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

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Summary

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 hypothesized 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 euthanized. 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 $P < 0.001$), 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 $P < 0.001$). In conclusion, phaseolin modulates mucin flow and Muc gene expression along the intestines differentially.

Introduction

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 sulfur 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, 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, the thickness and the protective effect of the mucus layer are determined by the dynamic balance between two processes: synthesis and secretion by goblet cells vs. 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 fiber 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 work 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 this study contained T phaseolin type. It was provided by the International Centre of tropical Agriculture (CIAT, 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 prior to 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 metabolic cages (Tecniplast 150-300; Buguggiate, Italy) for the whole experimental period. The control diet contained casein as the sole protein source. In the other three diets, protein was provided for 33 and 67 % (unheated form) and 67 % (heated form) 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 (Cr_2O_3) 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 BW 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 euthanized with an injection of Ketamin 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 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 minimize 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 nitrogen and finally stored in a deep freezer at -80 °C.

Enzyme-Linked Lectin Assay for high MW mucin along the gut

Ileal and colonic digesta and faeces were assayed for high MW glycosylated mucin by enzyme-linked lectin assay (ELLA) using wheat germ agglutinin (WGA) as the lectin, as described by

Trompette et al. [16]. Casein, phaseolin and the experimental diets were also checked by ELLA test for possible lectin binding. Porcine gastric mucin (Sigma, ref. M-1778) was used as the standard. Briefly, dilutions of standards and samples were prepared in carbonate buffer (0.5 M-Na_2CO_3, pH 9.6) prior to being coated on 96-well microtiter plates (NUNC microplates, ref. 469914, Roskilde, Denmark; 100 µl per well). After overnight incubation at 4 °C, the plates were washed four times with PBS Tween 1 g/l (PBS-T, pH 7.2). Microplate saturation was made with 250 µl per well of a PBS-T solution of bovine serum albumin (BSA, 20 g/l PBS-T) incubated for 1 h at 37 °C. Plates were washed again and 100 µl of biotinylated WGA (ref. B-1025, Abcys, Paris, France; 5 mg/l) in PBS-T-BSA was added and incubated for 1 h at 37 °C. Plates were washed and added with 100 µl per well of avidine-peroxidase conjugate (Sigma, ref. A-7419) for 1 h at room temperature. After washing, 100 µl per well of OPD solution (Fast OPD, Sigma, ref. P-9187) was incubated in the dark at room temperature for 5-10 min. The reaction was stopped by adding 25 µl per well of 3 M-H_2SO_4. Absorbance was read at 492 nm using an ELISA reader (Multiskan Spectrum ref. 5118550, Thermo Electron Corp., 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 homogenized in TRIzol reagent (1 ml/100 mg) with tissue lyser (Qiagen Inc., Valencia, CA) 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 ethanol 75 %, centrifuged at 7,500 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). Finally, samples were treated with DNase (DNA free kit, Applied Biosystems, Foster City, CA) 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 cDNA 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 retro-transcription, total RNA (2 µg) was added with RNase-free water to a final volume of 20 µl. The reaction mixture (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) had a final

volume of 20 µl and contained 4 µl of 10x RT buffer, 1.6 µl of 25x dNTP, 4 µl of 10x random primers, 2 µl of RT (50 U/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 cDNA was ready for use in real time PCR.

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 Uracyl-DNA glycosylase (UDG, 1 U/µl) (Applied Biosystems). The reaction mixture (20 µl) was mixed with sample cDNA (5 µl). The cycling conditions were as follows: 50 °C for 2 min for UDG action. Then, initial denaturation was conducted at 95 °C for 10 min and then followed by 40 amplification cycles of 95 °C for 15 s and 60 °C for 1 min. The expressions of Muc genes and Tff3 were expressed relatively to 18S RNA as reported previously [17].

Chemical analysis

Diets were analyzed for ash (AOAC 942.05), ether extract (AOAC 920.39) and neutral detergent fiber (AOAC 2002.04). Gross energy was determined in diets using a Parr 1342 calorimeter (Parr Instruments, Moline, IL). Diets, faeces and digesta were also analyzed 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 ELLA-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 - [(DMi or Ni / DMd or Nd) x (Crd / Cri)]) x 100

Apparent faecal digestibility of DM or N = (100 - [(DMf or Nf / DMd or Nd) x (Crd / Crf)]) x 100

where DMi, Ni and Cri are the DM, N and chromium contents of ileal digesta and DMf, Nf and Crf those in the faeces; DMd, Nd and Crd are the DM, N and chromium contents of the diet.

Statistical analysis

Two separate analyses of variance of data were conducted using the Mixed Model procedure of Statistical Analysis System software package version 8.0 (SAS Institute Inc., Cary, NC). In the first

179 analysis, the effect of unheated phaseolin level (0, 33 and 67 % of total N) in the diet was tested for
180 linear and quadratic variations using polynomial orthogonal contrasts [20]. The second analysis of
181 variance was conducted in order to test the effect of heat treatment of phaseolin and diets with
182 untreated phaseolin, heated phaseolin and casein were compared. When the *P* value of treatment
183 effects was ≤ 0.10, the diets were compared two by two using appropriate contrasts (P0 vs. P67; P0
184 vs. P67H and P67 vs. P67H).
185
186 **Results**
187 The voluntary DM and N intakes were on average across treatments 9.6 ± (SEM) 0.14 g/d and
188 151 ± 4 mg/d, respectively, and did not differ significantly between treatments ($P > 0.05$) (data not
189 shown).
190
191 *Flow and apparent digestibility of DM and N*
192 The flow of DM in the faeces, but not at the ileum and in the colon increased linearly ($P <$
193 0.001) with increasing the level of raw phaseolin in the diet (Table 3). As a result, the apparent
194 faecal digestibility of DM decreased quadratically with increasing the level of phaseolin ($P <$
195 0.001). The ileal digestibility of DM was not influenced by this factor. The flow of N at all
196 digestive sites increased linearly with increasing the level of unheated phaseolin in the diet ($P =$
197 0.011 to $P < 0.001$). As a consequence, both ileal and faecal digestibilities of N decreased linearly
198 with increasing dietary phaseolin level ($P < 0.001$). Heat treatment of phaseolin reduced DM and N
199 flows along the intestines and increased DM and N digestibilities ($P < 0.001$) to values close to
200 those observed with the casein-based control diet.
201
202 *Flow of mucin along the gastrointestinal tract and tissue expression of mucin family genes*
203 The ELLA test revealed no binding with casein or phaseolin or with the experimental diets,
204 indicating the lack of carbohydrate moieties recognized by WGA in these ingredients and diets. The
205 flow of mucin in the colon decreased linearly with increasing the dietary phaseolin level ($P < 0.001$)
206 (Table 3). This factor did not influence the flow of mucin at the ileum or in the faeces ($P > 0.05$).
207 The colonic flow of mucin was lower with P67-H than with the casein control ($P < 0.05$) but it was
208 not different from that with unheated phaseolin (P67) (probabilities of differences for P0 vs. P67,
209 P0 vs. P67-H and P67 vs. P67-H: $P = 0.001$, $P = 0.003$ and $P = 0.288$, respectively).
210 The expression of Muc3 and Muc4 mRNA tended to decrease quadratically ($P = 0.060$ and $P =$
211 0.090, respectively) in the ileal tissue and increased or tended to increase linearly ($P = 0.093$ and P
212 $= 0.023$, 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 was or tended to be influenced by heat treatment of phaseolin in colonic tissues, except for Muc1 gene (Figure 1). mRNA expressions of Muc2, Muc3, Muc4 and Tff3 were lower with heat-treated than unheated phaseolin ($P < 0.001$ to $P < 0.05$). 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 $P = 0.001$, 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 were or tended to be higher ($P = 0.058$ and $P = 0.005$) than comparing 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 gut tissues of rats.

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. Our 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 work and studies by Santoro et al. [6,22] may come from different experimental approaches (acute vs. 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 way 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-knocked out mice develop colitis spontaneously, indicating the important role of this mucin in colonic protection [23]. In the present work, 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 (Figure 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 work, 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 which 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.

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 defense [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 not significant. Heat-treated phaseolin, also reduced the expression of Muc4 mRNA.

Trefoil family 3 (Tff3) gene is also expressed in the mucin secretory cells. It helps to protect and stabilize 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 work 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 were varying in different ways along the gut according to the dietary treatments (e.g. in the ileum, casein-

based control diet had similar mucin flow while 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 mucin 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 *vs.* vegetal) of protein in milk formulas modulated mucin gut flow in baby calves [11]. Hydrolyzed casein increased ileal endogenous amino acid losses in humans [31] and gut tissue Muc3 and Muc4 mRNA levels in rats [32]. Hydrolyzed casein and related peptides (e.g. β-casomorphin-7) also induced mucin secretion in rat isolated and perfused 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 or higher to 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 may 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-7secretory properties on jejunal mucin with rat isolated and perfused jejunum [33] does not provide evidence for such effects on the ileum or the colon, the two sites under study in the present work. Finally, results from investigations with hydrolyzed casein [31, 33] do not mean that entire casein that is subsequently hydrolyzed by endogenous proteases and peptidases may have resulted in the same effects as exogenously hydrolyzed 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 [39,40] in rats and pigs. 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 this work. 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 short chain fatty acids which, in turn modulate the mucus layer [8,12] and the expression of secreted (Muc2) and membrane-bound (Muc1, Muc3, 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 fecal 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, this 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 vs. 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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Abbreviations used

BSA, bovine serum albumin; BW, body weight; ELLA, enzyme-linked lectin assay; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; Tff3, trefoil factor 3; WGA, wheat germ agglutinin

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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
Vegetal 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 sulfate	2	2	2	2
Chromium oxide	3	3	3	3
Analysis (g/kg DM)				
Dry matter (g/kg)	908	903	908	915
Protein (N x 6.25)	96	103	96	96
Ether extract	65	65	64	66
Ash	71	67	67	64
Neutral detergent fiber	63	70	70	67
Gross energy (MJ/kg DM)	16.3	16.3	16.4	16.3

* Diets: P0: casein control, P33, P67: diets with phaseolin T contributing to 330, 670 g/kg of total dietary protein. Casein was supplemented with 30 g DL-methionine per kg dry matter casein.

† P67-H: heat treated (121 °C, 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 D3; 15 mg vitamin E; 6 mg vitamin K; 10 mg vitamin B2; 35 mg calcium pantothenate; 75 mg niacin; 2.5 mg vitamin B6; 0.05 mg vitamin B12; 0.05 mg biotin; 200 mg choline; 150 mg Mn; 500 mg Zn; 40 mg Cu, 200 mg Fe; 2 mg I; 0.5 mg Se, 1 mg Co.

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

Gene	Primer	Sequences (5'- to -3')	Reference
18S	sense	ACGGAAGGGCACCACCAGGAG	(28)
	antisense	GCACCACCACCCACGGAAACG	
Muc1	sense	TCGACAGGCAATGGCAGTAG	(16)
	antisense	TCTGAGAGCCACCACTACCC	
Muc2	sense	GCCTCAAACCCGTGCGTGTC	(47)
	antisense	TCATTCACCAACCACTCATC	
Muc3	sense	CTTGAGGAGGTGTGCAAGAAA	(32)
	antisense	CCCCAGGGTGACATACTTTG	
Muc4	sense	GCTTGGACATTTGGTGATCC	(32)
	antisense	GCCCGTTGAAGGTGTATTTG	
Tff3	sense	CCTGGTTGCTGGGTCCTCTG	(28)
	antisense	GCCACGGTTGTTACACTGCTC	

Table 3. DM, N and mucin flows along the gastrointestinal tract and apparent ileal and fecal digestibilities of DM and N in rats fed graded levels of unheated phaseolin and of heat-treated phaseolin (n = 4-5 per treatment).

	Treatment*				Level of unheated phaseolin†			Heat treatment‡	
	P0	P33	P67	P67-H	SEM§	*P*- Contrast		SEM§	*P*‖
						Linear	Quadratic		
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

* P0 casein control; P33, 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 (H) 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.

§ SEM: standard error of the mean.

‖ Values with different letters (a,b,c) in the same row between P0, P67 and P67H treatments differ significantly at $P < 0.05$.

Table 4. Mucin gene expression in ileal and colic tissue in rats in rats fed graded levels of unheated phaseolin (n = 4-5 per treatment).

		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

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

† SEM: standard error of the mean.

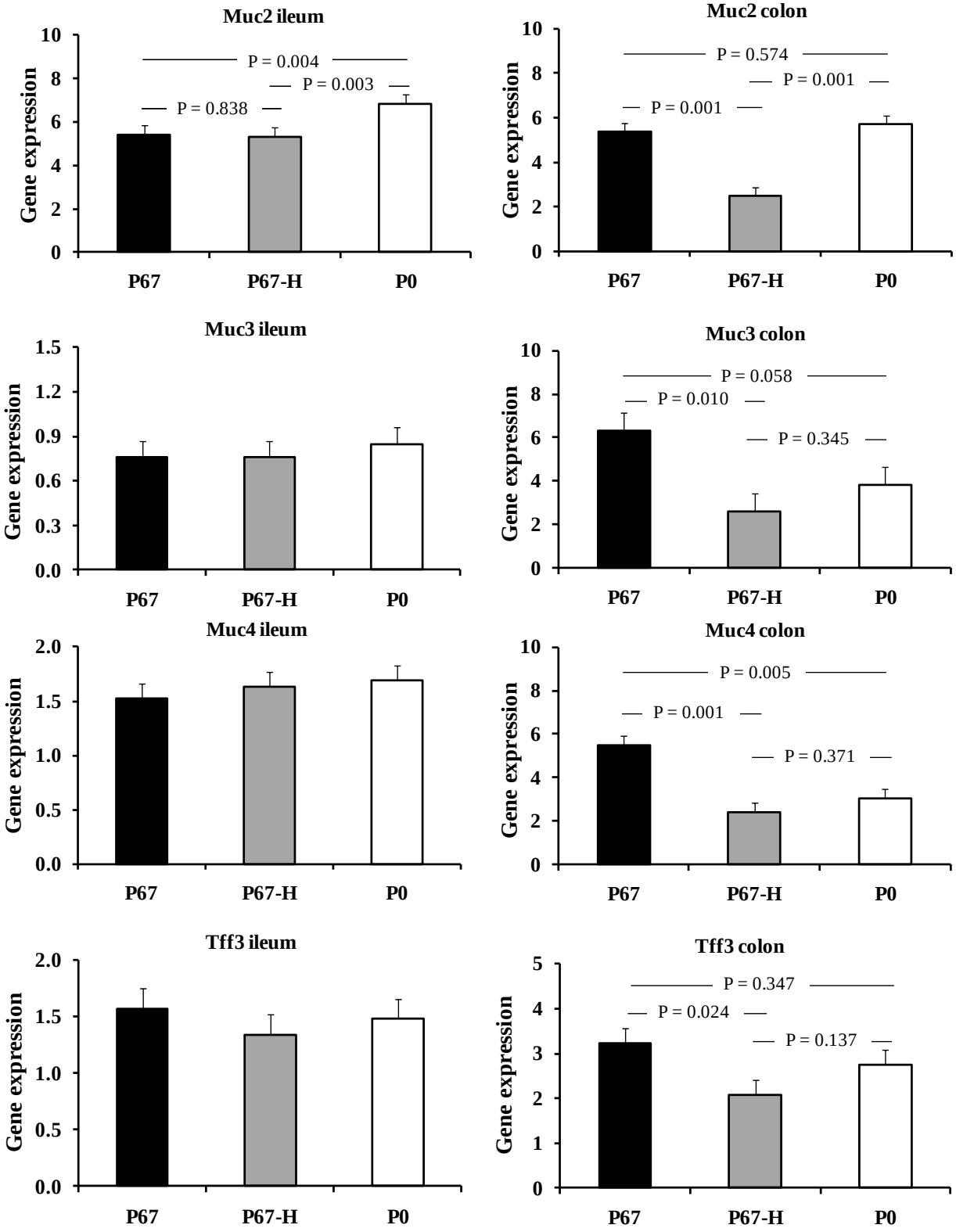

Figure 1. Influence of heat treatment of phaseolin on mucin gene expression in ileal and colonic tissues of rats (means and SEM, n = 4-5). The overall probabilities for treatment effects were for Muc2 (*P* =0.004 and 0.001), Muc3 (*P* =0.789 and 0.027), Muc4 (*P* =0.732 and 0.003) and Tff3 (*P* =0.432 and 0.062) in the ileum and in the colon, respectively.