

Quinoa (*Chenopodium quinoa* Willd.) seeds increase intestinal protein uptake

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Abbreviations used

BLG, β -lactoglobulin; BN, Brown Norway; Cdh1, cadherin-1; Cldn2, claudin 2; CT, cholera toxin; CRP, C-reactive protein; EtOH, ethanol; EPI, epithelium; FBS, fetal bovine serum; FITC, Fluorescein isothiocyanat; IFN- γ , interferon gamma; IL-1 β , interleukin 1 beta; IL-33, interleukin 33; LP, lamina propria; mLN, mesenteric lymph node; Muc2, mucin 2; Ocln, occluding; PAS, Periodic acid-Schiff; PBS, phosphate buffered saline; PP, Peyer's patches; TEER, transepithelial electrical resistance; Tjp1, tight junction protein 1; TNF- α , tumor necrosis factor alpha; TSLP, thymic stromal lymphopoietin; WPI, whey protein isolate.

Received: 04/02/2021; Revised: 07/03/2021; Accepted: 20/04/2021

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/mnfr.202100102](#).

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Keywords

Chenopodium quinoa, saponins, intestinal permeability, protein uptake, animal model

Abstract (200 words)

Scope: Within the last decade, quinoa seeds have gained much popularity as a new food and have recently been proposed as an appropriate food for early introduction in infants. Quinoa contains high levels of saponins, which are known for their adjuvant activity and effect on the intestinal barrier function. The aim of this study was to investigate the impact of quinoa on intestinal permeability and inflammation in comparison with the positive controls; cholera toxin (CT) and capsaicin.

Methods and results: The effect of quinoa on intestinal barrier function and inflammation was investigated *in vitro* using a Caco-2 cell line and *in vivo* using a Brown Norway rat model. Effects *in vivo* were analysed by protein uptake, histology, gene expression, antibody levels and flow cytometry. Quinoa and the positive controls all increased the intestinal permeability, but distinct patterns of absorbed protein were observed in the epithelium, Peyer's patches, lamina propria and serum. The quinoa-mediated effect on intestinal barrier function was found to be distinct from the effect of the two positive controls.

Conclusion: Our findings demonstrate the ability of quinoa to increase intestinal permeability and to promote compartment-specific protein uptake via mechanisms that may differ from CT and capsaicin.

1. Introduction

Securing a sufficient, healthy and climate friendly food supply is important and is foreseen to be a future challenge with the constantly growing population ^[1]. Quinoa (*Chenopodium quinoa* Willd.), originating from the Andean region of South America, is suggested as a crop of the future due to its high nutritional value, low water requirement and its tolerance to frost, drought, poor and saline soils as well as pests ^[1–5]. Quinoa is a pseudocereal belonging to the amaranth plant family (Amaranthaceae), and not the grass family (Poaceae) like the ‘true’ cereals ^[4,6]. Quinoa seeds have gained increasing popularity outside the Andean region and have been marketed as a protein-rich ‘superfood’ that is now consumed worldwide ^[7]. Quinoa seeds are mainly boiled and used in different dishes, or ground to flour and used as an ingredient in e.g. bread, cookies, breakfast cereals, pasta, noodles, and beers ^[5,6,8,9]. It has also found its way into newer commercial products like plant-based ‘milk’ ^[10]. Due to its natural gluten-free composition, it may constitute a large part of the diet for people suffering from coeliac disease or wheat allergy ^[8,9]. Additionally, porridge of quinoa is being suggested as one of the first solid foods to introduce to infants in e.g. Denmark ^[11]. Yet, quinoa contains high levels of the natural occurring bitter-tasting substances, saponins, that may possess inherent adjuvant capacity and influence antigen uptake from the intestinal lumen by possibly increasing intestinal permeability ^[12,13]. The latter has previously been demonstrated *in vitro* using rat intestinal tissue and saponin extracts from e.g. alfalfa and quillaja, as well as from quinoa ^[14,15]. Hence, before human consumption, it is recommended that the seeds are extensively washed in order to decrease the amount of saponins ^[16].

Several other food components have been investigated for their effect on intestinal permeability, including gliadin from wheat, the substance inducing the pathogenesis in celiac disease, extracts of sweet pepper and cayenne pepper, paprika, galangal, marigold and hops ^[17–19]. Especially, the active compound in cayenne pepper and paprika, capsaicin, has been investigated thoroughly and shown to induce functional and structural changes in the intestinal barrier ^[20,21].

Besides food components, microbial products such as cholera toxin (CT), a mucosal adjuvant, has been shown to facilitate antigen transport across the intestinal epithelial layer^[22].

The human intestinal cell line, Caco-2 expresses upon differentiation several morphological and biochemical characteristics of small intestinal enterocytes^[23]. However, studying intestinal uptake in animal models allows for more physiological relevant endpoints, as an intact gastrointestinal tract with the associated digestive system and all relevant cells and molecules as well as blood circulation are present. Thus, the present study investigated the impact of quinoa on intestinal permeability and immune regulation using the Caco-2 cell line and a rat animal model. The effect of quinoa was compared to two positive control substances, the mucosal adjuvant CT^[22,24] and the pungent-tasting substance capsaicin^[20,21], both known to increase intestinal permeability. For the *in vitro* assays, a quinoa extract containing the aglycones was used in order to study the isolated effects of aglycones (the non-glycosylated derivatives of saponins) on the epithelial monolayer. Intestinal permeability and immune regulations were evaluated *in vivo* using Brown Norway (BN) rats that were daily gavaged with quinoa flour for a week.

2. Experimental section

2.1 Products

White quinoa seeds (originating from Peru) were purchased from Procudan (Kolding, Denmark) and ground to flour. Capsaicin >97% pure (M2028, Sigma-Aldrich, St. Louis, MO, US) and CT (List Biological Laboratories, Campbell, CA, US) were included as control substances. Endotoxin level of CT was 12 EU/mg (according to the company). For protein uptake analyses, whey protein isolate (WPI) containing approximately 88% protein, with around 60% β -lactoglobulin (BLG), was used, and kindly provided by Arla Foods Ingredients (Videbæk, Denmark). Endotoxin levels were assessed in the quinoa flour and WPI, using PierceTM LAL Chromogenic Endotoxin Quantification Kit (Thermo Fisher

Scientific, MA, US) following the manufacturer's protocol, to be ~25 EU/mg and <0.1 EU/mg, respectively.

2.2 Extraction of quinoa aglycones

For the cell experiments, quinoa aglycones were extracted in order to assess the isolated effects on the intestinal transport. Half a gram of quinoa flour was weighed into a 50 mL centrifuge tube and suspended in 9 mL 50% methanol (Fluka, Steinheim, Germany) in MilliQ water (v/v) for 1 h at room temperature while shaking. The suspension was centrifuged at 4030 *g* for 5 min and subsequently mixed with 2 mL HCl (Emsure, Merch, Kenilworth, NJ, US) for hydrolysis at 80 °C for 90 min. Solid phase extraction (SPE) cartridges (Oasis HLB 200 mg, Waters, Etten-Leur, The Netherlands) were used for sample purification using a vacuum manifold. Columns were activated with 3 mL methanol followed by 3 mL MilliQ water. One mL of the quinoa extract was added to the columns followed by washing with 5 mL MilliQ water. The quinoa aglycones were eluted from the columns using 3 mL methanol. The eluates were dried under a stream of nitrogen at 50 °C for approximately 30 min. Quantification of quinoa aglycones were assessed by HPLC-ESI-QTOF-MS.

2.3 Caco-2 cell culture

Caco-2/TC7 cells were seeded in complete medium (DMEM high glucose, 10% (v/v) heat inactivated fetal bovine serum (FBS), 1% (v/v) HEPES (1 M), 1% (v/v) PenStrep (10,000 U/mL), 1% (v/v) L-glutamine (200 mM), all from Gibco, Life Technologies, Waltham, CA, US) at a density of 60,000 cells/cm² on semipermeable filters (Millicell Inserts 0.4 µM PET) in 12-well plates. Cells were cultivated for 21 days to fully differentiate and medium was replaced every 2-3 days. A fully differentiated monolayer was confirmed by measurement of transepithelial electrical resistance (TEER) with electrodes (Millicell ERS-2 Epithelial Volt-Ohm meter, Millipore, Burlington, MA, US) before initiation of experiments.

2.4 *In vitro* transport experiments

In order to assess apical to basolateral transport of 4 kDa fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich), culture medium was replaced with complete phenol red-free medium on day 21 of cultivation. Quinoa aglycones (25 μ M), CT (10 μ g/mL), or capsaicin (50 μ M) were dissolved in 0.2% (v/v) ethanol in phenol red-free medium and mixed with FITC-dextran to make a final concentration of FITC-dextran of 3 mg/mL that was placed in the apical compartment. After 0.5, 1, 3, and 6 h, 100 μ L medium was withdrawn from the basolateral compartment and fluorescence intensity measured by excitation at 485 nm and emission at 530 nm.

To assess apical to basolateral transport of BLG, culture medium was replaced with FBS-free medium on day 20 of cultivation. On day 21, quinoa aglycones (25 μ M), CT (10 μ g/mL), or capsaicin (50 μ M) were dissolved in 0.2% (v/v) ethanol in FBS-free medium and added together with WPI to make a final concentration of protein of 1.5 mg/mL in the apical compartment. After 1, 3, 6, 8, and 24 h, 100 μ L medium was withdrawn from the basolateral compartment and stored at -20 °C until further analysis. Basolateral BLG was determined by a commercial bovine BLG ELISA kit (Bethyl Laboratories, Montgomery, AL, US) in a 96-well Maxisorp plate (Nunc, Roskilde, Denmark) according to the manufacturer's protocol with the exception that plates were coated overnight. Each condition in both assays was performed in triplicates and independently repeated three times.

2.5 Animals

BN rats from the in-house breeding colony at the National Food Institute, Technical University of Denmark, were used. Rats were bred and raised for more than 10 generations on an in-house produced diet based on rice flour, potato protein and fish meal as protein sources, as previously described ^[25], with the exception of maize flour being substituted with rice flour. Rats were housed two per cage of same gender 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 observed twice a day. Diet and water were given *ad libitum* and water consumption was registered. Ethical approval for the animal experiment was given by the Danish Animal Experiments Inspectorate with the authorisation number 2015-15-0201-00553-C1. The experiment was overseen by the Institute's Animal Welfare Committee for animal care and use.

2.6 Dosage regime

BN rats with an age of 7-12 weeks were allocated into five groups of 24 rats (12/gender). Rats were daily administered by gavage for one week with 1 mL of either PBS (137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1 mM KH₂PO₄ in MilliQ water, pH 7.2) containing 0.2% (v/v) ethanol as control, or CT (10 µg), quinoa flour (300 mg) with or without co-administration of CT (10 µg), or capsaicin (12 mg) (Figure 1). At the start and at the end of the experiment blood was collected and converted into sera and stored at -20 °C until analysis of BLG uptake and total IgA. Faecal samples were additionally collected at the start and at the end of the experiment for quantification of total IgA. Intestinal uptake of BLG was assessed by administering animals by oral gavage with 100 mg of WPI in 0.5 mL PBS. After 5, 15, 30 or 60 min (six rats per time point) rats were euthanised by exsanguination using CO₂/O₂ as anaesthesia. Body weight of the animals and organ weights of the spleen and the cecum were registered.

2.7 *In vivo* intestinal protein uptake

To evaluate protein uptake in the small intestine BLG was quantified from all rats. The small intestine was excised and divided into fractions. The first 20 cm of the small intestine, 7 cm distal from the stomach, was rinsed in 0.9% (w/v) NaCl, then dissected into Peyer's patches (PP), epithelium (EPI) and lamina propria (LP). The different intestinal fractions were snap frozen in liquid nitrogen and stored at -80 °C until analysis. Proteins were extracted from the different tissues by mixing 10 µL

Tris-lysis buffer (150 mM NaCl, 20 mM Tris, 1 mM EGTA, 1% (v/v) Triton X-100, 1 mM EDTA) with 2% (v/v) Halt protease inhibitor cocktail (Thermo Fisher Scientific) per mg tissue. A stainless steel bead (Qiagen, Hilden, Germany) was added, and the samples were homogenised with a TissueLyserII (Qiagen) for 2 min at 30 cycles/s. Samples were immediately placed on ice and vortexed every 5 min for a total of 20 min. Samples were centrifuged at 15,000 *g* at 4 °C for 20 min, and supernatant was transferred to clean tubes and stored at -80 °C until analysis. Concentrations of BLG were quantified in the three different compartments of intestinal tissue and in the serum by a commercial bovine BLG ELISA kit (Bethyl Laboratories) according to the manufacture's protocol with the exception that the plates were coated overnight.

2.8 Histology

To evaluate histological changes, 1 cm of the small intestine, 38 cm distal from the stomach, and 1 cm of the large intestine, 1 cm distal from cecum, were collected (from 18 rats/group) and fixed overnight in 4% (w/v) paraformaldehyde (CellPath, Newtown, UK). Sections were dehydrated in a 77% to 99% graded ethanol series. Xylene (VWR Chemicals, Radnorm PS, US) was used as clearing agent to replace ethanol before embedding the tissue in paraffin (Hounisen, Skanderborg, Denmark). Sections of 3 µm were stained with Periodic acid-Schiff (PAS) (Merck) to identify goblet cells. Sections were examined using Leica DMR upright microscope (Leica Microsystems GmbH, Wetzlar, Germany). Goblet cells were counted from villus to crypt in two connected villi at two different places in the section of the small intestine, and at three different places in the section for the large intestine. Analysis of the sections were performed in a blinded manner.

2.9 Tissue RNA extraction, cDNA synthesis and RT-qPCR

To investigate mRNA changes in the small intestine, a 1 cm piece, 27 cm distal from the stomach, was excised (from 12 rats/group) and stored in RNeasy lysis buffer (Qiagen, Crawley, UK) at -80 °C until

extraction of RNA. RNA extraction, cDNA synthesis and RT-qPCR were performed as already described ^[26]. Taqman gene assays (Applied Biosystems, Thermo Fisher Scientific) used in the analysis were; *Cdh1* (cadherin-1 Rn00580109_m1), *Ocln* (occludin Rn00580064_m1), *Cldn2* (claudin 2 Rn02063575_s1), *Tjp1* (tight junction protein 1 (also known as ZO-1) Rn02116071_s1), *TSLP* (thymic stromal lymphopoietin Rn01761072_m1), *IL-33* (interleukin 33 Rn01759835_m1), *Muc2* (mucin 2 Rn01498206_m1), *TNF- α* (tumor necrosis factor alpha Rn99999017_m1), *IL-1 β* (interleukin 1 beta Rn00580432_m1), and *IFN- γ* (interferon gamma Rn00594078_m1).

2.10 Serum C-reactive protein (CRP)

In order to investigate the serum biomarker for inflammation, concentrations of CRP were measured in serum by a commercial ELISA kit (Bio-Techne, R&D Systems, Minneapolis, MN, US) according to the manufacture's instructions.

2.11 Total faecal and serum IgA

Total IgA was measured by sandwich ELISA in faecal water and in serum (from 18 rats/group). Faecal water was prepared from faecal pellets mixed with 10 μ L cold PBS containing 0.05% (w/v) NaN₃ (Sigma-Aldrich) per mg sample followed by homogenisation in a bead beater MM300 (Retsch) for 15 min at 30 cycles/s. Samples were centrifuged at 16,000 *g* at 4 °C for 10 min, and supernatant was transferred to clean tubes and stored at -80 °C until analysis. The sandwich ELISA was performed as previously described ^[26].

2.12 Preparation of single cell suspension of lymphoid tissue and small intestinal tissue

Lymphoid tissue and small intestinal tissue were excised (from 6 rats/group) and stored on ice immediately after dissection for flow cytometry analysis. The mesenteric lymph nodes (mLN) were excised and stored in PBS. PPs were collected from the part of the small intestine that was not used

for protein uptake analysis, washed in 0.9% (w/v) NaCl and stored in PBS. A 10 cm piece of the small intestine (without PP), 28 cm distal from the stomach, was rinsed with 0.9% (w/v) NaCl and stored in wash buffer (PBS, 2% (v/v) FBS, 15 mM HEPES). Preparation of single cell suspensions was performed as previously described ^[26].

2.13 Flow cytometry

One million cells were blocked with 10% (v/v) rat serum and 5 µg/mL anti-CD32 (D34-485, BD Biosciences, Franklin Lakes, NJ, US) in FACS buffer (PBS with 2% (v/v) FBS and 0.05% (v/v) NaN₃), and surface stained using the fluorochrome-conjugated antibodies anti-CD45/APC-eF780 (OX-1, Thermo Fisher Scientific), anti-CD3/PerCp-eF710 (eBioG4.18, Thermo Fisher Scientific), anti-CD4/PE-Cy7 (OX-35, BD Biosciences), anti-CD8/BV510 (OX-8, BD Biosciences), and anti-CD25/BV421 (OX-39, BD Biosciences) as previously described ^[26]. The cells were subsequently stained intracellularly by anti-FoxP3/FITC (JFK-16s, Thermo Fisher Scientific) using the BD Transcription Factor buffer set (BD Biosciences) according to the manufacture's recommendations. Samples were run on a BD FACSCanto II Cell Analyzer (BD Bioscience) and analysed using FlowJo (Version 10.4.2, BD Bioscience).

2.14 Statistics

Graphs were generated and statistically analyses performed with GraphPad Prism 8.4 (San Diego, CA, US). Normal distribution of data was tested by D'Agostino-Pearson normality test. For data that passed the normality test, differences between groups were analysed by one-way ANOVA followed by Tukey's multiple comparison test. For data that did not pass the normality test, differences between groups were analysed by non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. Differences in total IgA levels at different time points but within the same group were analysed by a paired t-test. Data for protein uptake were log-transformed and differences

within the different intestinal compartments at different time points were analysed by two-way ANOVA followed by Dunnett's multiple comparisons test. 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 No increase of protein transport in cells stimulated with quinoa aglycones, CT or capsaicin

In the present study, Caco-2 cells were cultured on semipermeable filters allowing investigation of epithelial transport *in vitro* by measuring FITC-dextran and BLG passage through the monolayer. Sampling from the basolateral compartment after 0.5, 1, 3 and 6 h of exposure to the test substances showed a time-dependent increase of FITC-dextran with the highest fluorescence measured after 6 h (Figure 2A). However, no statistically significant differences could be observed at any time points between the cells stimulated with the various substances and cells not stimulated. To evaluate protein transport through the monolayer, cells were incubated with WPI in addition to the different substances. BLG was quantified by ELISA from the basolateral compartment sampled after 1, 3, 6, 8 and 24 h. Similar to FITC-dextran transport, a clear time-dependent increase in BLG was observed with higher concentration of BLG detected after 24 h compared with 1, 3, 6 and 8 h (Figure 2B). A marginal higher concentration of BLG could be observed in the basolateral compartment of cells stimulated with quinoa aglycones and capsaicin compared with cells without stimulation or stimulated with CT, though not reaching statistical significance.

3.2 Rapid and time-dependent protein uptake in rats administered with quinoa in the presence of CT, or with capsaicin

In order to measure the impact of quinoa on intestinal protein uptake, rats were orally gavaged with WPI, after seven days of daily test substance administration, and euthanised at different time points. BLG was quantified in total protein extracts from three different compartments of the small

intestine, EPI, PP and LP, as well as in serum. Investigating the kinetics of protein uptake revealed an overall fast uptake in all compartments in all groups of animals except from the group administered with PBS (Figure 3). In general, a higher concentration of BLG was quantified in all compartments already 5 min after WPI gavage compared to 15, 30 and 60 min.

In EPI (Figure 3A), a statistically significant higher concentration of BLG was observed after 5 min in rats administered with quinoa in the presence of CT when compared with rats administered with PBS. To a similar extent, though without statistically significant differences ($p=0.1796$), a high BLG concentration was seen in rats administered with capsaicin compared with rats administered with PBS.

In PP (Figure 3B), a statistically significant higher concentration of BLG was measured after 5 min in rats administered with quinoa in the presence of CT, when compared with rats administered with PBS. An additive, or even synergistic, effect on protein uptake of quinoa co-administered with CT was revealed when combining the two substances. Also in rats administered with quinoa alone a slightly higher concentration of BLG could be observed after 5 min ($p=0.1865$) when compared with PBS. In rats administered with capsaicin, a statistically significant higher BLG concentration was seen after 5 min when compared with PBS, and a high concentration of BLG was also observed after 15 min ($p=0.2553$) and to a greater extent than in any of the other groups.

In LP (Figure 3C), a statistically significant higher concentration of BLG was observed after 5 min in rats administered with quinoa in the presence of CT, compared with rats administered with PBS. Indeed, higher concentration of BLG could also be observed after 5 min in rats administered with quinoa alone, CT alone or capsaicin, when compared with rats administered with PBS, but not in a statistically significant degree. In rats administered with quinoa, with or without CT, and in rats administered with capsaicin, substantial concentrations of BLG were still present in the tissue after 15 min. Only very low levels of BLG were present after 30 and 60 min in any of the groups no matter which intestinal compartment.

In serum (Figure 3D), a statistically significant higher BLG concentration was observed after 5 min in rats administered with quinoa with or without CT, CT alone or capsaicin when compared with PBS. In rats administered with capsaicin, BLG could additionally be observed in the serum after 15 and 60 min, showing a slightly different kinetic than the other groups. Collectively, these data demonstrates an extremely fast absorption of protein from the intestinal lumen to the circulating blood in animal exposed to quinoa, CT, and capsaicin (Figure 3E).

Evaluating the mean relative distribution between the three intestinal compartments, no statistically significant differences could be observed among the groups. The BLG was not evenly distributed in the three tissues of the intestine with a larger proportion of BLG present in EPI, compared with PP and LP (Figure 4).

3.3 No change in goblet cell numbers in rats administered with quinoa, CT or capsaicin

Besides epithelial cells, the intestinal EPI also contains mucus producing goblet cells. Investigating the number of goblet cells per villus showed similar numbers in the small intestine among all groups (Figure 5A, representative section Figure 5B). In the large intestine marginal lower numbers were seen in rats administered with quinoa, with and without CT, or capsaicin, when compared with rats administered with PBS or CT alone, however without statistical significant differences (Figure 5C, representative section Figure 5D).

3.4 Few alterations in junctional complex and inflammatory markers after administration of quinoa, CT or capsaicin

Relative expression of various tight junction complex protein genes and immune mediator genes was evaluated in small intestinal tissue. Among the tight junction complex protein genes, no statistically significant differences could be observed in rats administered with quinoa, neither in absence or in the presence of CT. Statistically significantly lower expression of the *Ocln* gene encoding occludin

was, however, observed in rats administered with capsaicin compared with the group administered with PBS (Figure 6A). Studying the expression of the immune mediators revealed that expression of *IFN- γ* in rats administered with quinoa, both with or without CT, was statistically significant higher than rats administered with PBS (Figure 6B). Statistically significant higher expression of *IL-33* was observed in rats administered with capsaicin compared with rats administered with PBS, showing a distinct mode-of-action than quinoa (Figure 6C). No differences were observed in the expression of *IL-1 β* , *TFN- α* and *TSLP* (Figure 6B, 6C).

Evaluating CRP concentrations in serum, a marginal increase could be observed in the group of rats administered with quinoa when compared to rats administered with PBS ($p=0.3340$). No differences could be seen in rats administered with CT or capsaicin (Figure 6D).

3.5 Small changes in humoral and cellular immune responses

Changes in humoral and cellular immune responses were investigated. A statistically significant higher total faecal IgA was observed in rats administered with CT (Figure 7A) after seven daily administrations. When comparing the levels on day 7, no differences between the groups were observed (Figure 7B). Total serum IgA levels were statistically significant lower day 7 compared to day 0 in rats administered with PBS, CT or quinoa alone (Figure 7C). In line with the total faecal IgA, no differences between the groups were observed in total serum IgA on day 7 (Figure 7D).

Intestinal tissue and lymphoid tissues were harvested and processed for analysis of T cell populations by flow cytometry. A statistically significant higher percentage of activated T cells was identified in rats administered with capsaicin when compared with rats administered with PBS in LP (Figure 8A), whereas no differences in activated T cells were found among the other groups. Similarly, a statistically significant higher percentage of cytotoxic T cells was identified in LP in rats administered with capsaicin in comparison to rats administered with PBS (Figure 8B), with no

statistically significant differences observed among the other groups. No differences were observed when evaluating the percentage of regulatory T cells (CD25+FoxP3+ of CD4+) (Figure 8C).

4. Discussion

Quinoa porridge has been suggested as one of the first solid foods for infants ^[11,27]. However, quinoa is known to contain high levels of saponins, which are known for their detergent, emulsifying and immune adjuvant properties ^[12,13,15]. These properties may be more problematic if introduced in infancy, as the immature gut ^[28] may be particularly susceptible to potential adverse effects of quinoa, and these properties may lead to increased intestinal permeability and an enhanced transport of macromolecules across the intestinal epithelial layer. Transepithelial transport of BLG across Caco-2 cells revealed a marginal increase in permeability when cells were stimulated with quinoa and capsaicin. This finding is in line with previous results showing increased *in vitro* epithelial permeability after stimulation with oat saponins ^[29], quillaja saponins ^[30] or capsaicin ^[20,21]. However, *in vitro* studies of intestinal permeability does not reflect the complex intestinal tissue with various cell types and distinct compartments. In the present study, we compared the effect of quinoa alone, quinoa in combination with CT, CT alone and capsaicin on intestinal permeability and inflammation after a week of daily exposure in BN rats.

We studied the ability of quinoa with and without CT, CT alone and capsaicin to promote intestinal permeability by characterising the BLG uptake in EPI, PP and LP, as well as in the serum. Quinoa, CT, and capsaicin were all able to promote a rapid detection of BLG in serum compared to the PBS control, demonstrating the compounds ability to increase intestinal permeability. However, the compounds showed different abilities to promote BLG uptake in the intestinal compartment, indicating different underlying mechanisms between the compounds. The epithelial barrier is highly regulated by transmembrane and intracellular proteins that form junctional complexes. Generally, the increased permeability was not associated with notable

changes in the gene expression of tight junction complex proteins in the intestine, indicating that the increased permeability was not mediated by alterations in the function of tight junctions. However, we cannot exclude alterations in function of tight junctions at the protein level. Immunofluorescence analysis could be considered in future studies exploring the distribution and rearrangement of junctional complexes as seen in a study investigating the effects of gliadin on intestinal permeability [17].

Secretory IgA and goblet cells play a central role in maintaining intestinal mucosal barrier function. IgA secreted into the intestinal lumen protect the gut from invading pathogens and control the commensal microbiota by binding to viruses, bacteria, and toxins in order to prevent their attachment to epithelial cells [31,32]. Goblet cells produce proteins and other factors that contribute to the mucus layer further protecting the intestinal EPI from the luminal content. Changes in IgA levels and goblet cell numbers can be used to evaluate changes in homeostatic maintenance of the intestinal mucus layer. CT was found to increase the level of faecal IgA, whereas limited effects on goblet cell numbers were seen for the quinoa, CT and capsaicin, indicating that the increased intestinal permeability mediated by these compounds may not arise from changes to goblet cells or the mucus layer. However, CT may specifically alter the homeostatic production of secretory IgA further highlighting different biological effects of the studied compounds.

Intestinal inflammation is a common cause of increased intestinal permeability. Inflammation was assessed by serum CRP and intestinal expression of the inflammatory cytokines *IFN- γ* , *TNF- α* , *TSLP*, *IL-33*, *IL-1 β* . Quinoa was found to significantly increase the intestinal expression of *IFN- γ* , as well as a marginal increase of serum CRP levels. Inflammation as a result of quinoa intake may have adverse health effects. *IFN- γ* has previously been shown to mediate paracellular intestinal permeability in 3D culture systems via a mechanism that are distinct from permeability mediated by *TNF- α* [33]. In the present study, quinoa did not upregulate the expression of *TNF- α* and *IL-1 β* . This finding may be contrary to previous reports of soy saponin-induced *TNF- α* and *IL-1 β* production in a

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mouse macrophage cell line ^[34]. However, this discrepancy highlights the importance of studying the immunological mechanisms of saponins in context of the complex *in vivo* setting comprising several different immune cells. Capsaicin specifically upregulated intestinal gene expression of *IL-33*, which was associated with increased numbers of activated (CD25+) helper (CD4+) and cytotoxic (CD8+) T cells in the LP. These findings indicate that quinoa, CT and capsaicin exhibit different pro-inflammatory properties in the intestine. No differences were observed in the frequency of CD4+CD25+FoxP3+ regulatory T cells within the PP, mLN or LP in response to quinoa with or without co-administered CT, CT alone or capsaicin, indicating that the compounds do not affect homeostatic immune regulatory mechanisms in the gut.

The present study demonstrates that seven daily administrations of quinoa flour, particular when co-administered with CT leads to increased intestinal permeability. This was observed even though the daily administration of quinoa corresponded only to 1-2% of the daily food intake for adult rats ^[35]. The immune adjuvant effect of saponins has previously been demonstrated by the ability to increase humoral vaccine responses via the subcutaneous, nasal, and oral routes ^[36,37]. Oral administration of saponins was associated with increased intestinal permeability demonstrated by increased levels of protein markers in serum ^[38,39]. These studies support a role for quinoa-derived saponins in rapid protein uptake in the intestinal compartments and dissemination into the serum. However, the underlying mechanism remains largely unknown, although the saponins have been shown to directly act on epithelial cells ^[29,30]. CT-induced intestinal permeability is likely mediated by cellular stress and inhibition of cell junction formation following the uptake in epithelial cells ^[40–42]. Similar, capsaicin has been shown to increase intestinal permeability by altering cell junction formation in epithelial cells ^[43]. Our results indicate that quinoa, CT, and capsaicin mediate intestinal permeability via different mechanisms although we cannot exclude potential differences arising from the doses administered and/or individual potency of the

compounds. Especially in relation to quinoa when taken into consideration the relative low dose administered.

In conclusion, this study showed that quinoa increased intestinal permeability. Increased intestinal permeability is a feature associated with several chronic diseases and has in recent years been a subject to intense study for potential intervention ^[44]. Thus, food components affecting intestinal permeability may have adverse health effects by contributing to disease development. Further studies are needed in order to advice on introduction of quinoa to the diet of infants.

Acknowledgement

The authors thank Elise Navntoft, Kenneth Rene Worm, Maja Danielsen, Camilla Bæhr Everaerts, and Anne Ørngreen for their great assistance with the animal experiment. A thank to Sarah Grundt Simonsen, Juliane Margrethe Gregersen, Nanna Jordahn Kristoffersen, Heidi Broksø Letting, and Marianne E. Jensen for their great assistance with the laboratory work. All authors declare no conflict of interest.

Author contributions

KLB, KP and PHR conceived the study. ASRB and PHR extracted the quinoa products. ASRB and EU designed and conducted the *in vitro* studies, whereas ASRB and KLB designed and conducted the *in vivo* experiment. ASRB performed and analysed *in vivo* protein uptake, histology, gene expression, CRP levels and antibody levels, whereas JML performed and analysed flow cytometry. ASRB presented results and drafted the manuscript. All authors made substantial intellectual contributions, revised and reviewed the manuscript critically, and approved the final version of the manuscript.

Supporting information

Supplementary figures are available.

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Figure legends

Figure 1

Animal experimental design. Naïve Brown Norway rats were administered by gavage daily for seven consecutive days with either PBS containing 0.2% (v/v) EtOH, cholera toxin (CT), quinoa with or without co-administrated CT, or capsaicin. At day seven all rats were gavaged with whey protein isolate and subsequently euthanised after 5, 15, 30 or 60 min (six rats per group per time point). Faecal and blood samples were collected at day 0 and 7 of the experiment.

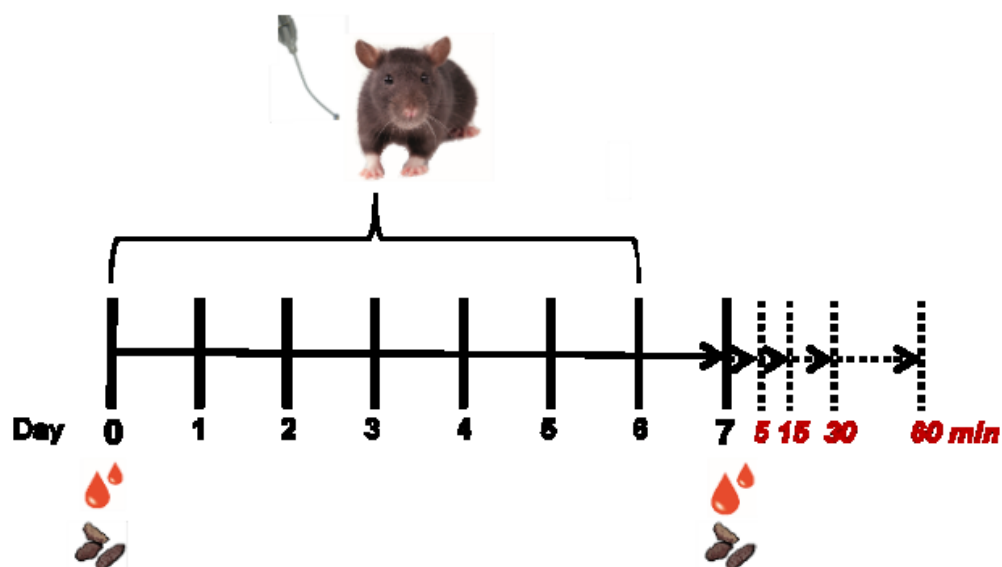


Figure 2

In vitro intestinal transport. A, Caco-2 cells grown on semipermeable filters and basolateral fluorescence intensity measured after incubation of a 4 kDa FITC-dextran together with 0.2% EtOH, cholera toxin (CT), quinoa aglycones (agl.), or capsaicin for 0.5, 1, 3, and 6 h. B, Caco-2 cells grown on semipermeable filters and basolateral β -lactoglobulin (BLG) concentrations measured after incubation of whey protein isolate (WPI) together with 0.2% EtOH, CT, quinoa agl., or capsaicin for 1, 3, 6, 8, and 24 h. Each bar represent three mean values from three different experiments $\pm$ SEM.

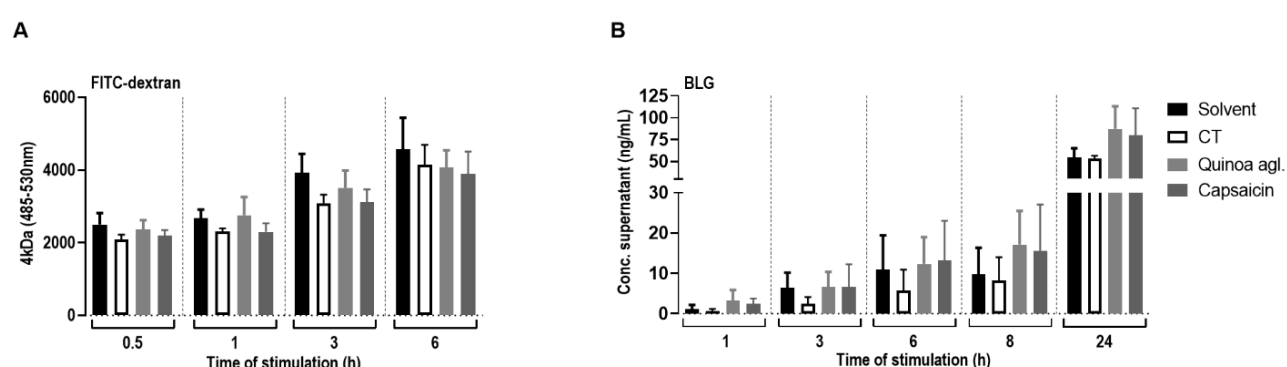


Figure 3

In vivo intestinal concentration of β -lactoglobulin (BLG). A, Epithelium (EPI). B, Peyer's patches (PP). C, Lamina propria (LP). D, Serum. Concentrations of BLG 5, 15, 30, and 60 min after gavage with whey protein isolate. E, schematic figure summarising protein uptake in the three intestinal compartments as well as in serum. Arrows represent statistical significant higher concentration of BLG when compared with the control group. The figure is created with BioRender.com. Each bar represent six rats per group per time point $\pm$ SEM. Statistical significant differences between indicated groups are shown as * $P \leq 0.05$, ** $P \leq 0.01$.

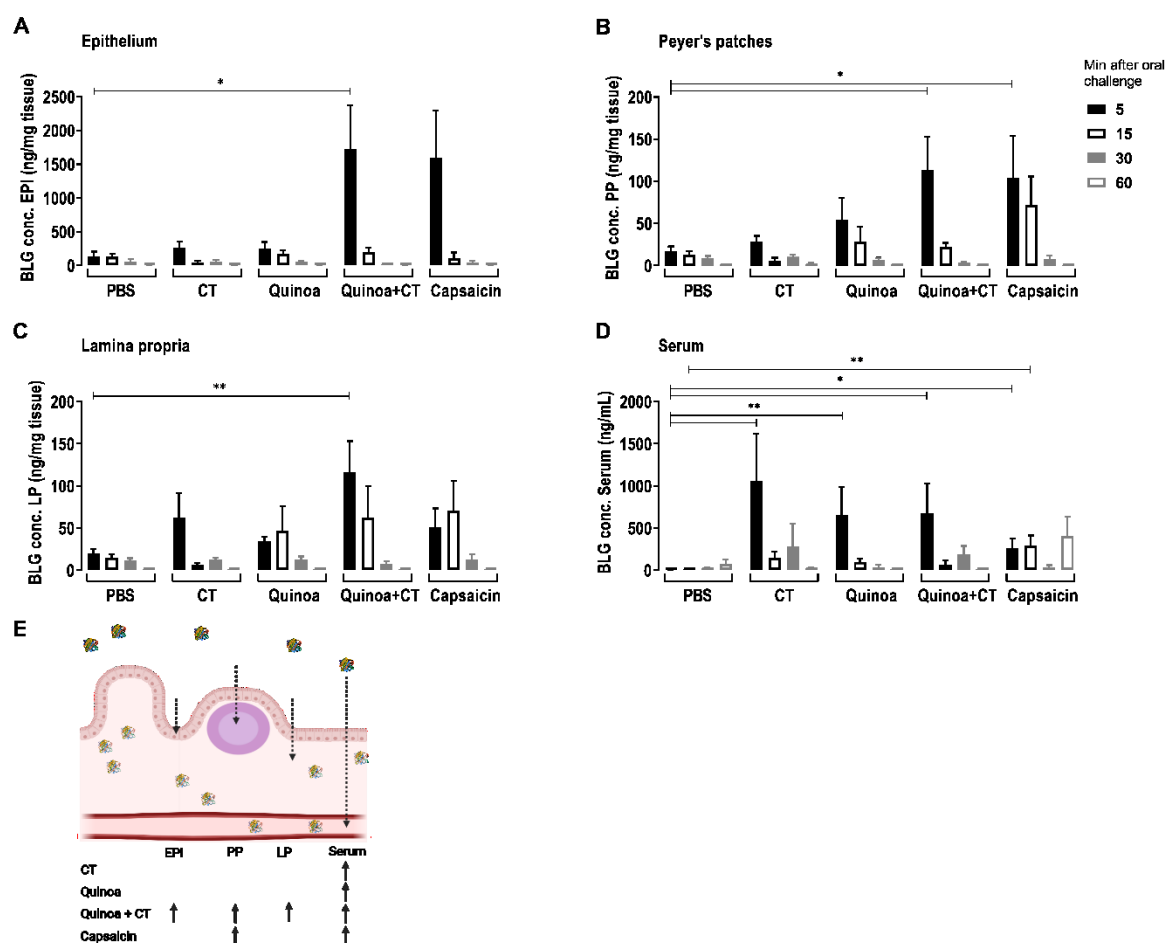


Figure 4

Mean relative distribution of β -lactoglobulin (BLG) in epithelium (EPI), Peyer's patches (PP) and lamina propria (LP) fractions per tissue weight.

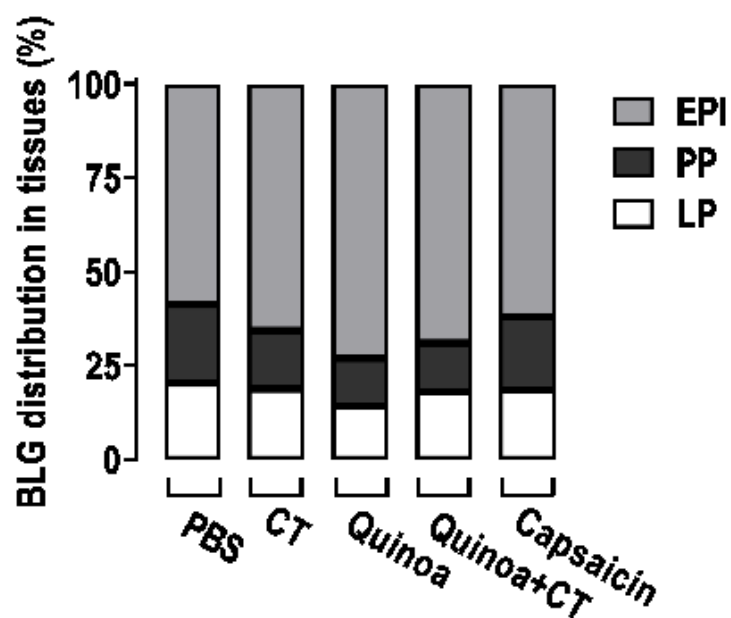


Figure 5

Goblet cell counts. Average goblet cells per villi in histological slides stained with Periodic acid-Schiff (PAS). A, Numbers of goblet cells measured in the small intestine. B, Representative slide from the small intestine. C, Numbers of goblet cells measured in the large intestine. D, Representative slide from the large intestine. Each symbol represent one animal and horizontal lines indicate median values.

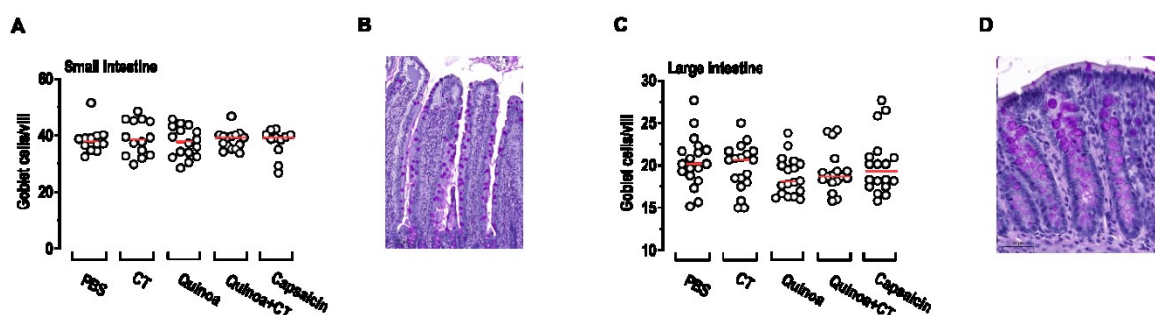


Figure 6

Intestinal gene expression and serum C-reactive protein (CRP). Relative gene expression is in accordance to the two reference genes, *B2m* and *SDHA*. A, Expression of junctional complex genes. B, Expression of inflammatory marker genes. C, Expression of interleukins. D, Concentration of CRP in serum. Each group represent 12 rats per group $\pm$ SEM. Statistical significant differences between indicated groups are shown as * $P \leq 0.05$, ** $P \leq 0.01$.

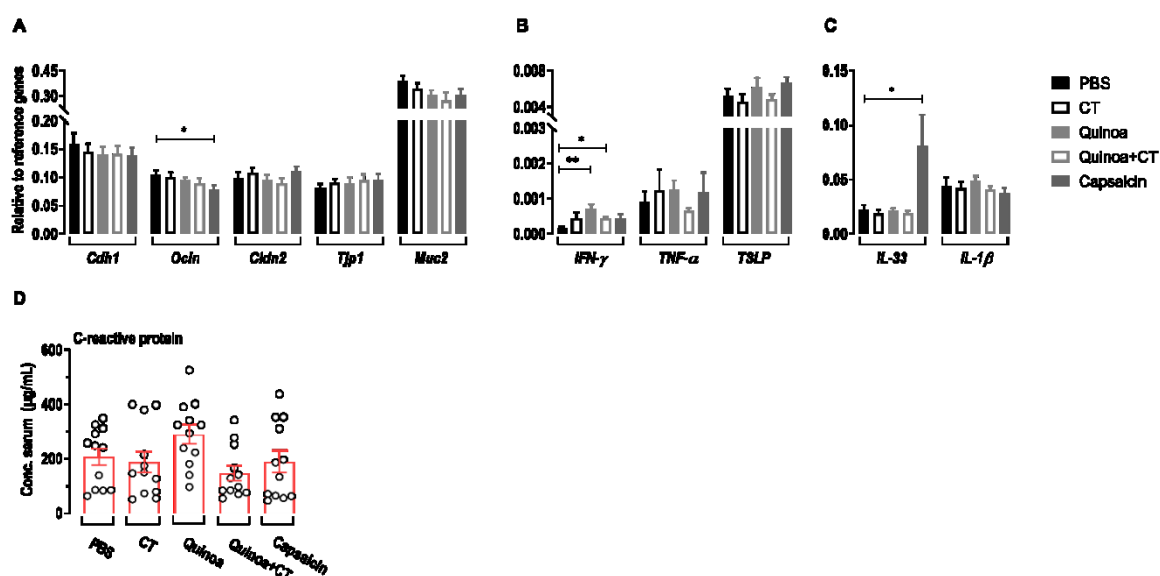


Figure 7

Total IgA titres. A, Total faecal IgA titres (log2) between day 0 and 7. B, Total faecal IgA titres (log2) day 7 compared between groups. C, Total serum IgA titres (log2) between day 0 and 7. D, Total serum IgA titres (log2) day 7 compared between groups. Each symbol represents one animal and horizontal lines indicate median values (18 rats per group). Statistical significant differences between indicated groups are shown as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

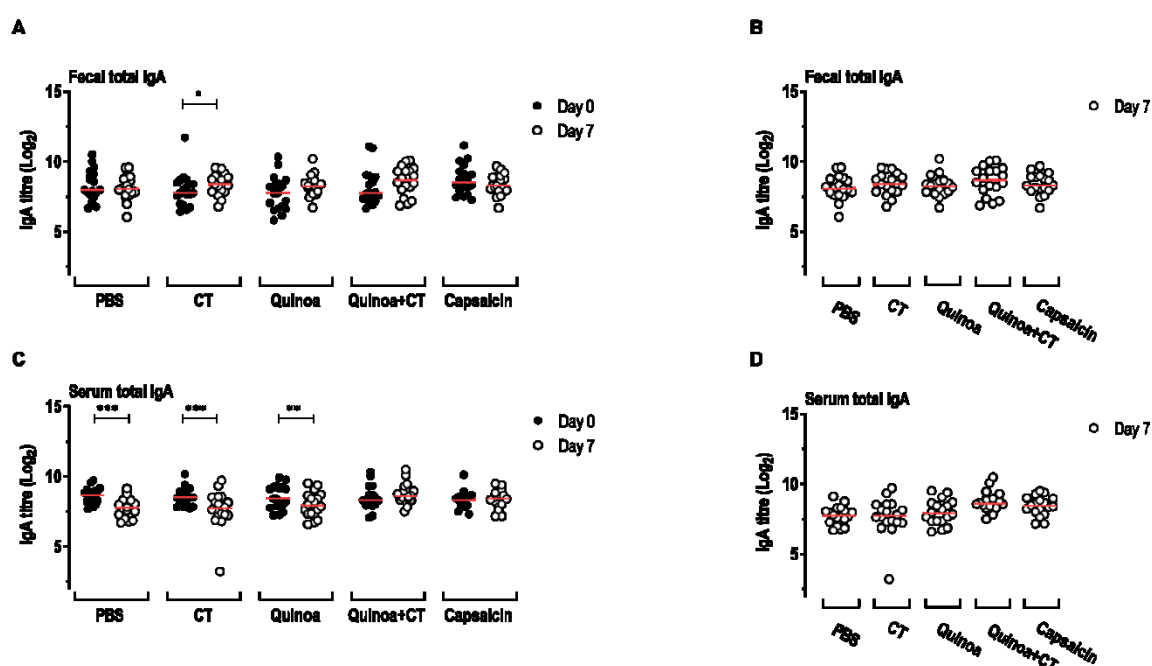
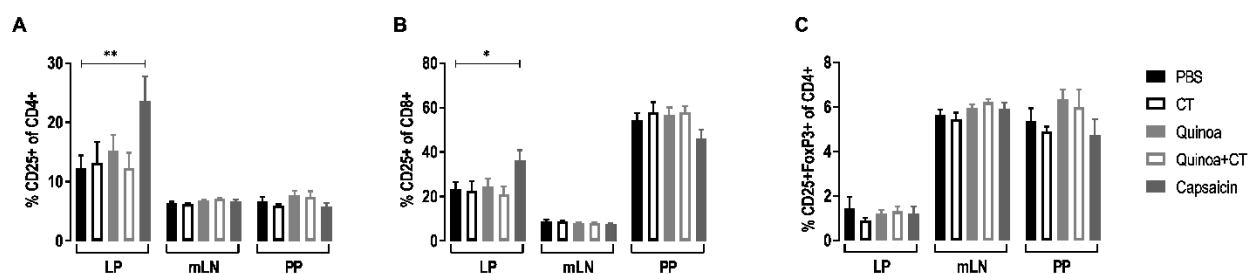
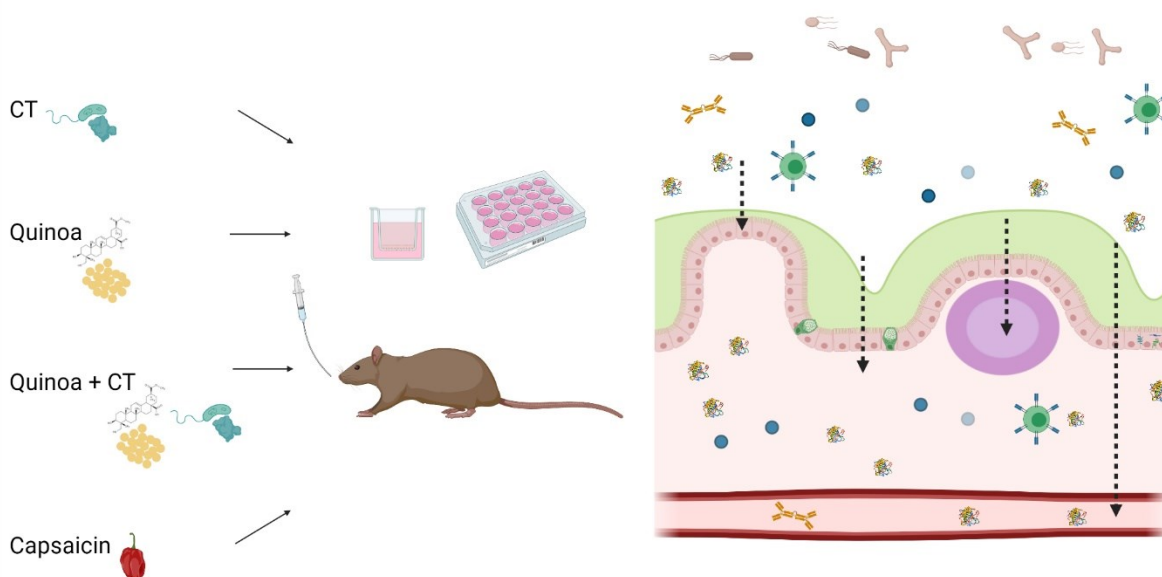


Figure 8

T cell population. A, Percentage of CD25+ cells of CD4+ T helper cells in lamina propria (LP), mesenteric lymph node (mLN), and Peyer's patches (PP). B, Percentage of CD25+ cells of CD8+ cytotoxic T cells in LP, mLN, and PP. C, Percentage of CD25+FoxP3+ cells of CD4+ T helper cells in LP, EPI, and PP. Each bar represent six rats per group $\pm$ SEM. Statistical significant differences between indicated groups are shown as * $P \leq 0.05$, ** $P \leq 0.01$.



High levels of saponins are present in quinoa seeds, which are known for their adjuvant activity and effect on the intestinal barrier and immune functions. In this study, in vitro and in vivo data demonstrated that quinoa seeds increase intestinal permeability and promote protein uptake in a manner that differ from the two positive controls; cholera toxin and capsaicin. On the contrary, quinoa seeds only had little effect on the immune function.



Supplementary figures

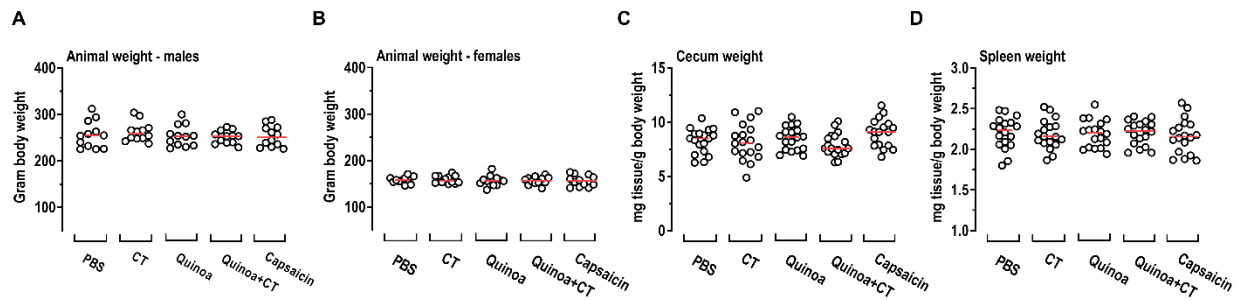


Figure S1

Weight of animals and dissected organs. A, animal weight of male rats. B, animal weight of female rats. C, weight of cecum. D, weight of spleen. Each symbol represent one animal and horizontal lines indicate median values.

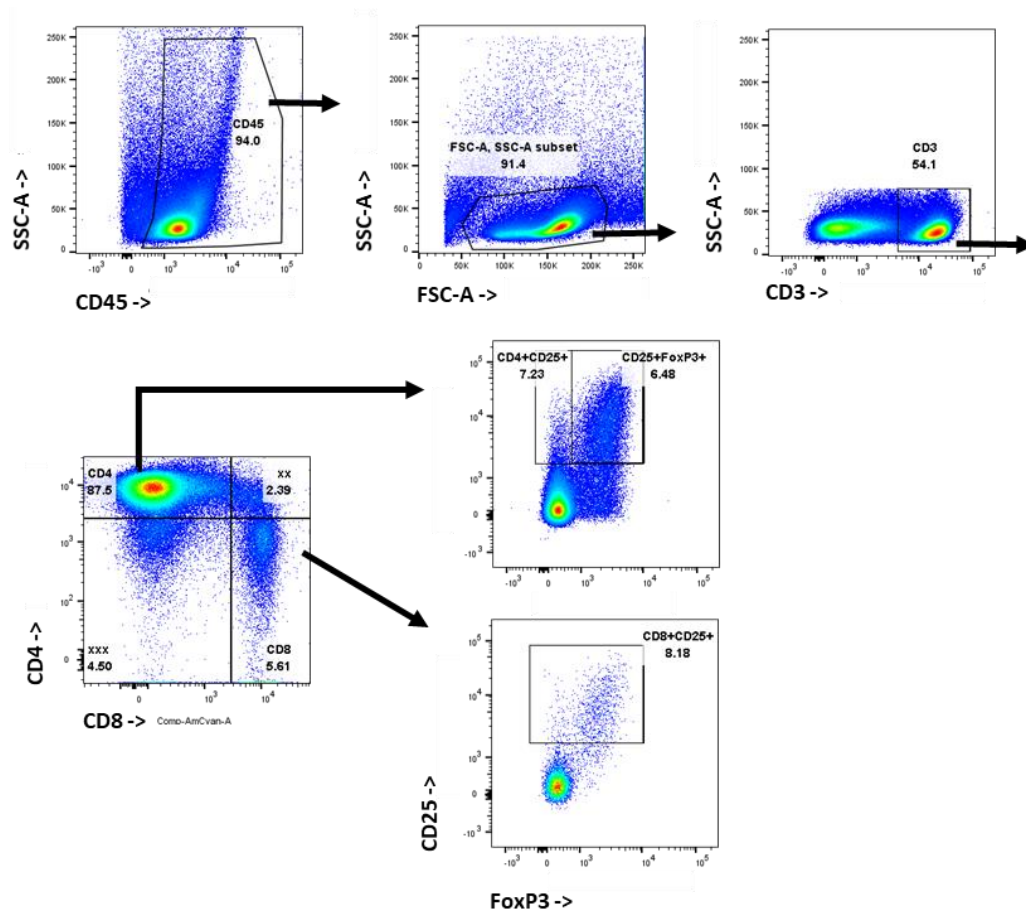


Figure S2

Gating strategy for flow cytometry. Leucocytes were identified by CD45 and lymphocytes by forward-side scatter. T cells were identified by CD3, and differentiated into CD4⁺ helper and CD8⁺ cytotoxic T cells. Conventional regulatory T cells were identified as CD3⁺CD4⁺CD25⁺FoxP3⁺. Activated helper T cells were identified as CD3⁺CD4⁺CD25⁺FoxP3⁻ and activated cytotoxic T cells as CD3⁺CD8⁺CD25⁺.