the two different products, the primary preventive capacity was evaluated by administering naïve rats with either water, WPI or HT-WPI *ad libitum* in their drinking bottles for 21 consecutive days before the rats were post-immunised i.p. by WPI (Fig. 4A). Only one rat in each prevention group had a detectable level of WPI-specific IgE after eight post-immunisations while all rats in the control group were sensitised (Fig. 4B). Analysis of WPI-specific IgG1 revealed that less rats had detectable levels of IgG1 after four post-immunisations in the group administered with HT-WPI compared with WPI (Fig. 4C).

### **Desensitisation**

To further investigate the tolerogenic properties of the two different whey products, the desensitising capacity was evaluated by administering rats that were pre-sensitised to WPI with either water, WPI or HT-WPI *ad libitum* in their drinking bottles for 21 consecutive days. Subsequently, the desensitised rats as well as naïve controls were post-immunised three times i.p. with WPI (Fig. 5A). It was confirmed that serum levels of WPI-specific IgE and IgG1 were not significantly different in the three groups before desensitisation was initiated.

While the natural decrease in WPI-specific IgE in the water control group was minimal, the IgE levels were dramatically decreased in both WPI and HT-WPI groups (Fig. 5B). WPI-specific IgE levels increased slightly with increasing number of i.p. post-immunisations in both WPI and HT-WPI groups, but remained significantly reduced compared with the water control group at all time points after desensitisation.

In the water control group, WPI-specific IgG1 levels decreased slightly after the desensitisation period and then increased with increasing numbers of immunisations (Fig. 5C). In both WPI and HT-WPI groups, levels remained constant through the study. Hence, there was a statistically significant difference in the levels compared with the water control group at all time points after desensitisation. Neither WPI-specific IgE nor IgG1 levels differed significantly between the WPI and HT-WPI groups at any time point. After the third post-immunisation, antibody levels in the WPI and HT-WPI groups did not differ significantly from naïve controls that were just i.p. immunised (not pre-sensitised and desensitised). Acute allergic skin response, measured by an ear swelling test, was significantly reduced in both WPI and HT-WPI groups compared with the water control group, and the response in the WPI and HT-WPI groups were not different from each other nor from that of naïve rats (Fig. 5D).

### ***In vivo intestinal uptake***

Naïve rats were orally gavaged with WPI or HT-WPI, and sacrificed at different time points to examine intestinal protein uptake. BLG was quantified in total protein extracts of three different tissue fractions (Peyer's patches, lamina propria and epithelium) from a piece of the proximal small intestine (Fig. 6A). The distribution of protein between the different tissue fractions differed between the two treatment groups with a larger proportion of BLG being present in epithelium of WPI dosed rats, while a larger proportion of BLG was present in Peyer's patches and lamina propria of HT-WPI dosed rats (Fig. 6B).

### ***In vitro epithelia transcytosis and uptake by DC***

*In vitro* epithelial transcytosis was assessed by measuring BLG transport across polarised Caco-2 cells grown on semi-permeable membranes. BLG concentrations were quantified in the basolateral compartments 24 h after apical application of the products. This experiment showed that the basolateral BLG concentration was higher in wells applied with WPI compared with HT-WPI (Fig. 7A). *In vitro* product uptake in DC was assessed by co-incubating murine BMDC with FITC-labelled product preparations. The cells were harvested and washed at different time points, and protein uptake was measured as the level of fluorescence. The experiment showed that BMDC uptake of HT-WPI was more pronounced than the uptake of WPI (Fig. 7B-C).

## DISCUSSION

There is a great interest in producing new and improved hypoallergenic infant formulas for prevention, management, and treatment of CMA. Such infant formulas should have a reduced allergenicity without compromising tolerogenicity. To accomplish this, we need a solid understanding of how structural changes induced by food processing, such as heat-treatment, affect immunological properties of food proteins. This study investigated how heat-treatment affects intestinal uptake, allergenicity and tolerogenicity of a whey protein product.

The whey product was subjected to mild heat-treatment at 90°C for 10 min, which induced partial protein denaturation and aggregation. Heat-treatment disrupts the secondary and tertiary protein structure by breaking hydrogen bonds, and hence cause an unfolding of proteins. In BLG, unfolding has been shown to result in exposure of hydrophobic sulfhydryl groups previously buried inside the folded protein[33]. Interactions of the sulfhydryl group with caseins or other unfolded whey proteins drives aggregation[34]. Conformational changes induced by heat-treatment may influence allergenicity directly by affecting IgE binding epitopes, or indirectly by affecting biophysical properties of proteins such as digestibility and solubility, or the route of intestinal uptake[10,35,36]. The heat-treatment may influence protein epitopes in various ways that either promote or prevent antibody binding: Unfolding may expose epitopes that were previously inaccessible inside the folded protein and/or dissociate conformational epitopes, while aggregation may cause formation of neo epitopes and/or masking of others. Previous studies have indicated that the effect of heat-treatment on epitope accessibility is temperature dependent, as they observed that heat-treatment below 90°C increased BLG antibody reactivity, while heating above 90°C reduced reactivity[37–39].

In the present study, heat-treatment reduced the inherent i.p. sensitising capacity, while the oral sensitising capacity was unaffected when the products were administered together with the mucosal adjuvant CT. However, HT-WPI induced significantly milder oral symptoms in sensitised rats compared with WPI upon oral challenge without adjuvant. Despite reduced allergenicity, heat-treatment did not reduce the tolerogenic properties as WPI and HT-WPI were equally good at preventing sensitisation of naïve rats and desensitising pre-sensitised rats. In line with our results, it was previously observed that heat-treatment reduced the i.p. sensitising capacity of BLG[20] and egg ovalbumin[40]. However, in contrast to our results, other studies found that heat-treatment increased the oral sensitising capacity of BLG[19,21], which might be a matter of the exact heat-treatment applied to the products or related to the use of adjuvants.

Tolerogenic properties of heat-treated milk are not well characterised. It has been reported that the introduction of baked milk in the diet of cow's milk allergic patients can accelerate the development of tolerance to fresh milk[13–15], but it has been questioned whether tolerance to baked milk is just a biomarker for spontaneous resolution of milk allergy, or if heat-treated milk has a true tolerogenic effect[18]. Evidence from controlled animal studies is scarce, but one study reported, in line with our results, that feeding of unmodified and heat-treated WPI protected mice against subsequent immunisation with unmodified WPI equally well[25]. To our knowledge, no published animal studies have until now investigated the desensitising effect of heat-treated milk proteins, but results with egg ovomucoid showed, in line with our results, that heated ovomucoid was as efficacious as unmodified ovomucoid for oral desensitisation of mice[41].

Currently, most hypoallergenic infant formulas are based on hydrolysed milk proteins[4]. Animal studies have shown that hydrolysed products have some capacity to prevent subsequent sensitisation[6,42–46]. Evidence on the desensitising capacity of hydrolysed milk products is scarce, but two studies found no desensitising capacity of hydrolysed whey products[6,43]. The present study suggests that heat-treatment could be used as an alternative or supplement to hydrolysis for production of hypoallergenic infant formulas. Future studies should aim for a direct comparison of the allergenic versus tolerogenic properties of milk proteins modified by heat-treatment and hydrolysis to investigate which type of modification is superior.

The present study showed that heat-treated products elicited milder oral symptoms in sensitised animals compared with the unmodified product, which is in agreement with results from previous studies with BLG[21] and egg white proteins[47,48]. This divergence between IgE levels and oral symptoms are hardly related to functionality of the raised IgE antibodies, since no difference in acute allergic skin response was observed, but could be explained by differences in intestinal degradation or intestinal uptake upon oral challenge. BLG is a relatively stable protein resistant to pepsin and acid proteolysis[49]. Previous studies showed that heating increased BLG digestibility[23,50,51] due to the exposure of peptic cleavage sites by unfolding[52]. In the small intestine, food proteins can be taken up through the layer of epithelial cells covering lamina propria by different transport mechanisms, or through M cells into Peyer's patches. The physicochemical features of proteins have been suggested to impact the route of uptake, and the uptake route has been suggested to impact the type of immune response that will be mounted[53,54].

The present study showed that heat-treatment altered the route of uptake, with less being absorbed through the epithelium but more into the Peyer's patches *in vivo* and into BMDC *in vitro* after heat-

treatment. This is in line with previous *in vivo* and *in vitro* uptake results for aggregated whey induced by heat-treatment[19] or by cross-linking through laccase treatment[32]. Despite that results from the *in vivo* and *in vitro* intestinal uptake in the present studies were in line with previous studies, this did not increase oral sensitisation as was previously observed[19,32]. This inconsistency could be due to differences in sensitisation study design, in particular dose and potency of CT. The adjuvant function of CT includes induction of the tight junction complex protein claudin-2 on intestinal epithelial cells, which promote antigen transport across the epithelia barrier[55] and could possibly mask differences in intestinal uptake of the products during the sensitisation phase. Alternatively, the inconsistency between the studies might indicate that differences in intestinal uptake are most important in the elicitation phase, for which the results are consistent, while other factors, such as differences in protein epitopes, which depends on the exact heat-treatment[37–39], are critical for sensitisation.

In the present study, results from inhibitory ELISA indicated that antibodies from rats dosed with HT-WPI react to epitopes that were not present or accessible in WPI under physiological conditions. However, considering the results from immunoblotting, performed under reducing conditions, the antibodies from HT-WPI sensitised rats reacted to the same protein bands in the two products, which indicated that the new epitopes were at least partly due to exposure of epitopes previously buried inside the folded proteins. Results from avidity ELISA suggested that the reactivity towards new epitopes increased the binding strength between HT-WPI and antibodies raised against HT-WPI.

In conclusion, this study showed that heat-treatment overall reduced the allergenicity without compromising immunogenicity and tolerogenic capacity of a whey protein product. This result could be related to differences in intestinal uptake since it was observed that WPI was transported more efficiently through the epithelia cell layer and that heat-treatment increased uptake into Payer's patches. These results highlight the potential for heat-treatment in the production of efficient and safe whey-based products as an alternative or supplement to whey-based hydrolysates for prevention and treatment of CMA in an attempt to accelerate the outgrowth of allergy and for tolerance development. However, clinical studies are needed to validate the safety and efficacy of heat-treated whey.

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## **CONFLICT OF INTEREST**

L.N.J., H.F.C. and L.V.S. are employees at Arla Foods Ingredients. K.L.B and K.B.G have ongoing collaboration with the company Arla Foods Ingredients, which supplied the whey protein products for the current study. Other authors declare no conflict of interest.

## **DATA SHARING**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## FIGURE LEGENDS

**FIGURE 1:** Physicochemical characterisation of whey protein isolate (WPI) and heat-treated WPI (HT-WPI).

(A) Native protein distribution determined by TSK high-performance liquid chromatography, (B) aggregation status determined by gel permeation chromatography, (C) sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and (D) native PAGE. RT, retention time; BLG,  $\beta$ -lactoglobulin; ALA,  $\alpha$ -lactalbumin; cGMP, glycomacropeptides; Lf, lactoferrin; BSA, bovine serum albumin.

**FIGURE 2:** Intraperitoneal sensitisation study. (A) Animal experimental design: Groups of eight Brown Norway rats were immunised three times, at day 0, 14 and 28, by intraperitoneal injections of whey protein isolate (WPI) or heat-treated WPI (HT-WPI), and sacrificed at day 35. (B) Product-specific IgE titres, (C) product-specific IgG1 titres, (D) binding strength between IgG1 and products, (E) competitive capacity of products by inhibitory IgG1 ELISA, and (F) immunoblotting based on sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) with pooled sera for detection of protein reactivity. Differences between the two blots are indicated by <. Analyses were performed on sera from sacrifice. Each symbol represents a single rat, with the exception of inhibitory ELISA where a symbol represents a group of rats and vertical lines indicate standard deviation of three independent technical replicates. Horizontal lines indicate mean value. Statistically significant differences between indicated groups are shown as \*\*:  $p \leq 0.01$ , \*\*\*:  $p \leq 0.001$ , ns: no statistically significant differences.

**FIGURE 3:** Oral sensitisation study. (A) Animal experimental design: Groups of 12 Brown Norway rats were gavaged with whey protein isolate (WPI) or heat-treated WPI (HT-WPI) three times per week for five weeks. At day 35, an oral challenge (OC), and on day 38, an ear swelling test (EST) were performed. (B) Product-specific IgE titres, (C) product-specific IgG1 titres, (D) binding strength between IgG1 and products, (E) competitive capacity of products by inhibitory IgG1 ELISA, (F) changes in core body temperature after OC, (G) acute allergic response from an EST, and (H) immunoblotting based on sodium dodecyl sulphate polyacrylamide gel electrophoresis with pooled sera for detection of protein reactivity. Differences between the two blots are indicated by <. Analyses were performed on sera from day 42. Each symbol represents a single rat, with the exception of inhibitory ELISA where a symbol represents a group of rats and vertical lines indicate standard deviation of three independent technical replicates. Horizontal lines indicate median (B-C) or mean (D, F-G) value. Statistically significant differences between indicated groups (B-D, F-G) or relative to naïve controls (G) are shown as \*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ , \*\*\*:  $p \leq 0.001$ , ns: no statistically significant differences.

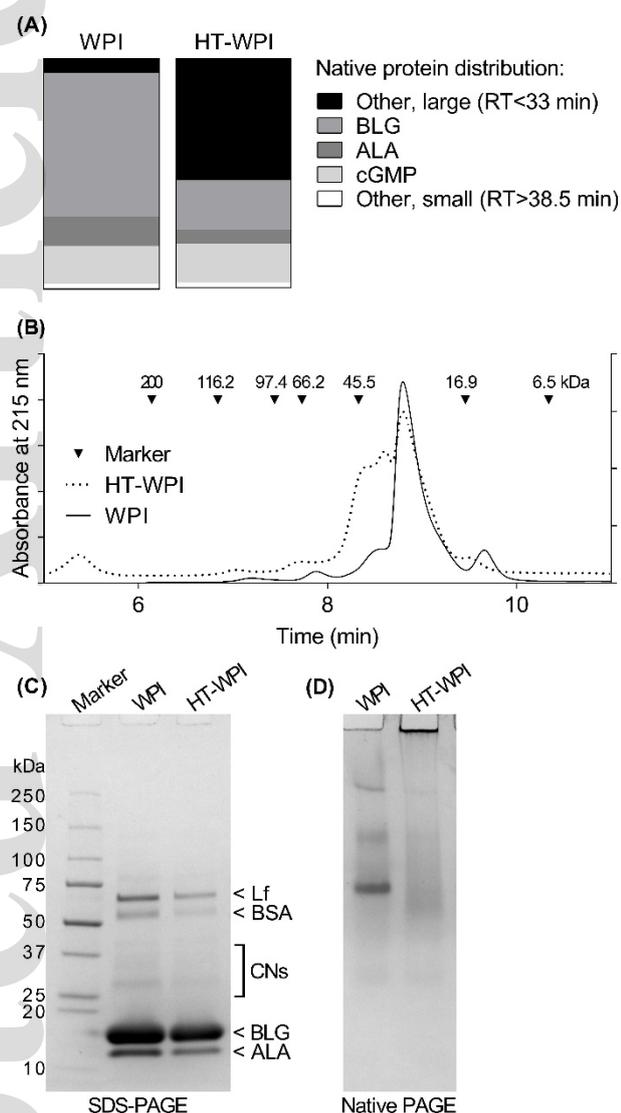
**FIGURE 4:** Primary prevention study. (A) Animal experimental design: Groups of eight Brown Norway rats were given whey protein isolate (WPI) or heat-treated WPI (HT-WPI) or water *ad libitum* in their drinking bottles for 21 days. After one week of rest, rats were post-immunised by intraperitoneal injection with WPI once a week for eight weeks. Rats were sacrificed one week after the last post-immunisation. (B) WPI-specific IgE titres and (C) WPI-specific IgG1 titres. Analyses were performed on sera from day 28, 56 and day 84 (after 0, 4 and 8 post-immunisations, respectively). Each symbol represents a single rat, and horizontal lines indicate group medians. Statistically significant differences relative to the water control group are shown as \*\*:  $p \leq 0.01$ , \*\*\*:  $p \leq 0.001$ , ns: no statistically significant differences.

**FIGURE 5:** Desensitisation study. (A) Animal experimental design: Groups of 12 Brown Norway rats were orally gavaged with whey protein isolate (WPI) three times per week for five weeks. WPI-sensitised rats were given water, WPI or heat-treated WPI (HT-WPI) *ad libitum* in their drinking bottles for 21 days. After one week of rest, the three groups of desensitised and one group of naïve rats were post-immunised by intraperitoneal injections with WPI once per week for three weeks. The day after the last post-immunisation, rats were subjected to an ear swelling test (EST). Blood samples were taken after sensitisation (day 42) after desensitisation (day 70) and after each post-immunisation (day 77, 84 and 91). (B) WPI-specific IgE titres, (C) WPI-specific IgG1 titres, and (D) acute allergic response from an EST. Each symbol represents a single rat and horizontal lines indicate mean values. Statistically significant differences relative to the water desensitisation control group (unframed) and relative to WPI desensitisation group (framed) are shown as \*:  $p \leq 0.05$ , p\*\*\*:  $p \leq 0.01$ , \*\*\*:  $p \leq 0.001$ , ns: no statistically significant differences.

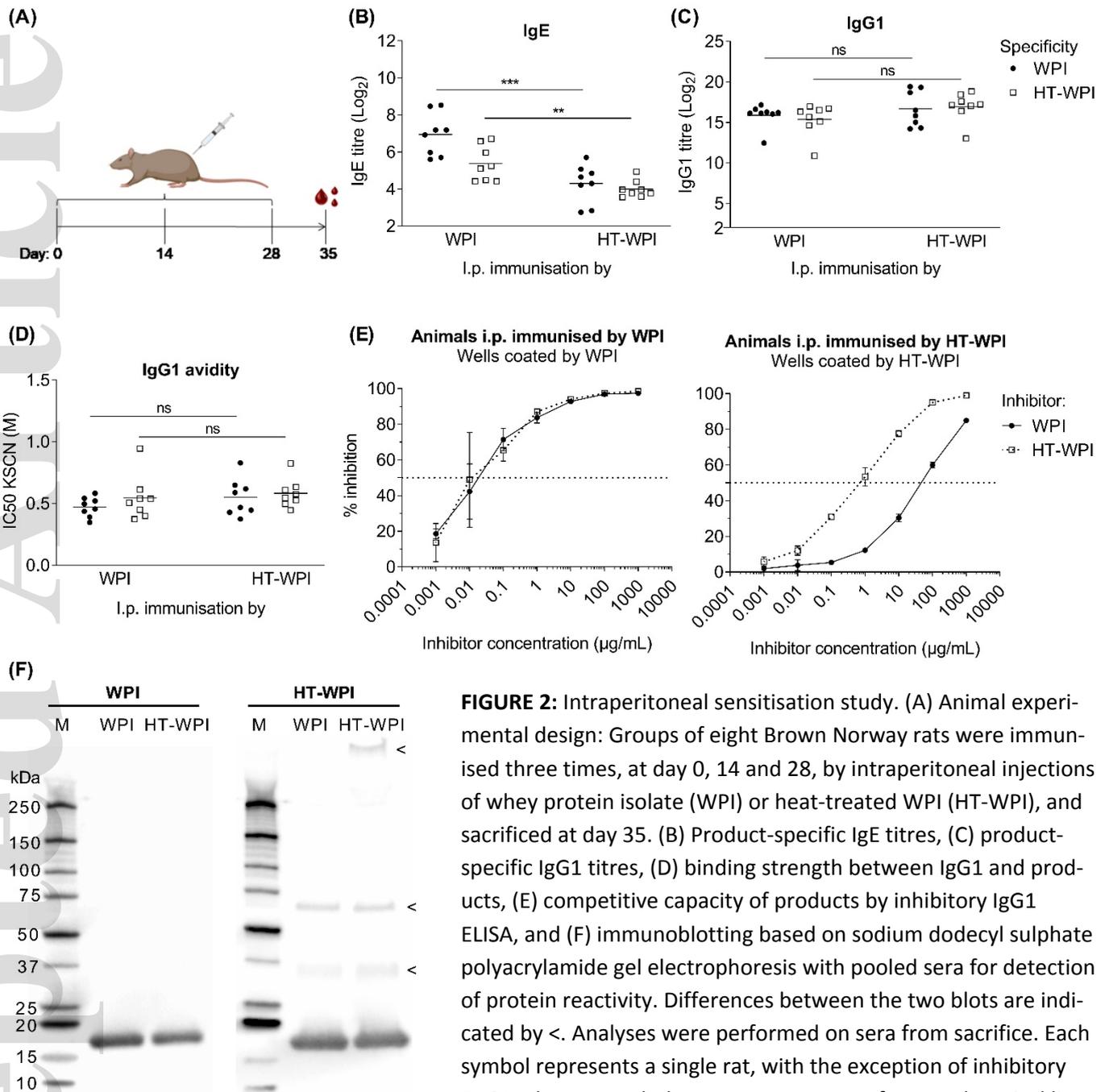
**FIGURE 6:** *In vivo* intestinal uptake. (A) Animal experimental design: Naïve Brown Norway rats were gavaged with either whey protein isolate (WPI) or heat-treated WPI (HT-WPI) and sacrificed at different time points from 15-90 min after dosing (four rats per treatment group per time point). (B) Mean relative distribution of  $\beta$ -lactoglobulin (BLG) between Peyer's patches (PP), lamina propria (LP), and epithelium (EPI) fractions per tissue weight.

**FIGURE 7:** *In vitro* epithelia transcytosis and protein uptake by dendritic cells. (A)  $\beta$ -lactoglobulin (BLG) concentrations in basolateral compartment of Caco-2 cells grown on semi-permeable membranes 24 hours after application of whey protein isolate (WPI) or heat-treated WPI (HT-WPI). (B) Mean fluorescent intensity and (C) fraction of fluorescein isothiocyanate positive (FITC+) murine bone marrow derived dendritic cell after incubation with FITC-labelled WPI or HT-WPI. Each symbol represents one replicate (A) or the mean of two replicates (B-C).

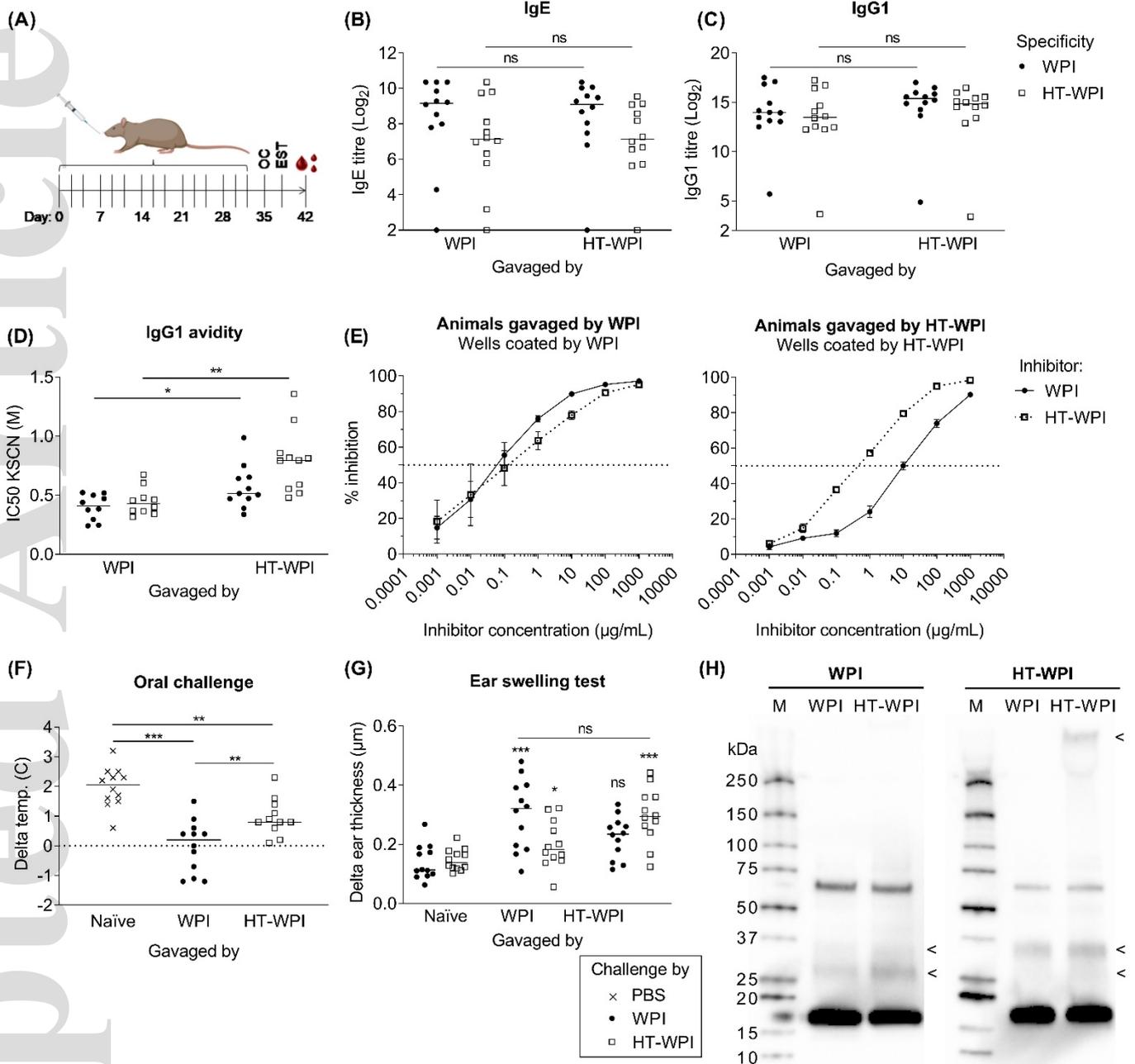
Appendix: Suggestion for arrangement of figure legends



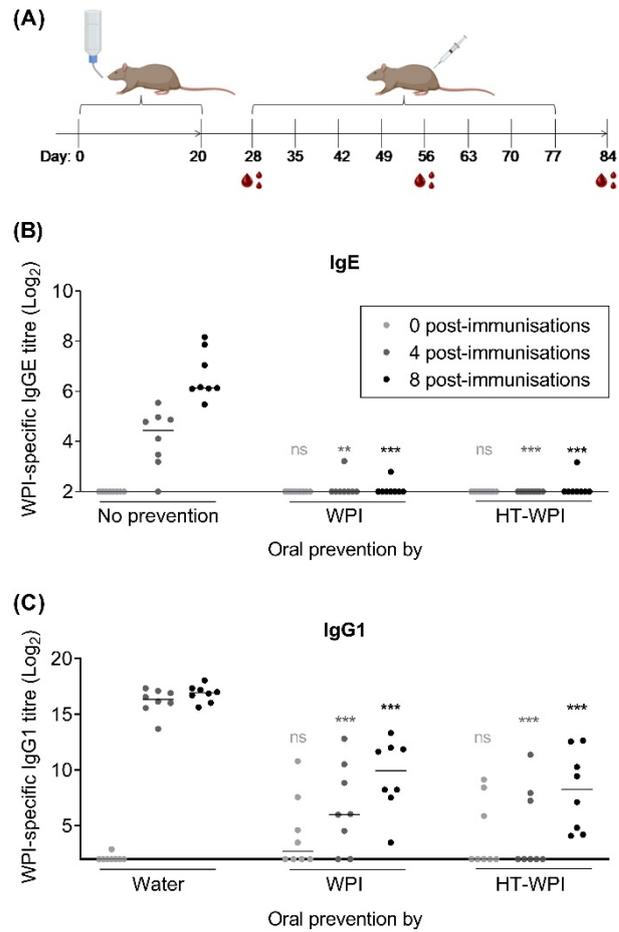
**FIGURE 1:** Physicochemical characterisation of whey protein isolate (WPI) and heat-treated WPI (HT-WPI). (A) Native protein distribution determined by TSK high-performance liquid chromatography, (B) aggregation status determined by gel permeation chromatography, (C) sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and (D) native PAGE. RT, retention time; BLG,  $\beta$ -lactoglobulin; ALA,  $\alpha$ -lactalbumin; cGMP, glycomacropeptides; Lf, lactoferrin; BSA, bovine serum albumin.



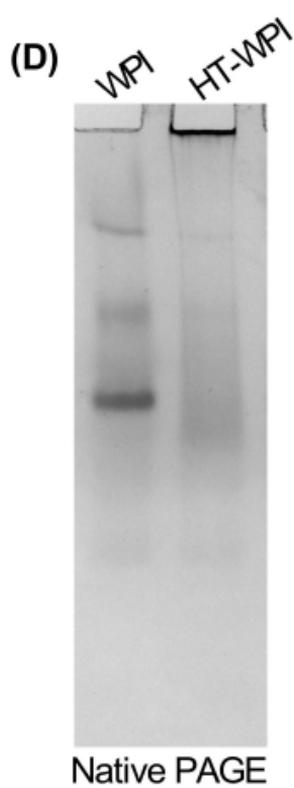
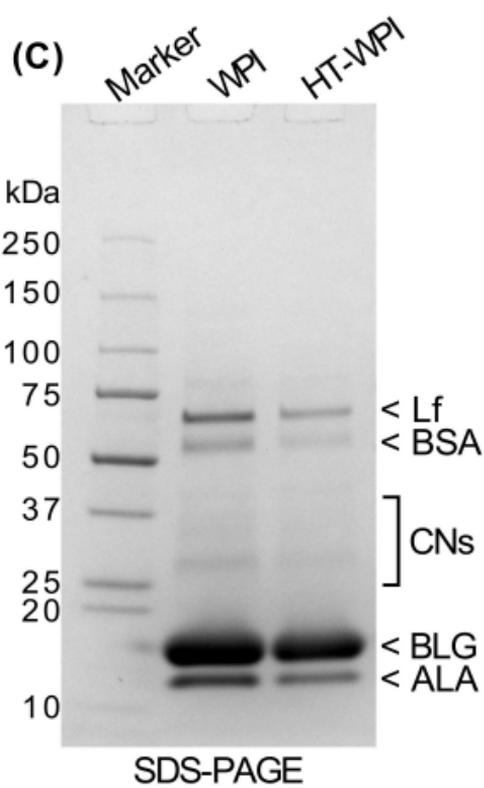
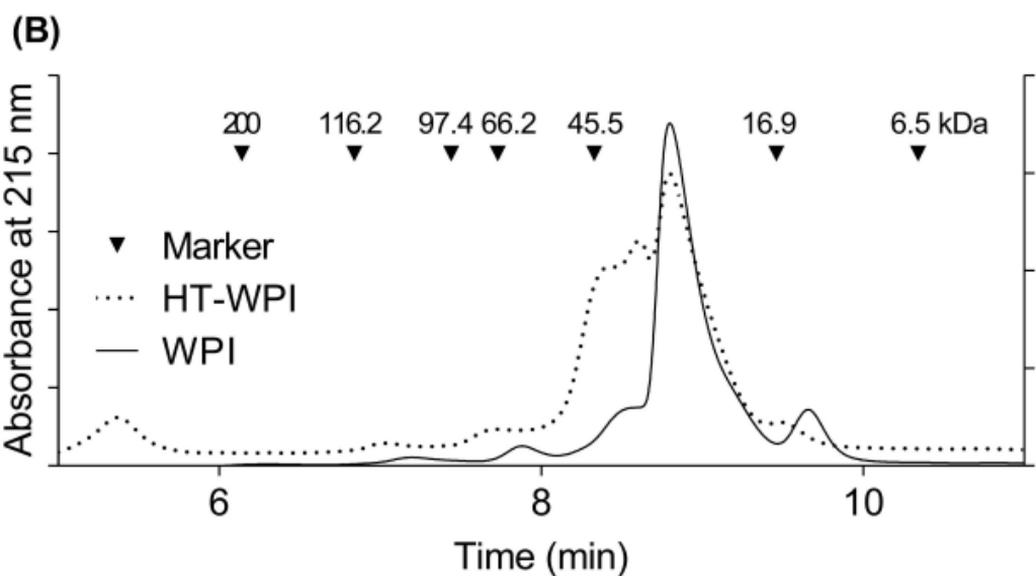
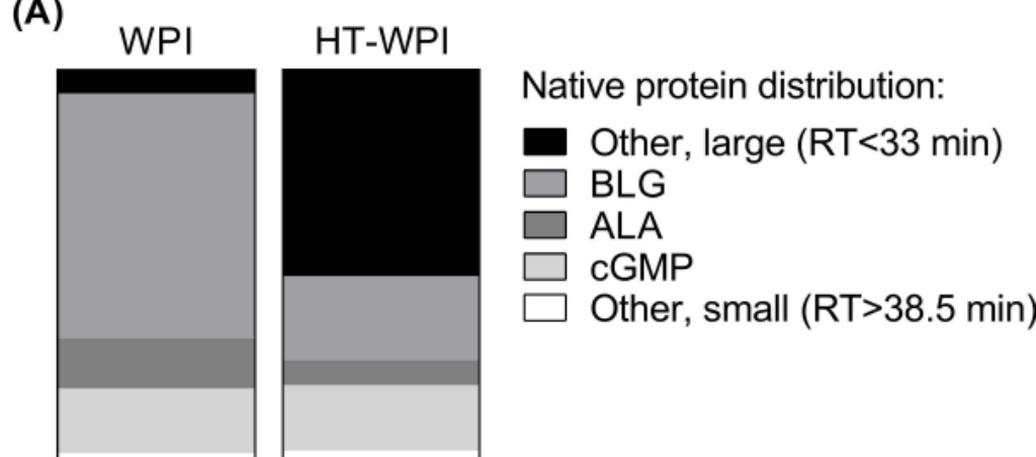
**FIGURE 2:** Intraperitoneal sensitisation study. (A) Animal experimental design: Groups of eight Brown Norway rats were immunised three times, at day 0, 14 and 28, by intraperitoneal injections of whey protein isolate (WPI) or heat-treated WPI (HT-WPI), and sacrificed at day 35. (B) Product-specific IgE titres, (C) product-specific IgG1 titres, (D) binding strength between IgG1 and products, (E) competitive capacity of products by inhibitory IgG1 ELISA, and (F) immunoblotting based on sodium dodecyl sulphate polyacrylamide gel electrophoresis with pooled sera for detection of protein reactivity. Differences between the two blots are indicated by <. Analyses were performed on sera from sacrifice. Each symbol represents a single rat, with the exception of inhibitory ELISA where a symbol represents a group of rats and vertical lines indicate standard derivation of three independent technical replicates. Horizontal lines indicate mean value. Statistically significant differences between indicated groups are shown as \*\*:  $p \leq 0.01$ , \*\*\*:  $p \leq 0.001$ , ns: no statistically significant differences.

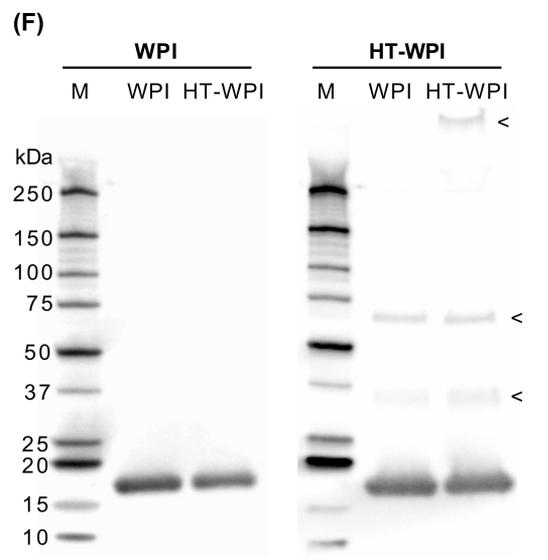
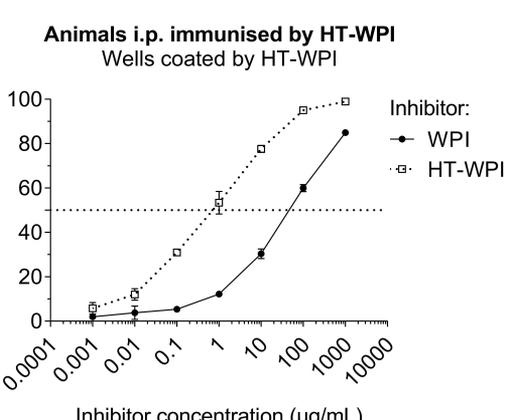
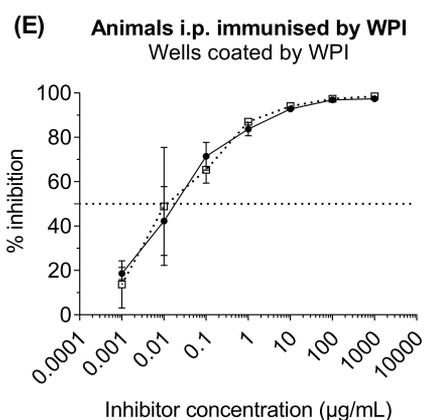
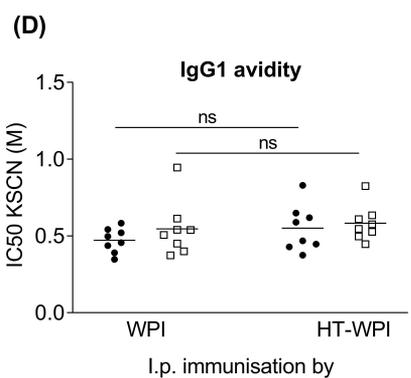
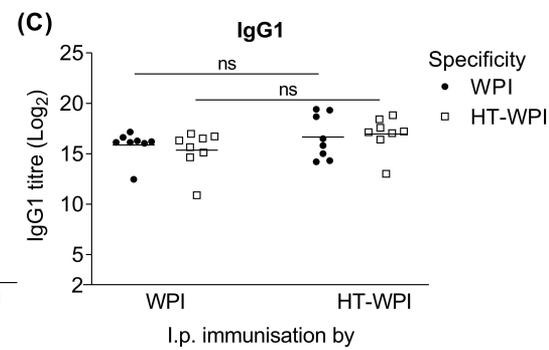
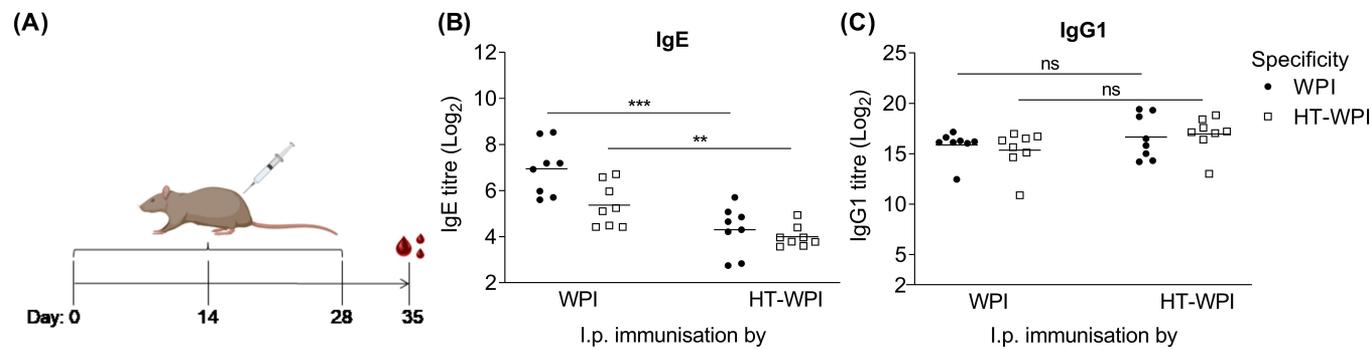


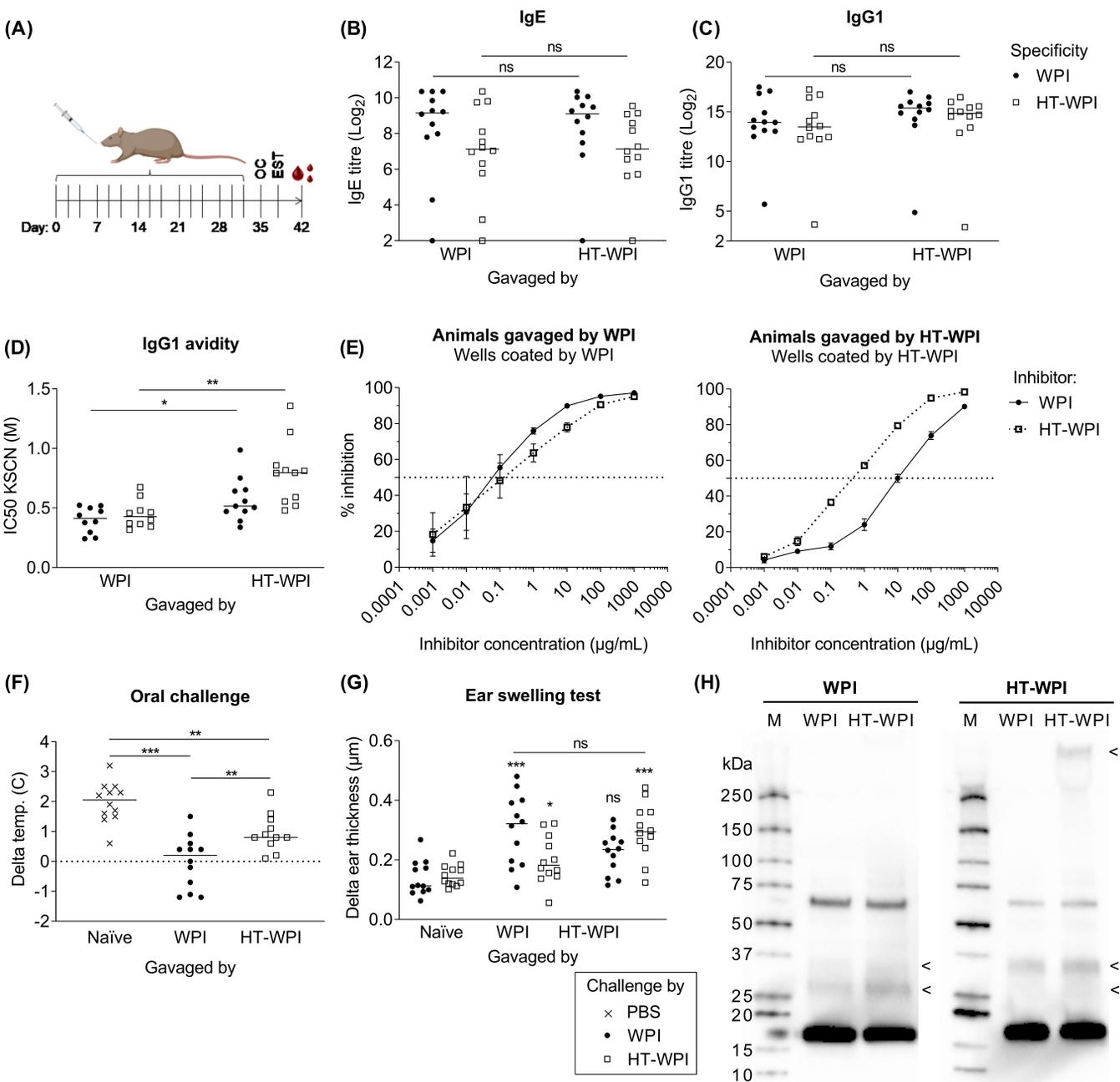
day 35, an oral challenge (OC), and on day 38, an ear swelling test (EST) were performed. (B) Product-specific IgE titres, (C) product-specific IgG1 titres, (D) binding strength between IgG1 and products, (E) competitive capacity of products by inhibitory IgG1 ELISA, (F) changes in core body temperature after OC, (G) acute allergic response from an EST, and (H) immunoblotting based on sodium dodecyl sulphate polyacrylamide gel electrophoresis with pooled sera for detection of protein reactivity. Differences between the two blots are indicated by <. Analyses were performed on sera from day 42. Each symbol represents a single rat, with the exception of inhibitory ELISA where a symbol represents a group of rats and vertical lines indicate standard deviation of three independent technical replicates. Horizontal lines indicate median (B-C) or mean (D, F-G) value. Statistically significant differences between indicated groups (B-D, F-G) or relative to naïve controls (G) are shown as \*: p < 0.05, \*\*: p < 0.01, \*\*\*: p < 0.001, ns: no statistically significant differences.

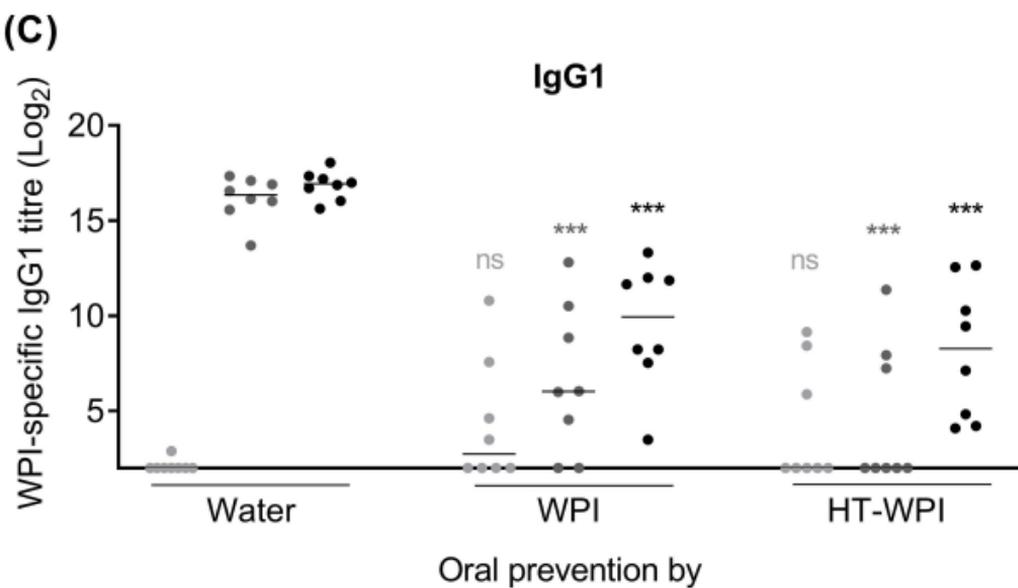
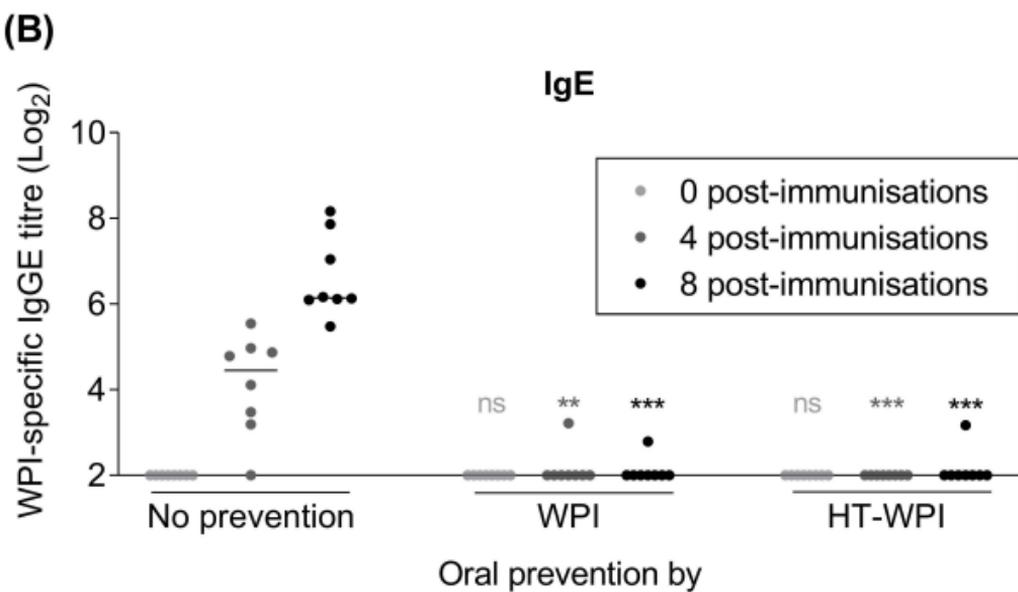
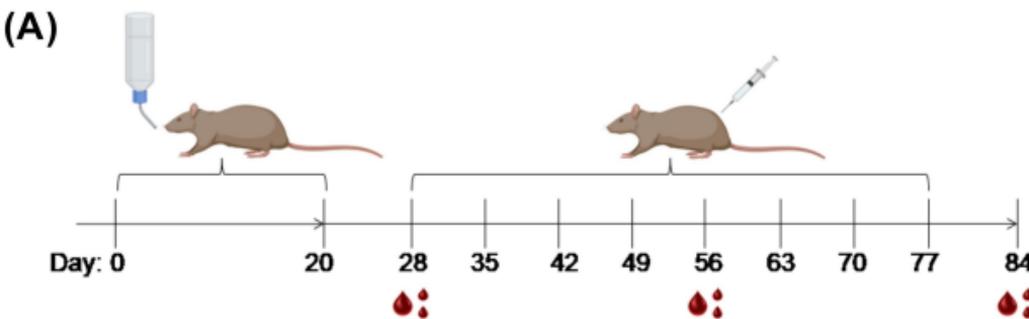


**FIGURE 4:** Primary prevention study. (A) Animal experimental design: Groups of eight Brown Norway rats were given whey protein isolate (WPI) or heat-treated WPI (HT-WPI) or water *ad libitum* in their drinking bottles for 21 days. After one week of rest, rats were post-immunised by intraperitoneal injection with WPI once a week for eight weeks. Rats were sacrificed one week after the last post-immunisation. (B) WPI-specific IgE titres and (C) WPI-specific IgG1 titres. Analyses were performed on sera from day 28, 56 and day 84 (after 0, 4 and 8 post-immunisations, respectively). Each symbol represents a single rat, and horizontal lines indicate group medians. Statistically significant differences relative to the water control group are shown as \*\*:  $p \leq 0.01$ , \*\*\*:  $p \leq 0.001$ , ns: no statistically significant differences.



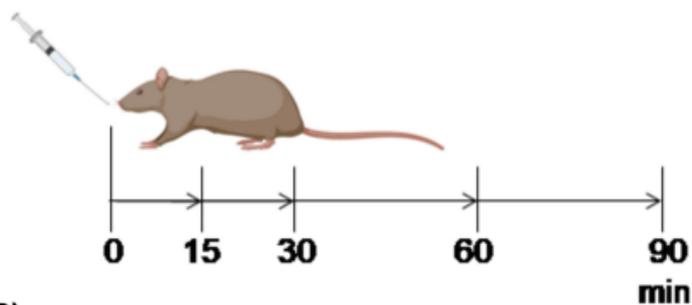




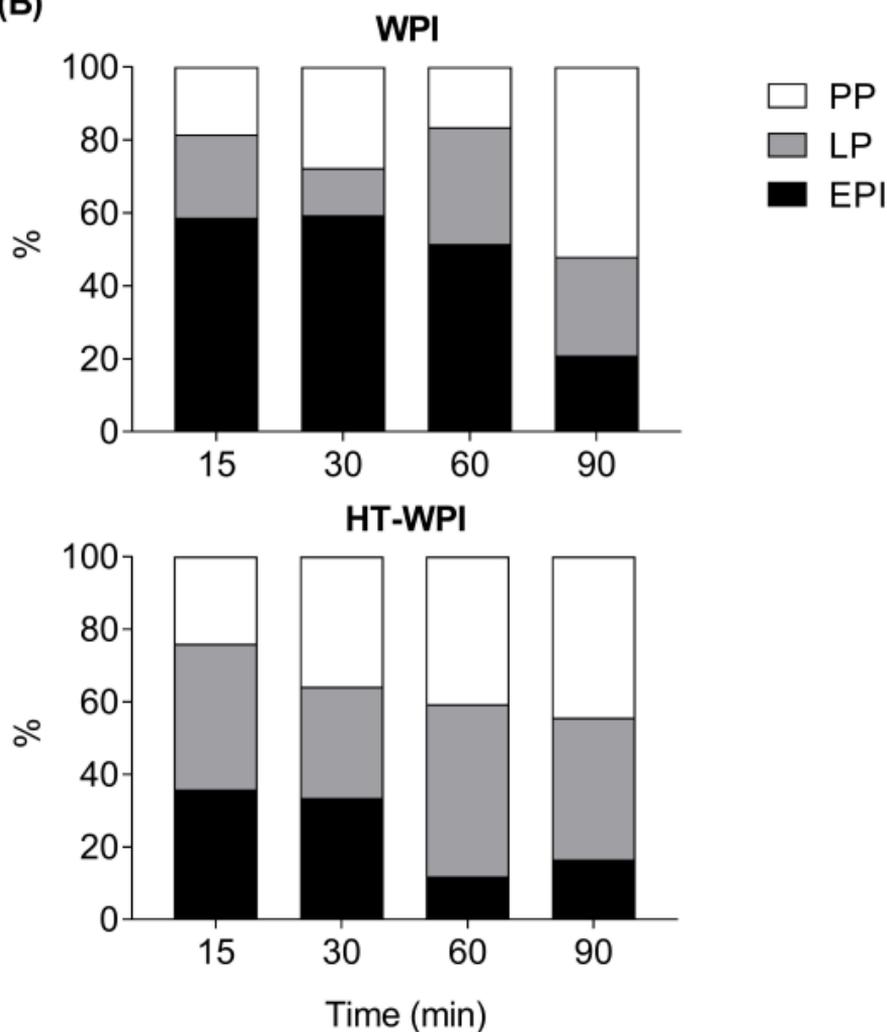


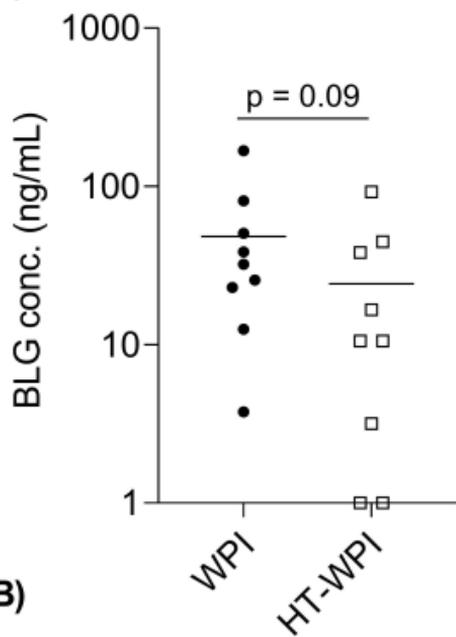
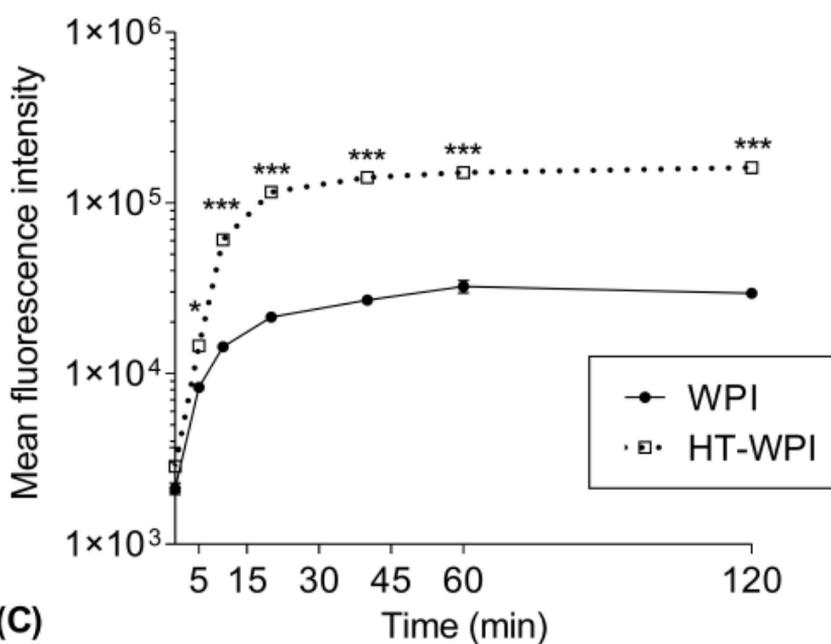


(A)



(B)



**(A)****(B)****(C)**