Phaseolin from *Phaseolus vulgaris* bean modulates gut mucin flow and gene expression in rats

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Dietary protein might modulate mucin flow and intestinal mucin gene expression. Since unheated phaseolin from *Phaseolus vulgaris* bean is resistant to digestion and increases gut endogenous protein losses, we hypothesised that unheated phaseolin influences mucin flow and gene expression, and that phaseolin heat treatment reverses these effects. The hypothesis was tested using a control diet containing casein as the sole protein source and three other diets with casein being replaced by 33 and 67 % of unheated and 67 % of heated phaseolin. The rats were fed for 6 d and euthanised. Digesta and faeces were collected for determining digestibility and mucin flow. Gut tissues were collected for mucin (*Muc1*, *Muc2*, *Muc3* and *Muc4*) and Trefoil factor 3 (*Tff3*) gene expressions. Colonic mucin flow decreased linearly with increasing the dietary level of unheated phaseolin ($P < 0.05$). Unheated phaseolin increased N flow in ileum, colon and faeces ($P < 0.05$), and reduced apparent N digestibility linearly ($P < 0.01$). Heat treatment reversed all these changes ($P < 0.05$ to < 0.001), except mucin flow. The expressions of *Muc* mRNA in gut tissues were influenced by dietary phaseolin level (ileum and colon: *Muc3* and *Muc4*) and thermal treatment (ileum: *Muc2*; colon: *Muc2*, *Muc3*, *Muc4* and *Tff3*) ($P < 0.05$ to 0.001). In conclusion, phaseolin modulates mucin flow and *Muc* gene expression along the intestines differentially.

Digestion: Mucin: Phaseolin: Rats

The storage globulin phaseolin represents about half of the total protein content of the common bean (*Phaseolus vulgaris*)[1]. The nutritive value of phaseolin is limited by its low content in sulphur amino acids and tryptophan and its high resistance to enzymatic hydrolysis[2,3]. However, phaseolin digestion is markedly improved with heat treatment due to alterations in phaseolin structure[4,5].

Unheated phaseolin and/or its digestion fragments exert a secretagogue activity on the gut of rats fed a single test meal since intestinal endogenous protein losses (e.g. shed cells, digestive enzymes, gastrointestinal mucus and blood serum proteins) increase when phaseolin intake increases[6]. These authors postulated that mucin could be a possible significant contributor to these losses[6]. By contrast, in a chronic feeding trial, the dietary level of unheated phaseolin had little effects on rat small intestine architecture and enzymatic activity[7].

The composition, thickness and protective effect of the mucus layer are determined by the dynamic balance between two processes: synthesis and secretion by goblet cells *v.* degradation by physical abrasion and proteolysis[8]. An intact mucus layer is required at the gut epithelial surface for optimal protection[9]. Mucin could represent 11 % of total endogenous N losses at the ileum of pigs[10]. Previous studies in single-stomached animals showed that food components, including fibre and protein source and their level of incorporation into the diet could stimulate mucin secretion in the small intestine[11,12]. However, no information is available on the effect of prolonged intake of diets differing in phaseolin level or with heated phaseolin on mucin flow in the gut lumen and on mucin gene expression in gut tissues of rats.

The aim of the present study was to test the hypothesis that unheated phaseolin modulates mucin flow and tissue gene expression in the intestines of rats and that heat treatment of phaseolin abolishes these effects.

Experimental methods

Phaseolin purification

The bean cultivar used in the present study contained phaseolin of the T type. It was provided by the International Centre

of Tropical Agriculture (Cali, Colombia). Phaseolin was isolated by successive protein solubilisation and precipitation steps as previously reported[7,13]. The final phaseolin precipitate was dialysed against distilled water, frozen and freeze-dried before being incorporated into the experimental diets. Phaseolin isolated with this protocol was found to be pure as revealed by SDS-PAGE[7].

Animals and diets

The experiment was conducted in agreement with the guidelines of the National University of Colombia for care and use of laboratory animals[14]. Twenty young adult Wistar male rats with an initial body weight of 110 (SD 5) g were randomly allocated to one of the four treatments described below, and placed in individual metabolism cages (Tecniplast 150-300, Buguggiate, Italy) for the whole experimental period. The control diet (P0) contained casein as the sole protein source. In the other three diets, protein was provided at 33 % (P33) and 67 % (unheated form; P67) and 67 % (heated form; P67-H) by purified phaseolin, respectively. No attempt was made to incorporate 100 % of protein as phaseolin because it was previously shown to drastically reduce food intake in our previous studies with rats[7]. The complement of protein in the diets was casein (Table 1). Heat treatment of purified phaseolin was carried out under pressure at 121°C for 15 min, as described previously[7]. Chromium oxide was added to the diets as an indigestible marker for determining the flow and the apparent digestibility of food components along the gut. The rats were fed the experimental diets for 6 d only because the amounts of purified phaseolin were limited. Food intake was fixed at 10 % of body weight in order to limit food refusals[7,15]. The rats had free access to water.

Collection and preparation of faeces, digesta and gut tissue samples

Faeces were collected during the last day of the feeding experiment and mixed (350 g/l) with cold saline (9 g NaCl/l) (4°C). At the end of the trial, rats had access to the experimental diets for 4 h and then were euthanised with an injection of Ketamine and Rompun® (1:1, v/v). Gut digesta and tissues were sampled as described previously[7,15]. All digesta present in the distal 20 cm of the small intestine (referred to as ileum) and in the whole colon were collected. Ileal and colonic digesta were gently flushed from the segments with 10 ml of cold saline (9 g NaCl/l) (4°C) using a syringe. Gut digesta and faeces were immediately mixed with 40 g NaN$_3$/l with a final concentration of 2 g/l in order to minimise protein degradation by bacterial enzymes. Digesta and faeces were fractionated into two aliquots that were frozen and stored at −20°C. An aliquot was kept frozen until mucin analysis, while the other aliquot was freeze-dried and ground (1 mm mesh screen) for digestion studies.

Whole tissue samples (1·5 cm in length) were collected in the middle of the ileum and colon, opened longitudinally and washed three times in cold saline (9 g NaCl/l) (4°C). They were stored immediately in cold TRIzol reagent (4°C), then frozen in liquid N$_2$ and finally stored in a deep freezer at −80°C.

Enzyme-linked lectin assay for high molecular weight mucin along the gut

Ileal and colonic digesta and faeces were assayed for high molecular weight glycosylated mucin by enzyme-linked lectin assay using wheat germ agglutinin as the lectin, as described by Trompette et al.[16]. Casein, phaseolin and the experimental diets were also checked by enzyme-linked lectin assay test for possible lectin binding. Porcine gastric mucin (reference M-1778; Sigma-Aldrich, Saiat-Quentin Fallavier, France) was used as the standard. Briefly, dilutions of standards and samples were prepared in carbonate buffer (0·5 M-Na$_2$CO$_3$, pH 9·6) before being coated on ninety-six-well microtitre plates (reference 469914; NUNC microplates, Roskilde, Denmark; 100 μl/ well). After overnight incubation at 4°C, the plates were washed four times with PBS Tween (1 g/l) (pH 7·2). Microplate saturation was made with 250 μl/well of a PBS Tween solution of bovine serum albumin (20 g/l PBS Tween) incubated for 1 h at 37°C. Plates were washed again, and 100 μl of biotinylated wheat germ agglutinin (reference B-1025; Abcys, Paris, France; 5 mg/l) in PBS Tween−bovine serum albumin was added and incubated for 1 h at 37°C. Plates were washed, and 100 μl/well of avidin-peroxidase conjugate (reference A-7419; Sigma) was added for 1 h at room temperature. After washing, 100 μl/well of o-phenylenediamine (OPD) solution (reference P-9187; Fast OPD, Sigma) was incubated in the dark at room temperature for 5−10 min. The reaction was stopped by adding 25 μl/well of 3 M-H$_2$SO$_4$. Absorbance was read at

Table 1. Ingredient and analytical composition of the experimental diets

	Experimental diet*			
	P0	P33	P67	P67-H†
Ingredients (g/kg DM)				
Casein	120	83	41	41
Phaseolin‡	0	35	70	70
Starch	599	601	608	608
Sucrose	100	100	100	100
Ground rice hulls	80	80	80	80
Vegetable oil	60	60	60	60
Vitamin trace elements§	10	10	10	10
Calcium phosphate	14	14	14	14
Calcium carbonate	3	3	3	3
Sodium chloride	2	2	2	2
Potassium chloride	7	7	7	7
Magnesium sulphate	2	2	2	2
Chromium oxide	3	3	3	3
Analysis (g/kg DM)				
DM (g/kg)	908	903	908	915
Protein (N × 6·25)	96	103	96	96
Ether extract	65	65	64	66
Ash	71	67	67	64
Neutral-detergent fibre	63	70	70	67
Gross energy (MJ/kg DM)	16·3	16·3	16·4	16·3

* Diets: P0, casein control; P33 and P67, diets with phaseolin T contributing to 330 and 670 g/kg of total dietary protein. Casein was supplemented with 30 g DL-methionine/kg DM casein.
† P67-H, heat-treated (121°C for 15 min) phaseolin.
‡ Purified phaseolin of the T type.
§ Mineral and vitamin mixture supplied per kg of diet: 7·5 mg vitamin A; 0·2 mg vitamin D$_3$; 15 mg vitamin E; 6 mg vitamin K; 10 mg vitamin B$_2$; 35 mg calcium pantothenate; 75 mg niacin; 2·5 mg vitamin B$_6$; 0·05 mg vitamin B$_{12}$; 0·05 mg biotin; 200 mg choline; 150 mg Mn; 500 mg Zn; 40 mg Cu; 200 mg Fe; 2 mg iodine; 0·5 mg Se, 1 mg Co.

492 nm using an ELISA reader (Multiskan Spectrum reference 5118550; Thermo Electron Corporation, Vantaa, Finland). Mucin concentration in samples was calculated from porcine gastric mucin standard curve. Data were expressed as mg or μg mucin per g digesta or faeces, depending on its concentration along the gut.

RNA extraction from gut tissues

Gut tissue samples were homogenised in TRIzol reagent (1 ml/100 mg) with tissue lyser (Qiagen Inc., Valencia, CA, USA) at room temperature. Then, 200 μl of chloroform was added, and the sample was mixed and centrifuged at 11 300 g for 15 min at 4°C. The chloroform upper phase was recovered, mixed with 500 μl of isopropanol and centrifuged at 11 300 g for 15 min at 4°C. The precipitated RNA was rinsed with 75 % ethanol, centrifuged at 7500 g for 5 min at 4°C, and dissolved into 100 μl of RNase-free water and stored at −80°C until further analysis. RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm using an Agilent 2100 bioanalyser (Agilent Technologies, Palo Alto, CA, USA). Finally, samples were treated with DNase (DNA-free kit, Applied Biosystems, Foster City, CA, USA) following the manufacturer's recommendations.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was conducted as previously reported[16] with slight modifications. Briefly, mucin complementary DNA rat *Muc1*, *Muc2*, *Muc3*, *Muc4*, trefoil factor 3 (Tff3) and *18S* mRNA were amplified by PCR using the primer sequences shown in Table 2. For retrotranscription, total RNA (2 μg) was added with RNase-free water to a final volume of 20 μl. The reaction mixture (High Capacity complementary DNA Reverse Transcription Kit; Applied Biosystems) had a final volume of 20 μl, and contained 4 μl of 10 × RT buffer, 1·6 μl of 25 × deoxy nucleotide triphosphate, 4 μl of 10 × random primers, 2 μl of RT (50 Iu/μl) and 8·4 μl of sterile water. The reaction mixture and the sample were mixed together (40 μl, final volume), then incubated at 25°C for 10 min, at 37°C for 2 h, and finally cooled on ice. Afterwards complementary DNA was ready for use in real-time PCR.

Table 2. Nucleotide sequences of the PCR primers used to measure the effect of phaseolin in rats

Gene	Primer	Sequences (5′–3′)	Reference
18S	Sense	ACGGAAGGGCACCACCAGGAG	Hoebler *et al.*[28]
	Antisense	GCACCACCACCCACGGAAACG	
Muc1	Sense	TCGACAGGCAATGGCAGTAG	Trompette *et al.*[16]
	Antisense	TCTGAGAGCCACCACTACCC	
Muc2	Sense	GCCTCAAACCCGTGCGTGTC	Lin *et al.*[47]
	Antisense	TCATTCACCAACCACTCATC	
Muc3	Sense	CTTGAGGAGGTGTGCAAGAAA	Han *et al.*[32]
	Antisense	CCCCAGGGTGACATACTTTG	
Muc4	Sense	GCTTGGACATTTGGTGATCC	Han *et al.*[32]
	Antisense	GCCCGTTGAAGGTGTATTTG	
Tff3	Sense	CCTGGTTGCTGGGTCCTCTG	Hoebler *et al.*[28]
	Antisense	GCCACGGTTGTTACACTGCTC	

Real-time PCR was performed in duplicate for each sample using ABI PRISM Sequence Detection System 7000 (Applied Biosystems). A reaction mixture containing the following components was prepared: 5·8 μl ultrapure water, 0·75 μl of forward and 0·75 μl of reverse primer (5 μmol/l), 12·5 μl SYBR Green PCR Master Mix kit and 0·2 μl uracil-DNA glycosylase (1 Iu/μl) (Applied Biosystems). The reaction mixture (20 μl) was mixed with sample complementary DNA (5 μl). The cycling conditions were as follows: 50°C for 2 min for uracil-DNA glycosylase action. Then, initial denaturation was conducted at 95°C for 10 min and then followed by forty amplification cycles of 95°C for 15 s and 60°C for 1 min. The *Muc* genes and *Tff3* were expressed relatively to *18S* RNA as reported previously[17].

Chemical analysis

Diets were analysed for ash (AOAC 942.05), diethyl ether extract (AOAC 920.39) and neutral-detergent fibre (AOAC 2002.04). Gross energy was determined in diets using a Parr 1342 calorimeter (Parr Instruments, Moline, IL, USA). Diets, faeces and digesta were also analysed for DM (AOAC 930.15) and N (Kjeldahl method). Chromium concentration in diets, ileal and colonic digesta and faeces was determined colorimetrically after nitro-perchloric acid hydrolysis[18].

Digesta flow and digestibility calculations

The flows of DM, N and enzyme-linked lectin assay mucin at the ileum, in the colon and in the faeces were calculated from marker concentrations in diets and digesta or faeces as reported previously[11]. Apparent ileal and faecal digestibilities of DM and N were calculated using the following equations[19]:

Apparent ileal digestibility of DM or N

$$= (100 - ((DM_i \text{ or } N_i/DM_d \text{ or } N_d) \times (Cr_d/Cr_i))) \times 100$$

Apparent faecal digestibility of DM or N

$$= (100 - ((DM_f \text{ or } N_f/DM_d \text{ or } N_d) \times (Cr_d/Cr_f))) \times 100$$

where DM_i, N_i and Cr_i are the DM, N and chromium contents of ileal digesta, and DM_f, N_f and Cr_f are those in the faeces; DM_d, N_d and Cr_d are the DM, N and chromium contents of the diet.

Statistical analysis

Two separate ANOVA of data were conducted using the Mixed Model procedure of Statistical Analysis System software package version 8.0 (SAS Institute Inc., Cary, NC, USA). In the first analysis, the effect of unheated phaseolin level (0, 33 and 67 % of total N) in the diet was tested for linear and quadratic variations using polynomial orthogonal contrasts[20]. The second ANOVA was conducted in order to test the effect of heat treatment of phaseolin, and diets with untreated phaseolin, heated phaseolin and casein were compared. When the *P* value of treatment effects was ≤0·10, the diets were compared two by two using appropriate contrasts (P0 *v.* P67, P0 *v.* P67-H and P67 *v.* P67-H).

Results

The voluntary DM and N intakes were on average across treatments 9·6 (SEM 0·14) g/d and 151 (SEM 4) mg/d, respectively, and did not differ significantly between treatments ($P > 0.05$) (data not shown).

Flow and apparent digestibility of DM and nitrogen

The flow of DM in the faeces, but not at the ileum and in the colon, increased linearly ($P < 0.001$) with increasing the level of raw phaseolin in the diet (Table 3). As a result, the apparent faecal digestibility of DM decreased quadratically with increasing the level of phaseolin ($P < 0.001$). The ileal digestibility of DM was not influenced by this factor. The flow of N at all digestive sites increased linearly with increasing the level of unheated phaseolin in the diet ($P = 0.011$ to < 0.001). As a consequence, both ileal and faecal digestibilities of N decreased linearly with increasing dietary phaseolin level ($P < 0.001$). Heat treatment of phaseolin reduced DM and N flows along the intestines and increased DM and N digestibilities ($P < 0.001$) to values close to those observed with the casein-based control diet.

Flow of mucin along the gastrointestinal tract and tissue expression of mucin family genes

The enzyme-linked lectin assay test revealed no binding with casein or phaseolin or with the experimental diets, indicating the lack of carbohydrate moieties recognised by wheat germ agglutinin in these ingredients and diets. The flow of mucin in the colon decreased linearly with increasing the dietary phaseolin level ($P < 0.001$) (Table 3). This factor did not influence the flow of mucin at the ileum or in the faeces ($P > 0.05$). The colonic flow of mucin was lower with P67-H than with

the casein control ($P < 0.05$), but it was not different from that with unheated phaseolin (P67) (probabilities of differences for P0 v. P67, P0 v. P67-H and P67 v. P67-H: $P = 0.001$, 0·003 and 0·288, respectively).

The expression of *Muc3* and *Muc4* mRNA tended to decrease quadratically ($P = 0.060$ and 0·090, respectively) in the ileal tissue and increased or tended to increase linearly ($P = 0.093$ and 0·023, respectively) in the colonic tissue as the dietary level of unheated phaseolin increased in the diet (Table 4). By contrast, the expressions of *Muc1*, *Muc2* and *Tff3* mRNA in the ileal and colonic tissue were not influenced by the level of unheated phaseolin in the diet ($P > 0.1$).

The expression of *Muc* genes and *Tff3* tended to be influenced by heat treatment of phaseolin in colonic tissues, except for the *Muc1* gene (Fig. 1). mRNA expressions of *Muc2*, *Muc3*, *Muc4* and *Tff3* were lower with heat-treated phaseolin than unheated phaseolin ($P < 0.001$ to < 0.05). mRNA expression of the *Muc* family genes and *Tff3* in ileal tissues was not influenced by heat treatment of phaseolin ($P > 0.05$). *Muc2* mRNA levels were lower in ileal and colonic tissues of rats fed the heat-treated phaseolin diet than in those fed the casein-based control diet ($P = 0.003$ and 0·001, respectively). Finally, *Muc2* gene expression in the ileal tissue was lower ($P = 0.004$), and those of *Muc3* and *Muc4* in the colonic tissue of rats fed unheated phaseolin tended to be higher ($P = 0.058$ and 0·005) when compared with the corresponding tissues of rats fed the casein-based control diet.

Discussion

The present investigation shows for the first time that phaseolin intake and heat treatment can modulate mucin flow and mRNA levels of various mucin genes in the gut tissues of rats.

Table 3. DM, nitrogen and mucin flows along the gastrointestinal tract, and apparent ileal and faecal digestibilities of DM and nitrogen in rats fed graded levels of unheated phaseolin and of heat-treated phaseolin (n 4–5)

| | Treatment* | | | | Level of unheated phaseolin† | | | Heat treatment‡ | |
| | | | | | | P contrast | | | |
	P0	P33	P67	P67-H	SEM	Linear	Quadratic	SEM	P
DM flow (mg/g DM intake)									
Ileum	221	230	275	215	21	0·130	0·512	26	0·280
Colon	110	123	116	117	10	0·664	0·432	7	0·740
Faeces	103[b]	102	140[a]	96[b]	7	0·001	0·008	12	0·001
N flow (mg/g N intake)									
Ileum	100[b]	300	416[a]	102[b]	29	0·001	0·598	16	0·001
Colon	14b[b]	139	254[a]	59[b]	39	0·004	0·871	26	0·001
Faeces	92[b]	173	427[a]	105[b]	20	0·001	0·007	19	0·001
DM apparent digestibility (%)									
Ileum	0·78	0·77	0·73	0·79	0·02	0·130	0·512	0·03	0·280
Faeces	0·90[a]	0·90	0·86[b]	0·90[a]	0·01	0·001	0·008	0·01	0·001
N apparent digestibility (%)									
Ileum	0·90[a]	0·70	0·59[b]	0·90[a]	0·02	0·001	0·160	0·02	0·001
Faeces	0·91[a]	0·83	0·57[b]	0·91[a]	0·02	0·001	0·007	0·02	0·001
Mucin flow									
Ileum (μg/g DM intake)	2149	2358	2443	2292	382	0·619	0·895	329	0·830
Colon (ng/g DM intake)	539[a]	238	171[b]	248[b]	34	0·001	0·025	120	0·002
Faeces (ng/g DM intake)	85	57	66	57	12	0·279	0·280	11	0·250

[a,b] Values within a row with unlike superscript letters between P0, P67 and P67-H treatments were significantly different ($P < 0.05$).
* P0, casein control; P33 and P67, diets with unheated phaseolin contributing to 330 and 670 g/kg of the total dietary protein in the diet; P67-H, diet with heated phaseolin contributing to 670 g/kg of total protein in the diet.
† Comparisons between diets P0, P33 and P67.
‡ Comparisons between diets P0, P67 and P67-H.

Table 4. Mucin (*Muc*) gene expression in ileal and colonic tissues of rats fed graded levels of unheated phaseolin (*n* 4–5)

	Treatment*				*P* contrast	
	P0	P33	P67	SEM	Linear	Quadratic
Ileum						
Muc1	1·74	1·25	1·82	0·41	0·866	0·342
Muc2	6·83	6·96	5·41	0·60	0·110	0·253
Muc3	0·85	1·08	0·76	0·10	0·540	0·060
Muc4	1·69	1·37	1·52	0·11	0·255	0·093
Tff3	1·48	1·28	1·57	0·17	0·697	0·253
Colon						
Muc1	3·80	3·06	2·14	0·93	0·171	0·928
Muc2	5·69	4·90	5·35	0·51	0·625	0·304
Muc3	3·82	5·95	6·32	1·03	0·090	0·434
Muc4	3·04	4·95	5·48	0·62	0·023	0·355
Tff3	2·75	3·40	3·23	0·39	0·421	0·407

Tff3, trefoil factor 3.

* P0, casein control; P33 and P67, diets with unheated phaseolin contributing to 330 and 670 g/kg of the total dietary protein in the diet.

Influence of phaseolin on mucin flow and gut tissue expression of Muc family genes

Santoro *et al.*[6] based on acute feeding experiments assumed that the poor nutritional value of unheated phaseolin was due to increased intestinal losses of endogenous N, suggestively mucin, when the level of phaseolin increased in the diet. The present results do not support this view because raw phaseolin intake did not increase mucin flow at the end of the small intestine and did not alter intestinal mRNA levels of *Muc2*, the main component of intestinal secreted mucin[21]. The discrepancies between the present investigation and studies by Santoro *et al.*[6,22] may come from different experimental approaches (acute *v.* repeated feeding experiments) or from methodologies for evaluating endogenous N losses. The present data are consistent with our previous observations showing limited effects of phaseolin on intestinal anatomy and enzyme activities[7,15].

We observed that dietary phaseolin level and heat treatment influenced the expression of *Muc2*, *Muc3* and *Muc4* and *Tff3* genes in different ways along the intestines. The actual reasons for these effects and the possible consequences in terms of gut protection are not fully understood yet. Mucin 2 is the major component of mucin secreted along the gastrointestinal tract[21]. *Muc2* knock out mice develop colitis spontaneously, indicating the important role of this mucin in colonic protection[23]. In the present study, both unheated and heat-treated phaseolin reduced *Muc2* gene expression in the ileum, and heat treatment of phaseolin reduced *Muc2* gene expression in the colon as compared to the casein control (Fig. 1). These observations may suggest a potentially weaker gut protection following phaseolin intake, with differential effects in the ileum and the colon depending on phaseolin cooking.

Muc3 contributes to the protection of the intestinal epithelium. A higher intestinal expression of *Muc3* mRNA following hypoxia suggests a protective mechanism during episodes of diminished oxygen delivery[24]. Conversely, a reduction in *Muc3* mRNA levels has been reported in Crohn's disease patients[25]. In the present study, the level of unheated phaseolin in diets affected *Muc3* mRNA levels in opposite ways depending on the intestinal segment: it

decreased in ileal tissue and increased in colonic tissue. These results suggest that ileal *Muc3* gene expression may be negatively regulated by ileal N flow, while that of colonic *Muc3* may be positively regulated by colonic N flow. Although the correlation between ileal *Muc3* mRNA level and ileal N flow did not reach significance, colonic *Muc3* mRNA level was found to be positively correlated to colonic N flow (*r* 0·70, *P* < 0·05). Heat treatment of phaseolin decreased the expression of *Muc3* mRNA in the colon, an observation that supports the latter assumption. Further work is needed to demonstrate causal links between mucin gene expression and digesta flow or fermentation in order to contribute to explain regional variations in the expression of these genes along the gut.

The *Muc4* gene is expressed in cells at the basement of crypts in the small intestine, but its expression is higher in the colon where it is located in goblet cells[26]. Muc4 appears to play important roles in epithelial growth, cell differentiation[27], mucosal defence[28] and intestinal lubrication[26]. In the present study, the increased expression of *Muc4* mRNA levels in colonic tissues and the trend in ileal tissues, in response to increased levels of dietary phaseolin, could not be explained by changes in the flow of DM (or fresh digesta) in the ileum and the colon that were NS. Heat-treated phaseolin also reduced the expression of *Muc4* mRNA.

The trefoil family 3 (*Tff3*) gene is also expressed in the mucin secretory cells. It helps to protect and stabilise the mucus layer and heal the epithelium[29]. As for the *Muc* family genes (except *Muc1*), a reduction in the expression of *Tff3* mRNA in the colonic tissue after thermal treatment of phaseolin was observed. A positive association between intestinal trefoil factor and *Muc3* expressions has been linked with mucosal hypoxia and altered epithelial barrier function[24].

The link between *Muc* gene tissue expressions and mucin flow along the intestines does not seem to be straightforward, because this flow reflects the balance between mucin production and luminal degradation by the microbiota[8] and also because *Muc* gene expression varies regionally along gut compartments for a given diet[30]. The most consistent point in the present study was the lower colonic mucin flow with heated phaseolin, which could reflect at least partly the lower colonic *Muc2* mRNA levels with this diet. By contrast, *Muc* gene levels and mucin flow varied in different ways along the gut according to the dietary treatments (e.g. in the ileum, the casein-based control diet had similar mucin flow and higher *Muc2* gene expression than phaseolin-containing diets).

In previous studies with longer periods of feeding (2 weeks) similar phaseolin-based diets, we did not observe health problems or important alterations in gut anatomy and enzyme activities[15]. Therefore, it can be suggested that the changes in the expression of *Muc* genes in gut tissues (mainly in colon) as noted here might have had little effect on gut function, at least in our experimental conditions.

Nutritional factors modulating Muc gene expression and mucin synthesis

A number of studies suggest that casein may influence mucin secretion and gene expression in the intestine[11,31–34]. Varying the level and the origin (animal *v.* plant) of protein in milk formulas modulated mucin gut flow in baby calves[11].
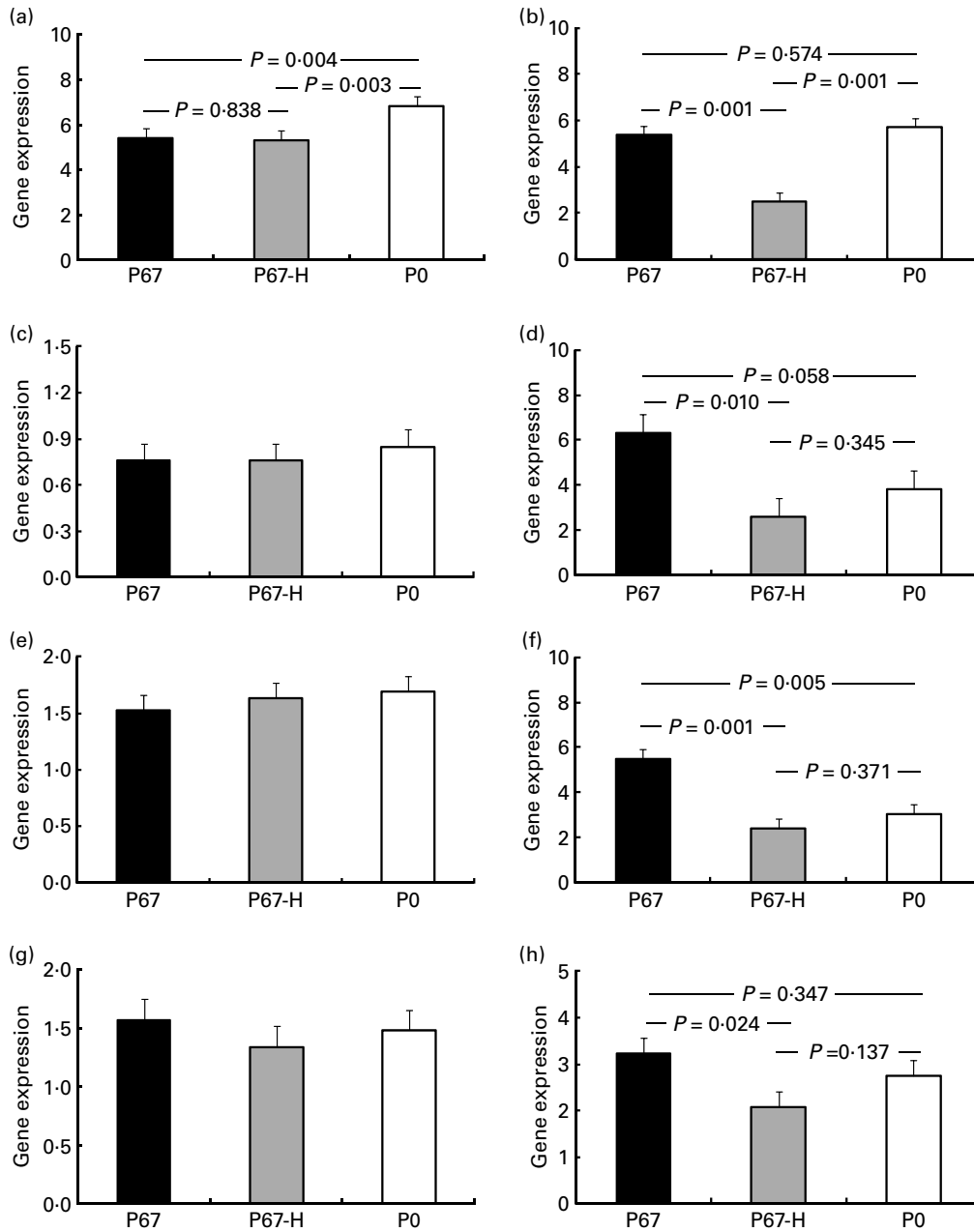
Fig. 1. Influence of heat treatment of phaseolin on mucin (*Muc*) and trefoil factor 3 (*Tff3*) gene expression in ileal and colonic tissues of rats: *Muc2* in the ileum (a); *Muc2* in the colon (b); *Muc3* in the ileum (c); *Muc3* in the colon (d); *Muc4* in the ileum (e); *Muc4* in the colon (f); *Tff3* in the ileum (g); *Tff3* in the colon (h). Values are means (n 4–5), with their standard errors represented by vertical bars. P0, casein control diet; P67, diet containing phaseolin at 670 g/kg total protein; P67-H, diet containing heat-treated phaseolin at 670 g/kg total protein. The overall probabilities for treatment effects were for *Muc2* ($P = 0.004$ and 0.001), *Muc3* ($P = 0.789$ and 0.027), *Muc4* ($P = 0.732$ and 0.003) and *Tff3* ($P = 0.432$ and 0.062) in the ileum and in the colon, respectively.

Hydrolysed casein increased ileal endogenous amino acid losses in human subjects[31] and gut tissue *Muc3* and *Muc4* mRNA levels in rats[32]. Hydrolysed casein and related peptides (e.g. β-casomorphin-7) also induced mucin secretion in isolated and perfused rat jejunum[33] and increased the expression of *Muc2* and *Muc3* genes in intestinal mucin-producing cells[34]. Here, casein may have been responsible for the higher *Muc2* mRNA levels observed in the ileal tissues. However, in the colon, *Muc* gene expression was similar to or higher than those observed with casein when the level of unheated phaseolin increased in the diet. Possible effects of casein peptides on intestinal mucin gene expression

and flow make the interpretation of data more difficult in studies where casein is used as the control and is substituted with other protein sources (e.g. phaseolin), as in the present study. However, it must be borne in mind that luminal concentrations of casein peptides, including that of β-casomorphin-7 after milk or casein intake, have never been determined *in vivo*[33]. Also, two independent studies revealed that the bioactive peptide β-casomorphin-7 is not detected in digests after casein digestion *in vitro*[35,36]. Additionally, the demonstration of β-casomorphin-7 secretory properties on jejunal mucin with isolated and perfused rat jejunum[33] does not provide evidence for such effects on the ileum or the colon,

the two sites under study in the present study. Finally, results from investigations with hydrolysed casein[31,33] do not mean that entire casein that is subsequently hydrolysed by endogenous proteases and peptidases may have resulted in the same effects as exogenously hydrolysed casein on mucin flow and gene expression. Collectively, there is no evidence to date showing that casein peptides (like β-casomorphin-7) are released during casein digestion and are bioactive *in vivo*. Therefore, the most reasonable interpretation of the present data is that phaseolin was responsible for the observed changes in intestinal mucin flow and gene expression.

Dietary threonine is also important to consider because it is highly represented in mucin, its dietary restriction reduces intestinal mucin synthesis specifically[37,38] and it may become a limiting amino acid for mucin synthesis in intestinal inflammation in rats and pigs[39,40]. Threonine restriction reduced *Muc* gene expression in the small and large intestines of rats[39]. According to the amino acid composition of casein and phaseolin[41] and the ileal digestibility of N in the present diets, the theoretical availability of amino acids decreased as the level of unheated phaseolin increased in the diet. The casein-based control diet provided 25 and 68 % more threonine than phaseolin P33 and P67 diets, respectively. These diets with unheated phaseolin did not lead to a reduction in ileal mucin flow. The limited threonine supply with the unheated phaseolin-containing diets may have been responsible at least partly for the changes recorded in the ileal *Muc* gene expression in the present study. In the colon, the mucin flow decreased in proportion to the unheated phaseolin included in the diet. This could have been caused by bacterial fermentation being stimulated by indigested phaseolin components, thus leading to enhanced mucin degradation in the colon. Dietary protein type and amino acid composition influence the colonic microbiota and resulting profiles of SCFA, which, in turn, modulate the mucus layer[8,12] and the expression of secreted (Muc2) and membrane-bound (Muc1, Muc3 and Muc4) mucins[30,42,43]. It can partially explain the increased proportion of *Muc3* and *Muc4* gene expressions as dietary unheated phaseolin was included in the diet.

Although the casein control diet and the heat-treated phaseolin diet had similar and high digestibilities, they displayed different *Muc2* mRNA levels in ileal and colonic tissues and different colonic mucin flow. Both of these observations could be explained by the β-casomorphin-7 peptides of casein[33] and/or changes in microbiota[30,42,43] as explained above. New investigations are needed to determine the influence of phaseolin on gut fermentation profiles and possible links with gut *Muc* gene expression.

Effect of phaseolin on digesta flow along the gastrointestinal tract and on digestibility

The present results revealed increased flows of DM and N that led to a reduction in apparent N digestibility in rats fed with the highest level of unheated phaseolin in the diet, in agreement with our previous studies[15]. The low nutritional value of unheated phaseolin consumed chronically may be due mainly to its high resistance to enzymatic hydrolysis, as evidenced by increased ileal and faecal output of undigested phaseolin polypeptides[44]. Heat treatment of phaseolin

reduced ileal and faecal N output, thus increasing the apparent digestibility of N. These observations are in agreement with previous investigations[2,7,45,46]. Recently, we showed that these improvements were related to the disappearance of undigested phaseolin polypeptides in ileal digesta following heat treatment of phaseolin[15,44].

In conclusion, the present study provides evidence that the level and the source of protein influence the flow of mucin and the expression of various *Muc* family genes in the ileal and colonic tissues. Additionally, we showed that different sources of protein (casein *v.* phaseolin) with similar digestibility could influence *Muc* gene expression in the intestines differentially. Further work is required to elucidate the actual mechanisms of *Muc* gene modulation by phaseolin and to evaluate the possible functional outcomes of phaseolin intake in terms of gut protection.

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