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Allergenicity evaluation of quinoa proteins – A study in Brown Norway rats

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ABSTRACT

The popularity of quinoa seeds has increased in the last decade due to their high nutritional value and natural gluten-free composition. Consumption of new proteins may pose a risk of introducing new allergies. In the present study the immunogenicity and sensitising capacity of quinoa proteins were assessed in a dose-response experiment in Brown Norway rats in comparison to proteins from spinach and peanut. Cross-reactivity between quinoa proteins and known allergens was evaluated by *in silico* analyses followed by analyses with 11 selected protein extracts and their anti-sera by means of ELISAs and immunoblotting. Further, an *in vitro* simulated gastro-duodenal digestion was performed. Quinoa proteins were found to have an inherent medium to high immunogenicity and sensitising capacity, being able to induce specific IgG1 and IgE levels higher than spinach but lower than peanut and elicit reactions of clinical relevance similar to peanut. Quinoa proteins were generally shown to resist digestion and retain capacity to bind quinoa-specific antibodies. Quinoa proteins were shown to be cross-reactive with peanut and tree nut allergens as high sequence homology and antibody cross-binding were demonstrated. Present study suggests that quinoa pose a medium to high level of allergenicity that should be further investigated in human studies.

1. Introduction

The seeds of quinoa (*Chenopodium quinoa* Willd.) have been consumed for thousands of years in the Andean region of South America (Repo-Carrasco et al., 2003), and have in the recent decade gained a profound popularity outside South America, especially as a substitute for wheat (Balize et al., 2015). Quinoa seeds are valued for their nutritional profile, with a high protein content of 11–18% comprising all essential amino acids (Angeli et al., 2020; Venlet et al., 2021), compared to a protein content of 7–10% for seeds of cereal crops such as rice, corn and millet (Charalampopoulos et al., 2002). Consumption of new proteins may pose a risk of introducing unexpected reactions from the immune system, including allergic reactions (Verhoeckx et al., 2019). To date, few cases of allergic reactions after consumption of quinoa have been reported, with the first case of anaphylaxis being described in France in 2009 (Astier et al., 2009). A similar case was described in the US (Hong et al., 2013), and a third case was reported in Italy confirming an IgE-mediated mechanism for quinoa-induced asthma (Guarnieri et al., 2019). Such allergic reactions may result from either *de novo* sensitisation and thereby induction of a new allergy or from cross-reactivity to known allergens in already allergic patients.

The major seed storage proteins of quinoa are 2S albumins and 11S globulins, constituting approximately 35% and 37% of the total protein content, respectively (Brinegar et al., 1996; Brinegar and Goundan, 1993). 2S albumins in quinoa are proteins with a molecular weight (MW) of 8–9 kDa, whereas 11S globulins are hexameric proteins with a

Abbreviations: BN, Brown Norway; DIG, digoxigenin; EST, ear swelling test; HRP, horseradish peroxidase; ip, intraperitoneal; MW, molecular weight; nsLTP, non-specific lipid transfer protein; OD, optical density; PVDF, polyvinylidene difluoride; SD, standard deviation; SGF, simulated gastric fluid; SMP, skimmed milk powder; RT, room temperature.

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MW of 320 kDa, of which each subunit consists of a pair of a basic (20–25 kDa) and an acidic (30–40 kDa) peptide covalently linked by a disulphide bond (Brinegar and Goundan, 1993; Thanapornpoonpong et al., 2008). Protein families such as the cupin superfamily, comprising vicilin (7S globulin) and legumin (11S globulin), and the prolamin superfamily, comprising 2S albumin as well as the non-specific lipid transfer protein (nsLTP), are known to contain a large number of allergens, and are families of proteins that may trigger severe allergic reactions (Radauer et al., 2008). Given that more than 70% of the total protein content of quinoa belongs to these important allergen protein families, alongside with the increased consumption in the Western world, makes it highly relevant to study the allergenicity of quinoa seeds.

Currently, no single risk assessment test can provide information about the allergenicity of a given protein. *In silico* analysis is a tool that for example can provide information about amino acid sequences and sequence homology with known allergens (Hayes et al., 2015). Based on the current guideline, a given protein should have a minimum of 35% sequence identity, over a sliding window of 80 amino acids, to be considered to pose a risk for inducing allergic reactions due to cross-reactivity (FAO/WHO, 2003). Yet, even though the threshold is set to 35%, it has been suggested that an allergic reaction caused by cross-reactivity requires more than 50–70% sequence identity (Aalberse, 2000). If *in silico* analysis suggests that a given protein can be recognised by IgE in already allergic patients, the next step in the allergenicity assessment would require *in vitro* or *in vivo* testing (Remington et al., 2018). For ethical reasons, *de novo* sensitisation cannot be studied in humans, thus animal models are a valuable tool for thorough allergenicity assessments. For evaluation of food allergy sensitisation, specific strains of mice and rats, genetically predisposed to develop allergy, are the most frequently used (Bøgh et al., 2016). The primary read-out for sensitisation is detection of allergen-specific IgE, often assessed concomitant with detection of specific IgG1, in the rodent models. For identification of reactive proteins, immunoblotting is an often used method (Castan et al., 2020). The aim of this study was to evaluate the inherent allergenicity of quinoa proteins as well as their potential cross-reactivity with known allergens, using the high IgE responder Brown Norway (BN) rat strain.

2. Material and methods

2.1. Products

White quinoa seeds purchased form Procudan (Kolding, Denmark) were ground to flour. A quinoa suspension was made for intraperitoneal (ip) solutions and a quinoa extract was made for ELISAs and gel electrophoresis. Peanut protein extract as control high-allergenic protein extract was made from peanut purchased from Morrisons market (Leeds, UK) and spinach protein extract as control low-allergenic protein extract was made from spinach leaves purchased from Grøntriget (Denmark) and used for ip solutions and ELISAs. Several protein extracts; peanut, hazelnut, walnut, almond, Brazil nut, birch pollen, and grass pollen were provided by ALK (Hørsholm, Denmark), whereas wheat (gluten) extract was kindly provided by Tereos (Alost, Belgium), cow's milk whey was kindly provided by Arla Foods Ingredients (Videbæk, Denmark), and soy protein extract was purchased from ADM (Chicago, IL, US). They were all included in the study for assessment of potential cross-reactivity.

2.1.1. Quinoa protein extraction

A quinoa suspension was prepared for ip dosing solutions for the animal experiment by suspending quinoa flour in phosphate buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4 in Milli Q water, pH 7.2). Further, a quinoa protein extract was made for ELISAs and gel electrophoresis by suspending quinoa flour in PBS containing 3% (w:v) NaCl. The suspension was placed on a magnetic stirrer for 2 h at 4 °C, followed by centrifugation at 4,500 *g* for 20 min at 4 °C. The supernatant was transferred to clean tubes and subsequently centrifuged at 12,000 *g* for 15 min at 4 °C. Protein concentration was measured in both the suspension and the extract using Pierce™ 660 nm Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, US) according to the manufacturer's protocol.

2.1.2. Peanut protein extraction

A peanut protein extract was prepared for ip dosing solutions for the animal experiment as described in Sztuk et al. (2023). In brief, defatted peanut flour (prepared from deskinned peanuts, grinded and extracted with hexane (5:1 w:w hexane to flour, repeated once)) was stirred in water for 1 h (10:1 w:w). The pH of the extraction mixture was maintained at 10 by the addition of 1 M NaOH and the solubilised protein collected by centrifugation (3000 *g*, 20 min, 20 °C). The pH of the solubilised protein supernatant was adjusted to 4.5 with 1 M HCl and incubated at 20 °C for 1 h. The precipitated protein was collected by centrifugation (3000 *g*, 5 min, 20 °C) and washed twice by resuspension, mixing and centrifugation (3000 *g*, 5 min, 20 °C) with 2 volumes of water (pH 4.5, adjusted with 1 M HCl). The resulting material was freeze dried and finely ground to produce a powdered peanut extract. Protein concentration was determined by the Pierce™ BCA Protein Assay Kit (Fisher Scientific UK Ltd, Leicestershire, UK) according to the manufacturer's protocol.

2.1.3. Spinach protein extraction

A spinach protein extract was prepared for ip dosing solutions for the animal experiment. A total of 250 g of fresh spinach leaves were mixed with 500 mL of PBS in a leaf-to-PBS ratio of 1:2 (w:w). The mixture was mechanically separated in a laboratory-scale twin-screw-press (Angelia 8500S juicer ,Angel Juicer Co., Queensland, Australia), at low turning speed, to obtain a green juice and press-cake. The green juice was subsequently heat-treated for 20 s at 60 °C using a heat exchanger (Ultraterm-TFT-200, J.P. Selecta, Spain) prior to centrifugation at 12,000 *g* for 10 min at room temperature (RT) to remove insoluble compounds, resulting in separation of a green pellet and a cleared juice. The cleared green juice was acidified with 1 M citric acid solution to pH 4.0 to precipitate the soluble leaf proteins. The precipitated proteins were harvested by centrifugation at 12,000 *g* for 10 min at RT and subsequently freeze dried in a Telstar LyoQuest freeze drier (Azbil Co., Japan) at −30 °C, 0.500 mBar for around 24 h and milled in a Pulverisette tabletop ball-mill, at 600 RPM (Fritsch, Germany). The protein concentration was quantified according to the total nitrogen concentration using an elemental analyzer (FlashSmart, 2000; Thermo Scientific), based on the total nitrogen concentration and a conversion factor of 6.25 for nitrogen to protein. The spinach extract was kept at RT in a tightly closed container until further analysis.

2.2. Animals

BN rats, from the in-house breeding colony at the National Food Institute, Technical University of Denmark, were housed 2–4 per cage in macrolon cages with a 12-h light/dark cycle at a temperature of 22 ± 1 °C, with a relative humidity of 55 ± 5%. Rats were bred and raised for more than 10 generations on an in-house diet based on rice flour, potato protein and fish meal as protein sources, as previously described (Bøgh et al., 2009), with the exception of maize flour being substituted with rice flour. Diet and water were given *ad libitum*. Rats were inspected twice a day and body weights recorded weekly. Ethical approval was given by the Danish Animal Experiments Inspectorate and the authorisation number given 2015-15-0201-00553-C1 and 2020-15-0201-00500-C1. The experiments were overseen by the Institute's in-house Animal Welfare Committee for animal care and use.

2.2.1. Animal experiment

Male and female BN rats with an age of 5–10 weeks were allocated into groups of 6–8 rats. Rats were weekly ip immunised for a total of 5

times, at Day 0, 7, 14, 21 and 28 with 0.5 mL PBS alone (as control), or with 4 different doses, 12.5 μg, 50 μg, 125 μg, or 500 μg, of quinoa, peanut, or spinach protein in PBS without the use of adjuvant (Fig. 1). At Day 33 or 34, rats were subjected to an ear swelling test (EST) with the corresponding protein as the rats were ip immunised with as previously described (Locke et al., 2022). In brief, rats were anaesthetised with hypnorm-dormicum and the thickness of the ear was measured using a digital micrometer, at the same spot prior to and 15 min after intradermal injection of 10 μg protein in 20 μL PBS. At Day 35, rats were euthanised by exsanguination using CO_2/O_2 as anaesthesia. Blood was collected prior to immunisation at Day 14, 21, 28, and at sacrifice, converted into serum and stored at −20 °C until analysis. In order to test for potential cross-reactivity, anti-sera towards soy, peanut, hazelnut, birch pollen, walnut, cashew nut, almond, Brazil nut, grass pollen, wheat gluten and cow's whey were obtained in-house from BN rats ip immunised with 50 μg protein as described above.

2.3. In vitro gastro-duodenal digestion

In vitro simulated gastro-duodenal digestion of quinoa flour (1.5 g) was performed at 37 °C, essentially as described by Moreno et al. (2005). In brief, the gastric digestion phase was performed in simulated gastric fluid (SGF; 150 mM NaCl, pH 2.5) with porcine pepsin (activity of ~3300 U/mg of enzyme; EC 3.4.23.1, Sigma-Aldrich. Saint-Louis, MO, US) added to give an activity of ~165 U per mg of quinoa protein at a ratio of quinoa protein:pepsin of 1:0.05 (w:w). Aliquots were taken at 0, 1, 2, 5, 10, 20, 40, 60 and 120 min for further analysis, and the gastric digestion was stopped by raising pH to 7 by adding 40 mM NH_4HCO_3 (Sigma-Aldrich). Control samples with no added pepsin were also prepared. The duodenal digestion was performed by mixing samples from 2 h of gastric digestion with: 0.5 M Bis-Tris pH 6.5; a bile salt mixture containing 0.125 M of sodium taurocholate (Sigma-Aldrich) and 0.125 M of glycodeoxycholic acid (Sigma-Aldrich); trypsin (activity of 13,800 U/mg; EC 3.4.21.4, Sigma-Aldrich) and α-chymotrypsin (activity of 40 U/mg; EC 3.4.21.1, Sigma-Aldrich) in 0.15 M NaCl at pH 6.5. The solution was prepared to give an activity of ~30.7 U trypsin and 0.36 U chymotrypsin per mg of quinoa protein at a ratio of quinoa protein: trypsin:chymotrypsin of 1:0.0022:0.008 (w:w:w). Aliquots were taken at 0, 1, 2, 5, 10, 15 and 30 min for further analysis, and the duodenal digestion was stopped by adding Bowman-Birk trypsin-chymotrypsin inhibitor from soybean (Sigma-Aldrich) at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix.

2.4. ELISAs

Various ELISAs were performed in order to analyse specific antibody responses and assess the inherent allergenicity and immunogenicity of quinoa proteins in comparison to the high-allergenic peanut proteins and the low-allergenic spinach proteins as well as the level of cross-reactivity between quinoa proteins and a range of selected food and pollen protein extracts. General for all ELISAs were that plates were incubated at RT in the dark on a shaking table, unless otherwise depicted. Between each step, plates were washed five times in PBS-T (0.01% (w:v) Tween-20 (Sigma-Aldrich) in PBS). Before visualisation of the reaction, plates were additionally washed twice in running tap water and subsequently incubated for 12 min with 100 μL/well of TMB-one™ (Kem-En-Tec Diagnostics, Taastrup, Denmark). The reaction was stopped with 100 μL/well of 0.2 M H_2SO_4. Absorbance was measured at 450 nm with a reference wavelength of 630 nm. Detection limits were determined as the mean absorbance for a negative control serum sample plus three times the standard deviation (SD).

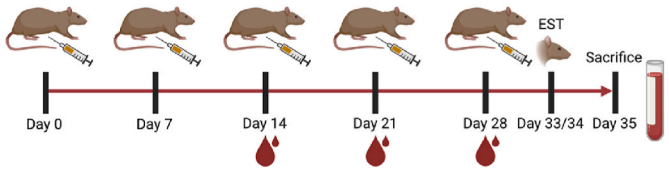


Fig. 1. Animal experimental design. Groups of 6–8 Brown Norway rats were immunised 5 times, at Day 0, 7, 14, 21 and 28 by intraperitoneal injection of either PBS (control), or with 12.5 μg, 50 μg, 125 μg or 500 μg of protein in spinach protein extract, quinoa flour suspension or peanut protein extract. An ear swelling test (EST) was performed on Day 33 or 34, and rats were euthanised at Day 35. Blood samples were collected throughout the study. Figure created with BioRender.com.

2.4.1. IgG1 ELISA for protein extracts

Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 100 μL/well of 2 μg/mL of quinoa, peanut or spinach protein extract in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Plates were incubated for 1 h with 50 μL/well of 2-fold serial diluted serum samples from rats immunised with the individual protein extracts in PBS-T starting at 1:8. Subsequently, plates were incubated for 1 h with 100 μL/well of the secondary antibody, horseradish peroxidase (HRP)-labelled mouse anti-rat IgG1 (Southern Biotechnology, Birmingham, AL, US) diluted 1:20,000 in PBS-T. Results are expressed as $\log_2$ titres and defined as the interpolated dilution of the given serum sample leading to the mean optical density (OD) for the negative control serum plus three times the SD corresponding to an OD of 0.1. IgG1 ELISAs for evaluation of cross-reactivity were performed similarly.

2.4.2. IgG1 ELISA for digestion products

Maxisorp 96-well plates (Nunc) were coated overnight at 4 °C with 100 μL/well of 5 μg/mL of either quinoa protein extract in SGF, extract from gastric digestion at time 0, extract from after gastric digestion (120 min) or extract from after gastro-duodenal digestion (30 min), all diluted in carbonate buffer. Plates were incubated for 1 h with 50 μL/well of 2-fold serial diluted serum samples from rats immunised with 50 μg quinoa protein in PBS-T starting at 1:8. Afterwards, the procedure was as described above.

2.4.3. IgE ELISAs for protein extracts

Maxisorp 96-well plates (Nunc) were coated overnight at 4 °C with 100 μL/well of 0.5 mg/mL mouse anti-rat IgE (HydriDomus, Nottingham, UK) in carbonate buffer. Plates were blocked for 1 h at 37 °C with 200 μL/well of 5% (v:v) rabbit serum (BioWest, Nuaillé, France) for quinoa, of 3% (v:v) rabbit serum (BioWest) for peanut or of 3% (w:v) skimmed milk powder (SMP, BCCB3332, Sigma) for spinach in PBS-T. After blocking, plates were incubated for 1 h with 50 μL/well of 2-fold serial diluted serum samples from rats immunised with the individual protein extracts in PBS-T starting at 1:8. Subsequently, plates were incubated with 50 μL/well of 0.2 μg/mL digoxigenin (DIG)-coupled protein extract (coupled 10:1) diluted in 5% (v:v) rabbit serum for quinoa, in 3% (v:v) rabbit serum for peanut or in 3% (w:v) SMP for spinach in PBS-T. Afterwards, plates were incubated for 1 h with 100 μL/well of HRP-labelled sheep-anti-DIG-POD (Roche Diagnostics GmbH, Mannheim, Germany) diluted 1:1,000 in PBS-T for quinoa and peanut or in PBS-T containing 3% (w:v) SMP for spinach. Results are expressed as $\log_2$ titres and defined as the interpolated dilution of the given serum sample leading to the mean OD for the negative control serum plus three times the SD.

2.4.4. IgG1 inhibition ELISA

In order to examine the competitive capacity for antibody binding between quinoa and the individual potential cross-reacting protein extracts, IgG1 inhibition ELISAs were performed. Plates were coated in the same manner as for the IgG1 ELISA. The ELISAs were performed on sera pooled group-wise. Pooled sera were diluted in PBS-T to reach an OD of around 1 in the absence of inhibitor. Sera were pre-incubated for 1 h

with 10-fold serial diluted protein extracts (0–1,000 μg/mL) as inhibitor. After pre-incubation of sera/inhibitor mixture, duplicates of 50 μL/well with the sera/inhibitor mix were added to the plates and incubated for 1 h. Subsequently, this assay followed the same procedure as for IgG1 ELISAs previously described. The inhibition ELISA was performed three times, and results are expressed as percentage of inhibition against concentration of inhibitor.

2.5. In silico analyses

Bioinformatics tools were used to identify quinoa protein sequences. *Chenopodium quinoa* was typed as organism and a search for protein sequences were performed using the NCBI database (National Library of Medicine, 2000). An advanced search was performed to identify albumins, globulins, profilins, oleosins and nsLTPs. Amino acid sequences of the identified proteins were compared with sequences of known allergens using AllergenOnline (Goodman et al., 2016). A full alignment and an 80mer sliding window alignment were performed.

2.6. SDS-PAGE and immunoblotting

Protein extracts or digestion products were separated under either reduced or non-reduced conditions in 4–20% Tris-Glycine Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad, Hercules, CA, US) according to the manufacturer's instructions. Proteins were visualised by Bio-Safe Coomassie Stain (Bio-Rad) using the Imager ChemiDoc XRS+ (Bio-Rad). For peptide analysis, digestion samples were separated under reducing conditions on a 16.5% Mini-PROTEAN® Tris-Tricine Gel (Bio-Rad) according to the manufacturer's instructions and peptides visualised by Bio-Safe Coomassie Stain (Bio-Rad) using the Imager ChemiDoc XRS+ (Bio-Rad).

After SDS-PAGE, proteins from the quinoa extract were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using the trans-blot turbo system (Bio-Rad). After blocking with 5% (w:v) egg white from chicken (Sigma-Aldrich) in PBS-T for 1 h at RT, membranes were washed with PBS-T (0.05% (v:v) Tween-20 (Sigma-Aldrich) in PBS). Membranes were incubated overnight at 4 °C with pooled rat sera raised against either walnut (1:100), hazelnut (1:200), soy, almond, peanut (1:500), cashew nut, Brazil nut, birch pollen (1:750), grass pollen (1:1,000) or quinoa (1:20,000) diluted in the blocking solution. After washing, specific IgG1 binding was detected with HRP-labelled mouse anti-rat IgG1 diluted in blocking solution (1:15,000 for almond, 1:30,000 for quinoa or 1:10,000 for the rest of the samples) for 1.5 h at RT. StrepTacin-HRP conjugate (1:15,000) (Bio-Rad) was used for detection of Precision Plus Protein™ Unstained Standard (Bio-Rad) according to the manufacturer's instructions. Membranes were washed, incubated with peroxidase substrate (Clarity™ Western ECL Substrate, Bio-Rad) and photographed using Imager ChemiDoc XRS+ (Bio-Rad).

Further, after SDS-PAGE, proteins from soy, peanut, hazelnut, birch pollen, walnut, cashew nut, almond, Brazil nut and grass pollen extracts were transferred to a PVDF membrane as described above. Membranes were incubated with pooled BN rat sera raised against quinoa and diluted in the blocking solution overnight at 4 °C as follows: walnut (1:100), soy (1:400), hazelnut, almond, peanut (1:500), cashew nut, Brazil nut, grass pollen (1:750), walnut or birch pollen (1:1,000). Specific IgG1 binding was detected as described above (1:10,000 for all samples except for almond and peanut 1:20,000) for 1.5 h at RT.

In addition, after SDS-PAGE, protein samples collected prior to digestion, after gastric digestion (0 and 120 min) and after gastro-duodenal digestion (0 and 30 min) were transferred to a PVDF membrane as described above. Membranes were incubated with pooled rat sera raised against quinoa (1:10,000 for gastric digested samples and 1:2,000 for gastro-duodenal digested samples) for protein analysis, or 1:1,000 (for gastric digestion) and 1:200 (for gastro-duodenal digestion) for peptide analysis. Sera were diluted in the blocking solution overnight at 4 °C. Specific IgG1 binding was detected as described above (1:15,000 for protein analysis and 1:5,000 for peptide analysis).

2.7. Statistical analysis

Graphs, curve analyses and statistical analyses were performed using GraphPad Prism v8.4 (San Diego, CA, US). Curves obtained from inhibition ELISA were compared by one-way ANOVA. Differences in antibody levels or ear swelling responses between groups were evaluated using non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test. To determine the significance between the association of dose and the level of IgG1 or IgE the non-parametric Spearman's correlation was applied. Asterisks indicate statistically significant differences between the given groups: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$.

3. Results

3.1. Medium to high immunogenicity and sensitising capacity of quinoa proteins

To assess the immunogenicity and sensitising capacity of quinoa proteins a dose-response study was conducted, where the levels of specific IgG1 and IgE raised against quinoa were compared to the levels of specific IgG1 and IgE raised against the known low-allergenic spinach and the known high-allergenic peanut, in a BN rat model of food allergy. As expected, peanut was shown to have an inherent high immunogenicity and sensitising capacity, whereas spinach was shown to have an inherent low immunogenicity and sensitising capacity, showing statistically significant lower antibody levels, irrespectively of type of antibody, dose and amount of ip immunisations (Fig. 2). Quinoa was shown to have an inherent medium to high immunogenicity with a clear and statistically significant dose-response relationship after both two, three, four and five ip immunisations, where increasing doses resulted in higher IgG1 levels (Fig. S1A). Overall, quinoa was shown to have an inherent immunogenicity higher than that of spinach but lower than that of peanut. Yet, in general the higher the doses of quinoa and the more ip immunisations the lesser the differences between the levels of quinoa- and peanut-specific IgG1 (Fig. 2). Similarly, quinoa was shown to have a higher sensitising capacity than spinach but a lower sensitising capacity than peanut (Fig. 2). Yet, whereas quinoa was shown to most closely resemble the sensitising capacity of spinach after few ip immunisations, quinoa seemed more closely to resemble the sensitising capacity of peanut upon further ip immunisations. Interestingly, a dose-response related sensitising capacity with higher doses promoting higher levels of IgE was seen for quinoa upon two to four ip immunisations but reversed after five ip immunisation with higher doses of quinoa providing lower IgE levels similar to what was seen for peanut already after three ip immunisations (Fig. S1B).

To assess the functionality of the specific IgE raised in the rats and thus the clinical relevance of the sensitisation, an *in vivo* ear swelling test was performed with intradermal injection of quinoa protein in the ear (Fig. 2E). The ear swelling test confirmed the functionality of the IgE raised in the rats ip immunised with quinoa and peanut contrary to IgE raised in spinach. The three highest doses of quinoa and all four doses of peanut induced specific IgE of a clinical relevance to a statistically significant degree when compared to the control PBS. Interestingly, no differences in the clinical relevance of the induced allergy were observed between quinoa and peanut.

3.2. Immune reactive proteins in quinoa

A search for proteins assigned to the species *Chenopodium quinoa* resulted in 55,440 hits in the NCBI database when adjusted for identical protein groups. A more detailed search was performed in order to investigate whether proteins belonging to important allergenic protein families such as the albumins, globulins, profilins, oleosins and nsLTPs

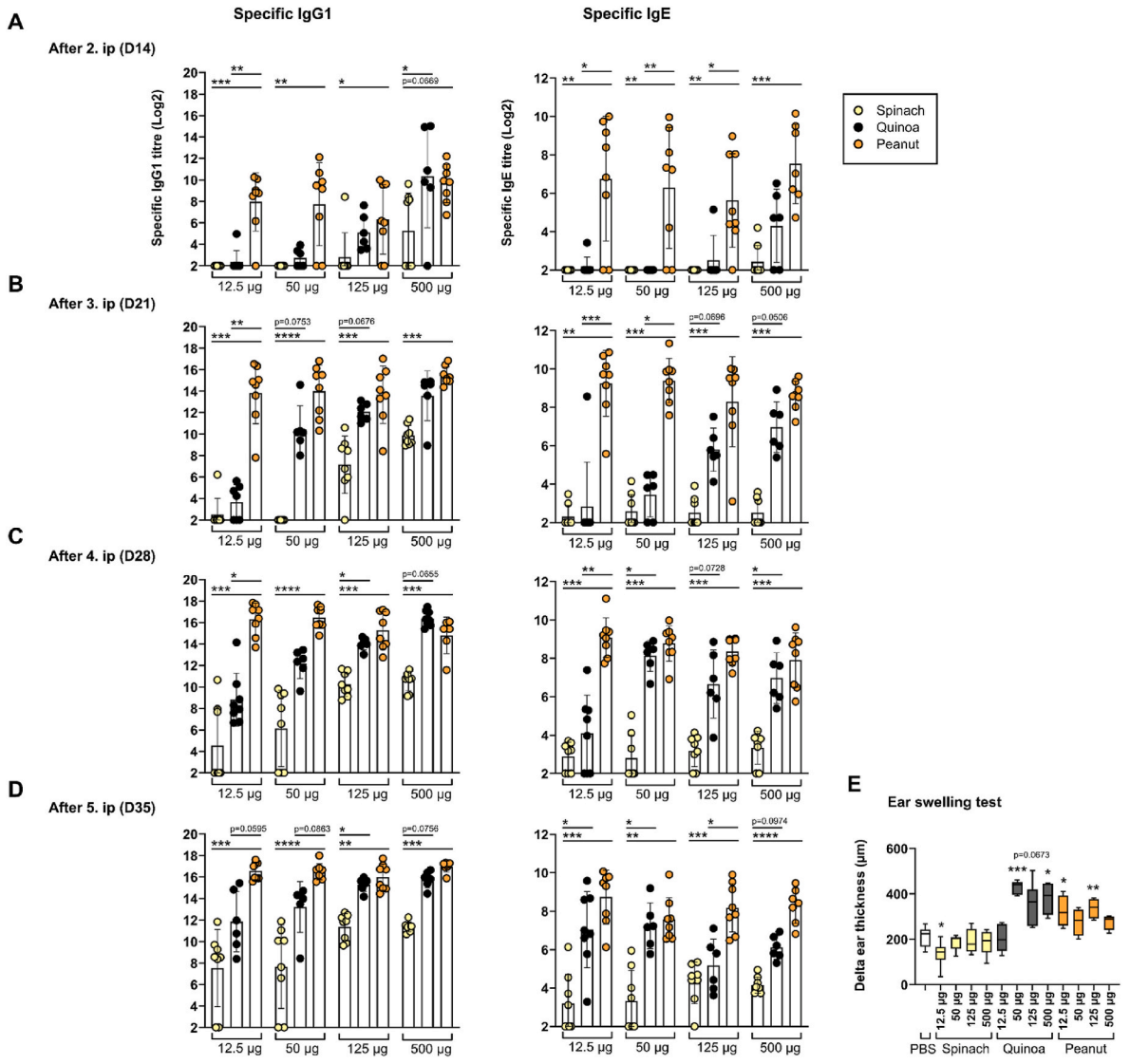


Fig. 2. Comparison of immunogenicity and sensitising capacity between spinach, quinoa and peanut protein. Spinach, quinoa and peanut-specific IgG1 and IgE repsonses presented as titres (log2) after (A) the 2nd immunisation, (B) the 3rd immunisation, (C) the 4th immunisation, or (D) the 5th immunisation. (E) Acute allergic response from an ear swelling test indicating clinical relevance of the sensitisation. Horizontal lines indicate median, and each symbol represents a single rat. Statistically significant differences between the indicated groups (A, B, C, D) or between the PBS control group and individual groups (E) are shown as.: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

are present in quinoa. Three amino acid sequences could be identified as 2S albumins, three as 7S globulins, 11 as 11S globulins, two as profilins, nine as oleosins, and 12 as nsLTPs. To investigate reactive proteins in quinoa SDS-PAGE was performed. In reduced condition, a range of proteins between 10 kDa and 100 kDa with several pronounced bands were observed (Fig. 3A), whereas in non-reduced condition a range of proteins between 10 kDa and 250 kDa were seen (Fig. 3B). Slightly fewer intense bands were observed in the non-reduced gel compared to the reduced gel. Yet, in both gels a band around a MW of 50 kDa was observed; likely the 7S globulin, as well as a lower MW band around 10 kDa; likely the 2S albumin. Bands probably corresponding to 11S globulin could be observed with a basic (20–25 kDa) and an acidic (30–40 kDa) subunit in the reduced gel, whereas in the non-reduced gel, the 11S globulin was seen as one band just below 50 kDa. Immunoblotting was performed in order to evaluate the IgG1 reactivity in serum from rats immunised with the quinoa proteins. Whereas the immunoblot revealed several faint reactive bands, one dominant reactive band was observed with a MW of around 25 kDa, likely corresponding to the basic subunit of 11S globulin (Fig. 3C).

3.3. Low digestibility of quinoa proteins

Simulated gastro-duodenal digestion was performed to assess the resistance of the quinoa proteins when exposed to *in vitro* gastric and duodenal digestion. Evaluating the gastric digestion at pH 2.5 with pepsin for up to 120 min, showed that several of the quinoa proteins remained intact throughout the digestion process (Fig. 4A). Yet, it could be observed that during the digestion more low MW peptides emerged on the gel, as well as a high MW band around 100 kDa. Additionally, the band around 20 kDa seemed to be reduced during the digestion phase. Evaluating the gastro-duodenal digestion at pH 6.5, showed that a large amount of the proteins still remained intact (Fig. 4B). The amount of low MW peptides was slightly increased, whereas the high MW band around 100 kDa seemed to be reduced after 30 min. The small increase in low MW peptides and the disappearance of the band around 20 kDa could clearly be observed and was further confirmed by the peptide gel (Fig. 4C). It was clearly demonstrated that both the gastric as well as the gastro-duodenal digests retained ability to react with quinoa-specific IgG1, though in a statistically significant reduced manner compared to

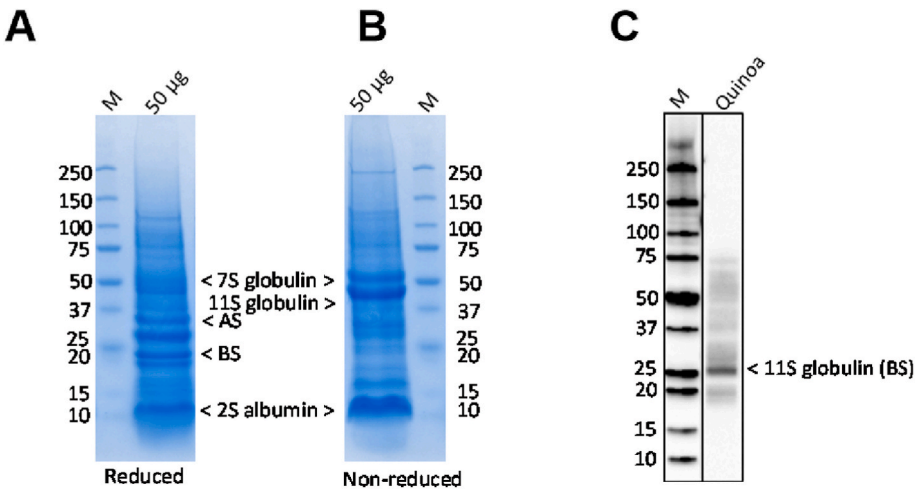


Fig. 3. Proteins in quinoa. (A) Reduced SDS-PAGE with 50 µg quinoa protein extract. (B) Non-reduced SDS-PAGE with 50 µg quinoa protein extract. (C) Immunoblot from reduced SDS-PAGE with quinoa protein extract incubated with pooled sera from rats immunised 5 times with 50 µg quinoa protein. Suggested proteins are indicated on the gels and blot. AS: acidic subunit of 11S globulin, BS: basic subunit of 11S globulin, M: marker.

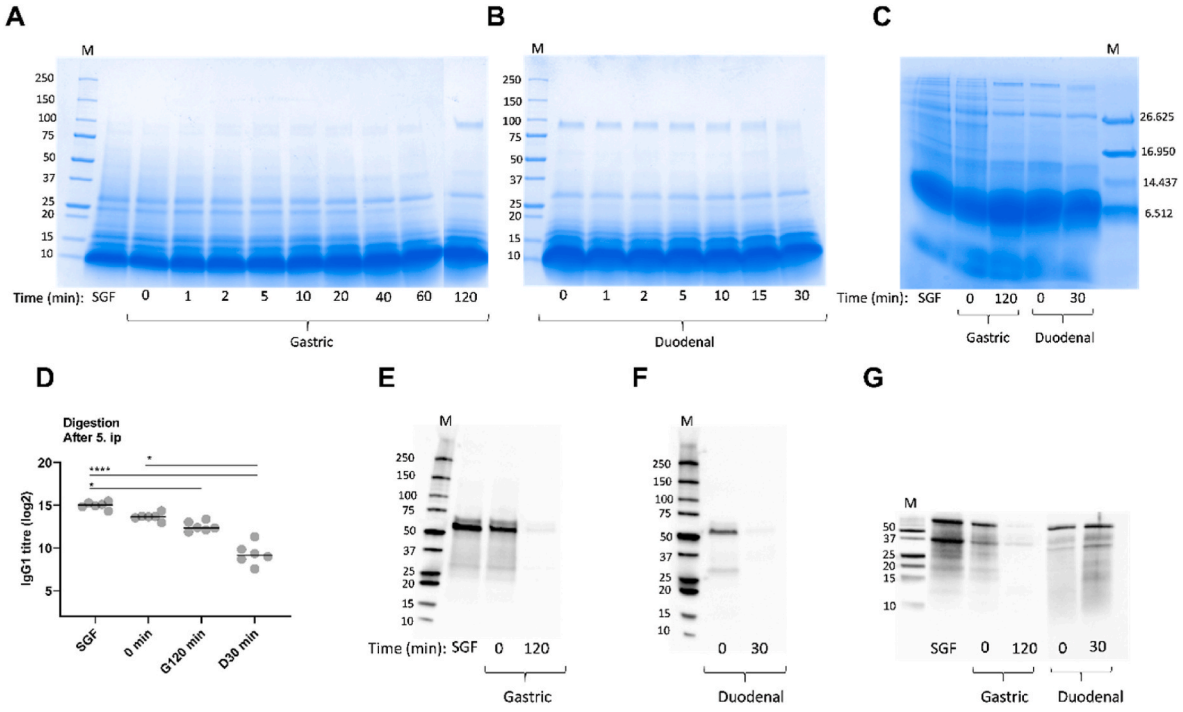


Fig. 4. Gastric and gastro-duodenal digestibility of quinoa proteins. (A) Reduced SDS-PAGE showing simulated *in vitro* gastric digestion of quinoa protein extract digested with pepsin in simulated gastric fluid (SGF) for 0, 1, 2, 5, 10, 20, 40, 60 or 120 min. (B) Reduced SDS-PAGE showing simulated *in vitro* gastro-duodenal digestion of gastric digested quinoa protein extract further digested with trypsin and chymotrypsin for 0, 1, 2, 5, 10, 15 or 30 min. (C) Peptide SDS-PAGE with quinoa protein extract from both gastric and gastro-duodenal digestion. (D) Quinoa-specific IgG1 titres (log2) from rats immunised five times with 50 µg quinoa protein and tested against quinoa proteins in SGF at time 0 and after 120 min of gastric digestion (G120 min) and after additional 30 min of duodenal digestion (D30 min). Horizontal lines indicate median, and each symbol represents one rat. Statistically significant differences between the indicated groups are shown as: *$p < 0.05$, ****$p < 0.0001$. (E) Immunoblot of proteins from gastric digestion with pooled sera from rats immunised 5 times with 50 µg quinoa protein. Note the change in reactivity when quinoa proteins were suspended in SGF compared to the PBS and NaCl solubilised extract. (F) Immunoblot of proteins from gastro-duodenal digestion with pooled sera from rats immunised five times with 50 µg quinoa protein. (G) Immunoblot of proteins from both gastric and gastro-duodenal digestion with sera from rats immunised 5 times with 50 µg quinoa protein. M: marker.

the non-digested version of quinoa (Fig. 4D). For evaluation of the digestibility at the protein level, the pattern of antibody recognition of the quinoa proteins was assessed by immunoblotting, where a change in reactivity could already be observed when quinoa proteins were suspended in SGF, compared to the PBS and NaCl solubilised extracts (Figs. 3C and 4E). Greatest reactivity was found towards the protein just above 50 kDa, though a faint band was still present around 25 kDa (Fig. 4E). Thus, a change in pH and hence a change in protein solubility seemed to affect the reactivity. After both phases of digestion, the reactivity was decreased indicated by the presence of only faint bands (Fig. 4E and F). No immune reactivity was observed towards proteins and peptides below 10 kDa (Fig. 4G).

3.4. In silico predicted cross-reactivity between quinoa proteins and known allergens

In order to identify potential cross-reactivity of quinoa proteins with known allergens, *in silico* analyses were performed. All amino acid sequences identified from the NCBI search were further evaluated and compared with sequences of known allergens via AllergenOnline (Goodman et al., 2016). The top 10 identity hits, or all hits if less than 10, are included in Table 1. The table includes hits from the quinoa protein giving rise to the highest percentage of identity (in the 80mer sliding window) with known allergens within each protein family. The expected (E)-value, indicative of the number of hits one can expect to observe by chance when searching a database of a particular size, and the full alignment are included, thus increasing the sensitivity of the analysis (Herman et al., 2015; Pearson, 2016; Silvanovich et al., 2009). An E-value lower than 10e-6 reflects high identity between the sequences (Pearson, 2016). For quinoa 2S albumin, five hits appeared with an identity of more than 35% in the 80mer sliding window, with the highest being 38%. High E-values were observed and identity percentage between 31% and 32% for the full alignment. No proteins with more

Table 1
Top ten hits, or all if less, of identity greater than 35% (in the 80mer sliding window) between quinoa proteins and known allergens. Data obtained from AllergenOnline.org[a].

Quinoa protein (accession number[b])	Matched species	Protein	Assigned allergen	Accession number[b]	Alignment 80mer sliding window	E-value	Alignment full
2S albumin (XP_021758543.1)	Brazil nut (*Bertholletia excelsa)*	2S sulfur-rich seed storage protein	Ber e 1	P04403.2	38%	4.8e-006	32%
	Hazelnut (*Corylus avellana*)	2S albumin	Cor a 14	ACO56333.1	36%	6.2e-005	31%
	Sesame (*Sesamum indicum*)	2S albumin	Ses i 1	AAK15088.1	36%	0.0029	31%
	Sesame (*Sesamum indicum*)	2S albumin	Unassigned	ACI41244.1	36%	0.0038	31%
	Common walnut (*Juglans regia*)	Albumin seed storage protein	Jug r 1	AAB41308.1	36%	1.1e-005	32%
11S globulin (ABI94735.1)	Pistachio (*Pistacia vera*)	11S globulin	Pis v 2	ABG73110.1	73%	5.8e-047	49%
	Pistachio (*Pistacia vera*)	11S globulin	Unassigned	ABU42022.1	73%	5.8e-047	49%
	Hazelnut (*Corylus avellana*)	11S globulin	Cor a 9	AHA36627.1	73%	1.4e-046	53%
	Kiwi (*Actinidia chinensis*)	11S globulin-like protein	Act d 1	ABB77213.1	71%	2.7e-065	54%
	Pistachio (*Pistacia vera*)	11S globulin	Pis v 2	ABG73109.1	71%	8.7e-098	50%
	Hazelnut (*Corylus avellana*)	11S globulin-like protein	Cor 1 9	AAL73404.1	71%	8.7e-047	53%
	Sesame (*Sesamum indicum*)	11S globulin	Ses i 7	AAK15087.1	70%	8.3e-073	50%
	Pecan nut (*Carya illinoinensis*)	11S legumin protein	Unassigned	ABW86979.1	69%	2.6e-048	53%
	Pecan nut (*Carya illinoinensis*)	11S legumin protein	Car i 4	ABW86978.1	69%	1.2e-048	52%
	Black walnut (*Juglans nigra*)	Legumin	Jug n 4	APR62629.1	69%	5.0e-048	54%
Profilin (XP_021741387.1)	Goosefoot (*Chenopodium album*)	Pollen allergen	Che a 2	AAL92870.1	99%	4.6e-067	98%
	Lychee (*Litchi chinensis*)	Profilin	Unassigned	ABC02750.1	91%	1.5e-059	82%
	Lychee (*Litchi chinensis*)	Profilin	Lit c 1	AAL07320.1	91%	4.8e-060	83%
	Potato (*Solanum tuberosum*)	Profilin-like protein	Unassigned	ABB16985.1	90%	3.3e-060	85%
	Peanut (*Arachis hypogaea*)	Profilin	Unassigned	AGA84056.1	90%	1.2e-059	84%
	Potato (*Solanum tuberosum*)	Profilin-like	Unassigned	ABA81885.1	90%	8.4e-060	82%
	Peanut (*Arachis hypogaea*)	Profilin	Unassigned	ADB96066.1	90%	2.1e-059	84%
	Banana (*Musa acuminata*)	Profilin	Mus a 1	AAK54834.1	89%	7.2e-057	83%
	Soybean (*Glycine max*)	Profilin	Gly m 3	CAA11756.1	89%	8.0e-059	84%
	Common ragweed (*Ambrosia artemisiifolia*)	Profilin-like protein	Unassigned	AAP15202.1	89%	1.0e-059	85%
Oleosin (XP_021752156.1)	Sesame (*Sesamum indicum*)	Oleosin	Ses i 5	AAD42942.1	86%	4.4e-043	67%
	Sesame (*Sesamum indicum*)	Oleosin	Unassigned	ACH85188.1	86%	3.7e-043	67%
	Hazelnut (*Corylus avellana*)	Oleosin	Cor a 13	AAO65960.1	80%	2.0e-040	65%
	Peanut (*Arachis hypogaea*)	Oleosin 1	Ara h 11	AAZ20276.1	75%	1.3e-036	58%
	Peanut (*Arachis hypogaea*)	Oleosin 2	Ara h 11	Q45W86	74%	2.2e-035	56%
	Peanut (*Arachis hypogaea*)	Oleosin 3	Ara h 15	AAU21501.1	66%	3.0e-027	52%
	Hazelnut (*Corylus avellana*)	Oleosin	Cor a 12	AAO67349.2	53%	1.0e-018	37%
	Sesame (*Sesamum indicum*)	Oleosin	Ses i 14	AAG23840.1	49%	1.9e-017	34%
	Peanut (*Arachis hypogaea*)	Oleosin 1	Ara h 10	AAU21499.2	46%	1.1e-018	36%
	Peanut (*Arachis hypogaea*)	Oleosin 2, partial	Ara h 10	AAU21500.1	46%	8.0e-018	41%
LTPs (XP_021747529.1)	Common mugwort (*Artemisia vulgaris*)	Precursor	Art v 1	AAO24900.1	36%	1.8	41%
	Pear (*Pyrus communis*)	Lipid transfer protein isoform c	Unassigned	AET05732.1	36%	1.9e+002	35%
	Orange (*Citrus sinensis*)	Lipid transfer protein	Cit s 3	CAH03799.1	36%	0.29	34%
	Western mugwort (*Artemisia ludoviciana*)	Lipid transfer protein	Art v 1	AHF71025.1	35%	6.2	39%

[a] The table includes homology hits from one chosen protein from quinoa giving rise to the highest percentage of identity (in the 80mer sliding window) with known allergens. The E-value and the full alignment is included, where an E-value lower than 10e-6 reflects high identity between the sequences (Pearson, 2016).
[b] Accession number according to NCBI.

than 35% identity, could be identified in the search of identity between quinoa 7S globulin and known allergens. For quinoa 11S globulin, a large number of hits were found. The top seven hits were found to have more than 70% identity (in the 80mer sliding window) to known allergens from pistachio, hazelnut, kiwi and sesame. Low E-values were observed for all hits and full alignment identity between 49% and 54%. Also, for quinoa profilin, a large number of hits were found with more than 35% identity (in the 80mer sliding window). The top 10 hits revealed a high percentage of identity, with the highest being 99% identity to profilin from goosefoot (*Chenopodium album*), a species closely related to quinoa. Other related profilins were from lychee, potato, peanut, banana, and soybean. Low E-values and high percentage of identity was found for the full alignment for all hits. For quinoa oleosin, 10 hits were identified from the search, and the top five hits had a high identity percentage (in the 80mer sliding window) of more than 70% to oleosins from sesame, hazelnut and peanut. Low E-values were observed for the first five hits, whereas the value increased for the last five hits. Lastly, for quinoa nsLTPs only few hits were observed and all with less than 40% identity and high E-values.

Eleven highly relevant allergenic foods and pollen were chosen for further investigation of potential cross-reactivity with quinoa proteins (see Fig. 5 for an overview of the foods and pollen included). In Table 2, the top five hits, for these 11 selected food and tree pollen within each of the allergenic protein families are included. For quinoa 2S albumin, low identity was found to Brazil nut, hazelnut and walnut with a identity percentage of less than 40% and high E-values. For quinoa 11S globulin, identity was found to hazelnut, walnut, cashew nut and Brazil nut with percentages between 66 and 73% and low E-values. For quinoa profilin, high identity between 89 and 90% was found to peanut, soy and hazelnut and with low E-values. For quinoa oleosin, identity of 66–75% and 53%, respectively, was found to peanut and hazelnut and with low E-values. None of the 11 selected allergenic foods and pollen were found to have an identity higher than 35% for quinoa nsLTPs. Collectively, sequence identities were found between quinoa proteins and already known allergens with regard to the primary structures, however, serological studies of antibody binding from allergic patients are needed to further confirm potential cross-reactivity.

3.5. Serological analyses demonstrate cross-reactivity between quinoa proteins and known allergens

Various ELISAs were performed with serum samples from rats immunised with quinoa protein or each of the selected food and pollen protein extracts. The antibody binding capacity towards the different protein extracts was evaluated for IgG1 raised against quinoa (Fig. 6A), showing that the quinoa-specific IgG1 was able to react with several of the protein extracts, with the most pronounced responses seen for peanut (6AIII), hazelnut (6AIV), almond (6AVIII) and Brazil nut (6AIX). No reactivity could be observed towards grass pollen (6AX), wheat gluten (6AXI) and cow's whey (6AXII). For comparison, the binding capacity between the different protein extracts and IgG1 raised against these were included, revealing, as expected, a higher response towards the given extract when their specific anti-sera were used, than for quinoa-specific anti-sera.

For rats immunised with one of the 11 selected individual protein extracts, different IgG1 binding capacities toward quinoa were observed (Fig. 6B). Reaction towards quinoa was seen for IgG1 raised against peanut (6BIII), hazelnut (6BIV), walnut (6BVI), cashew nut (6BVII), almond (6BVIII), Brazil nut (6BIX) and grass pollen (6BX). No reaction was seen towards quinoa for IgG1 raised against wheat gluten (6BXI) and cow's whey (6BXII). Noteworthy is the higher level of response (higher titre values) for rats immunised with peanut, hazelnut, cashew nut, Brazil nut and grass pollen towards quinoa, than the level of response towards these protein extracts for rats immunised with quinoa, indicating that the same level of cross-reactivity may not necessarily be obtained for quinoa allergic individual exposed to tree nuts as the tree nut allergic individuals may experience upon exposure to quinoa (Fig. 7).

In order to further assess the potential cross-reactivity between quinoa and the selected 11 protein extracts, IgG1 inhibition ELISA was performed (Fig. 8). When evaluating the capacity of the different products to inhibit the binding between quinoa and quinoa-specific IgG1, only walnut was seen to have an inhibitory effect but this was only to a level of ~35% inhibition (Fig. 8A). When evaluating the capacity of quinoa to inhibit the binding between the individual protein extracts and the IgG1 raised against these, quinoa revealed the highest inhibitory effect for hazelnut (8D) and walnut (8F), and a marginal inhibitory capacity of quinoa for peanut (8C), cashew nut (8G), almond (8H) and Brazil nut (8I).

Whereas ELISAs reveal the overall antibody binding cross-reactivity, immunoblotting gives the opportunity to investigate the specific proteins involved in the cross-reactions. Based on the results from the ELISAs, demonstrating the absence of cross-reactivity between quinoa, and wheat gluten and cow's whey, respectively, these allergenic foods were excluded from the immunoblot analyses. An SDS-PAGE was performed with the nine protein extracts, which revealed distinct protein profiles for the different protein extracts (Fig. 9A). The proteins were transferred to a membrane and incubated with sera from rats immunised

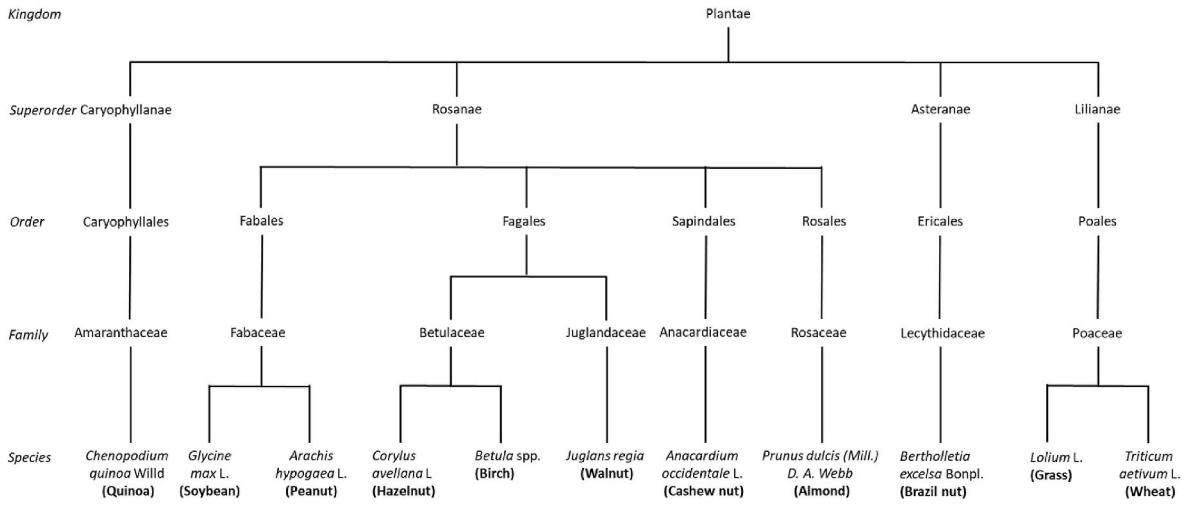


Fig. 5. Overview of the 11 selected foods and pollen, divided into superorder, order, family and species. Constructed with data from Integrated Taxonomic Information System (ITIS).

Table 2
Top five hits of identity greater than 35% (in the 80mer sliding window) between quinoa proteins and known allergens from 11 selected foods and pollen. Data obtained from AllergenOnline.org[a].

Quinoa protein (accession number[b])	Matched species	Protein	Assigned allergen	Accession number[b]	Alignment 80mer sliding window	E-value	Alignment full
2S albumin (XP_021758543.1)	Brazil nut (*Bertholletia excelsa)*	2S sulfur-rich seed storage protein	Ber e 1	P04403.2	38%	4.8e-006	32%
	Hazelnut (*Corylus avellana*)	2S albumin	Cor a 14	ACO56333.1	36%	6.2e-005	31%
	Common walnut (*Juglans regia*)	Albumin seed storage protein	Jug r 1	AAB41308.1	36%	1.1e-005	32%
11S globulin (ABI94735.1)	Hazelnut (*Corylus avellana*)	11S globulin	Cor a 9	AHA36627.1	73%	1.4e-046	53%
	Hazelnut (*Corylus avellana*)	11S globulin-like protein	Cor 1 9	AAL73404.1	71%	8.7e-047	53%
	Cashew nut (*Anacardium occidentale*)	11S globulin	Ana o 2	AAN76862.1	69%	3.3e-076	52%
	Walnut (*Juglans regia*)	Seed storage protein	Jug r 4	AAW29810.1	68%	3.6e-048	53%
	Brazil nut (*Bertholletia excelsa*)	11S globulin	Unassigned	6B4S_A	66%	5.3e-098	53%
Profilin (XP_021741387.1)	Peanut (*Arachis hypogaea*)	Profilin	Unassigned	AGA84056.1	90%	1.2e-059	84%
	Peanut (*Arachis hypogaea*)	Profilin	Unassigned	ADB96066.1	90%	2.1e-059	84%
	Soybean (*Glycine max*)	Profilin	Gly m 3	CAA11756.1	89%	8.0e-059	84%
	Hazelnut (*Corylus avellana*)	Profilin	Cor a 2	AAK01236.1	89%	9.6e-059	81%
	Soybean (*Glycine max*)	Profilin	Gly m 3	CAA11755.1	89%	6.3e-058	82%
Oleosin (XP_021752156.1)	Hazelnut (*Corylus avellana*)	Oleosin	Cor a 13	AAO65960.1	80%	2.0e-040	65%
	Peanut (*Arachis hypogaea*)	Oleosin 1	Ara h 11	AAZ20276.1	75%	1.3e-036	58%
	Peanut (*Arachis hypogaea*)	Oleosin 2	Ara h 11	Q45W86	74%	2.2e-035	56%
	Peanut (*Arachis hypogaea*)	Oleosin 3	Ara h 15	AAU21501.1	66%	3.0e-027	52%
	Hazelnut (*Corylus avellana*)	Oleosin	Cor a 12	AAO67349.2	53%	1.0e-018	37%

[a] The table includes identity hits from one chosen protein from quinoa giving rise to the highest percentage of homology (in the 80mer sliding window) with the 11 selected foods and pollen. The E-value and the full alignment is included, where an E-value lower than 10e-6 reflects high identity between the sequences (Pearson, 2016).
[b] Accession number according to NCBI.

with quinoa (Fig. 9B). Two dominating bands could be observed for soy in the area of 50 and 70 kDa, whereas three distinct areas of bands were seen for the related species peanut. Similar, three areas were observed for hazelnut, with intense bands around 20, 37 and 70 kDa. Two bands in the area of 20 and 37 kDa could be seen for birch pollen. A slightly different pattern was observed for walnut, with high MW dominating bands, as well as bands around 20 and 30 kDa. Three intense bands were seen for cashew nut, whereas one single intense band was seen for almond and Brazil nut, but with very different sizes. Similar to peanut and hazelnut, three areas of intense bands were observed for the grass pollen. Further, SDS-PAGE with quinoa proteins (Fig. 9C) were transferred to a membrane and incubated with sera from rats immunised with the nine selected protein extracts (Fig. 9D). In the blot, several distinct bands were observed for each individual anti-sera, and in general, intense reactivity towards high MW aggregates was detected in the area of 100–200 kDa. For all anti-sera, apart from birch pollen-specific IgG1, an intense band was observed around 25 kDa, similar to the dominating band after incubation with sera from quinoa-immunised rats. For birch pollen and Brazil nut anti-sera an intense band was observed around 20 kDa. Overall, evidenced from the blots there is a great diversity in cross-reactivity between quinoa proteins and the food and pollen allergens.

4. Discussion

Quinoa is a high stress-tolerant crop, making it a good choice as a worldwide cultivated crop (S.-E. Jacobsen, 2003a; S. E. Jacobsen et al., 2003b). The seeds of quinoa have a high nutritional value with high protein content (Dakhili et al., 2019; Venlet et al., 2021). The consumption of quinoa has increased in the last decade, and the seeds may serve as an alternative to stable global food products such as rice and wheat. Additionally, it may serve as a gluten-free food for those wanting to avoid gluten (Balize et al., 2015). Furthermore, proteins from quinoa are potential new sources of protein used in functional foods or plant-based protein foods (López-Castejón et al., 2020). Thus, investigating the inherent immunogenicity, allergenicity and cross-reactive potential of quinoa proteins are highly relevant. The immunogenicity and allergenicity of quinoa protein were assessed in a dose-response study in BN rats in comparison to the low-allergenic spinach and the high-allergenic peanut, with subsequent evaluation by means of ELISAs, immunoblotting and *in vivo* analysis. Further, an *in vitro* simulated gastro-duodenal digestion analysis was performed. For evaluation of the cross-reactivity between quinoa proteins and known allergens, *in silico* analyses were conducted followed by analyses with 11 selected protein extracts and their anti-sera by means of ELISAs and immunoblotting.

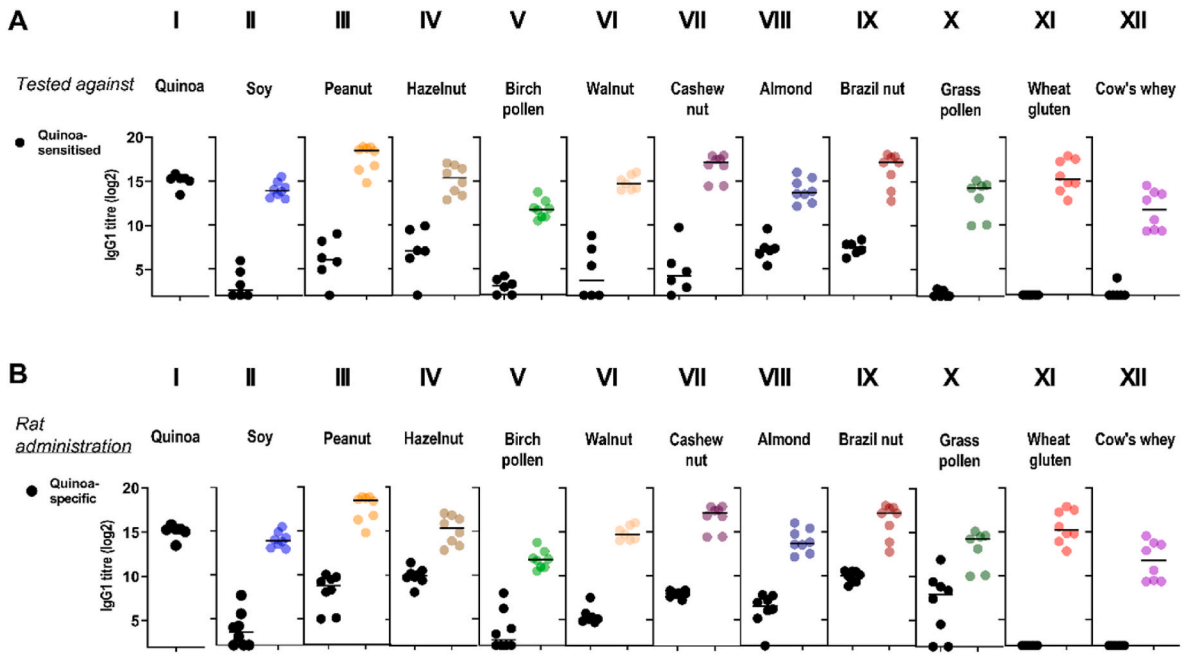


Fig. 6. Cross-reactivity by means of IgG1 ELISAs. ELISAs were performed with sera from in-house ip Brown Norway rat sensitisation experiments with 11 selected protein extracts as well as quinoa. (A) Plates were coated with one of the selected protein extracts and incubated with sera from rats immunised with quinoa. Furthermore, plates were individually incubated with sera from rats immunised with the same protein extract as coated with: I, quinoa. II, soy. III, peanut. IV, hazelnut. V, birch pollen. VI, walnut. VII, cashew nut. VIII, almond. IX, Brazil nut. X, grass pollen. XI, wheat gluten. XII, cow's whey. (B) Plates were coated with either quinoa protein extract or each of the individual protein extract and incubated with sera from rats immunised with: I, quinoa. II, soy. III, peanut. IV, hazelnut. V, birch pollen. VI, walnut. VII, cashew nut. VIII, almond. IX, Brazil nut. X, grass pollen. XI, wheat gluten. XII, cow's whey. Horizontal lines indicate median, and each dot represents one rat. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

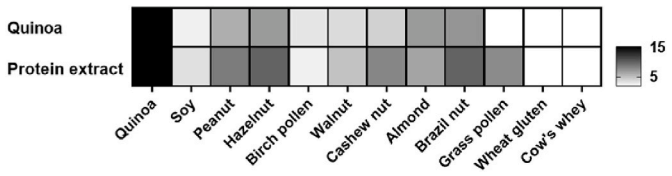


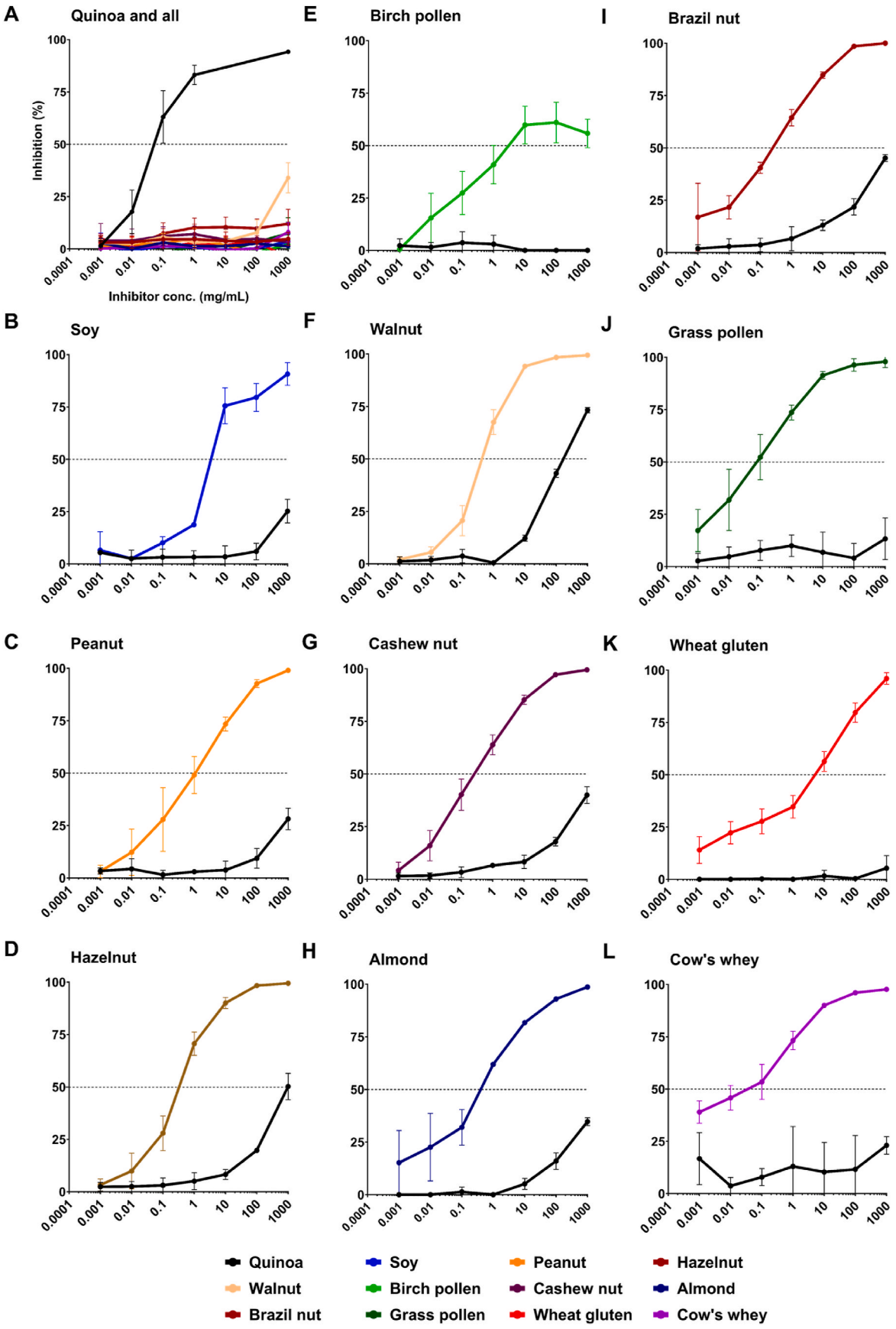
Fig. 7. Heat map of cross-reactivity. Heat map based on IgG1 titres from quinoa-immunised rats tested against the specific protein extracts (upper row), or IgG1 titres from rats immunised with the selected protein extracts and tested against quinoa (lower row). The highest titre is 15 and the lowest is 2, which corresponds to below detection limit.

BN rats are known as a high IgE-responder strain (Knippels et al., 1998), enabling the investigation of the allergenicity of new food protein sources. Using the ip route of administration, proteins bypass the oral cavity, the digestive system and the gut-associated lymphoid tissue, and thereby avoid the induction of oral tolerance (Dearman et al., 2003), allowing the investigation of the inherent immunogenicity and sensitising capacity. When evaluating the potential risk of inducing *de novo* sensitisation, studies should be performed in comparison to known non/low-allergenic as well as high-allergenic food sources, where both the doses and the number of immunisations are relevant parameters to consider. In the present study, the low-allergenic spinach was included, which hase previously been reported to be capable of inducing allergic reactions, though only rarely and often due to cross-reactivity with latex proteins (Ferrer et al., 2011; Maillard et al., 2000; Sanchez et al., 1997), and with no allergens identified in the Allergen Nomenclature database (WHO/IUIS Allergen Nomenclature Sub-Committee, 1994), as well as the high-allergenic peanut, known as the most potent allergenic food giving rise to persistent and severe allergy (Bock et al., 2001; Dyer et al., 2015; Umasunthar et al., 2015), and with a total of 17 proteins identified as allergens in the Allergen Nomenclature database (Sudharson et al., 2021; WHO/IUIS Allergen Nomenclature Sub-Committee, 1994).

The present study demonstrated that quinoa contains an inherent medium to high immunogenicity and sensitising capacity, inducing higher levels of specific IgG1 and IgE than spinach but lower levels of specific IgG1 and IgE than peanut. Yet, upon evaluating the clinical relevance of the sensitisation induced in the BN rats using an ear swelling test, it was revealed that the three highest doses of quinoa induced clinical reactions similar to peanut. That quinoa exhibits the potential to induce a *de novo* sensitisation of clinical relevance may not be astounding due to the very high content of proteins belonging to allergen superfamilies of prolamins and cupins, constituting 2S albumins and 11S globulins, respectively (Brinegar et al., 1996; Brinegar and Goundan, 1993).

A clear dose-dependent response was observed for quinoa-specific IgG1 throughout the experiment, with the highest dose giving rise to the highest level of antibodies. This was also seen for the IgE response, but only until the fourth immunisation, where the pattern shifted, and subsequently revealed the lowest dose to be the most sensitising one. Yet, the dose-dependent sensitisation response is most likely bell shaped, as has previously been indicated in animal sensitisation studies (Bøgh et al., 2009; Kroghsbo et al., 2014; Sztuk et al., 2023), and supported by the present study. This also highlights that investigations of the *de novo* sensitising capacity of new foods should always include dose-response relationships, as different foods may reveal different dose-dependent responses.

Cases of allergic reactions toward quinoa are emerging (Astier et al., 2009; Chatain et al., 2020; Guarnieri et al., 2019; Hong et al., 2013), however, only limited information is provided on potential quinoa allergens. In the present study, immunoblotting was performed with sera from rats immunised with quinoa proteins and revealed especially one dominant band, likely the basic subunit of 11S globulin. In one case-study, patient reactivity was demonstrated towards a protein of 35 kDa, possibly corresponding to the 11S globulin, but most likely the acidic subunit (Astier et al., 2009), and in another case-study including three patients, patient reactivity was demonstrated towards a band of



(caption on next page)

Fig. 8. IgG1 inhibitory ELISA. (A) Inhibitory capacity of all 11 selected protein extracts on the binding between quinoa and quinoa-specific IgG1. (B) Inhibitory capacity of quinoa and soy on the binding between soy and soy-specific IgG1. (C) Inhibitory capacity of quinoa and peanut on the binding between peanut and peanut-specific IgG1. (D) Inhibitory capacity of quinoa and hazelnut on the binding between hazelnut and hazelnut-specific IgG1. (E) Inhibitory capacity of quinoa and birch pollen on the binding between birch pollen and birch pollen-specific IgG1. (F) Inhibitory capacity of quinoa and walnut on the binding between walnut and walnut-specific IgG1. (G) Inhibitory capacity of quinoa and cashew nut on the binding between cashew nut and cashew nut-specific IgG1. (H) Inhibitory capacity of quinoa and almond on the binding between almond and almond-specific IgG1. (I) Inhibitory capacity of quinoa and Brazil nut on the binding between Brazil nut and Brazil nut-specific IgG1. (J) Inhibitory capacity of quinoa and grass pollen on the binding between grass pollen and grass pollen-specific IgG1. (K) Inhibitory capacity of quinoa and wheat gluten on the binding between wheat gluten and wheat gluten-specific IgG1. (L) Inhibitory capacity of quinoa and cow's whey on the binding between cow's whey and cow's whey-specific IgG1. Analyses were performed in duplicates with pooled sera and repeated three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

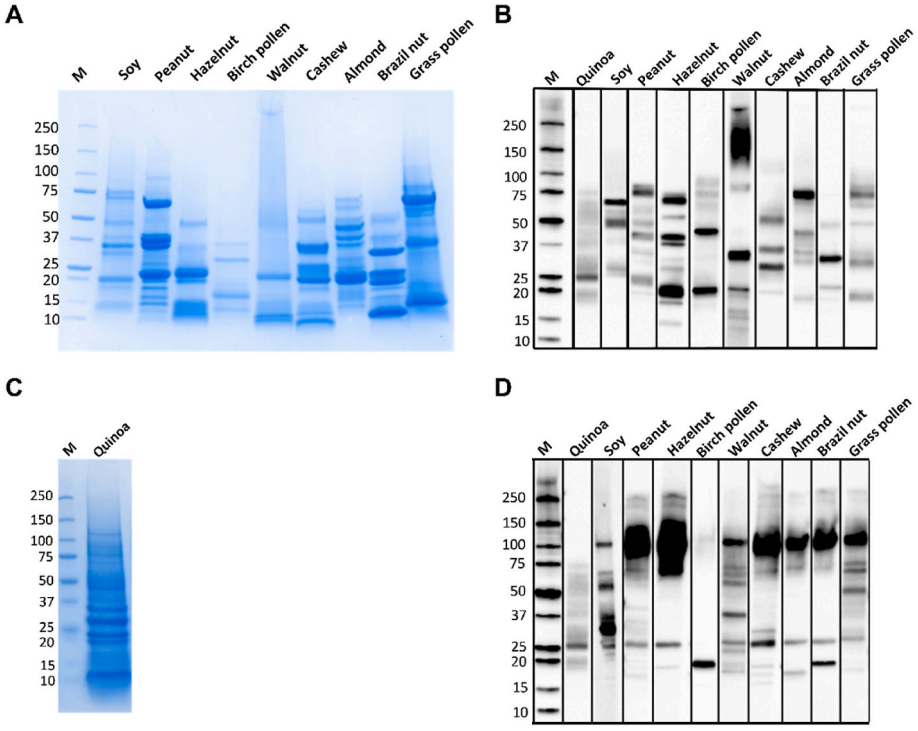


Fig. 9. SDS-PAGE and immunoblot of the selected protein extracts and quinoa. (A) Reduced SDS-PAGE with the nine selected protein extracts. (B) Immunoblot based on the gel with the selected protein extracts and incubated with sera from rats immunised with quinoa protein (5 times with 50 μg protein). (C) Reduced SDS-PAGE with quinoa protein extract. (D) Immunoblot based on the gel with quinoa proteins and incubated with sera from rats immunised with either quinoa, soy, peanut, hazelnut, birch pollen, walnut, cashew nut, almond, Brazil nut or grass pollen. M: marker.

51 kDa for all three patients, probably corresponding to the 7S globulin (Chatain et al., 2020).

Digestive resistance is an important aspect when considering potential allergenicity (Bøgh and Madsen, 2016). Evaluating genetically modified crops, protein resistance to digestion is a key element in the allergenicity risk assessment (FAO/WHO, 2003; Goodman et al., 2008). It is generally well-accepted that proteins susceptible to digestion are less allergenic than those resistant to digestion, as the proteins will need to survive the digestion process as intact proteins or large peptide fragments to induce an allergic response (Astwood et al., 1996; Bøgh and Madsen, 2016; Verhoeckx et al., 2019). However, exceptions exist, where non-allergenic proteins are demonstrated to be relative stable to digestion and where known allergens are rapidly degraded (Bøgh and Madsen, 2016; Fu et al., 2002; Herman et al., 2007; Verhoeckx et al., 2019). In the present study, quinoa proteins were in general observed to contain a high level of resistance to gastric as well as duodenal digestion, thus, based on their digestibility the proteins in quinoa may be regarded as potential allergens.

Proteins belonging to the allergenic protein superfamilies, like 11S globulins and 2S albumins, are the major proteins found in quinoa, and may be of concern in the case of possible cross-reactivity to known allergens. 11S globulin, 2S albumin and 7S globulin remain the three classes of seed storage proteins responsible for most clinical reactions in patients suffering from peanut and tree nut allergies (Bublin and Breiteneder, 2014; Geiselhart et al., 2018). Several studies have investigated possible cross-reactivity between peanut and tree nuts due to the presence of proteins with homologous amino acid sequences and structures (Bublin and Breiteneder, 2014; De Leon et al., 2003; Kulis et al., 2009). Thus, in the present study, an evaluation of potential cross-reactivity between quinoa proteins and several known allergens were investigated. Based on the *in silico* analyses, comparing quinoa proteins with known allergens, the highest percentage of identity was found for 11S globulin, profilin and oleosin. Low identity was on the other hand found for the 2S albumin, which is in line with previous results revealing low sequence homology between 2S albumin allergens from different plant families (Radauer et al., 2008).

Using serum samples from rats immunised with different known food and pollen allergens, ELISAs revealed a possible cross-reactivity between quinoa, peanut and the tree nut protein. This may be of relevance for patients suffering from quinoa allergy as well as for patients suffering from peanut or tree nut allergies. Based on the present data, a potential higher risk of reaction towards quinoa would be expected for patients suffering from peanut, hazelnut, cashew nut, Brazil nut or grass pollen allergy, than the risk of reaction towards these allergens in quinoa

allergic patients. As demonstrated from the ELISA results, no reaction with wheat gluten was found suggesting that inclusion of quinoa in the diet should not pose a risk of cross-reactions in wheat allergic patients. With *in silico* analyses and IgG1 ELISA results as a basis, immunoblotting demonstrated reactivity towards a broad range of proteins. For quinoa, the various anti-sera were demonstrated to react with 11S globulin, likely the basic subunit. This has likewise been described for hazelnut (Beyer et al., 2002) and almond (Albillos et al., 2008), where the highest IgE reactivity was observed towards the 11S globulins. In the present study, immunoblotting with the different products and incubation with sera from rats immunised with quinoa, showed potential cross-reactivity with hazelnut 11S globulin (Cor a 9). This correlated with the *in silico* analyses demonstrating high identity of quinoa and hazelnut 11S globulin, despite the great taxonomic distance between quinoa and hazelnut. Potential cross-reactivity could furthermore be suggested for cashew nut, revealing potential cross-reactivity with 11S globulin (Ana o 2). As seen for all products, a range of potential cross-reactive proteins was demonstrated, regardless of the taxonomic distances. In order to further identify specific cross-reactive proteins, identification of the reactive proteins should be confirmed with either immunoblotting using antibodies developed against a specific protein or by MS/MS analysis of the relevant bands (Mazzucchelli et al., 2018).

Collectively, the present study showed that quinoa proteins contain immunogenicity as well as sensitising capacity, being able to induce medium to high level of specific antibodies in BN rats without the use of an adjuvant. Further, the study showed that quinoa may contain proteins with potential cross-reactivity to certain tree nut allergens. However, these results should be confirmed in human studies.

CRediT authorship contribution statement

Anne-Sofie Ravn Ballegaard: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Ana Isabel Sancho:** Methodology, Investigation, Visualization, Writing – review & editing. **Cui Zhou:** Methodology, Writing – review & editing. **Niels-Peter Hell Knudsen:** Writing – review & editing, Methodology. **Neil Marcus Rigby:** Writing – review & editing, Methodology. **Claus Heiner Bang-Berthelsen:** Writing – review & editing. **Shashank Gupta:** Methodology, Writing – review & editing. **Alan Robert Mackie:** Methodology, Writing – review & editing. **Mette Lübeck:** Methodology, Writing – review & editing. **Kirsten Pilegaard:** Investigation, Visualization, Writing – review & editing, Conceptualization. **Katrine Lindholm Bøgh:** Investigation, Visualization, Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: NPHK and SG are employees at ALK. KLB has ongoing collaboration with ALK. 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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2023.114118.

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