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Investigation on the allergen profile of the soluble fraction of autoclaved peanuts and its interaction with Caco-2 cells

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Peanuts are a source of proteins and fats but they are also considered a harmful food for individuals who are allergic to them due to their ability to trigger severe and life-threatening reactions. Strict avoidance of peanuts is the most effective means to prevent the development of an allergic reaction. Physical or chemical strategies employing autoclaving can represent a valid alternative to produce a final food with a decreased allergenic power as in the case of peanuts. Thermal processing might induce protein modifications in foods and affect protein digestibility or absorption of nutrients across the intestinal mucosa. Besides, the type of processing could also alter food protein allergenicity thus influencing the interplay with the biological system at the gut level. In this paper, we investigated the influence of autoclaving based treatments on the proliferation of epithelial cells at the intestinal level. Extractable proteins of raw and autoclaved peanuts were analysed by SDS-PAGE and untargeted LC-high resolution-MS/MS to investigate the peptide composition. Our findings show that when raw peanuts were assayed on Caco 2 cell lines, an antiproliferative effect was observed. By contrast, peanuts subjected to hydration and autoclaving did not show an inhibition of proliferation on Caco-2 cells. In parallel, extensive fragmentation induced by autoclaving treatments on the original peanut proteins was also recorded by LC-MS/MS analysis with a consequent increase in the number of peptides detected. These results indicate that the processing applied to peanuts can have an influence on both the nutritional and allergological sides, and more investigations will be required on this issue to understand the alteration of inflammatory mediators induced by the treatment applied.

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1. Introduction

Food allergy is a widespread health problem especially affecting young children and an increase in its prevalence has been recorded over the past decade.¹ Peanut allergies represent one of the most harmful types of food allergies due to the high number of anaphylactic shocks recorded throughout the population after peanut ingestion.²

Recent data have shown that the prevalence of sensitisation to peanuts in the adult population is approximately 9.3% in the US and between 0.8% and 4.2% in European countries.³ Conversely, this type of food allergy is infrequent in China and Asian countries, despite the high rate of peanut consumption.^{4,5}

Strict avoidance of allergenic foods remains the most effective means to prevent the onset of allergic reactions, although the development of physical or chemical strategies aimed to reduce food or protein allergenicity could represent a practical approach to the problem. As far as peanuts are concerned, it has been reported that the varietal diversity or the different processing conditions applied to peanuts can alter their protein structure in different ways (including unfolding, aggregation, cross-linking between the ingredients and chemical modifications, such as oxidation and glycosylation)^{6,7} and can have a deep impact on the sensitivity recorded throughout consumers. Notably, sensitivity response can depend on the structure and/or the chemical properties of the allergen, the thermal processing applied (whether dry or wet heat), and the duration and temperature of the heating. Besides, the interaction with other food matrix components or the influence of the specific variety may have a major impact on the overall allergenicity of foods.⁸

It is noteworthy that protein modifications induced by food processing also affect protein digestibility^{9,10} and consequently its absorption across the intestinal mucosa. Indeed, many

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food allergens are stable proteins that are very resistant to digestion by gastrointestinal enzymes^{11–13} and/or can be digested into high molecular weight peptide fragments which retain IgE binding and T-cell stimulating capacities.^{13,14}

Exposure of the immune system to the raw or processed matrix can trigger local gastrointestinal (GI) immune events in the intestinal mucosa, such as the activation of lymphocytes and cytokine release consequent to food allergen ingestion.

A deeper understanding of the causes triggering food hypersensitivity upon allergen ingestion is needed due to the complex mechanism of immunomodulation occurring after the interaction of allergenic food proteins with the intestinal epithelium that is crucial for the subsequent development of an immune response. Indeed, from a nutritional standpoint, foods contain a rich and varied source of peptides bearing valuable and diverse biological functions as anti-hypertensive, antimicrobial, and immunomodulatory activities. MS is currently the elective technique to profile food compounds and/or characterize the entire panel of peptides related to a specific protein matrix or organism. Many fragments released by the proteolysis of milk, meat or cereal proteins and identified by MS analysis have been claimed as biologically active.¹⁵ However, the persistence and bioavailability of peptides *in vivo* are still under debate, because to reach the epithelial barrier and exert physiological functions, they must be in sufficient concentrations. The epithelial barrier does not represent only a physical barrier with the external environment or for nutrient uptake, but also plays an active role in the immunological process, driving innate immune responses, modulating adaptive immunity and ultimately, therefore, playing an essential role in the pathogenesis and development of food allergy.¹⁶

In vitro models could be of some utility to a better understanding of these processes. In this regard, previous studies have demonstrated how the exposure of Caco-2 cells to heated Ara h 1 (one of the major allergenic peanut proteins) was able to promote the proliferation of these cells along with a significant secretion of IL-8.¹⁷ Caco-2 cells are generally used as an *in vitro* model for human epithelium cells, since they functionally and morphologically resemble human enterocytes¹⁸ and allow the investigation of the effect of biological activity of food components on different functions, including cell proliferation and secretion of key mediators. Besides, this model is used to show that the different type of food processing can not only modify the allergenic food proteins but can also interplay with the biological system at the gut level modulating inflammatory events due to the development of an allergic reaction.

In this framework, this study aimed at investigating whether new physical treatments that couple pressure and temperature can influence the composition of the allergenic proteins and the peptides produced during the applied processes, and whether it can affect the biological activity at the intestinal level. In particular, the effect of the processing used on peanuts on the growth of human Caco-2 cells was investigated as well as the change in the levels of CD23 expressed at the cellular level, to provide some understanding of their possible implications on the gut mucosa.

2. Experimental

2.1 Chemicals

Trizma-base, sodium chloride, urea, ammonium bicarbonate, iodoacetamide (proteomic grade, IAA) along with other chemicals for electrophoresis (dithiothreitol-DTT, sodium dodecyl-sulfate-SDS, glycine, glycerol, Coomassie brilliant blue-G 250 and methanol-HPLC grade) were obtained from Sigma-Aldrich (Milan, Italy). Bromophenol blue was provided by Carlo Erba Reagents (Cornaredo, MI, Italy). Electrophoresis experiments were accomplished on Mini Protean Tetra Cell equipment provided by Bio-Rad Laboratories (Segrate, MI, Italy). Syringe filters in cellulose acetate (CA), 1.2 μm , were obtained from Labochem Science S.r.l. (Catania, Italy) while those in polytetrafluoroethylene (PTFE), 0.45 μm , were purchased from Sartorius (Gottingen, Germany).

Acetonitrile (Gold HPLC ultragradient) and trifluoroacetic acid (TFA) were purchased from Carlo Erba Reagents (Cornaredo, Milan, Italia) and ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Formic acid (MS grade) was provided by Fluka (Milan, Italy) while trypsin (proteomic grade) was purchased from Promega (Milan, Italy). PTFE syringe filters (4 mm, 0.2 μm) and ultrafiltration (UF) tubes with 10 kDa cut-off membranes were purchased from Sartorius (Sartorius Stedim Italy S.p.A., Antella-Bagno a Ripoli, FI, Italy).

2.2 Peanut samples

Raw peanut seeds (*Arachis hypogaea* var. Virginia) used in the present study were provided by Besana s.p.a. (San Gennaro Vesuviano, NA, Italy). A total of 8 seeds (corresponding to approximately 10 g) were placed into a centrifuge tube and subjected to autoclaving treatments. Two processing schemes were investigated including or not preliminary hydration of peanuts. The hydration of whole peanut seeds was performed for 2 hours at room temperature in an orbital shaker (KS 4000 i-control shaker, IKA Works GmbH & Co. KG, Staufen, Germany) with ultrapure water, before autoclaving. Autoclave settings were: a temperature of 134 °C at a pressure of 2 atm for 10 minutes. After that, raw and thermally processed peanut seeds were milled and subjected to protein extraction.

2.3 Protein extraction

Raw and autoclaved peanuts were ground to a fine powder by using an electric grinder (Mulinex, Milan, Italy). Proteins were extracted into TBS buffer (50 mM Tris-HCl, 150 mM NaCl) containing 1 M urea (pH 8), according to the following protocol: 4 ml of extraction buffer were added to 1.4 g of sample and left shaking for 1 h at a temperature of 26 °C in an orbital shaker (KS 4000 i-control shaker, IKA Works GmbH & Co. KG, Staufen, Germany). After that, the samples were centrifuged for 20 min at 1734g at a temperature of 18 °C. The central phase was carefully collected and filtered through a 1.2 μm CA syringe filter. The material was stored at –20 °C until its use and filtered on a 0.45 μm PTFE filter just before electrophoretic analysis.

2.4 Bradford quantification

The protein content of raw and thermally processed peanuts was measured as mg/albumin equivalent using the Bradford method (Quick Start™ Bradford Protein Assay). Bovine serum albumin (BSA), in the range comprised between 1 mg ml⁻¹ and 0.125 µg ml⁻¹, was used as a standard, and the absorbance at 595 nm wavelength was measured.

2.5 SDS-PAGE analysis

Protein extracts were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8–16% polyacrylamide pre-cast gels (8.6 cm × 6.7 cm × 1 mm). Before gel analysis, samples were mixed (1:1 ratio) with a Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, 100 mM DTT) and then kept at 100 °C for 5 min to allow denaturation. Specifically, 10 µg of proteins were loaded in each lane. PAGE gels were run in the Mini-Protean Tetra Cell equipment (Bio-Rad Laboratories, Segrate, MI, Italy) with TGS (25 mM Tris, 192 mM glycine, 0.1% SDS) running buffer under the following conditions: 50 V for 15 min and 80 V until the end of the run. Gels were stained using a solution of Coomassie Brilliant Blue G-250, and the bands were detected on a Gel Doc EZ Imager system (Bio-Rad Laboratories, Segrate, MI, Italy). For protein molecular weight referencing, a Precision Plus Protein™ All blue standard (10–250 kDa, Bio-Rad Laboratories, Segrate, MI, Italy) was loaded on each gel.

2.6 Cell culture conditions

The human colon adenocarcinoma-derived Caco-2 cell line was obtained from the Interlab Cell Line Collection (Genoa, Italy). Cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin in monolayer culture and incubated at 37 °C under a humidified atmosphere containing 5% CO₂ in the air. At confluence, the grown cells were harvested using trypsinisation and serially subcultured with a 1:4 split ratio. All cell culture components were purchased from Sigma-Aldrich (Milan, Italy).

2.7 Peanut treatments

Caco-2 cells were seeded at a density of 2×10^5 cells per 5 ml of supplemented RPMI-1640 medium in 60 mm tissue culture dishes (Corning Costar Co., Milan, Italy). After 24 h, the extracts containing 1 mg ml⁻¹ of raw and thermally processed peanuts (raw, autoclaved, hydrated-autoclaved) were filtered on sterile filters (Millex-GS pore size 0.22 µm, Millipore, Bedford, MA, USA) before supplementing the culture medium for 1 h, 3 h, 6 h, 24 h, and 48 h. Triplicate cultures were set up for each treatment and the control, and each experiment was repeated three times. Cell viability, determined using the Trypan blue exclusion test, always exceeded 90%.

2.8 Assessment of cell proliferation

After Caco-2 had been cultured for 1 h, 3 h, 6 h and 24 h with 1 mg ml⁻¹ of raw and thermally processed peanuts (the ratio 8.4 g matrix per 16 ml buffer was used for protein extraction for this experiment), the proliferative response was measured by a colorimetric 3-(4,5 di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. To determine cell growth by the colorimetric test, MTT stock solution (5 mg ml⁻¹ in medium) was added to each dish at a volume of one-tenth the original culture volume and incubated for 2 h at 37 °C under a humidified CO₂ atmosphere. At the end of the incubation period, the medium was removed, and the blue formazan crystals were solubilised with acidic isopropanol (0.1 N HCl absolute isopropanol). A spectrophotometer was used to monitor MTT conversion to formazan by metabolically viable cells at an optical density of 570 nm.

2.9 Effects of peanut treatment extracts on the inflammatory marker level

The effects of raw and thermally processed peanuts administered for 3 h on the CD23 protein levels of Caco-2 cells were evaluated using western blot analysis.

Caco-2 cells were collected and lysed on ice in RIPA buffer (Pierce Ripa buffer, Thermo Scientific, Rockford, IL, USA). After homogenization and centrifugation at 14 000 rpm for 15 min at 4 °C, the protein concentration was measured using a standard Bradford assay (Bio-Rad, Milan, Italy). Aliquots of 50 µg of total proteins were separated on 4–12% pre-cast polyacrylamide gels (Bio-Rad, Milan, Italy) and transferred onto a PVDF membrane (Bio-Rad, Milan, Italy) by using a Transblot Turbo (Bio-Rad, Milan, Italy). CD23 and β-actin protein expressions were evaluated by using 1:500 diluted CD23 and a β-actin antibody (Immunological Sciences, Rome, Italy). After overnight incubation, the membrane was further incubated with a horseradish peroxidase-conjugated goat secondary antibody (Bio-Rad, Milan, Italy). The proteins were detected by chemiluminescence (ECL, Thermo Scientific, Rockford, IL, USA) and the densitometric analysis of each protein-related signal was obtained using the Molecular Imager Chemidoc™ (Bio-Rad, Milan, Italy) and normalised against the β-actin expression.

2.10 LC-HRMS/MS analysis

2.10.1 Sample preparation for MS analysis. Protein extracts from untreated and treated peanuts were subjected to tryptic digestion before LC-MS/MS analysis. To this aim, an aliquot of each extract corresponding to 50 µg of proteins was firstly denatured at 95 °C for 15 min and then reduced by adding a calculated volume of 50 mM DTT solution. Protein reduction was obtained by keeping samples at 60 °C for 30 min under constant agitation. Successively, after cooling the samples at room temperature, a calculated amount of IAA solution (100 mM) was added in order to alkylate the reduced disulfide bonds. The reaction was carried out for 30 min leaving the samples under dark conditions. Finally, 1 µl of a trypsin solu-

tion ($1 \mu\text{g } \mu\text{L}^{-1}$ in 50 mM acetic acid) was added to the sample so that a ratio of 1/50 (enzyme/protein based on the results of the Bradford protein assay) was obtained to facilitate complete enzymatic digestion. The reaction was stopped after 14 h by acidifying the sample with 1 M HCl. Before LC/MS analysis, tryptic digests were centrifuged at 10 000 rpm for 5 min and the supernatant was filtered on RC 0.45 μm syringe-filters.

2.10.2 Isolation of low molecular weight components of raw and autoclaved peanuts. Peanut extracts were subjected to purification in ultrafiltration tubes with cut-off membranes (10 kDa) to collect the fraction enriched with low MW peptides. In particular, 500 μL of sample was loaded onto ultrafiltration tubes previously conditioned with ultrapure water. After 1 h of centrifugation (14 000g), the sample fraction with a MW lower than 10 kDa was collected, filtered through a cellulose syringe filter (0.2 μm), subjected to tryptic digestion and analysed by untargeted HPLC-HRMS/MS.

2.10.3 Untargeted HR MS/MS analysis. Peptide mixtures that arose from untreated and processed peanut digestion were analysed by LC-MS/MS using an Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer coupled to a UHPLC pump system (Thermo Fisher Scientific, Bremen, Germany). For chromatographic separation, a reversed phase Aeris peptide analytical column (internal diameter 2.1 mm, length 150 mm, particle size 3.6 μm , porosity 100 Å, Phenomenex, Torrance, CA, US) was used. The flow rate and volume of injection were set to 200 $\mu\text{L mL}^{-1}$ and 20 μL respectively. The elution gradient started with 5% B increased to 40% B in 45 min and then to 60% in 20 min. An isocratic step at 85% B continued for 15 min, followed by a return to 5% B in 5 min. At the end of the run, the column was allowed to equilibrate at 5% B for 25 min. As solvent A, $\text{H}_2\text{O} + 0.1\%$ FA was used, while solvent B was acetonitrile + 0.1% FA. The MS spectrometer was run in data dependent (FullMS-dd2) positive ion mode and the spectra were acquired in the range of 200–2000 m/z . MS parameters, along with ESI conditions, were the same as described in Bavaro *et al.*, 2018 except for the dd-setting maximum AGC target that was here decreased to 5.00 e1. Moreover, in the current work, ions with a charge equal or higher than four were excluded. Each sample was injected twice in the MS equipment.

The obtained data set (two analytical replicates for each sample) was simultaneously processed *via* the commercial software Proteome Discoverer™ version 2.1 (Thermo-Fisher-Scientific, San José, US) and protein identification was achieved by SequestHT search against a database extracted by Swiss Prot DB on the basis of the taxonomy code of *Arachis hypogaea* (ID: 3818) and contains about 2362 sequences. Although peanut extracts were subjected to tryptic digestion before LC/MS analysis, unspecific enzyme cleavage was set to generate theoretical peptides, to take into account also those peptides originating from protein fragmentation induced by thermal/pressure treatments applied to the peanut samples. Mass tolerance on the precursor and fragment ions was set to 5 ppm and 0.05 Da, respectively. Moreover, only peptides exhibiting an “unambiguous” PMS (peptide match spectrum)

were considered by setting the confidence to medium ($\text{FDR} < 5\%$) and high ($\text{FDR} < 1\%$).

2.11 Statistical analysis

Due to the non-normal distribution of the data, non-parametric tests were performed. Data were analysed by Kruskal–Wallis analysis of variance and Dunn's multiple comparison test. All data are expressed as mean $\pm$ standard error of the mean (SEM). Differences were considered significant at $P < 0.05$. A specific software package (SigmaStat for Windows version 3.00 SPSS Inc. San Jose, CA, USA) was used.

3. Results and discussion

3.1 Effects of treatments on the peanut protein profiles

Raw and autoclaved peanuts were extracted according to the protocol described in the “Experimental” section and separated on an SDS-PAGE gel as pictured in Fig. 1. As shown in the figure, the protein profiles of autoclaved peanuts appear considerably different from raw peanuts. In particular, raw peanuts' protein bands with a MW between 75 and 150 kDa (lane 1), attributed to Ara h 1 in our previous study,¹⁹ were significantly reduced when the samples were autoclaved for 10 minutes at 134 °C (lane 2). By contrast, a thermal resistance

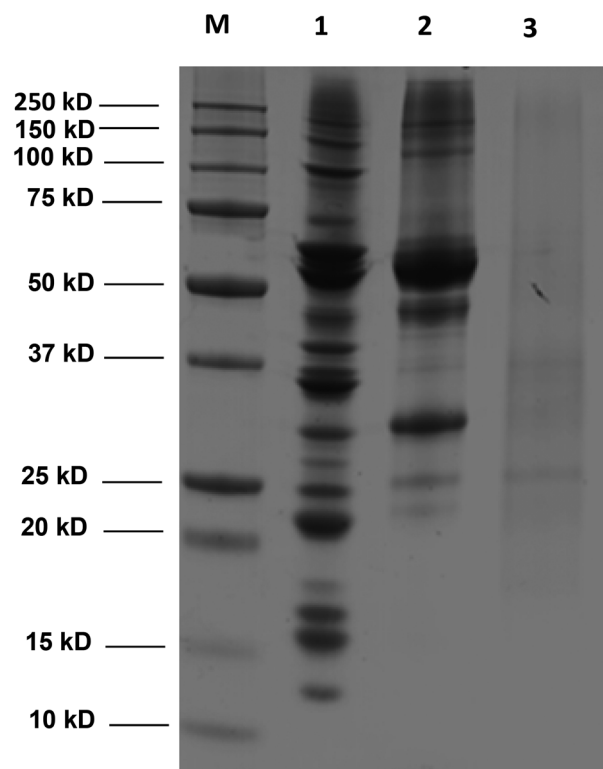


Fig. 1 SDS-PAGE gel separation of raw, autoclaved and hydrated-autoclaved peanuts. Lane 1, raw peanuts; Lane 2, peanuts autoclaved at 134 °C, 2 atm, for 10 min; Lane 3, peanuts pre-hydrated and autoclaved at 134 °C, 2 atm, for 10 min. For each sample, 10 μg of proteins were loaded.

was observed for bands with a MW slightly above 50 kDa and between 50 and 45 kDa, which exhibited a signal intensity similar to the corresponding bands of raw peanuts. These protein bands were attributed both to Ara h 1 and Ara h 3 allergen proteins. Previous studies have already described the resistance of peanut proteins after conventional heat treatments.^{20,21} In particular, heating was reported to induce fewer changes in Ara h 2 and Ara h 3 than in Ara h 1. In this case, a loss of the original 63.5 kDa polypeptide was recorded after heating, with the consequent formation of two new polypeptides of about 23 and 33 kDa (previously attributed to Ara h 3).²² Heating has, in general, a substantial effect on food allergenicity. This effect is due to the loss of secondary and tertiary protein structures, and the consequent increase in the percentage of extended β -sheet structures along the moiety promoting the formation of large protein complexes or aggregates.^{23,24} Besides, heating induces lower protein solubility with damage to lysine, arginine, and cysteine residues, change in protein digestibility to some degree and reduction of recovery during protein extraction.²⁵

In particular, lane 2 of Fig. 1 shows the pattern of autoclaved peanuts where protein bands comprised in the range 30–40 kDa and assigned to Ara h 3 (from the corresponding unheated sample) proved to disappear after the thermal treatment. In stark contrast with this, a protein banding at 30 kDa showed to be stable and to persist after autoclaving. As for proteins with MW ranging between 20 and 25 kDa only a partial reduction of band intensities was displayed after autoclaving. As previously reported by Bavaro *et al.* in 2018, in the raw samples these protein bands after LC-MS/MS analysis were mainly attributed to Ara h 2 and Ara h 7 along with Ara h 3 allergens, while in the autoclaved samples the bands were putatively assigned to Ara h 3. This evidence suggests that Ara h 2 and Ara h 7 allergens are likely to be affected by thermal/pressure treatment.¹⁹

Our results are in agreement with other studies carried out on different foods subjected to autoclaving. In this regard, Cabanillas *et al.* reported a decrease in Ara h 2 and Ara h 6 levels in the three types of peanuts analysed (raw, roasted and fried) subjected to different autoclaving conditions.²⁶ On the other hand, Husband *et al.* demonstrated that neither a thermal treatment alone (115 °C) nor the sole application of high pressure (700 MPa) at an initial temperature of 20 °C caused modifications in the protein immunoreactivity of the apple allergen.²⁷ This event would explain the results obtained in the present study, where only a combination of pressure and heat (autoclaving) proved to be sufficiently efficient to change the protein profile. Indeed, in pre-hydrated and autoclaved peanuts at 134 °C for 10 min (lane 3), a drastic reduction in the protein intensity was highlighted by the SDS-PAGE gel and it is worth noting that no protein bands were detectable in the corresponding electrophoretic patterns, with the exception of a faint band displayed at approximately 25 kDa and putatively assigned to the Ara h 3 group. Parallel analysis carried out on the protein extracts of raw and autoclaved peanuts pointed out a decrease in protein levels that

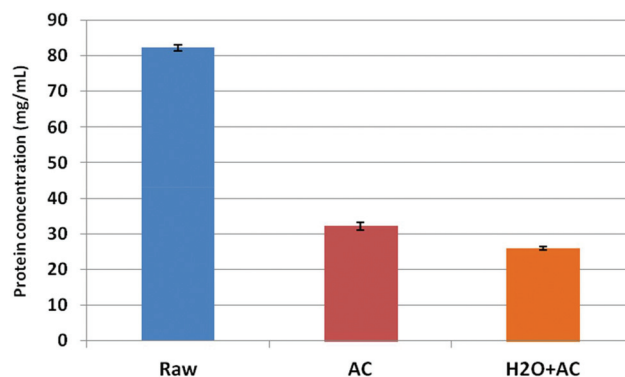


Fig. 2 Protein profile of raw, autoclaved (AC) and hydrated-autoclaved (10'-AC) peanuts. Total protein content of peanuts referred to as raw samples autoclaved for 10 min (AC) at 134 °C and samples pre-hydrated and autoclaved for 10 minutes at 134 °C with error bars ($n = 3$ replicates).

dropped down to 40% and 30% compared to the control, as reported in Fig. 2. The reduction of total proteins recorded was even more enhanced in samples exposed to preliminary hydration (for 2 hours) before autoclaving at 134 °C and 2 atm. In particular, for both autoclaved samples, the extensive reduction recorded in the protein content was in line with the disappearance of the major allergenic proteins as depicted in Fig. 1 (lane 3 of the SDS-PAGE gel). It is conceivable that the treatment applied to peanuts, using a combination of pressure and temperature, induced the disruption of most proteins due to a synergistic action, both mechanical and physical, thus causing a decrease in protein recovery. These data are confirmed by a similar work carried out by Zhang *et al.* that highlighted a change in protein recovery when dry almond powder samples underwent autoclave sterilisation (121 °C and 0.15 MPa for 10 min).²⁸ In this work, the reduction in protein recovery appeared to be enhanced when samples were autoclaved in PBS compared to dried samples. Moreover, the almond protein profiles of autoclaved samples without any exposure to PBS were similar to those of unprocessed samples, whereas samples autoclaved in the presence of PBS solution showed the disappearance of protein bands in the molecular range 20–22 kDa.

3.2 Effects of peanut treatment on the cell proliferation of Caco2 cells

Protein stability during food processing and digestion is an important factor in determining the allergenic potential of ingested food.¹⁰ The effect of processing on the food matrix can have a massive impact on the final uptake at the cell level and on the interaction with the intestinal epithelium with consequences on the sensitising capacity. It is not clear how individuals become sensitised to selected foods or why proteins encountered along the GI tract normally result in oral tolerance (actively down-regulated immune responses).²⁹ However, the mechanisms explaining how food exposed to different processing methods promote or inhibit cell proliferation form the

basis to understand the expression and consequent release of all those molecules (*e.g.* cytokines, chemokines, galectins and growth factors) that drive immune polarisation by affecting dendritic cell function and immune response.

In this regard, we investigated the ability of peanut seeds subjected to different treatments to inhibit Caco-2 cell line growth. Both raw peanuts and peanuts autoclaved for 10 minutes or autoclaved after a preliminary hydration step were administered to the cell cultures at the concentration of

1 mg mL⁻¹ for different incubation times (namely 1 h, 3 h, 6 h, and 24 h). As shown in Fig. 3a, a significant inhibition effect on the growth rate was observed up to 6 h of incubation. In detail, raw peanuts (Fig. 3A) caused a significant ($P < 0.05$) reduction in the conversion of the MTT tetrazolium salt in the tested cells compared to the untreated ones, reaching, after 3 h of administration, a viability as low as 49.3% (Fig. 3, panel A). Previous studies demonstrated the antiproliferative effects of purified peanut allergens when tested on Caco-2 and

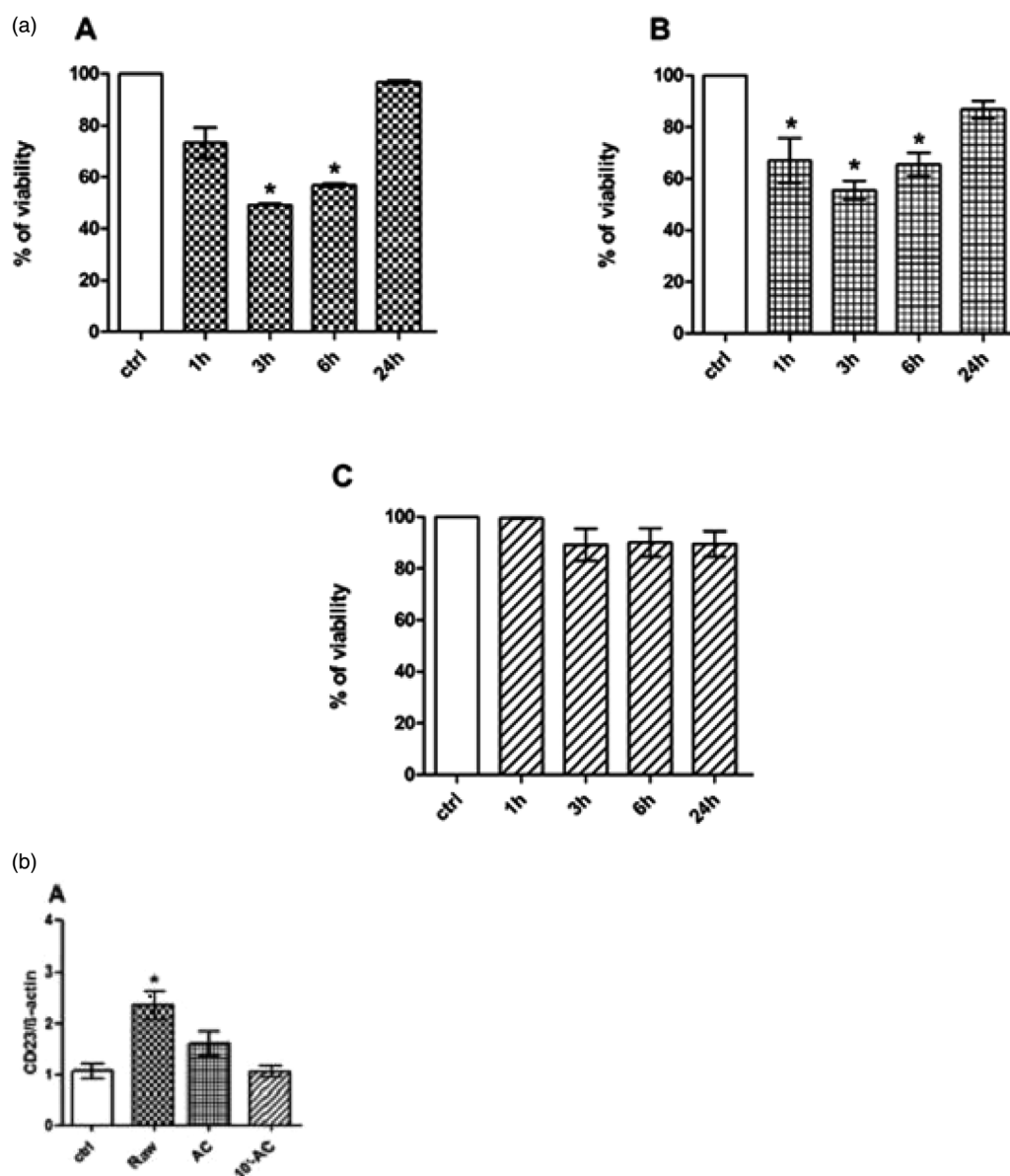


Fig. 3 (a) Proliferative response of Caco-2 cells exposed for 1 h, 3 h, 6 h and 24 h to raw, autoclaved and hydrated-autoclaved peanuts (1 mg mL⁻¹). Effects of raw (Panel A), autoclaved (Panel B), and hydrated-autoclaved (Panel C) peanuts (1 mg mL⁻¹) (Panel C) on the conversion of MTT tetrazolium salt in Caco-2 cells after 1 h, 3 h, 6 h, 24 h and 48 h of treatment. All data represent the result of three different experiments (mean $\pm$ SEM). Data were analyzed by the Kruskal–Wallis analysis of variance and Dunn's multiple comparison test. (*) $P < 0.05$ compared to untreated control cells. (b) Western blot analysis of CD23, IL-10 and TGF- β 1 in Caco-2 cells exposed for 3 h to raw, autoclaved (AC) and hydrated-autoclaved (10'-AC) peanuts (1 mg mL⁻¹). Immunoreactive bands were quantified using the Quantity One Programme. The diagrams show the quantification of the intensity of bands, calibrated to the intensity of the β -actin band. All data represent the results of three different experiments (mean $\pm$ SEM). Data were analyzed by the Kruskal–Wallis analysis of variance and Dunn's multiple comparison test. (*) $P < 0.05$ compared to untreated control cells.

HT29 cell lines. Teodorowicz and coll. showed that the incubation of Caco-2 cells in the presence of non-hydrolysed and unmodified Ara h 1 allergen caused an inhibition of proliferation of approximately 51%. However, no differences were observed in the proliferation rate of cells incubated with hydrolysed or glycated Ara h 1 heated both at 37 °C and 60 °C compared to the control.³⁰ In stark contrast with these findings, we reported that autoclaving peanuts for 10 min led to a significant ($P < 0.05$) antiproliferative effect compared to the control. Indeed, after 3 h of treatment, the viability was reduced to 55.5%, (Fig. 3, panels B). No significant effect of proliferation inhibition was observed when peanuts autoclaved for 10 minutes after hydration were assayed (Fig. 3, panel C). This result is likely to be due to protein fragmentation, subsequent combined treatment based on hydration and autoclaving (based on the application of temperature and pressure) and the consequent release of small presumably bioactive peptides while boosting cell growth. With the aim to investigate whether the treatment applied to peanuts might also influence the inflammatory network of epithelial cells causing a proliferative or antiproliferative effect, we monitored the change in the CD23 protein levels by performing western blot analysis. Three hours of incubation was chosen as the reference time point to evaluate the levels of such mediator release since it represented the best compromise between the time of exposure to activate cell mediators and the time length of the digestive process. According to the results obtained, a significant increase ($P < 0.05$) in the CD23 protein levels of Caco-2 cells treated with raw peanut extracts was recorded while no significant differences in the autoclaved or in the hydrated-autoclaved samples were observed (Fig. 3b) compared to the other samples.

It is well known that CD23, the low-affinity IgE receptor (FcεRII), is expressed constitutively on human intestinal epithelial cells, which play an essential role in the transport of antigens across human intestinal epithelial cell monolayers. In a study conducted on mice sensitised to peanuts, an increase in the absorption of peanut allergens and the overexpression of the CD23 protein on the apical membrane of intestinal epithelial cells have been shown.³¹

3.3 Evaluation of the effect of thermal/pressure treatment on the peanut allergen composition by LC-MS/MS analysis

In order to gain more insight into the proteins involved in cell proliferation, extracts of untreated and treated peanuts were subjected to tryptic digestion and analysed by HPLC-MS/MS. Peptides emerged from enzymatic digestion together with those generated by physical fragmentation induced by autoclaving and autoclaving preceded by hydration were identified using software as previously detailed. In Table 1, all allergenic proteins identified in raw and processed peanuts are listed. As shown in the table, the composition of the allergenic fraction seems to be very similar across the different types of peanuts, the more representative classes being Ara h 1, Ara h 2, Ara h 3 and Ara h 6 as demonstrated by the high number of peptides retrieved by the software. Some other minor allergens, such as

Ara h 7, Ara h 8, Ara h 9, Ara h 10, Ara h 12, Ara h 13 and Ara h 15, were also identified both in raw and processed samples. While the allergen composition seems to be unaffected by the treatment applied, the number of peptides attributed to each protein proved to be susceptible to processing, and this is more evident in the case of peanuts incubated with water before autoclaving. Fig. 4 depicts, panel A and B, the change in the peptide number observed for each allergen in variously treated peanuts (raw, autoclaved, prehydrated and autoclaved). As for the main peanut allergens (Ara h 1, Ara h 2, Ara h 3 and Ara h 6, Fig. 4 panel A), a consistent increase in the peptide number was displayed for water-incubated autoclaved samples estimated to be approximately 55% compared to raw peanuts' extracts.

In contrast, a very slight increase (ranging from 4 to 20%) was observed for Ara h 1, Ara h 2 and Ara h 3 in autoclaved samples whereas Ara h 6 peptides decreased their number by approximately 12%. A more heterogeneous trend was, instead, obtained for other allergens both in autoclaved or prehydrated autoclaved samples (Fig. 4, panel B). Compared to the untreated sample, in autoclaved peanuts the number of peptides attributed to Ara h 7 (Fig. 4, panel A) and Ara h 8 decreased by approximately 40 and 50% respectively, while a greater reduction (around 90%) was observed for Ara h 9 and Ara h 13. The peptide number estimated for Ara h 10 remained unaltered concerning raw peanuts, while no peptides were identified for Ara h 12. Irrespective of what is observed for other allergens, peptides assigned to Ara h 15 increased by approximately 20%. Similarly, a reduction in the peptide number attributed to Ara h 7, Ara h 9, Ara h 12 and Ara h 13 (ranging from 18 to 50%) was recorded in peanuts exposed to hydration before autoclaving. Opposite results were obtained for Ara h 8, Ara h 10 and Ara h 15 in which the peptide number increased by 125, 80 and 140%, respectively, compared to untreated peanuts.

The increase in the peptide number observed for the different allergenic proteins upon food processing seems to confirm the fragmentation phenomenon that occurs during the autoclaving treatment, likely due to the combined effect of heat and pressure. This phenomenon appeared to strengthen in peanuts hydrated before autoclaving, suggesting that probably the water absorbed by seeds facilitates heat propagation in the inner part of the seed as well as exerts a mechanical effect during high pressure autoclaving, thus promoting an enhanced protein fragmentation. Our results are in agreement with those reported in our previous study, where a progressive reduction in the intensity of the major allergen proteins was observed in the protein fraction extracted from autoclaved peanuts when samples were preliminarily exposed to 2 h hydration, which was attributed to the occurrence of the fragmentation phenomenon upon processing.¹⁹ Similarly, Cabanillas and coworkers found that the combination of pressure and heat under specific conditions during autoclaving led to protein fragmentation in peanut samples.²⁶

These results are consistent with those observed both in the SDS-PAGE protein profile and cell viability experiments. In

Table 1 Allergenic proteins identified by LC/MS analysis and via commercial software (Proteome Discoverer 2.1) in peanuts untreated (raw peanuts), autoclaved and prehydrated/autoclaved along with the relevant parameters

Protein accession	Type of protein	Allergen	Peptides (unique)	Coverage (%)	Score
Raw peanuts					
E5G076	Ara h 1 allergen	Ara h 1	78 (9)	58	608.83
N1NEW2	Seed storage protein Ara h 1	Ara h 1	100 (9)	71	834.58
Q6PSU4	Conarachin	Ara h 1	73 (9)	76	730.38
Q6PSU6	Conarachin	Ara h 1	46 (1)	67	469.22
Q6PSU2	Conglutin-7	Ara h 2	28 (26)	78	221.50
A1DZF0	Arachin 6	Ara h 3	127 (9)	87	1275.27
O82580	Glycinin (fragment)	Ara h 3	80 (1)	71	921.58
Q0GM57	Iso-Ara h 3	Ara h 3	107 (64)	88	881.46
Q647H2	Arachin Ahy-3	Ara h 3	33 (11)	54	139.69
Q647H3	Arachin Ahy-2	Ara h 3	123 (4)	86	1229.81
Q8LKN1	Allergen Ara h 3/Ara h 4	Ara h 3	122 (11)	86	1241.22
Q9FZ11	Gly1	Ara h 3	121 (11)	85	1086.87
Q9SQH7	Glycinin	Ara h 3	100 (2)	73	904.05
Q647G9	Conglutin	Ara h 6	17 (14)	77	128.59
B4XID4	Ara h 7 allergen	Ara h 7	21 (6)	65	111.26
Q647G8	2S protein 2	Ara h 7	15 (1)	54	85.36
B0YIU5	Ara h 8 allergen isoform	Ara h 8	4 (6)	56	0.00
B6CEX8	Non-specific lipid-transfer protein	Ara h 9	6 (3)	47	10.35
B6CG41	Non-specific lipid-transfer protein	Ara h 9	8 (3)	73	20.15
Q647G5	Oleosin 1	Ara h 10	9 (10)	44	18.71
B3EWP3	Defensin 1	Ara h 12	4 (4)	58	6.58
B3EWP4	Defensin 2 (fragment)	Ara h 13	5 (3)	57	11.78
C0HJZ1	Defensin 3	Ara h 13	6 (4)	54	11.45
Q647G3	Oleosin	Ara h 15	5 (5)	38	8.47
Autoclaved peanuts					
E5G076	Ara h 1 allergen	Ara h 1	89 (11)	61	596.19
N1NEW2	Seed storage protein Ara h 1	Ara h 1	113 (7)	73	830.62
Q6PSU4	Conarachin	Ara h 1	78 (8)	75	622.42
Q6PSU6	Conarachin	Ara h 1	52 (1)	71	462.33
Q6PSU2	Conglutin-7	Ara h 2	34 (33)	81	221.43
A1DZF0	Arachin 6	Ara h 3	135 (15)	88	1585.08
Q0GM57	Iso-Ara h 3	Ara h 3	95 (52)	85	777.56
Q5I6T2	Arachin Ahy-4	Ara h 3	125 (2)	86	1482.55
Q647H2	Arachin Ahy-3	Ara h 3	28 (9)	41	128.60
Q647H3	Arachin Ahy-2	Ara h 3	134 (3)	83	1523.52
Q8LKN1	Allergen Ara h 3/Ara h 4	Ara h 3	140 (15)	87	1543.15
Q9FZ11	Gly1	Ara h 3	134 (15)	82	1388.16
Q9SQH7	Glycinin	Ara h 3	102 (2)	72	1104.45
Q647G9	Conglutin	Ara h 6	15 (11)	63	80.45
B4XID4	Ara h 7 allergen	Ara h 7	13 (3)	59	53.41
Q647G8	2S protein 2	Ara h 7	11 (1)	49	39.52
B0YIU5	Ara h 8 allergen isoform	Ara h 8	2 (2)	11	0.00
B6CEX8	Non-specific lipid-transfer protein	Ara h 9	1 (1)	10	0.00
Q647G5	Oleosin 1	Ara h 10	9 (9)	37	24.00
C0HJZ1	Defensin 3	Ara h 13	1 (1)	51	0.00
Q647G3	Oleosin	Ara h 15	6 (6)	25	8.95
Autoclaved prehydrated peanuts					
E5G076	Ara h 1 allergen	Ara h 1	137 (13)	71	880.41
N1NEW2	Seed storage protein Ara h 1	Ara h 1	164 (11)	79	1391.25
Q6PSU4	Conarachin	Ara h 1	112 (8)	76	1157.06
Q6PSU6	Conarachin	Ara h 1	78 (1)	79	790.92
Q6PSU2	Conglutin-7	Ara h 2	44 (38)	81	379.26
A1DZF0	Arachin 6	Ara h 3	176 (20)	86	1749.31
Q0GM57	Iso-Ara h 3	Ara h 3	141 (79)	79	1245.56
Q5I6T2	Arachin Ahy-4	Ara h 3	172 (1)	87	1579.16
Q647H2	Arachin Ahy-3	Ara h 3	36 (10)	43	178.62
Q647H3	Arachin Ahy-2	Ara h 3	183 (6)	91	1744.96
Q647H4	Arachin Ahy-1	Ara h 3	184 (16)	88	1719.85
Q9FZ11	Gly1	Ara h 3	185 (19)	90	1585.63
Q9SQH7	Glycinin	Ara h 3	131 (4)	76	1197.61
Q647G9	Conglutin	Ara h 6	27 (23)	78	139.50
B4XID4	Ara h 7 allergen	Ara h 7	18 (15)	62	64.10
B0YIU5	Ara h 8 allergen	Ara h 8	9 (9)	58	0.00
B6CG41	Non-specific lipid-transfer protein	Ara h 9	5 (6)	66	8.16

Table 1 (Contd.)

Protein accession	Type of protein	Allergen	Peptides (unique)	Coverage (%)	Score
Q647G5	Oleosin 1	Ara h 10	16 (16)	56	35.19
B3EWP3	Defensin 1	Ara h 12	2 (2)	24	0.00
B3EWP4	Defensin 2	Ara h 13	3 (1)	30	9.24
C0HJZ1	Defensin 3	Ara h 13	3 (3)	51	7.28
Q647G3	Oleosin	Ara h 15	12 (12)	37	9.41

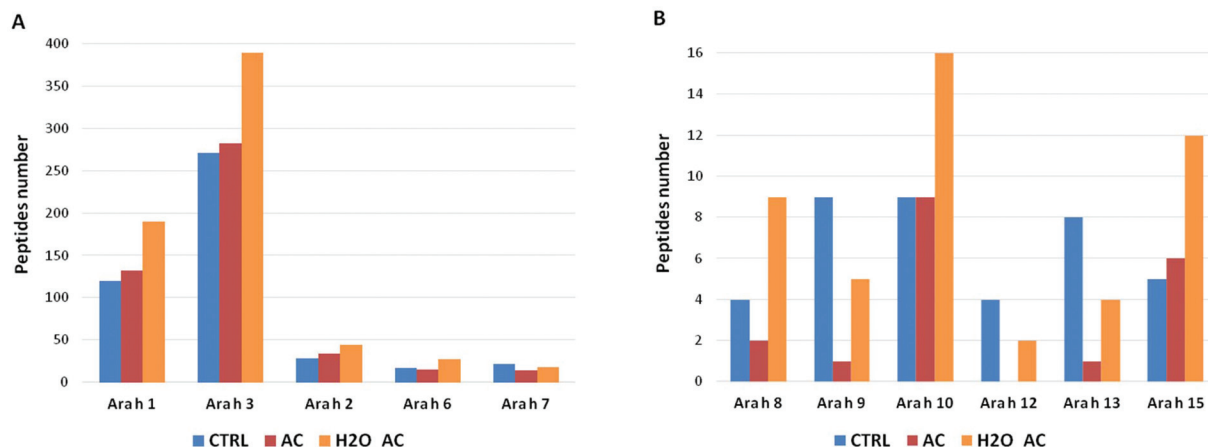


Fig. 4 (Panel A and B) Variation of the peptide number upon autoclaving and prehydration/autoclaving treatments for each allergen identified in untreated and (Ctrl) treated samples (AC: autoclave; H₂O_AC: water incubation followed by autoclave) via proteome discover 2.1.

particular, in the protein profiles of prehydrated and autoclaved peanuts (Fig. 1, lane 3) no protein bands with MW comprised between 250 and 10 kDa were observed, and also no difference was found in cell viability compared with the control (Fig. 3, panel C), likely due to the extensive fragmentation of original proteins as confirmed by LC-MS/MS analyses. In contrast, by looking at the protein profile of the autoclaved samples, some intense bands attributed to Ara h 3 and Ara h 1 (MW comprised between 30 and 60 kDa) persisted after the treatment and appeared to affect cell viability. These findings are in agreement with the level of protein fragmentation that was comparable to that found for raw peanuts despite the significant increase in the number of peptides generated after autoclaving.

3.4 Evaluation of the effect of thermal/pressure treatment on the lower MW fraction of processed peanuts by LC-MS/MS analysis

In order to gain deeper insight into the identity of peanut peptides, which could influence the growth and the activities of inflammatory markers in the intestinal epithelium, peanut extracts were passed through an ultra-filtration tube (10 kDa cut off) and the peptide fraction collected with a molecular weight lower than 10 kDa was analyzed by HPLC-MS/MS. As depicted in Fig. 5, although both treated and untreated peanuts showed approximately the same allergen composition (according to the results of the proteomics analysis), a decrease in the peptide number was recorded in the low MW

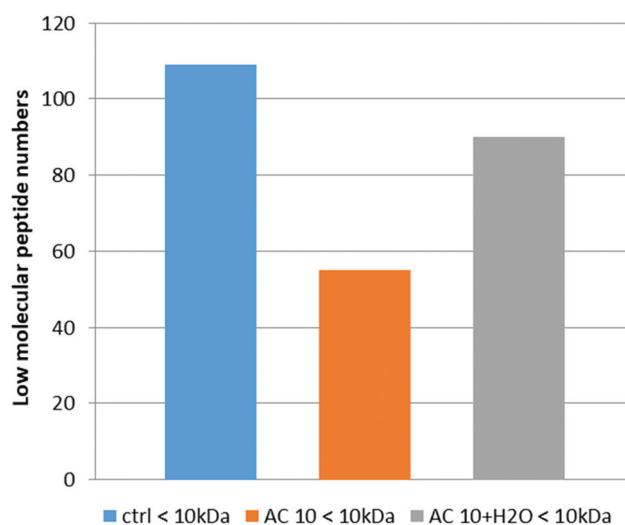


Fig. 5 Variation of the total low molecular peptide number (<10 kDa) in untreated and (Ctrl) treated samples (AC: autoclave; H₂O_AC: water incubation followed by autoclave) via proteome discover 2.1.

fraction of the extracts of autoclaved and prehydrated and autoclaved peanuts compared to the raw samples. As for the main peanut allergens (Fig. 6), the consistent decrease recorded in the peptide number of Ara h 1 and Ara h 3 was calculated to be around 25% and 75% in autoclaved samples compared to the raw sample, while for Ara h 2 and 6 these values were ranging between 20% and 30%. By contrast, a

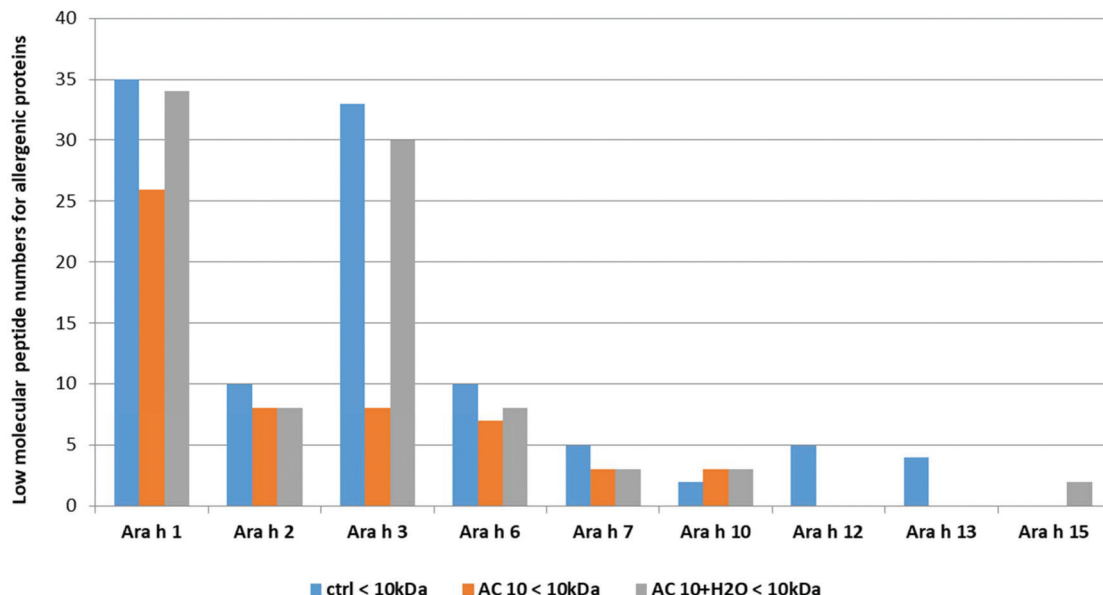


Fig. 6 Variation of the low molecular peptide number (<10 kDa) upon autoclaving and prehydration/autoclaving treatments for each allergen identified in untreated and (Ctrl) treated samples (AC: autoclave; H₂O_AC: water incubation followed by autoclave) via proteome discover 2.1.

lower decrease (ranging from 3 to 10%) was observed for Ara h 1 and Ara h 3 in the fraction <10 kDa of prehydrated and autoclaved peanuts compared to the raw fraction, while for Ara h 2 and Ara h 6, the peptide number decreased in both cases by approximately 20%.

These data are interesting since they highlight that a reduction in the total number of peptides attributed to Ara h 1, Ara h 2 and Ara h 6 occurs in autoclaved-based peanuts giving rise to a decrease in the number of peptides traceable to the three allergenic protein families considered of primary concern. A more different trend was, instead, obtained for other allergens in both autoclaved and prehydrated autoclaved samples with total disappearance of peptides belonging to Ara h 12 and 13 compared to raw fractions. The increase of peptide number within the major allergenic proteins in the lower MW fraction of raw extracts could account for the higher level of CD23 protein displayed compared to the autoclaved samples. Deeper investigations will be required on this matter to confirm the preliminary results herein obtained on the Caco-2 cell line tested, in order not only to assess the interactions between allergens and the intestinal epithelium, but also to evaluate their capacity to interact and play a role in the downstream immune response to dietary allergens through their association with the intestinal epithelium.

4. Conclusions

In this paper, we demonstrated that while the allergen composition seems to be unaffected by autoclaving treatments, the number of peptides released by each identified protein is instead closely related to the type of processing applied, and

this is more evident in the case of peanuts exposed to water before autoclaving. On the other hand, it was also highlighted that the type of processing applied might modulate the biological activity of peanuts at the gut level through the release of specific mediators. Our results demonstrate that hydration before autoclaving induces a proliferative effect on Caco-2 cells irrespective of what was found for raw peanuts. This is probably due to a more extensive fragmentation of the proteins observed and a consequent production of peptides as assessed by LC-MS/MS analysis which might also account for the proliferative effect exerted on the Caco cells according to our findings.

Secondly, our results also demonstrate that autoclaving peanuts causes a reduction in the levels of CD23 protein irrespective of what was observed for raw peanuts and this might account for reduced allergenicity of the processed samples. A deeper investigation into this aspect is required for extending the study to allergic consumers to assess any reduction or loss of the allergenic power of treated peanuts towards sensitive patients.

Conflicts of interest

There are no conflicts to declare.

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