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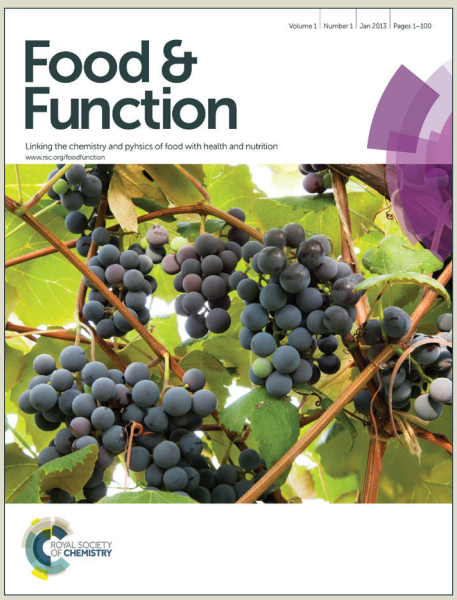

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The anti-inflammatory potential of a moderately hydrolysed casein and its 5kDa fraction in *in-vitro* and *ex-vivo* models of the gastrointestinal tract.

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Abstract

Bioactive peptides from milk can impart a wide range of physiological benefits without the allergies and intolerance associated with the consumption of whole milk. The objective of this study was to characterise the anti-inflammatory properties of intact sodium caseinate (NaCAS), a moderately hydrolysed NaCAS enzyme hydrolysate (EH) and its 5-kDa fraction (5kDaR), in both *in-vitro* and *ex-vivo* systems. *In-vitro,* Caco-2 cells were stimulated with tumor necrosis factor (TNF) α and co-treated ± casein hydrolysates or dexamethasone (control). The inflammatory marker interleukin (IL)-8 was measured by ELISA in the supernatant at 24 h. *Ex-vivo*, porcine colonic tissues were stimulated with lipopolysaccharide (LPS) and co-treated with casein hydrolysates for 3 h from which the relative expression of a panel of cytokines was measured *in-vitro*. While the steroid dexamethasone brought about a 41.6 % reduction in the IL-8 concentration in the supernatant, the 5kDaR reduced IL-8 by 59 % ($P < 0.05$) when compared to the TNFα stimulated Caco-2 cells. In the *ex-vivo* system, 5kDaR was associated with decreases in *IL-1α, IL-1β, IL-8* and *TGF-β* expression and an increase in *IL-17* expression ($P < 0.05$) relative to the LPS challenged tissues. We concluded, that a 5kDa casein fraction demonstrates potent anti-inflammatory effects both in *in-vitro* and *ex-vivo* models of the gastrointestinal tract.

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Introduction

Bioactive proteins and peptides present in milk have a range of health promoting properties [1; 2]. Whole milk facilitates the postnatal adaptation of newborns by providing essential nutrients as well as promoting and regulating the growth, maturation and functionality of the intestine [3]. The health promoting effects of milk exert their effects in the intestine of the host inhibiting the growth of pathogenic organisms while simultaneously providing a more optimal substrate for the non-pathogenic symbiotic gut microflora [4]. The colonization of the gut microflora plays an important role in development and functional adaptation of the gut associated lymphoid tissue (GALT) [5]. The bioactive milk peptides present within whole milk are released as a consequence of proteolytic digestion, a process that takes place naturally within the gastrointestinal tract. This process can be achieved artificially on an industrial scale using either enzymatic digestion or bacterial cultures [1].

The gastrointestinal tract (GIT) has a major role in the maintenance of immune homeostasis and is of particular interest when evaluating dietary ingredients with immuno-modulatory potential. Human colonic adenocarcinoma (Caco-2) cell lines are widely used as *in-vitro* models of the small intestinal epithelial cells, an important part of the GIT, as they are functionally similar to fully differentiated enterocytes. Caco-2 cells

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have the capacity to elicit pro-inflammatory responses in response to stimuli such as bacterial endotoxin lipopolysaccharide (LPS) [16] and tumor necrosis factor alpha (TNF-α) [17]. Hence the Caco-2 cell line is extensively used to study the effects of bioactive ingredients in both normal and inflamed conditions [15]. Caco-2 cells lack the cellular diversity present within the baso-lateral side of the intestinal epithelium of intact tissue [18] and hence, explants from intestinal tissue with their inherent heterogeneity, are also informative in-terms of studying the effects of bioactive compounds on both tissue physiology and immune responses under normal conditions and following microbial infection [19; 20]. Total RNA has previously been reported to remain intact in porcine colonic explants for up to 3 h in the presence of LPS and hence the effects of the bioactives on the transcriptome can be explored using this *ex-vivo* system [21].

Bovine milk hydrolysates exhibit clinically observable effects on conditions such as type 2 diabetes mellitus [7], obesity [8] and hypertension [9] as well as influencing plasma cholesterol levels [10] and the activity of angiotensin-converting enzyme (ACE) [11] and immune responses [12]. The degree of hydrolysis has been shown to influence the biological activities of the hydrolysates, as the size and amino acid composition of the hydrolysate is altered [13]. In contrast to this, another *in-vitro* study indicated that the degree of hydrolysis had no effects on both the

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cytotoxic and immunomodulatory properties of NaCAS in human Jurkat cells [14]. Hence, the thorough evaluation of the bioactivity of individual hydrolysates generated under different physico-chemical conditions is essential, as the findings cannot be generalised.

Casein is one of the most abundant proteins present in mammalian milk. Acid precipitation of milk casein results in the formation of sodium caseinate (NaCAS). Sodium caseinate has a wide range of bioactive properties as well as low levels of lactose, making it a suitable supplement for lactose intolerant individuals [6]. In a previous experiment, a 1-kDa retentate of NaCAS hydrolysate generated by a low degree of hydrolysis (5-10%) demonstrated anti-inflammatory activity in both *in-vitro* and *ex-vivo* systems (Mukhopadhya et al. in press). Hence, the objective of this study was to evaluate the anti-inflammatory effects of intact NaCAS, a NaCAS enzyme hydrolysate generated by a moderate degree of hydrolysis (11-16%) as well as its 5-kDa fraction (5kDaR) in two experimental systems; an *in-vitro* system using TNF-α stimulated Caco-2 cells and in an *ex-vivo* system using LPS stimulated porcine colonic explants.

Materials and Methods

Generation of sodium caseinate hydrolysate

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Bovine milk derived sodium caseinate (NaCAS) (90 % w/w protein, Kerry Food Ingredients, Listowel, Ireland) was suspended at 10 % (w/w) on a protein basis in distilled water and dispersed under agitation at 50 °C for 1 h using an overhead stirrer (Heidolph RZR 1, Schwabach, Germany). The pH was adjusted to 7.0 using a NaOH 4.0 N solution (VWR, Dublin, Ireland). A bacterial food-grade enzyme preparation, was added to the protein solution and hydrolysis was carried out at constant pH (7.0) by manual titration of 4.0 N NaOH, until a moderate degree of hydrolysis (11-16%) was achieved. The enzyme was inactivated by heat-treatment of the hydrolysate sample at 85 °C for 25 sec. All hydrolysis experiments were conducted in triplicate. The hydrolysate (50 L) described above was dehydrated in a pilot scale Anhydro Lab 3 spray drier (SPX Flow Technology A/S, Soeborg, Denmark) at an inlet temperature range of 185 – 190 °C and outlet of 85 – 90 °C. The enzyme hydrolysate (EH) was further concentrated (to approximately 40 % total solids) before spray drying, as outlined above, in a Anhydro F1 Lab single effect falling film evaporator (SPX Flow Technology A/S, Soeborg, Denmark).

Membrane processing of the milk hydrolysate

The milk hydrolysate was subjected to microfiltration, to remove any aggregates after enzymatic hydrolysis, using a GEA Model F unit (GEA

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Process Engineering A/S, Skanderborg, Denmark). This unit was fitted with three 0.14 µm ceramic membranes (Tami Industries, Nyons Cedex, France) having a nominal molecular weight cut off of 30, 10 and 5 kDa. Microfiltration was carried out at 50 ºC and pH 7.0 to a volume concentration factor of 8.0. A feed recirculation rate of 1500 L h^{-1} at 1 bar and membrane inlet pressure of 4.2 bar were maintaned throughout processing. The permeate stream prepared above was then subjected to ultrafiltration using the same GEA model F unit fitted with a spiral wound membrane (Koch Membrane Systems, Wilmington, MA, US). This membrane had a nominal molecular weight cut off of 5 kDa. The 5 kDa retentate stream was dehydrated in a pilot scale Anhydro Lab 3 spray drier (SPX Flow Technology A/S, Soeborg, Denmark) at an inlet temperature range of 185 - 190 °C and outlet of 85 - 90 °C.

Compositional analysis

The lipid content of the powder samples was determined using the Röse-Gottlieb method for lipid determination [22]. Ash was determined gravimetrically through a modification method outlined by the International Dairy Federation [23] where > 1 g of powder was weighed to the nearest 0.1 mg. Dry matter was determined according to a method employed by the International Dairy Federation for milk and milk products [24]. Protein content was determined by Kjeldahl on a Foss

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Kjeltec™ 8400 (Foss, Hillerød, Denmark). The procedure was modified from Koops et al. [25] where a protein conversion factor of 6.38 was used [26].

Chromatography

High performance liquid chromatography (HPLC) was carried out using a Waters 2695 separation module, a Waters 2487 dual wavelength absorbance detector running on Waters Empower® software (Milford, MA, USA). Size-exclusion chromatography (SEC) was carried out on a TSK Gel G2000SW, 7.8 mm x 600 mm, column (TosoHaas Bioscience GmbH, Stuttgart, Germany) using an isocratic gradient of 30 % acetonitrile (ACN) containing 0.1 % trifluoroacetic acid (TFA) (v/v) at a flow-rate of 0.5 mL min^{-1} over 60 min. Samples of Alpha lactoalbumin (α-la), Beta lactoglobulin (β-lg) A and B, Bovine Serum Albumin (BSA), Lactoferrin, and Caseinomacropeptide (CMP) (Sigma-Aldrich, Dublin, Ireland) were used as protein standards. Ribonuclease A, Cytochrome C, Aprotinin, Bacitracin, His-Pro-Arg-Trp, Leu-Trp-Met-Arg, Bradykinin, Leu-Phe, and Tyr-Glu (Bachem AG, Bubendorf, Switzerland) were used as molecular weight (M_w) standards. All chromatography test samples and standards were made up in Milli-Q water (2.5 g L^{-1} solutions) pre-filtered through 0.2 µm low protein binding membrane filters (Sartorius Stedim Biotech, GmbH, Goettingen, Germany) and 20 µL applied to the

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column. The column elute was monitored at 214 nm and 280 nm and all solvents were filtered under vacuum through 0.45 μm high velocity filters (Millipore Ltd., Durham, UK).

Caco-2 cell culture

The human colonic adenocarcinoma cell line (Caco-2) was sourced from American Type Culture Collection and was maintained in 75 cm^2 cell culture flasks, in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen Corp., San Diego, CA, USA) supplemented with 10 % (v/v) foetal bovine serum (Invitrogen Corp.), 1 % sodium pyruvate, 1 % non-essential amino acids and 1 % penicillin-streptomycin (Sigma-Aldrich Corp., St. Louis, MO, USA) at 37 °C in a humidified 5 % CO_2 incubator. Media was changed on alternate days, with cells trypsinised and sub-cultured at regular intervals. Caco-2 cells with passage 53 to 65 were used in this study. In preparation for experimental analyses, cells were plated in a 24 well plate at a plating density of 10^6 cells/ml and maintained for 21 d until fully differentiated.

Dose dependent anti-inflammatory activity of milk hydrolysates

To identify the concentration of milk hydrolysate with optimal anti-inflammatory properties, Caco-2 cells were stimulated with *TNFα* (10 nM). The *TNFα* stimulated Caco-2 cells were then exposed to the milk

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hydrolysates at a range of concentrations including; 0.01, 0.02, 0.05, 0.1, 0.5, 1, 2.5 and 5 mg/ml and incubated at 37 °C for 24 h in a humidified 5% CO_2 incubator. Following this, the media was collected and the IL-8 concentrations were measured using a Human CXCL8/IL-8 ELISA kit (R&D Systems Europe, Ltd. Abingdon, UK) following the manufacturers protocol.

Comparison of the anti-inflammatory activity of casein hydrolysates

Caco-2 cells were challenged with TNFα (10 nM) (Sigma-Aldrich Corp., St. Louis, MO, USA) to stimulate a pro-inflammatory response (control). Caco-2 cells challenged with TNFα and simultaneously co-treated with the commercially available anti-inflammatory steroid, Dexamethasone (10 nM) (Sigma-Aldrich Corp., St. Louis, MO, USA), was used as a positive control for this experiment. Similarly, Caco-2 cells were challenged with TNFα and co-treated with 1 mg/ml of NaCAS or EH or 5kDaR to evaluate their anti-inflammatory property. The cell culture plates, after stimulating with TNFα and co-treating with/without either dexamethasone or NaCAS hydrolysates and fractions, were incubated at 37 °C for 24 h in a humidified 5% CO_2 incubator. After 24 h the media was collected and IL-8 levels were measured using Human CXCL8/IL-8 ELISA kit, as mentioned in above section.

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Ex-vivo challenge of porcine colonic tissues

Following euthanasia, colonic sections from three pigs were dissected along the mesentery and rinsed with sterile PBS. Tissue sections of approx. 1 x 1 cm were stripped of overlying muscle and placed in 1 ml of DMEM containing 10 µg/ml bacterial LPS (Sigma-Aldrich Corp., St. Louis, MO, USA) and NaCAS or EH or 5kDaR (1 mg/ml). DMEM containing LPS served as a carrier control. All tissue explants were incubated at 37 ºC for 90 min before being removed and transferred to 5 ml of RNAlater™ (Applied Biosystems, Foster City, CA), which following an overnight incubation at room temperature, the RNAlater™ was removed and samples were stored at -80 °C.

Gene expression analysis

RNA extraction and cDNA synthesis

Total RNA was extracted using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Corp.) according to the manufacturer's instructions. Total RNA was subjected to DNAse I (Sigma-Aldrich Corp.) treatment, followed by further purification using a phenol-chloroform extraction method. The total RNA was quantified using a NanoDrop-ND1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The quality of the total RNA was evaluated on a 1% agarose gel stained with ethidium bromide. Total RNA

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(1µg) was used for the synthesis of first strand cDNA using the First Strand cDNA Synthesis Kit (Qiagen Ltd. Crawley, UK) and oligo dT primers according to the manufactures instructions. The final volume of cDNA was adjusted to 120 µl with nuclease free water.

Quantitative Real-Time PCR (qPCR)

A panel of 12 cytokines were evaluated using qPCR. This panel included interleukins (*IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17, IL-21*), interferon (*IFN-γ*), tumor necrosis factor (*TNFα*), transforming growth factor (*TGF-β*) and Forkhead box P3 (*FOXP3*). Primers used for these targets are presented in Table 1. Primer efficiencies were determined using a serial dilution (1:4 dilution series over 7 points) of a cDNA pool from all of the experimental samples and were in the range of 90 to 110 %. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β_2 microglobulin (*B2M*), Beta-actin (*ACTB*), Peptidylprolyl isomerase A (*PPIA*) and 14-3-3 protein zeta/delta (*YWHAZ*) were used as endogenous controls as described by Ryan et al. [27]. All primers were designed using Primer Express ™ software and were synthesised by MWG Biotech (Milton Keynes, UK). Assays were carried out using 96 well fast optical plates on a 7500HT ABI Prism Sequence Detection System (PE Applied Biosystems, Foster City, CA) using Fast SYBR Green PCR Master Mix (Applied Biosystems). All reactions were performed in triplicate in a final

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volume of 20 µl containing 10 µl Fast SYBR PCR Master mix, 1 µl forward and reverse primer mix (100µM), 8 µl nuclease free water and 1µl of cDNA. The thermal cycling conditions were 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15 s and 65 ºC for 1 min followed by a dissociation curve analysis to confirm specificity.

Normalisation of qPCR data

Mean C_t values were converted to relative quantities using the formula, relative quantity = (PCR efficiency) $^{-\Delta Ct}$, where ΔC_t is the C_t values of the sample minus the C_t of the sample with the highest expression (minimum C_t value). Relative quantities of the endogenous controls were analysed using geNorm [28] from which a normalisation factor (geometric mean of the relative quantities) was obtained from the four most stable ($M < 1.5$) reference genes (*GAPDH, B2M, ACTB* and *PPIA*). The relative quantities of the target genes were then divided by the normalisation factor to give normalised relative quantity for each sample.

Statistical analysis

The *in-vitro* and *ex-vivo* experiments used a complete randomised design and the data was analysed using the general linear model procedure of the Statistical Analysis Institute [29]. All data was checked for normality and the presence of outliers using the PROC univariate procedure in SAS [29].

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The values from the treatment groups were compared to unchallenged group using contrast statements. Probability values of < 0.05 were used as the criterion for statistical significance. All results are presented in the tables as least square means ± standard error of the means (SEM).

Results

Compositional analysis and molecular weight distribution

The compositional analysis profiles of NaCAS, EH and 5kDaR are presented in Table 2. The spray drying process decreased the moisture content of the EH ($P < 0.01$) compared to the commercial NaCAS powder. The addition of NaOH during the hydrolysis process increased the ash content in EH and 5kDaR compared to NaCAS substrate ($P < 0.001$). The increased ash levels introduced during the hydrolysis process was associated with a decrease in lipid content of the EH (0.62 vs. 0.80 ± 0.06 g $100g^{-1}$, $P<0.05$), while no lipids were detected in 5kDaR fraction due to the use of 0.14 µm MF membrane. The protein content was analysed by Kjeldahl method and the samples had a protein content in the range of 88 to 90 g $100g^{-1}$.

The molecular weight distribution of the NaCAS, EH and 5kDaR is presented in Table 3. The NaCAS substrate consisted mostly of material with a molecular weight of > 30 kDa compared to only 14.55 % present in 30 – 10 kDa and traces with a molecular weight of < 10 kDa. The

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enzymatic hydrolysis of NaCAS increased the abundance of material with molecular weight in the range of 5 - <1 kDa ($P < 0.001$). Whereas, the size fractionation increased the abundance of the material in the range of 5-1 kDa, reducing the presence of fractions of < 1kDa ($P < 0.001$). The technique used in this process enriched the fractions based on size ranges rather than accurately separating the proteins and the peptides according to discrete sizes (Table 3).

Dose dependent responses and optimisation of working concentration of milk hydrolysates

The dose dependent anti-inflammatory responses of NaCAS, EH and 5kDaR were evaluated by quantifying IL-8, with all samples displaying a dose dependent anti-inflammatory effect in Caco-2 cells (Figure 1). The NaCAS, EH and 5kDaR, displayed maximum anti-inflammatory effects within the range of 0.5 to 1.0 mg/ml and based on this, a final working concentration of 1 mg/ml were used for subsequent experiments.

Inhibition of IL-8 production by milk hydrolysate in Caco-2 cells

IL-8 secretion in response to exposure of Caco-2 cells to TNFα □□□□□□□ (positive control), □□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□ □□□□□□ (negative control)□□NaCAS, EH or 5kDaR is presented in

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Table 4. Co-treatment of TNFα challenged Caco-2 cells with NaCAS, EH or 5kDaR resulted in significant reductions in IL-8 production relative to the TNFα exposed control cells.

Effects of LPS and milk hydrolysates on a select panel of cytokine gene expression in porcine colonic tissue explants

The effect of the LPS challenge on the expression of a selected panel of cytokine genes from the *ex-vivo* colonic tissues are summarised in Fig 2. The treatment with LPS resulted in increases in the transcript abundance of a number of inflammatory cytokines including; *IL-1α* (0.181 vs 0.068 ± 0.026 RQ, $P < 0.05$), *IL-1β* (0.218 vs 0.062 ± 0.010 RQ, $P < 0.01$), *IL-8* (0.185 vs 0.064 ± 0.030 RQ, $P < 0.05$) and *TNFα* (0.210 vs 0.024 ± 0.029 RQ, $P < 0.001$) relative to the unchallenged colonic tissues. The LPS-challenged tissues had reduced *IFN-γ* expression (0.644 vs 0.0762 ± 0.033 RQ, $P<0.001$) while there were no significant effects on *IL-6, IL-10, IL-17A* and *TGF-β* expression. The expression of *IL-4*, *IL-21* and *FOXP3* were undetectable by qPCR in this study.

Effect of NaCAS, NaCAS enzyme hydrolysate and 5kDaR on LPS stimulated and un-stimulated porcine colonic tissue

The effect of NaCAS, EH and 5kDaR on the expression of a panel of cytokines is summarised in Fig 3. Co-treatment of the colonic tissue with

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NaCAS and LPS resulted in an up-regulation of *IL-8* (0.301 vs 0.185 ± 0.030 RQ, $P < 0.05$) with no significant alterations in expression of the other cytokines relative to the control.

Co-treatment of the colonic tissue with EH and LPS did not significantly alter the expression of the cytokines relative to the control (containing LPS).

Presence of 5kDaR resulted in down-regulation of *IL-1α* (0.066 vs 0.181 ± 0.032 RQ, $P < 0.05$), *IL-1β* (0.103 vs 0.218 ± 0.035, $P < 0.05$) and *IL-8* (0.058 vs 0.185 ± 0.030 RQ, $P < 0.05$) expression relative to the control (containing LPS).

The presence of EH did not have significant effects. While the 5kDaR did not alter the expression of *IL-10*, the abundance of *IL-17* was increased (0.388 vs 0.121 ± 0.048 RQ, $P < 0.01$) and the expression of *TGF-β* was decreased (0.043 vs 0.160 ± 0.040 RQ, $P < 0.05$) relative to the carrier control (no LPS) (data not presented).

Discussion

This study was designed to evaluate the anti-inflammatory effects of intact NaCAS as well as two enzyme hydrolyates of NaCAS i.e. whole enzyme hydrolysate and a 5-kDa retentate generated by a moderate degree of hydrolysis (11-16%) of bovine milk derived NaCAS substrate. The anti-inflammatory activity was evaluated in both an *in-vitro* Caco-2

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cell based system and an *ex-vivo* colonic explant system. Results indicate that the 5kDaR had the most promising anti-inflammatory effects based on the observed reduction in IL-8 concentration in the TNF-α stimulated Caco-2 cells and also the reduced expression of a sub-group of pro-inflammatory cytokines in LPS stimulated porcine colonic explants.

TNF-α is a critical pro-inflammatory mediator that is up-regulated during both the acute and chronic stages of gut inflammatory conditions [30; 31]. Similarly, IL-8, which is produced by immune cells such as macrophages, endothelial cells and epithelial cells in the gut, is another established marker for inflammation of the gastrointestinal system [32]. IL-8 exhibits chemotactic activities against T lymphocytes, basophils and neutrophils as well as inducing release of lysosomal enzymes through neutrophils, hence playing causal role in establishing acute inflammation [32]. The 5kDaR co-treatment of TNF-α stimulated Caco-2 cells resulted in a reduction in IL-8 concentration. A similar reduction of 50.1% in IL-8 concentration was seen in Caco-2 cells stimulated with H_2O_2 and treated with high hydrostatic pressure treated whey protein hydrolysate [33]. The results of this study and previous research indicated that these milk hydrolysates may exert inhibitory effects on the NF-κβ pathway or may actively re-establish homeostasis following inflammation e.g. following stimulation with either TNF-α or H_2O_2. While the NF-κβ pathway does regulate pro-inflammatory cytokine production and leukocyte recruitment

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which are both important contributors to the inflammatory response, NF-κβ can also promote leukocyte apoptosis in certain contexts and therefore contribute to the resolution of inflammation [34]. Therefore, further investigation would be required to fully elucidate which pathways are being altered and by what mechanism following exposure to the milk bioactives.

To further validate the anti-inflammatory properties of the milk hydrolysates, an *ex-vivo* system of porcine colonic explants were used. While, the *in-vitro* Caco-2 cell model is efficient for initial screening, the monolayer of a single cell type acts as a limiting factor in evaluation of food bioactives in a complex, *in-vivo* like environment because of the absence of a heterogeneous mixture of cells [18]. Thus, *ex-vivo* models of the colon are useful in providing greater insight as they have the advantage of better reflecting the *in-vivo* complexity of colonic tissue [20]. Another advantage of *ex-vivo* tissue explants is that they can be used to model responses to a microbial challenge i.e. using LPS. LPS exerts its effects via the TLR4/MD-2 complex and is capable of stimulating pro-inflammatory cytokines in the tissue explants [35], many of which would otherwise have negligible expression levels. In this study the comparison of gene expression between the control unchallenged and the control LPS challenged tissues confirmed the success of the LPS challenge in eliciting a pro-inflammatory response in the colonic tissue.

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The anti-inflammatory activity of the 5kDaR in response to the external LPS challenge was validated in the *ex-vivo* system. Co-treatment of the LPS challenged colonic tissues with 5kDaR was associated with the down-regulation of the pro-inflammatory cytokines *IL-1α*, *IL-1β* and *IL-8*. The increase in expression of *IL-1α*, *IL-1β* and *IL-8* has been reported to be associated with metabolic syndromes and the progression of inflammatory diseases of the GIT such as inflammatory bowel disease (IBD) [36].

The cytokines *IL-1α* and *IL-1β* are members of the interleukin 1 family and have been associated with increases in inflammatory lesions in patients with IBD [37]. There were increases in both *IL-1α* and *IL-1β* expression in response to LPS challenge in our study as would occur during an inflammatory condition. Monocytes and macrophages are the main sources of IL-1 that subsequently activates IL-1 converting enzyme and releases active IL-1β in the colonic mucosa [38]. While IL-1α is produced by macrophages, monocytes, neutrophils and endothelial cells and mediates immune and inflammatory cells, IL-1β plays an important role in the Th17 mediated immune response and induces Th17 cell differentiation [39; 40]. A decrease in circulating IL-1α and IL-1β concentrations have been observed in mice supplemented with whey protein [41]. Also, in previous experiments, a 5kDaR and a 1-kDa retentate (1kDaR) of NaCAS enzyme hydrolysate generated by low degree of

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hydrolysis, was associated with reductions in *IL-1α* and *IL-1β* expression (Mukhopadhya et al., in press). This indicates that the enzymatic hydrolysis process generates peptides that down-regulate *IL-1α* and *IL-1β* expression in the presence of LPS.

Similarly, the reduction in *IL-8* expression as a consequence of exposure to the 5kDaR indicates its anti-inflammatory effects. Interestingly the parent NaCAS lacks this anti-inflammatory property, indicating that the enzymatic hydrolysis is an important contributor to its bioactivity. Human ulceritive colitis (UC) patients receiving partition-herb moxibustion experienced a reduction in *IL-8* expression in the mucosa combined with improved histology of the colon [(42)]. The correlation between IL-8 and intestinal inflammation has established this cytokine as a clinically reliable marker of inflammation [(43)]. *IL-8* is a chemokine, the primary function of which is to mediate the activation and migration of neutrophils into the tissue from the peripheral blood [(44)]. Since macrophages, epithelial cells and fibroblasts produce IL-8 in the GIT [(45)], the suppression of IL-8 by 5kDaR in LPS stimulated colonic tissues may indicate that it possesses inhibitory activity that acts on a pathway relevant to IL8 production.

The regulation of the growth, function and differentiation of T cells is mediated by *TGF-β* [(46)]. Down-regulation of *TGF-β* is desirable during inflammation as *TGF-β* overproduction has been associated with tissue

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scars as well as increased fibrosis and related carcinogenesis in the intestinal tissues [47].

The basic definition of a bioactive food ingredient is that it not only supplies nutrients, but also exerts positive effects on the physiological functioning of the body [48]. In this study we have demonstrated that a casein fraction (5kDaR) derived from NaCAS, which has been generated from a moderate degree of hydrolysis (11-16%) of bovine milk, comprises of anti-inflammatory properties. It has previously been demonstrated that a 1-kDa retentate derived from NaCAS generated using a low degree of hydrolysis had anti-inflammatory activity in a colonic model (Mukhopadhya et al., in press). It is currently unknown whether the 5 kDa retentate presented in this manuscript shares a portion of the active fractions contained within the previously reported 1 kDa retentate or if they are completely distinct. This study provides evidence that the generation of bioactive fractions using the controlled hydrolysis of NaCAS is commercially viable on an industrial scale. However, while we have demonstrated the anti-inflammatory effects of 5 kDa retentate in both *in-vitro* and *ex-vivo* systems, further exploration of this bioactive fraction is required to fully explore its molecular composition, its mechanisms of action and overall effects *in-vivo*.

4.6. Conclusion

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The current study demonstrated the anti-inflammatory effects of a novel 5 kDa retentate fraction derived from the controlled hydrolysis of NaCAS in both *in-vitro* and *ex-vivo* systems. The anti-inflammatory properties of the 5-kDa retentate, evidenced by the reduction in IL-8 production in Caco-2 cells challenged with TNFα and the reduced gene expression of pro-inflammatory cytokines in the porcine colon challenged with LPS *ex-vivo* highlight its potential as a functional food, particularly relevant to inflammatory conditions of the GIT.

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Figure 1: Dose dependent reduction of IL-8 concentration by milk hydrolysate series (NaCAS, EH and 5kDaR).

Figure 2: Difference in expression of a selected panel of cytokine genes between unchallenged and LPS challenged ex-vivo colonic tissues. The red dots represent significant up-regulated genes (P<0.05), the blue dot represents significant down-regulated gene (P<0.05) and black dots represent genes not affected by LPS (P>0.05).

Figure 3: Effect of NaCAS, EH and 5kDaR on relative quantity (RQ) of a selected panel of cytokine genes stimulated by LPS challenge.

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Table 1: Oligonucleotide sequences of forward and reverse primers used for amplification of porcine cDNA targets by qPCR.

	Accession Number	Forward Primer (5'-3')	Tm (°C)	Reverse Primer (5'-3')	Tm (°C)	Product Length (bp)	Efficiency (%)
Reference genes							
ACTB	XM_001928093.1	GCACGGCATCATCACCAA	52.75	CCGGAGCTCGTTGTAGAAGGT	55.99	70	95.02
PPIA	NM_214353.1	CGGGTCCTGGCATCTTGT	62.1	TGGCAGTGCAAATGAAAAACT	60.7	75	100.26
GAPDH	AF017079.1	CAGCAATGCCTCCTGTACCA	62.2	ACGATGCCGAAGTTGTCATG	62.1	72	104.15
Cytokine genes							
IL-1α	NM_214029.1	CAGCCAACGGGAAGATTCTG	63.0	ATGGCTTCCAGGTCGTCAT	60.49	76	106.6
IL-1β	NM_001005149.1	TTGAATTCGAGTCTGCCCTGT	60.59	CCCAGGAAGACGGGCTTT	60.94	76	104
IL-4	HQ236500.1	CCAACCCTGGTCTGCTTACTG	61.8	TTGTAAGGTGATGTCGCACTTGT	58.9	71	95
IL-6	AB194100	AGACAAAGCCACCACCCCTAA	55.27	CTCGTTCTGTGACTGCAGCTTATC	59.92	69	99.99
IL-8	NM_213867.1	TGCACTTACTCTTGCCAGAACTG	61.9	CAAACTGGCTGTTGCCTTCTT	61.7	82	95.7
IL-10	NM_214041.1	GCCTTCGGCCCAGTGAA	63.4	AGAGACCCGGTCAGCAACAA	63.1	71	95.7
IL-17A	NM_001005729.1	CCCTGTCACTGCTGCTTCTG	60.57	TCATGATTCCCGCCTTCAC	60.40	57	111.2
IL-21	NM_214415	GGCACAGTGGCCCATAAATC	57.38	GCAGCAATTCAGGGTCCAAG	61.51	124	120
IFN-γ	NM_213948.1	TCTAACCTAAGAAAGCGGAAGAGAA	61.12	TTGCAGGCAGGATGACAATTA	61.54	81	94.4
FOXP3	NM_001128438.1	GTGGTGCAGTCTCTGGAACAAC	60.57	AGGTGGGCCTGCATAGCA	61.18	68	94
TNF-α	NM_214022.1	TGGCCCCTTGAGCATCA	62.5	CGGGCTTATCTGAGGTTTGAGA	62.8	68	91.5
TGF β	NM_214015.1	AGGGCTACCATGCCAATTTCT	60.63	CGGGTTGTGCTGGTTGTACA	61.68	101	93

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Table 2: Compositional characteristics of NaCAS, EH and 5kDaR protein powders.

Test sample	Moisture (%)	Ash g $100g^{-1}$	Lipid g $100g^{-1}$	Protein* g $100g^{-1}$
NaCAS	3.11±0.09	3.90±0.10	0.80±0.06	88.66±0.10
EH	1.87±0.18[b]	5.41±0.01[c]	0.62±0.01[a]	89.38±0.08[b]
5kDaR	3.50±0.39	7.15±0.12[c]	ND	89.99±0.09[b]

* Kjeldahl conversion factor used was 6.38, ND- not detected, [a] $P<0.05$, [b] $P<0.01$ and [c] $P<0.001$ in comparison to NaCAS

Table 3: Molecular weight distribution of NaCAS, EH and 5kDaR protein powders.

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Test sample	Molecular weight distribution (%)*				
	> 30 kDa	30 – 10 kDa	10 – 5 kDa	5 – 1 kDa	< 1kDa
NaCAS	83.59±0.22	14.55±0.01	1.20±0.01	0.61±0.02	0.06±0.01
EH	1.90±0.01[a]	1.28±0.01[a]	3.33±0.06[a]	46.17±0.01[a]	47.33±0.01[a]
5kDaR	1.77±0.01[a]	2.19±0.01[a]	6.20±0.12[a]	62.78±0.02[a]	27.06±0.01[a]

[*] Molecular weight distribution was determined by SEC (TSK G2000SW) where the powders were reconstituted in distilled H_2O to 2.5 g L^{-1} protein and subsequently filtered through a 0.45 μm filter prior to the application of 20 μL of this solution to the column. [a] $P<0.001$ in comparison to NaCAS

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Table 4: Difference in *IL-8* levels in fully differentiated Caco-2 cells stimulated with *TNFα* and co-treated with dexamethasone or NaCAS or EH or 5kDaR for 24 h. Cell lysate was collected after 24 h of treatment and ELISA was performed. Values are means of 3 independent experiments. SEM: Standard error mean, [+] The % reduction values are relative to control (TNFα stimulated) Caco-2 cells.

Treatment	IL-8 conc. (pg/ml)	SEM	% reduction[+]	Significance
TNFα	110.0	6.60		
TNFα + Dexamethasone	68.4	5.87	41.6	a
TNFα + NaCAS	75.8	3.35	31.1	a
TNFα + EH	47.8	4.82	56.6	b
TNFα + 5kDaR	45.1	3.81	59.0	b

a P<0.05 and b P<0.01 in comparison to TNFα, n=3

Figure 1

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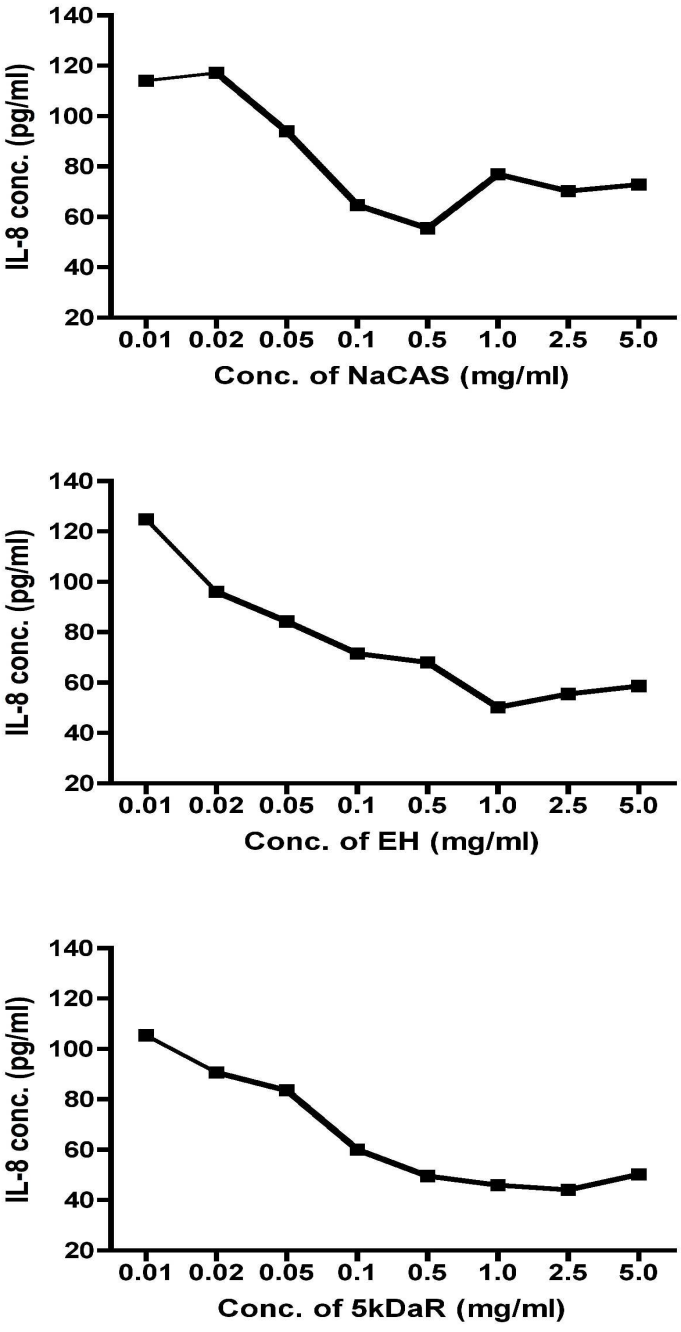

Figure 1: Dose dependent reduction of IL-8 concentration by a milk hydrolysate series (NaCAS, EH and 5kDaR). Each data point indicates means ± SE of 3 independent experiments.. The fully differentiated Caco-2 cells were challenged with *TNFα* and co-treated with milk hydrolysates for 24 h, cell lysate collected after 24 h and ELISA performed.

Figure 2

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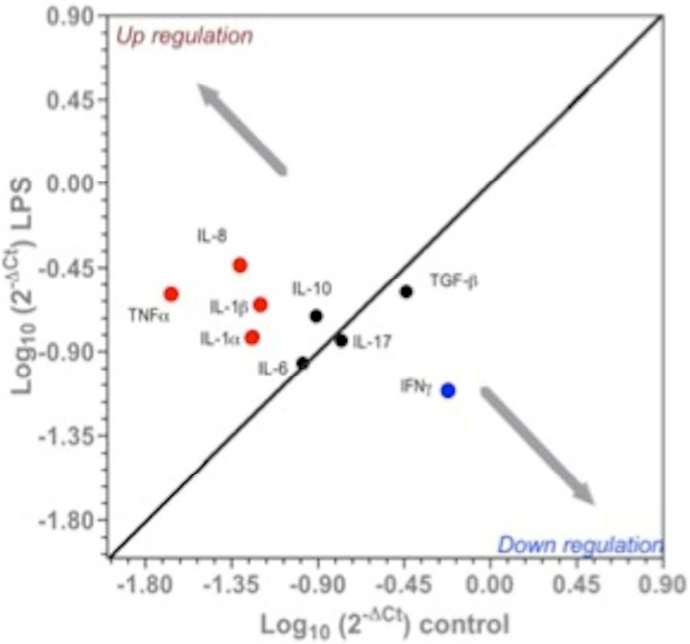

Figure 2: Difference in expression of a selected panel of cytokine genes between unchallenged and LPS challenged ex-vivo colonic tissues. The red dots represent significant up-regulated genes ($P<0.05$), the blue dot represents significant down-regulated gene ($P<0.05$) and black dots represent genes not affected by LPS ($P>0.05$).

Figure 3

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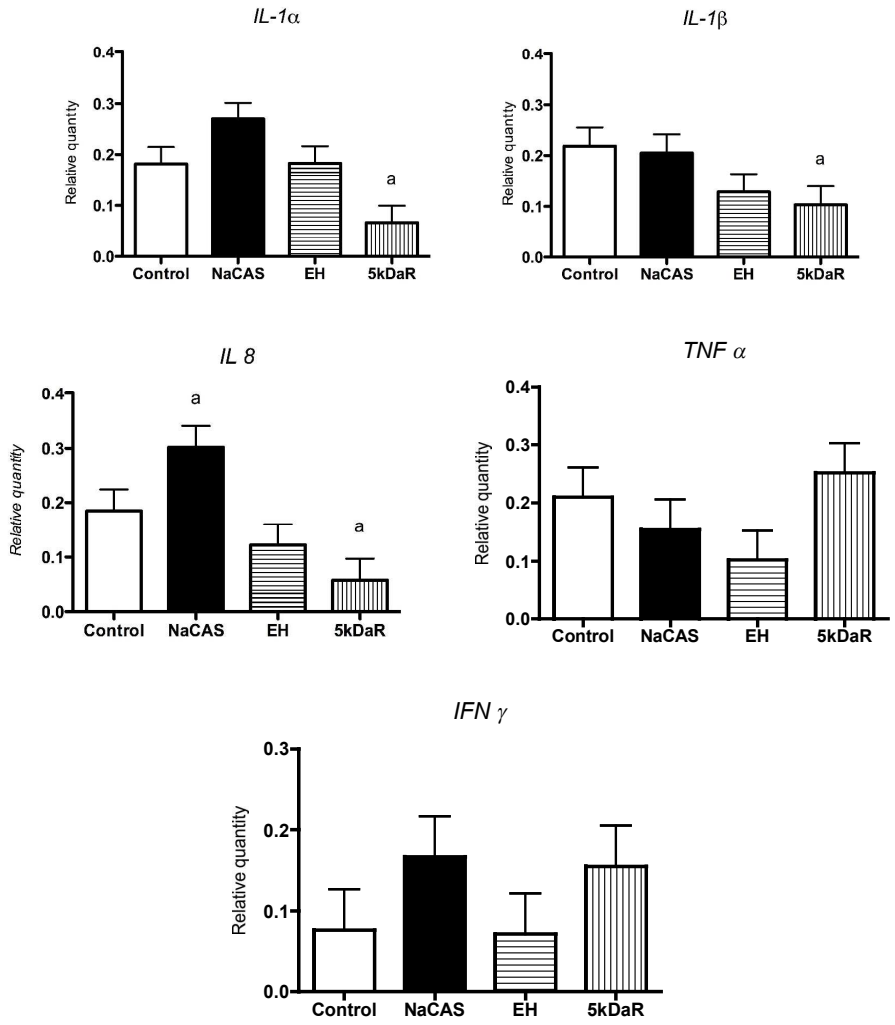

a P<0.05 in comparison to Control

Figure 3: Effect of NaCAS, EH and 5kDaR on relative quantity (RQ) of a selected panel of porcine cytokine genes stimulated by LPS challenge for 90 mins in porcine colonic tissue explant. After challenge with LPS and treatment with milk hydrolysates, total RNA was extracted, cDNA synthesised and real time qPCR was performed to quantify the selected panel of genes. Each data point indicates means ± SE of 3 independent experiments. RQ values of treatments with NaCAS, EH, 5kDaR, 1kDaR or 1kDaP compared to LPS challenged control, error bars indicate SE.

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