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Prolyl endopeptidase-degraded low immunoreactive wheat flour attenuates immune responses in Caco-2 intestinal cells and gluten-sensitized BALB/c mice

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ABSTRACT

Targeted degrading *Aspergillus niger*-derived prolyl endopeptidase (AN-PEP) is promising in gluten hydrolysis because it specifically cleaves the proline-rich sites in gluten. The current study aims to understand the safety aspects of AN-PEP hydrolysed low immunoreactive wheat flours by testing immune responses using cell line and animal models. In the AN-PEP hydrolysed wheat flour (AN-PEP HWF) gliadin extract, there was no increase in the levels of zonulin-1 (Zo-1) and pro-inflammatory cytokines (IL-6 and IL-8) but a significant increase was noted in the control wheat flour (CWF) gliadin-treated Caco-2 cells. The Zo-1 localization in Caco-2 cells was significantly noted in the reacted positive fluorescence cells that were treated with the control wheat flour. Further, a safety evaluation of HWF was carried out in gluten-sensitized BALB/c mice. Mouse anti-gliadin (IgG, IgA and IgE) antibodies were significantly generated in the CWF treated animals rather than the AN-PEP HWF groups. The serum pro-inflammatory (IL-1β, IL-4, IL-6, IL-15, TNF-α and IFN-γ) markers were observed in significant levels in CWF challenged mice and a similar trend was observed in ex-vivo splenocyte cells. A small intestine histopathological sectioning revealed that there are no abnormalities or structural changes in AN-PEP HWF challenged mice.

1. Introduction

Wheat is one of the major dietary sources for humans. However, wheat proteins are associated with different types of adverse reactions such as celiac disease (CD), food allergy, baker's asthma, non-celiac gluten sensitivity (NCGS) and gluten intolerance. The number of patients who have been adversely affected has increased up to 2% around the world (Shewry, 2009). Gluten is made up of alcohol-soluble gliadins and insoluble glutenins. The gliadins contain four types of major subunits such as α, β, γ and ω-gliadin proteins. Some glutenins have high molecular weight (HMW-GS) and some have low molecular weight (LMW-GS). The gluten proteins are cohesive and viscoelastic in nature and help in the formation of dough for bakery products and gives structure (Gujral et al., 2012). The water insolubility of gluten proteins has created extensive use for it in food processing. These gluten proteins are highly resistant to intestinal digestive enzymes. The partially-digested gluten proteins result in long immunogenic peptides, which trigger the immune system in the case of celiac and wheat allergies of genetically-predisposed people.

Celiac disease is an autoimmune disorder having positive HLA-DQ2/DQ8 allelic individuals (Denham and Hill, 2013). Gluten proteins are partially hydrolysed by the protease present in the gastrointestinal (GI) tract. The partially-hydrolysed peptides increase the intestinal permeability of the gut by releasing the tight junction (TJ) protein zonulin; this leads to a leaky gut. As a result, these gluten peptides enter into the lamina propria by transcellular transport, where they are deamidated by tissue transglutaminase (tTG) (Balakireva and Zamyatnin, 2016). These deamidated peptides have more affinity to MHC II molecules and they become immunogenic in HLA-DQ2/DQ8-containing patients. These gluten-derived peptides stimulate both innate and adaptive immune responses, with the production of IFN-γ and IL-15 in CD patients. The immune-mediated response leads to intestinal inflammation, atrophy of villi and an increase in crypt hyperplasia in intraepithelial lymphocytes (Escudero-Hernández et al., 2016).

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Abbreviations

TNF-α	Tumour necrosis factor-alpha
IL-1β	Interleukin-1beta
IFN-γ	Interferon-gamma
IL	Interleukin
COX-2	Cyclooxygenase 2
iNOS	inducible Nitrogen oxide synthase
NF-kB	Nuclear factor-kappa-light chain enhancer of activated B cells
Zo-1	Zonulin-1
IP	Intraperitoneal
HWF	Hydrolysed wheat flour
WF	Wheat flour
TJ	Tight junction
CD	Celiac disease
WA	Wheat allergy

The allergy to wheat is due to the stimulation of wheat gluten-derived peptides to the IgE receptor. The α/ω-gliadin sub-units are the main allergens that trigger IgE-mediated immune response. The exposure or binding of gliadin sub-units to IgE on mast cells or basophils activates histamine and other chemicals that secrete allergic reactions. Baker's asthma and wheat-dependent exercise-induced anaphylaxis (WDEIA) are also IgE-mediated hypersensitive immune responses. NCGS patients do not show an IgE reaction nor an autoimmune reaction; they suffer an innate immune response but one that is less severe than CD patients. Gluten ingestion has been linked to a range of clinical disorders.

Many processing techniques are utilized for reduction of the gluten immunoreactive epitopes present in wheat flour such as malting, germination, fermentation and microbial enzymatic hydrolysis; these techniques have been proven to be effective methods for the reduction of immunoreactivity (Kucek et al., 2015). According to Gianfrani et al. (2007) (Gianfrani et al., 2007), microbial transglutaminase (mTG) can reduce immunogenicity by the transdeamination of gluten proteins and peptides. A combined usage of glutamine-specific endoprotease and prolyl endopeptidase can effectively nullify the immunoreactive epitopes in wheat flour (Gass et al., 2007). Marti et al. (2004)9 demonstrated that the prolyl endopeptidase from *F. meningosepticum* and aminopeptidase N from the rat brush-border membrane are capable of detoxifying potent immunogenic gliadin epitopes such as PQPQLPYPQPQLP and 33-mer α-gliadin (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF). However, a combination of protease from enterococci and fungi (*Rhizopus oryzae*) can reduce gluten content concentration up to 98% after fermentation (M'hir et al., 2009). According to Heredia-Sandoval et al. (2018) (Heredia-Sandoval et al., 2018), AN-PEP specifically cleaves proline and glutamine sequences; there is up to 99.9% reduction in the bread prepared with AN-PEP treated wheat flour. Many methods or processes have been tried to reduce the gluten in wheat flour; the low-gluten and hypoallergenic products are prepared using hydrolysed wheat flour. Nevertheless, there is a lack of research findings of immunoreactivity in the hydrolysed wheat flour with *in-vitro* and *in-vivo* models. With this background information and existing knowledge, the aim of the present study is to understand the immunogenicity of low immunoreactive HWF by the AN-PEP enzyme and its safety evaluation in intestinal cell line models and gluten-sensitized BALB/c mice models.

2. Materials and methods

2.1. Wheat flour, enzymes, chemicals, antibodies and animal diet ingredients

The wheat grain sample (HD-2851) was collected from the National Seeds Corporation Limited (New Delhi, India). The animal (AIN-93) diet ingredients such as corn-starch and groundnut oil were purchased from the local market (Mysuru, Karnataka, India). Other diet ingredients such as dextrinized starch, choline bitartrate and L-cysteine were procured from Hi-Media Laboratories (Mumbai, India). The minerals and vitamin mix (AIN-93 MX) was procured from MP Biomedicals (Santa Ana, United States). The salt mixture (Bernhart Tommarelli) and sucrose were purchased from SRL Laboratories (Mumbai, India). The wheat gliadin, gluten, secondary antibodies (goat anti-mouse IgG-HRP, goat anti-mouse IgA (α-chain specific)-HRP), primers, RNaseZap and TRI-reagent were procured from Sigma-Aldrich (St. Louis, Missouri, United States). The goat anti-mouse IgE-HRP was obtained from Thermo Fisher Scientific (Waltham, Massachusetts, United States). The BALB/c mice (4–6 weeks old; female) were approved by the Institutional Animal Ethics Committee (IAEC No CFT/IAEC/33/2015) and procured from Adita Biosys (P) Ltd, (Tumakuru, Karnataka, India). The SYBR green master mix and qRT-PCR plates were obtained from Biorad (Hercules, California, United States). Apart from these, all other chemicals that were used for the study are of analytical grade.

2.2. AN-PEP hydrolysis of wheat flour for low immunoreactive characteristics

The wheat flour hydrolysis was carried out using the AN-PEP enzyme according to Luoto et al. (2012) (Luoto et al., 2012) with slight changes. According to the authors' earlier study (Mohan Kumar et al., 2017), the low immunoreactive HD-2851 wheat flour was selected for enzymatic modification. The flour was taken in 0.1 mol/L sodium acetate buffer (pH 4.0) with an enzyme concentration of 4 PPU/g and incubated up to 5 h at 40 °C. The modified wheat flour was dried and stored at −20 °C for further use.

2.3. In-vitro toxicological evaluation of AN-PEP-HWF in Caco-2 cells

2.3.1. Cell culture and maintenance

The Caco-2 cells (ATCC-36; American Type Culture Collection, Virginia, USA) were cultured in DMEM (Dulbecco Modified Eagle Medium) with 10% fetal bovine serum (FBS), 4 mM L-glutamine, antibiotic antimycotic solution (100 U/mL penicillin, 100 μg/ml streptomycin, and 25 μg Amphotericin B) from Hi-Media Laboratories (Mumbai, India) at 37 °C in a carbon dioxide (CO_2) incubator (5% CO_2, 95% humidity). The cultured cells were allowed to grow up into a monolayer of epithelial cells, and the media change was carried out three times per week. The sub-culture was performed after 80% confluence. All the experiments were performed after the passage of 62–74 h.

2.4. Zonulin and pro-inflammatory cytokines determination in treated cells

Wheat flour and AN-PEP HWF were subjected to peptic and tryptic digestion according to standardized protocol (Cabrera-Chávez et al., 2008). The wheat flour was incubated with pepsin from porcine gastric mucosa (at 37 °C with pH 2.0 for 4 h) followed by trypsin treatment (at 37 °C with pH 7.8 for 4 h). After digestion, the samples were freeze-dried and stored at −20 °C. The AN-PEP HWF was extracted in 60% ethanol according to van den Broeck et al. (2009) (van den Broeck et al., 2009) for gliadin recovery and the sample were suspended in DMEM media. The Caco-2 monolayer was stimulated with 1 mg/ml of the sample such as PT-digested wheat flour, AN-PEP HWF protein extract, gliadin and 19-mer peptide (LGQQQPFPPQQPYPQPQPFK),

exposed for 4 h at 37 °C in 5% CO_2 atmosphere. Cell viability of Caco-2 cells were determined by MTT (5-3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay according to Ravi et al. (2018) (Ravi et al., 2018). After treatment, the cell supernatants were collected and stored at −80 °C. The levels of pro-inflammatory cytokines (IL-6 and IL-8) were measured in the cell supernatant by the sandwich ELISA kit (Abcam, Cambridge, United Kingdom). The protocol was performed according to the manufacturer's instructions. The Zonulin level was determined in the supernatant by using the sandwich ELISA kit (Cloud-clone corp, Houston, United States) and the assay was performed as per manufacturer's instructions. All the results were expressed in terms of pg/mL.

2.5. Zonulin localization in Caco-2 cells by immunofluorescence

The localization of zonulin in the Caco-2 cells were performed by the immunofluorescence technique according to Sander et al. (2005) (Sander et al., 2005). The caco-2 cells were grown in the eight-well chamber glass slide. After 72 h the monolayer was washed with the HBSS (Hank's Balanced Salt Solution) and incubated overnight with in MEM supplemented with sodium pyruvate, sodium bicarbonate, 1% FBS and non-essential amino acids. The cell monolayer was washed with PBS before exposure of PT-digested extracts. The monolayers were exposed to a pepsin-trypsin (PT)-digested protein extracts (AN-PEP HWF, and HD-2851 digested WF) along with the control (PBS) in up to 4 h. After incubation, the monolayers were washed with phosphate buffer saline (PBS), and the cells were fixed by adding 1 mL of 4% ice-cold paraformaldehyde for 20 min at room temperature (RT). The cell surface was covered with 1 ml of chilled acetone and methanol (1:3) for permeabilization for 20–30 min.

Non-specific blocking was carried out with 3% bovine serum albumin (BSA) in PBS (0.1% Tween-20) for 1 h at room temperature. The monolayer was incubated with primary antibody ZO-1 (D6L1E) rabbit mAb diluted by 1/400 (Cell signaling technology, Danvers, Massachusetts, United States) and incubated for 2 h. The monolayer was washed and incubated with secondary anti-rabbit Alex-Fluor-647 (dilution 1/100; Thermo Scientific, Waltham, Massachusetts, United States) for up to 2 h. The DAPI (Dakocytomation) (Sigma Aldrich, St. Louis, Missouri, United States) was used for counterstaining the nuclei. The monolayer was then covered with gold antifade reagent (Thermo Scientific, Waltham, Massachusetts, United States). The slides were visualized using confocal microscopy (LSM 700 Carl Zeiss, Denmark) with the respective excitation and emission ranges.

2.6. In-vivo study for AN-PEP HWF in BALB/c mice

2.6.1. Experimental plan

The BALB/c mice (female; 4–6 week old) were randomly grouped into three groups such as: (i) vehicle control – PBS, pH 7.4; (ii) hydrolysed flour – AN-PEP enzymatic treated flour (HD2851) and (iii) positive control – untreated wheat flour (HD2851). Each group contained eight mice (n = 8/group) and the animals were approved by the Institutional Animal Ethical Committee. The BALB/c mice were maintained at 23 ± 2 °C in the animal house facility at the Central Food Technological Research Institute (CFTRI), Mysuru, Karnataka under pathogen-free conditions. The mice were fed with AIN-93G, formulated for growth of the mice according to Reeves et al. (1993) (Reeves et al., 1993). All the animals were taken care of throughout the study and allowed access to food and water *ad libitum*.

2.6.2. Gluten sensitization schedule for BALB/c mice

The gluten sensitivity for BALB/c mice for was carried out according to Chen et al. (2011); Gourbeyre et al. (2012); Vijaykrishnaraj et al. (2017) (Chen et al., 2011; Gourbeyre et al., 2012; Vijaykrishnaraj et al., 2017) with slight modifications. The mice were sensitized through intraperitoneal (IP) means; 0.2 mg/0.025 kg of wheat gluten proteins were administered with alum (aluminum hydroxide 2.52 mg/mL in sterile PBS). The sensitization was carried up to 35 days, with 7 days intervals (0, 7, 14, 21, 28 and 35 days). Comparatively, the control group mice received sterile PBS with alum. The pre- and post-immune responses in the blood were analyzed by drawing blood from each animal from the retro-orbital venous plexus every 15 consecutive days. Depending on the antibody titer in sera, the IP was stopped and the mice were orally challenged with: 40 mg/0.025 kg of wheat flour (0.5 ml PBS, pH 7.4) per mice (group 3); AN-PEP HWF (group 2) and sterile PBS (pH 7) (vehicle control group 1); given daily up to 3 weeks. The animals were sacrificed on the 60th day depending on the serum antibody titer level. Throughout the study, the body weight and diet intake were monitored. The diagrammatic representation of the sensitization schedule is shown in the graphical abstract.

2.7. Analysis of mouse anti-gliadin antibodies and pro-inflammatory cytokines

The gluten immunization efficacy of mice was determined in all the groups. The pre- and post-immune responses in the sensitized animal's sera were analyzed for IgG, IgE and IgA antibody titers against wheat gliadin by indirect enzyme-linked immunosorbent assay (ELISA) according to Bodinier et al. (2009) (Bodinier et al., 2009). The titer of anti-gliadin IgG was determined by serial diluting the sera (1:50 up to 1:12800) in gliadin-coated 96 well plates (Thermo Scientific, Waltham, Massachusetts, United States). The anti-gliadin (IgA and IgE) titers was determined by diluting the sera (1:50 and 1:20 respectively). The respective secondary antibodies (peroxidase-labelled) were used. The assay was developed by adding TMB solution and 2N sulphuric acid (H_2SO_4) was added to stop the reaction. The values were read at 450 nm by the ELISA plate reader (Multiskan Go, Thermo Scientific, Waltham, Massachusetts, United States). The expression levels of pro-inflammatory cytokines (IL-1β, TNF-α, IFN-γ, IL-4, IL-6, and IL-15) in sera were measured using eBioscience sandwich ELISA kit (Affymetrix, San Diego, United States) according to manufacturer's instructions. The sera samples were diluted to 1:25 ratio in antibody-diluting buffer for the assay.

2.8. Ex-vivo cell culture of splenocytes and in-vitro treatment of hydrolysed flours

The splenocytes were isolated from the sensitized animals according to Vijaykrishnaraj et al. (2017) (Vijaykrishnaraj et al., 2017). The isolated splenocytes (1.5×10^6 cells/ml) were incubated in RPMI-1640 media (Sigma-Aldrich, St. Louis, Missouri, United States) with 10% fetal bovine serum in 6-well culture plates (Eppendorf, Hamburg, Germany). The respective group cells were exposed to PBS (control), AN-PEP HWF, untreated wheat flour (WF) protein extract (100 μg/mL), PT-digested wheat flour and AN-PEP HWF extract (1 mg/mL). The cells were exposed up to 72 h at 37 °C (under 5% CO_2) and every 24 h intervals, samples were collected for quantification of the interleukins released. The cell supernatant was analyzed for the expression levels of the pro-inflammatory cytokines (IL-4, IL-6, IL-15, IL-1β, TNF-α, and IFN-γ) as mentioned above.

2.9. Histopathology evaluation of small intestine jejunum sections

The collected small intestine (jejunum) sections were preserved in 10% formaldehyde solution. Paraffin blocks were cut into 4 μm cross-sections and fixed onto a glass slide and the fixed sections were stained with hematoxylin and eosin. The section morphological changes and villi structure were observed under light microscopy (EVOS XL, Thermo Fisher Scientific, Waltham, Massachusetts, United States) in 10× and 40× magnifications.

2.10. Pro-inflammatory cytokines mRNA expression levels in jejunum sections and spleen

The messenger ribonucleic acid (mRNA) was isolated from the jejunum sections and spleen samples using the TRI reagent (Sigma-Aldrich, St. Louis, Missouri, United States) according to manufacturer's instructions. The isolated mRNA was quantified by the Nano dropper plate reader (Thermo Scientific, Waltham, Massachusetts, United States). One μg of mRNA was used to synthesize complementary deoxy ribonucleic acid (cDNA) by using the cDNA synthesis kit (Verso cDNA kit, Thermo Scientific, Waltham, Massachusetts, United States) according to manufacturer's instructions. The pro-inflammatory markers (IL-1β, IL-4, IL-6, IL-15, TNF-α, IFN-γ, COX-2, iNOS, and NF-kB) and primers were procured from Sigma Aldrich (St. Louis, Missouri, United States) and the details are given in the supplementary datasheet. The reverse transcription-polymerase chain reaction (RT-PCR) was performed in the BIO-RAD CFX96 system (BioRad, Hercules, California, United States) by using the master mix-universal SYBR green, primers (forward and reverse) and ten ng of isolated cDNA as template in a total of 20 μL reaction volume. The melting curve analysis was done and the crossing point (Cp) of the long linear portion of the amplification curve was determined. The relative induction of gene mRNA expression was calculated using the expression EΔCp (control samples − treated samples) and normalized to the expression of β-actin.

2.11. Statistical analysis

The values were mentioned as mean ± standard deviation (SD) with analysis of quadruplicates of the samples. A significant difference ($p < 0.05$) between the groups was analyzed through analysis of the variance (ANOVA) and the Graph Pad Prism version 5 software.

3. Results

3.1. Response to hydrolysed wheat flour proteins in Caco-2 cell line

After 21 days of the confluence of the Caco-2 monolayer, it was exposed to HF extract, Peptic tryptic (PT)-digested WF extract, PT-gliadin, gliadin and immunogenic peptides for *in-vitro* safety evaluation. The Caco-2 cells were grown up to 21 days and the cells were checked for viability after treatment. Finally, the viability of Caco-2 cells was evaluated by the MTT assay and there were no significant differences among the treated and untreated cells. The cell viability is shown in Fig. 1A. The zonulin level was determined in the treated cell supernatant after 4 h incubation using sandwich ELISA. The zonulin level increased significantly ($p < 0.05$) in the groups PT-digested WF (HD-2851) (2.029 ng/ml), PT-gliadin (2.049 ng/ml), gliadin (2.585 ng/ml) and Pep-II (2.078 ng/ml) as compared to the untreated, i.e., control group (0.9733 ng/ml) and PT-hydrolysed (0.958 ng/ml). The results are shown in Fig. 1B. From these results, it can be understood that the PT-digested WF extract and gliadin extract were inducing the apical zonulin release from the Caco-2 monolayer. The AN-PEP HWF extract did not have any effect in the membrane of the intestinal epithelial structure and the expression of zonulin was found to be absent. Hence, it might be safe for gluten-sensitive people.

3.2. Pro-inflammatory cytokine secretion in Caco-2 cells

Most wheat proteins and partially-digested peptides, having immunogenicity in the intestine, lead to innate immune responses. To determine the innate immune response to wheat-derived peptides and gliadin, the pro-inflammatory interleukins IL-8 and IL-6 were determined in the challenged Caco-2 cells. The IL-6 is associated with increased TJ permeability; it is a pleiotropic interleukin and is expressed in the host cell response with antigen or infection and cause-specific immune responses (Suzuki et al., 2011). The PT-digested WF, gliadin and gliadin peptides stimulated the production of IL-6

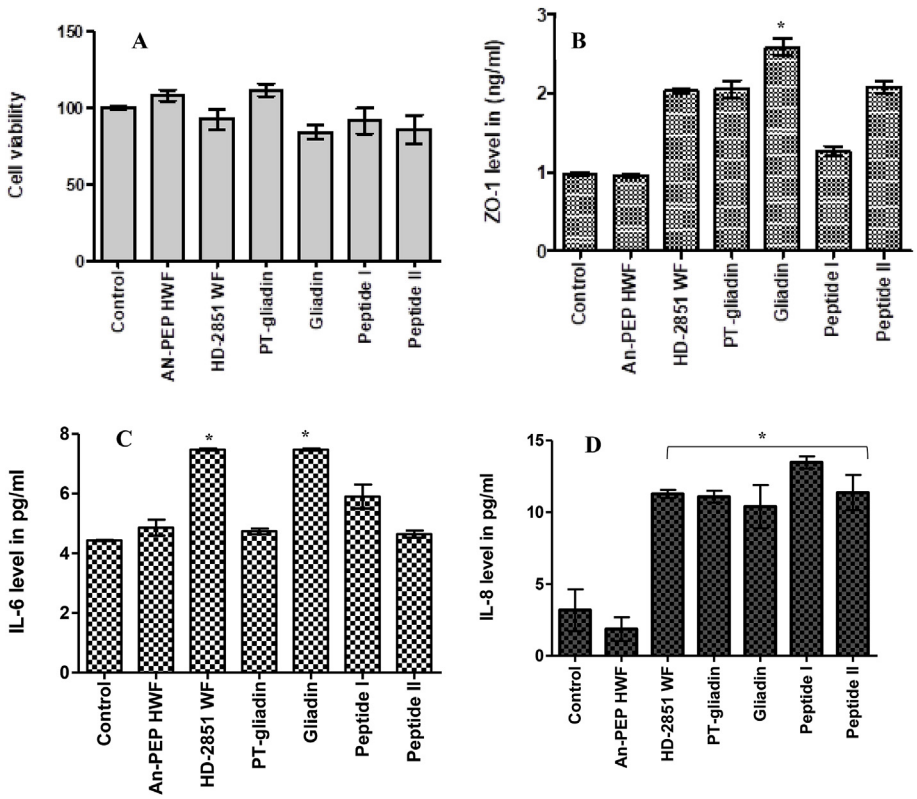

Fig. 1. Cell viability and pro-inflammatory cytokines detection in treated Caco-2 cells. (A) Effect of the in vitro PT-digest of wheat flour and modified flour treated on the growth of the Caco-2 cells. (B) Effect of the zonulin release from Caco-2 cells exposed to PT-gliadin, PT-WF gliadin extract, gliadin, AN-PEP HWF, peptide-I, and peptide-II. (C) IL-6 levels in the treated Caco-2 cell supernatant (D) IL-8 levels in the treated Caco-2 cell supernatant.

significantly ($p < 0.05$) compared to the HF extract and untreated cells (Fig. 1C). There are no significant levels of IL-6 expression in PT-gliadin treated cells; Ortiz et al. (2016) (Ortiz Sánchez et al., 2016) reported a similar kind of observation.

The interleukin is expressed from intestinal epithelial cells and stimulates the immune cells beneath the small intestine (SI) epithelial monolayer in response or triggered by food-derived substances (Satsu et al., 2004). The IL-8 level significantly increased ($p < 0.05$) in the PT-digested WF, PT-gliadin, gliadin and peptides (9-mer and 19-mer) as shown in Fig. 1D. The PT-digested WF, PT-gliadin and gliadin-derived peptides stimulate the release of IL-8 from the intestinal absorptive cells (Caco-2). It leads to neutrophils in the lamina propria. The intestinal cells contribute to the regulation of immune response (mucosal) by secreting cytokines and chemokines. Further, they lead to activation of the inflammatory cascade and the immune cells move towards the mucosa. Gronert et al. (1998) (Gronert et al., 1998) elucidated a possible hypothesis on the immune response in the mucosa. The gliadin epitopes interact with the intestinal mucosa, releasing IL-8 from the intestinal cells and activate neutrophils recruitment in the lamina propria. According to Fasano (2011) (Fasano, 2011), the gliadin peptides initiate intestinal cell permeability by releasing zonulin; this leads to paracellular translocation of gliadin and the subsequent interaction of peptides with macrophages in the SI sub-mucosa.

3.3. Immunofluorescence of ZO-1 localization in Caco-2 cells

The integrity of the intercellular barrier depends on the multiple TJ protein complex. The main TJ proteins are zonulin, occludens, occludin and claudins family. The zonula occludens (ZO)-1 is one of the well-characterized TJ proteins in the junctional complex and it localizes in the intercellular membrane TJ. The treated Caco-2 monolayers showed typical ZO-1 localization at the periphery of the cells in the vehicle control. However, in the monolayers exposed to digested and native prolamins, different degrees of ZO-1 redistribution were observed with areas having reduced or damaged zonulin staining at the edge of the cells. No changes were found in zonulin localization in the monolayers exposed to the hydrolysed flour prolamin extract. The observed results of the immunofluorescence images are depicted in Fig. 2. By an *in-vitro* model of the Caco-2 cell (intestinal epithelium), the present study confirmed that AN-PEP HWF does not induce cell toxicity nor pro-inflammatory effect.

3.4. Bodyweight, diet intake ratio and organ indices in experimental BALB/c mice

The animal body weight and diet intake ration were monitored throughout the experiment; no significant differences were observed in the diet intake and body weight between the groups (control, wheat flour and AN-PEP HWF treated mice). After 60 days, the mice were sacrificed. The body weight, diet intake and organ indices of the animal groups are shown in Fig. 3 (A, B and C).

3.5. Hydrolysed wheat flour and mouse anti-gliadin antibody response in BALB/c mice

The pre-immune sera of mice were checked initially for serum antibody cross-reactivity to gliadin. The result indicates that there is no cross-reactivity with gliadin. After the subsequent intraperitoneal (IP) immunization of gluten protein to mice, there was an increase in the IgG titer up to 3200 titers at the end of the 35th day of IP sensitization in both groups 2 and 3. The control group mice sera did not show reactivity to gliadin/gluten proteins. The animals were orally challenged with control-PBS (group 1), AN-PEP HWF (group 2) and WF (group 3) up to 3 weeks. After the oral challenge, the IgG titer value in the AN-PEP HWF group (group 2) decreased significantly ($p < 0.05$) compared to the wheat flour group (group 3). The IgG titer value of the wheat flour-orally challenged mice group increased two-fold (6400 titers) than IP sensitization at the end of 60 days. The result is shown in Fig. 4A. The result indicates that AN-PEP digested wheat flour does not show immune stimulation (IgG titer) after the oral dose compared to untreated wheat flour.

The reactivity of the gliadin-specific IgE and IgA antibodies was checked in sensitized sera through indirect ELISA. The gliadin-specific IgE and IgA levels increased on the 35th day after sequential IP immunization in the AN-PEP and control wheat flour groups. The control group did not show reactivity to gliadin/gluten proteins. The titer IgE (anti-gliadin specific) level significantly increased ($p < 0.05$) in the wheat flour (HD-2851) challenged group compared to the control and AN-PEP groups. The results are shown in Fig. 4 (B and C). A similar serum antibody titer trend was observed in the anti-gliadin specific-IgA antibodies in the wheat flour challenged group. The overall results of the humoral response shows that AN-PEP treated wheat flour does not stimulate/induce the immune system in gluten-sensitized animals.

3.6. Serum cytokines levels in hydrolysed wheat flour treated animals

The serum cytokine level was determined in the sensitized animal by using a sandwich ELISA kit. The pro-inflammatory cytokine IFN-γ, TNF-α, IL-6, IL-15, IL-1β and IL-4 levels increased significantly ($p <$

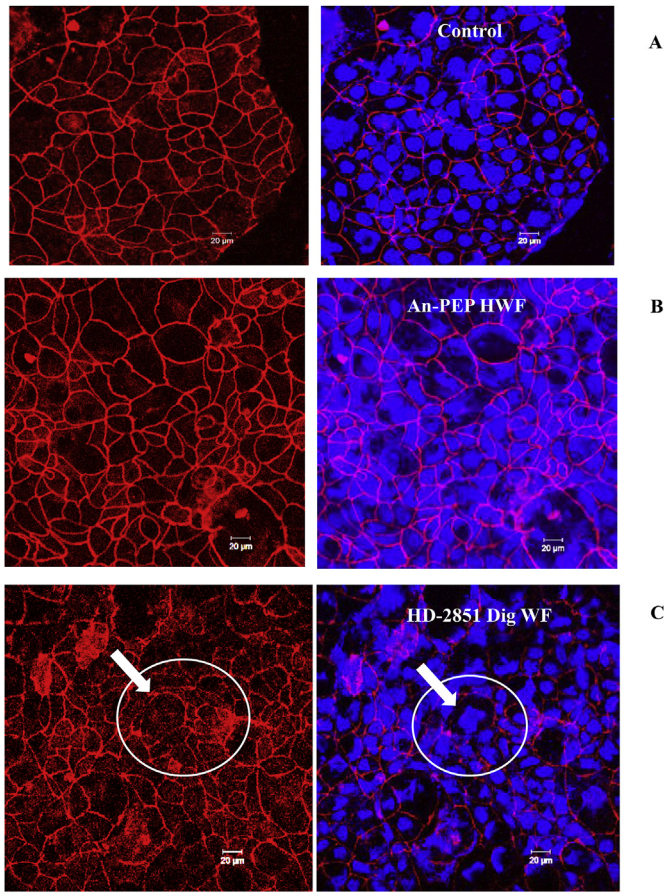

Fig. 2. Immunofluorescence analysis of ZO-1 localization in Caco-2 cells tight junctions. (A) Control cells (B) AN-PEP HWF treated cells (C) HD-2851 Dig WF treated cells. The control cells and AN-PEP HWF, treated cells shown the typical zo-1 localization at the edges, periphery of the cells. The HD-2851 PT-digest of WF shown altered ZO-1 redistribution due to the loss of ZO-1 at the periphery of the cell edges. The DAPI (Dakocytomation) was used for counterstaining of the nuclei, and it was observed in Blue color. Whereas, Alex-Fluor-647 was used for the targeted Zonulin localization and it was noted in red color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

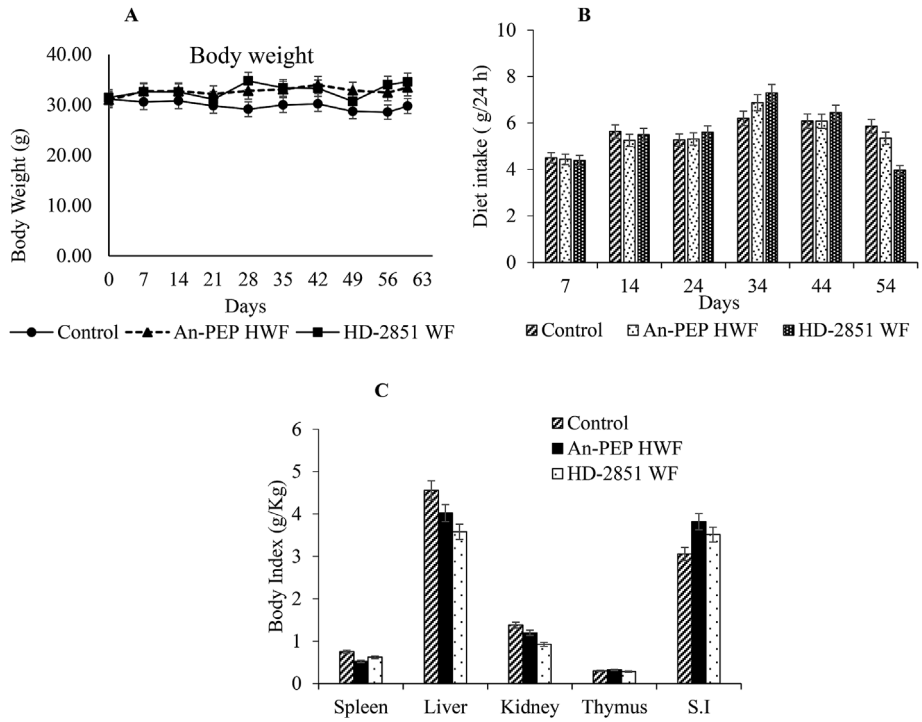

Fig. 3. Body parameters of the hydrolysed wheat flours administered animals. (A) BALB/c mice body weight results were taken regular intervals (B) Diet intake results were monitored every week intervals (C) Internal organ indices of the experimental animals.

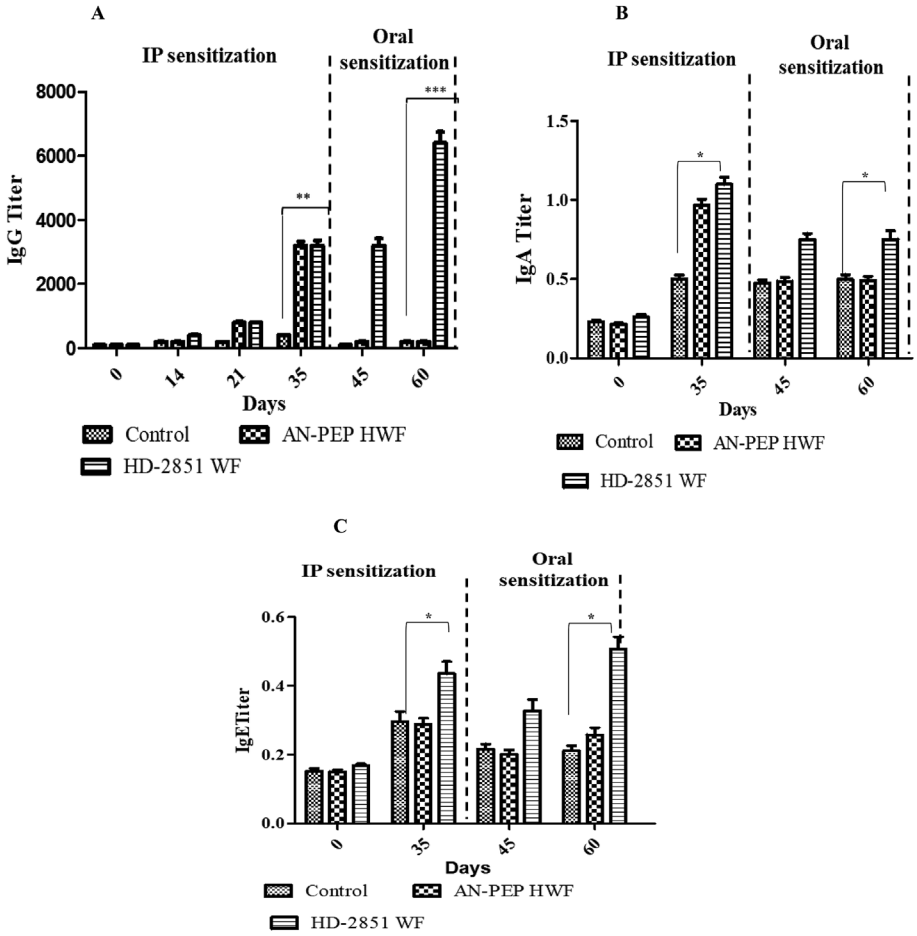

Fig. 4. Humoral response of the sensitized and hydrolysed wheat flours treated animals. (A) Anti-gliadin specific IgG titer response in IP sensitization and oral challenge (B) Anti-gliadin IgA level in serum (C) Anti-gliadin IgE level in serum. The serum anti-gliadin antibodies were significantly increased after oral challenge in the WF (HD- 2851) group animal's sere. The AN-PEP-HWF and control group did not show any significant anti-gliadin level after the oral challenge.

0.05) in the control wheat flour (HD-2851) challenged group compared to the AN-PEP HF and control (PBS) groups. IFN-γ is critical for the development of the T-helper cell-1 (T_H1) cell-mediated immune response. The IFN-γ level (T_H1 response) significantly increased in the CWF group compared to the AN-PEP HF orally administrated animal sera. IL-4 represents the T-helper cell-2 (T_H2) mediated immune response, which initiates the differentiation of native T cells towards a T_H2 phenotype. However, the IL-4 level significantly increased in the CWF group compared to the AN-PEP HF administrated mice sera. The pro-inflammatory cytokine levels in the sera are shown in Fig. 5 (A to F). The overall cytokines results reveal that AN-PEP HWF does not show any T_H1/T_H2 mediated immune responses after oral administration.

3.7. Inflammatory response and expression of pro-inflammatory cytokines in BALB/c mice

After completion of the IP and oral sensitization study, based on serological results, the mice were sacrificed and the splenocytes were isolated for *ex vivo* primary cell culture. The spleen is a secondary lymphoid organ of the lymphatic system and has mature native lymphocytes and serves to initiate adaptive immune responses. The sensitized spleen cells can easily recognize gluten proteins and these cells will be activated by specific immune cells to respective antigens. The respective splenocytes were further treated/exposed with/to peptic-tryptic digested extracts of wheat flour and AN-PEP HWF as well as undigested protein extracts of both. The pro-inflammatory cytokine markers (IL- 1β, IL-4, IL-15, IL-6, TNF-α and IFN-γ) were quantified using a sandwich ELISA kit. There was an increase in pro-inflammatory cytokines in the wheat flour group compared to the control and the AN-PEP treated groups. The IL-6 level significantly increased ($p < 0.05$) in the native and digested gluten proteins (CWF) treated in spleen cells after 24 h and 48 h of incubation; the level was downregulated at 72 h (Fig. 6 (C)). The IL-6 level was initially 61.97 pg/ml and after treatment with gluten protein (CWF), the level increased to 355.9 pg/ml; the PT-digested gluten increased the level to 562 pg/ml. At the same time, there were no significant changes in the level of IL-6 in the AN-PEP HWF extract. The TNF-α level significantly increased in both the digested (211 pg/ml) and undigested (HD-2851) (475 pg/ml) WF after 24 h of incubation. The AN-PEP HWF extract did not show any increase in the TNF-α level and it was found to be the same as that of the control. The TNF-α levels at different time intervals is shown in Fig. 6 B. Similarly, the IL-4 level also increased in both the digested (80 pg/ml) and undigested (HD-2851) (66 pg/ml) gluten after 24 h of incubation. Then, it was compared to the control and the AN-PEP treated flour extract (Fig. 6 (F)). The IL-1β level increased in gluten (HD-2851) treated splenocytes (148 pg/ml) and the PT-digested extract (145 pg/ml) (Fig. 6 (G)). However, there was no increase in the IL-1β level in the AN-PEP treated flour protein extract as compared to the control group. The IFN-γ level remained the same in the control and AN-PEP treated spleen cells, but there was an increase in the gluten treated group after 48 h of incubation. There was a slight increase in IL-15 level in the digested and undigested gluten (HD-2851) after 48 h of incubation. However, there was no significant increase in IL-15 level in the AN-PEP treated cells. From these study results, it is understood that the AN-PEP treated flour extract does not show any immunostimulatory effect on exposure up to 72 h.

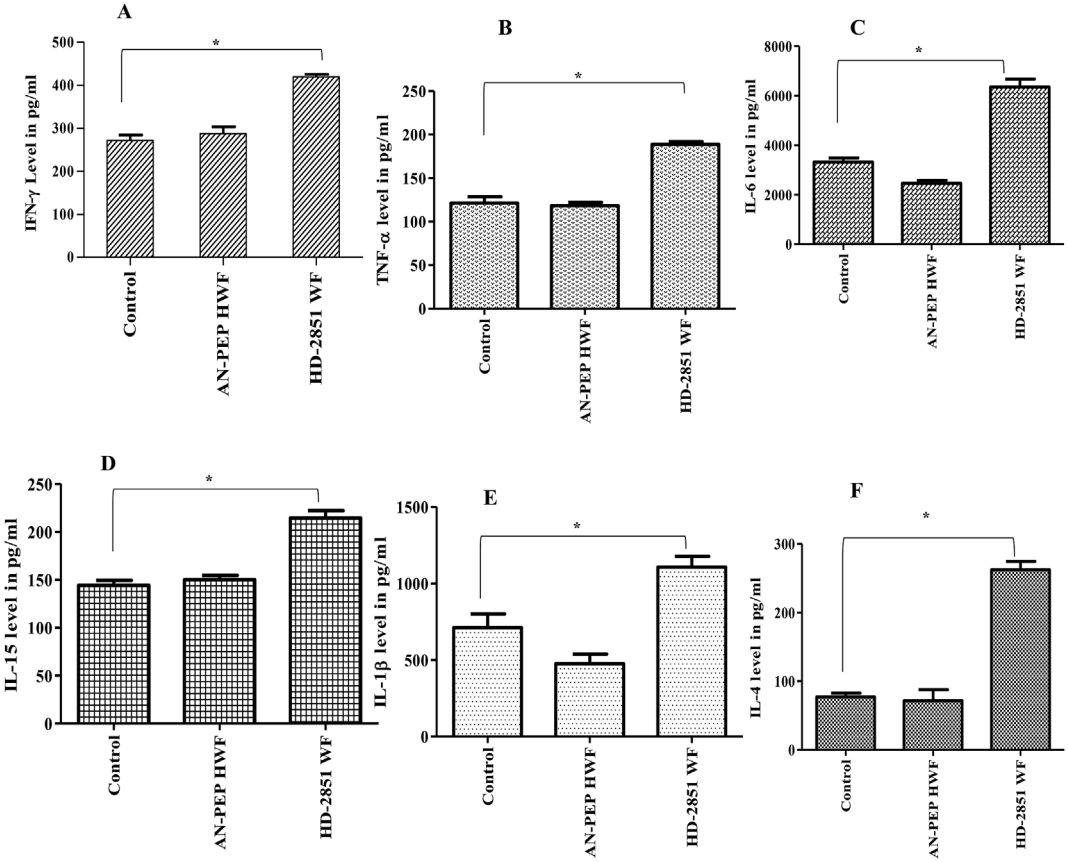

Fig. 5. Quantification of pro-inflammatory cytokines profiles in the treated animal's sera. The sera were collected from the sacrificed animal after the experiment (Day 60). (A) IFN-ϒ levels in the 60th day of mice sera (B) TNF-α levels in the 60th day of mice sera (C) IL-6 levels in the 60th day of mice sera (D) IL-15 levels in the 60th day of mice sera (E) IL-1β levels in the 60th day of mice sera (F) IL-4 levels in the 60th day of mice sera.

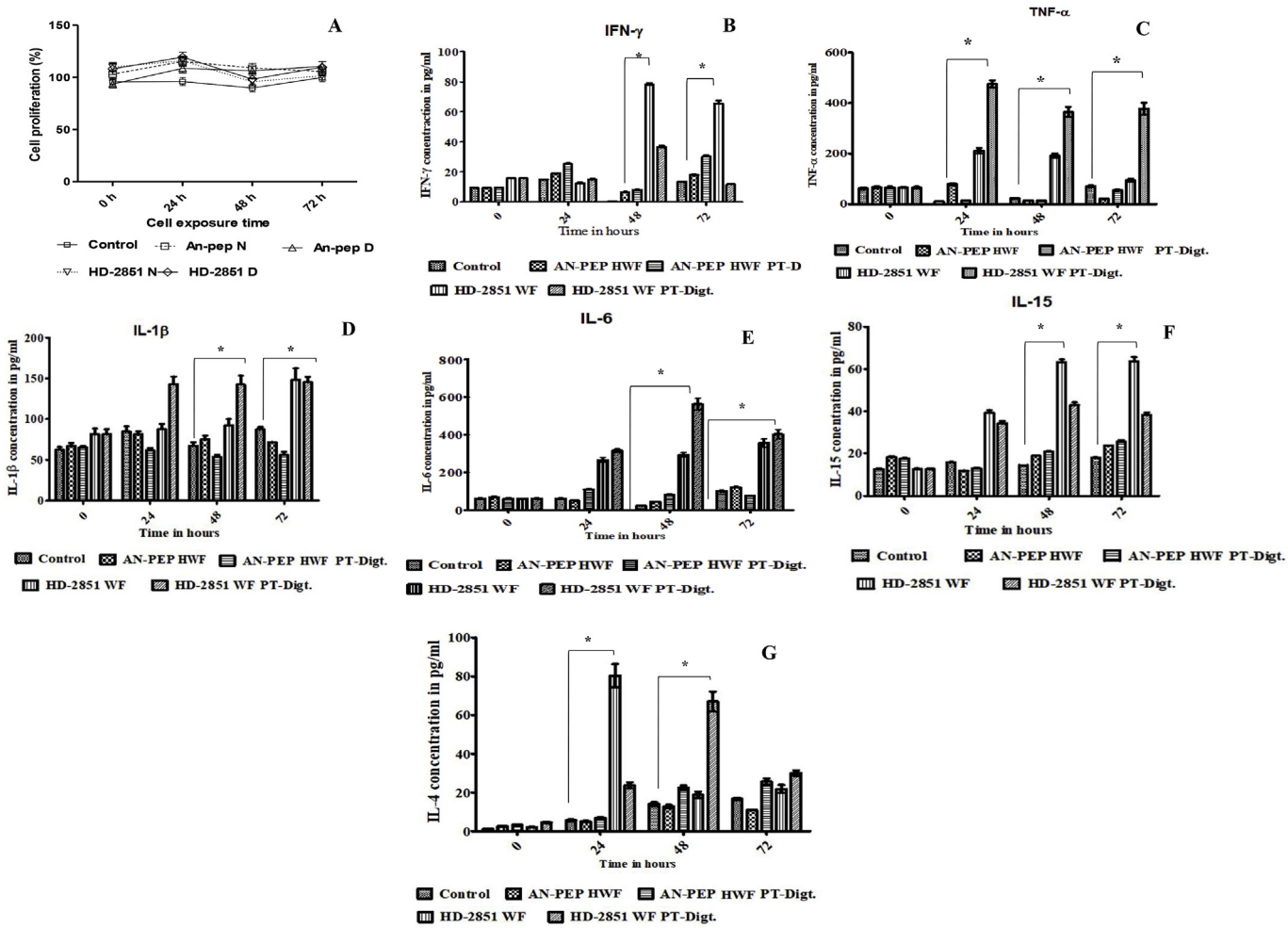

Fig. 6. Splenocytes response to the treated hydrolysed wheat flours in ex-vivo. The spleen cells were exposed to the unmodified wheat flour protein (gluten extract), ANPEP treated wheat flour extract, and HD-2851 digest extracts up to 72 h. (A).Ex-vivo cultured splenocytes proliferation assay and cells exposed with different treatments (B) Expression level of IL-6 in the cell supernatant (B) Expression level of TNF-α in the cell supernatant (C) Expression level of IL-4 in the cell supernatant (D) Expression level of IL-1β in the cell supernatant (E) Expression level of IFN-ϒ in the cell supernatant (F) Expression level of IL-15 in the cell supernatant. The result was expressed in terms of pg/ml (mean ± S.D).

3.8. Pro-inflammatory markers expression profile in intestinal jejunum sections by qRT-PCR

The intestine is affected greatly in gluten disorders; it may be an increase in the gut linking, permeability, destruction of the villi or stimulation of immune response after interaction with wheat gluten. The pro-inflammatory markers gene expression levels were analyzed in the small intestine from isolated mRNA samples. The cDNA of pro-inflammatory markers IL-1β, IL-4, IL-6, IL-15, TNF-α, IFN-γ, COX-2, iNOS and NF-kB were amplified and their expression profiles are shown in Fig. 7 (B). However, the wheat flour challenged (HD-2851) mice group small intestine mRNA exhibited upregulation (by a significant fold) in IL-15, IL-4, IL-6, IL-1β, IFN-γ, and TNF-α compared to the control and AN-PEP HWF groups. The result indicates that AN-PEP HWF does not show inflammation in the SI.

3.9. Histopathological features of small intestine microvilli sections

AN-PEP HWF along with the control wheat flour was orally administered to sensitized animals for intestinal morphological observations. The AN-PEP HWF treated intestinal group section was found to be healthy, similar to the control mice section (Fig. 7 (A)). The Marsh ratio was found to be normal in the control and AN-PEP HWF treated mice. The gluten protein creates a problem in digestion because the gluten network is more difficult to break down. This protein weakens the intestinal wall. Its effect on the tiny absorption villi in the small intestine may be compared to the action of sandpaper on wood. Later, they become blunt and broad, with a much-reduced ability to absorb. The histological examination of the jejunum segments revealed villi inflammation, lymphocytes infiltration and goblet cells hyperplasia in the mice that were sensitized and orally challenged wheat flour (40 mg/day) group.

4. Discussion

The purpose of the present study was to evaluate prolyl endopeptidase hydrolysed low immunoreactive wheat flour for safety aspects through sensitized animal and Caco-2 cell line immune responses. Wheat gluten is one of the main reasons for wheat related disorders. To overcome these gluten related disorders, many attempts were made to reduce the gluten immunoreactivity by treating wheat with microbial protease and endoprotease enzymes to degrade the immunoreactive epitopes (Tanabe et al., 1996; Takács et al., 2008). Currently, there are many enzymes that can be used to degrade the immunoreactive gluten epitopes. Different enzymes have been used to reduce immunoreactivity in wheat flour such as protease, transglutaminase and endoprotease but there is a lack of *in vivo* studies after enzymatic modification of the flour (Rey et al., 2016).

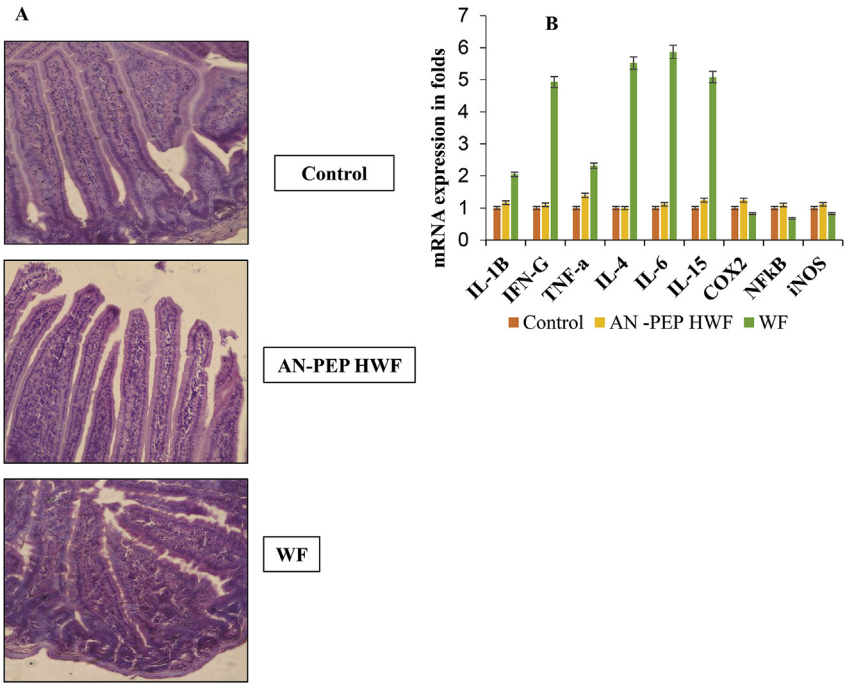

Fig. 7. Morphological observations and gene expression of small intestine sections of treated animals. (A) Histopathological features of small intestine jejunum sections of control, AN-PEP HWF and WF challenged mice (B) mRNA expression profile of inflammatory markers in small intestine section. mRNA expression represented in terms of their fold change.

In this investigation, AN-PEP HWF was analyzed for safety and immune responses by *in-vitro* and *in-vivo* assays. The AN-PEP digested wheat flour did not show permeation effect and pro-inflammatory responses in the Caco-2 intestinal cell line; there was typical ZO-1 localization at the periphery of the cells, which was confirmed using immunofluorescence, compared to the WF group. The intestinal barrier function was affected by WF gliadin and PT-digested gliadin through release of the TJ protein zonulin; this leads to an increase in the paracellular permeability and alters cellular localization. A similar report (Sander et al., 2005) states that exposing wheat gliadin to Caco-2 cells leads to a reduction in ZO-1 at the TJ and alters ZO-1 localization. According to Stenman et al. (2009) (Stenman et al., 2009) the germinated wheat seed protease hydrolysed gluten small peptide fragments did not alter or increase epithelial permeability and there was no change in the ZO-1 expression level and cytoskeletal rearrangement. According to Fasano (2012)[31], zonulin pre-hapto globin is a precursor molecule for zonulin proteins in celiac disease patients. However, zonulin associated with a series of immune-mediated disorders and also act as a biomarker for gastrointestinal diseases (Fasano, 2012; Sturgeon and Fasano, 2016) (Fasano, 2012; Sturgeon and Fasano, 2016). Based on the existing research findings, Zo-1 protein has been chosen for tight junction protein for the cellular epithelial permeability studies. Hence, the caco-2 cell line was used to study the epithelial permeability by localization of Zo-1 antibody in the hydrolysed wheat flours treated cells. These previous reports suggest that enzyme-modified wheat flour gluten loses its increased intestinal permeability because of hydrolysis of the protein. The study results also support the fact that AN-PEP HWF, upon oral administration in sensitized BALB/c mice, did not exhibit T_H2/T_H1immnoresponses.

The gliadin specific (IgG, IgE and IgA) levels were found to be quite high in the WF administrated group compared to the control (PBS) and HF groups. The cytokine IL-4 represents the T_H2 immune response, which was found normal in the HF challenged animal sera; but, in four cases of WF challenged animals, it was found to be high. A significant T_H2 response was found in WF challenged animals whereas, the HF group did not show T_H2 response after oral administration. These study results complement the earlier study report of deamidated gliadins sensitized BALB/c mice (Gourbeyre et al., 2012), which states that deamidated and native gliadin sensitized mice show higher concentrations of specific IgG and IgE levels in the sera. It also says that the WF orally challenged mice showed a significantly high level of IFN-γ (T_H2 response) as compared with the control and HF animal groups.

The other pro-inflammatory markers IL-1β, IL-6, IL-15 and TNF-α levels increased in WF challenged mice. According to Bodinier et al. (2009) (Bodinier et al., 2009) in a relative study model for sensitization of BALB/c mice and elicitation by gliadins, the animals showed specific IgE responses to α, β, γ, ω1,2- and ω5-gliadin (Bodinier et al., 2009). Papista et al. (2012) (Papista et al., 2012) also reported that the gluten challenge leads to the development of villous atrophy; the infiltration of T cells and macrophages in the small intestine region enteropathy can change the morphology of the small intestine jejunum; these mice also display an increased level of pro-inflammatory markers (IL-15) as well as gliadin specific and tTG autoantibodies (Papista et al., 2012). According to Adachi et al. (2012) (Adachi et al., 2012) hydrolysed wheat protein can induce IgE mediated immune responses in the BALB/c mice model; they also confirmed this by the production of IL-4, IL-5 and IL-10 by splenocyte stimulation assay using HWP (Adachi et al., 2012). The previous study by the current authors (Vijaykrishnaraj et al., 2017) showed that prolonged gluten administration during sensitization of BALB/c mice leads to the T_H1/T_H2 response. All these results demonstrate that WF oral administration was effective in stimulating antibody (IgG and IgE) production (T_H2 response) and cell-mediated immune responses (T_H1). The AN-PEP HF administrated animals did not exhibit T_H1/T_H2 mediated immune responses nor pro-inflammatory marker expression. The *in-vivo* studies confirm that AN-PEP HWF does not induce/trigger intestinal cell permeability nor cytoskeletal rearrangement in sensitized BALB/c mice.

The *ex-vivo* primary cell culture studies also support the fact that the HF extract and PT-digest do not stimulate the production of any pro-inflammatory markers. However, in the case of WF protein exposed spleen cells, there was an increase in pro-inflammatory markers between 24 and 48 h of incubation. The mRNA expression level in the SI reveals that there is an increase (2–5 fold) in pro-inflammatory expression levels in WF challenged BALB/c mice. The control (PBS) and AN-PEP HF challenged mice did not show overexpression of pro-inflammatory genes in the small intestine section. The overall results reveal that AN-PEP HWF does not stimulate neither humoral nor cell-mediated immune responses and so, it can be taken up for low

immunogenic or low gluten food preparations. AN-PEP HWF was found safe by confirmation using both *in-vitro* and *in-vivo* assays.

5. Conclusions

The present study states that wheat immunoreactive proteins were effectively reduced by AN-PEP enzymatic hydrolysis and this offers a promising tool to nullify the gluten protein. The *in-vitro* model of the intestinal epithelium demonstrates that the AN-PEP HWF protein extract does not stimulate nor induce cell cytotoxicity as compared to the WF gliadin extract. The AN-PEP effective hydrolysis or chapping of epitopes of wheat gluten leads to a reduction in the immunoreactive proteins. AN-PEP HWF does not induce cell permeability nor ZO-1 localization when compared to WF. The *in-vivo* challenge studies reveal that AN-PEP HWF does not stimulate any T_H2 (IgG, IgA and IgE) responses and the *ex-vivo* studies further confirm that AN-PEP HWF does not show any stimulation or overexpression of the pro-inflammatory markers (IL-1β, IL-4, IL-6, IL-15, TNF-α, IFN-γ, COX-2, iNOS and NF-kB). The histopathology of small intestine sections reveals that there is a structural dysregulation in villi architecture in the wheat flour treated group as compared to the control and AN-PEP HWF groups. AN-PEP HWF does not show immunostimulatory responses (both humoral and cell-mediated). AN-PEP HWF is found safe through *in-vitro* and *in-vivo* studies. Further, HWF can be used for the development of hypo-immunogenic food products and should be studied through pre-clinical trials or human volunteer approaches.

Conflict of interest

The authors of the article wish to declare that there is no conflict of interest.

Acknowledgments

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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.2019.05.011.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.05.011

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Table 1. Specific genes primers for CDNA amplification

S.No	Genes	Primer details	Accession No
1	β actin	5′ -TATAAAACCCGGCGGCGCA-3′ 5′ -GTCATCCATGGCGAACTGGTG-3′	NM_007393.5
2	IL-1β	5′ -GCCACCTTTTGACAGTGATGAG-3′ 5’ -ATGTGCTGCTGCGAGATTTG-3′	NM_008361.4
3	TNF-α	5′ -TAGCCCACGTCGTAGCAAAC-3′ 5′ -ACAAGGTACAACCCATCGGC-3′	NM_013693.3
4	IFN-ϒ	5′ -GAGGTCAACAACCCACAGGT-3′ 5′ -ATCTCTTCCCCACCCCGAAT-3	NM_008337.4
5	IL-4	5′ -GTACCAGGAGCCATATCCACG-3′ 5′ -TCTTCGTTGCTGTGAGGACG-3′	NM_021283.2
6	IL-6	5′ -ACCCCAATTTCCAATGCTCTCC-3′ 5′ -AACGCACTAGGTTTGCCGA-3′	NM_031168.2
7	IL-15	5′ -AGTGCATCTCCTTACGCGCT-3′ 5′ -GGATTCTTTCCTGACCTCTCTGAGC-3′	NM_008357.2
8	iNOS	5′ -GGTGAAGGGACTGAGCTGTT-3′ 5′ -ACGTTCTCCGTTCTCTTGCAG-3′	NM_010927.4
9	COX-2	5′ -TGAGTACCGCAAACGCTTCT-3′ 5′ -CAGCCATTTCCTTCTCTCCTGT-3′	NM_011198.4
10	NF-$_k$B	5′ - GCCCAGAAGCACCTGTGATA -3′ 5′ - TACGCTTCCCGGCTTTTACA -3′	NM_025719.3