Eur J Nutr (2012) 51:293–299
DOI 10.1007/s00394-011-0214-3

ORIGINAL CONTRIBUTION

A gluten metabolism study in healthy individuals shows the presence of faecal glutenasic activity

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Received: 4 March 2011 / Accepted: 27 May 2011 / Published online: 14 June 2011

Abstract
Purpose To study the gluten metabolism in healthy individuals and its effect over the intestinal microbial activity.
Methods The faeces of eleven healthy subjects were analysed under 4 diet regimens: their normal gluten diet, a strict gluten-free diet (GFD), a GFD with a supplemental intake of 9 g gluten/day and a GFD with a supplemental intake of 30 g gluten/day. Gluten content, faecal tryptic activity (FTA), short-chain fatty acids (SCFAs) and faecal glutenasic activity (FGA) were analysed in faecal samples.
Results Faecal gluten contents, FTA, SCFAs and FGA varied significantly with different levels of gluten intake in the diet. When high gluten doses (30 g/day) were administered in the diet, SCFA concentrations (70.5 mmoles/kg faeces) were significantly different from those from the GFD period (33.8 mmoles/kg faeces) of the experiment. However, the FTA showed significant differences between the GFD (34 units) and the normal gluten-containing diet (60 units) and also between the GFD and the GFD + 30 g of gluten/day (67 units). When gluten was present in the diet, gluten was detected in the faeces, showing that at least a portion of the gluten ingested is eliminated in the large intestine, providing a substrate for intestinal microbial proteases. We have also shown the presence of faecal glutenasic activity that increased proportionally with the gluten intake in the diet, showing an enzymatic activity of 993 units in DSG, 2,063 units in DSG + 9 g and 6,090 units in DSG + 30 g.
Conclusions The activity of the intestinal microbiota is modified by gluten intake in the diet. The incorporation of gluten in the diet increases the activity of a gluten proteolytic activity in the faeces.

Keywords Gluten · Coeliac disease · Faecal glutenasic activity · Gut microbiota

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Introduction

Coeliac disease (CD) is a condition traditionally characterised by chronic inflammation of the proximal small intestine, resulting in villous atrophy and malabsorption. It can develop in genetically susceptible individuals ingesting wheat storage proteins and/or storage proteins from barley and rye [1]. CD is a unique autoimmune disorder because

the environmental precipitant is known [2]. Thus, it provides an enormously valuable model for understanding autoimmune disorders because it is the only example in which the addition or removal of a single environmental component, gluten, can turn the symptoms of the disease process on and off [3].

Gluten can be defined as the rubbery mass that remains when wheat dough is washed to remove starch granules and water-soluble constituents. In practice, the term gluten refers to proteins, although lipids (up to 10%) and some starches are also present [4]. Gluten contains hundreds of proteins, included in two major protein types: the gliadins and the glutenins. The closely related proteins in barley and rye are the hordeins and the secalins. These proteins (gliadins, glutenins, hordeins and secalins) have a high proline and glutamine content and can trigger CD [5].

The nutritional requirements for amino acids in humans are met by assimilation of dietary proteins in the small intestine. Digestion of the proteins in the intestinal lumen by pancreatic proteases releases primarily large peptides that are not absorbable as such. It is the action of the membrane-bound peptidases in the brush-border membrane of the enterocyte that generates a major proportion of the absorbable products, namely amino acids, dipeptides and tripeptides. Free amino acids are absorbed into the enterocyte across the brush-border membrane via multiple amino acid transport systems. Small peptides, primarily those consisting of two or three amino acids, are transported across the brush-border membrane via specific peptide transport system [6]. The small intestine is the principal site of protein absorption, but proteins are not completely absorbed; direct determination of the ileal protein digestibility in vivo shown that the assimilation of even easily digestible protein is incomplete [7–9]. Non-absorbed dietary protein enters the human large intestine in the form of a complex mixture of proteins and peptides. Protein and peptides reaching the colon are fermented to SCFA, branched fatty acids such isobutyrate, isovalerate and some end products that may be toxic to the host, e.g., certain amines and phenolic compounds [10].

Two major factors govern the amount of protein reaching the colon: the total amount of the protein in the diet and the digestibility of this protein in the upper gut [7, 8, 10]. A normal Western diet includes 13–15 g of gluten/day [11]; in vitro studies have shown that the high proline content (around 15%) of gluten and related proteins renders these proteins resistant to complete digestion by gastric, pancreatic and brush-border enzymes in the human small intestine because those enzymes are deficient in prolyl endopeptidase activity. As a result, many high-molecular weight oligopeptides (with 10 to $\geq$30 amino acid residues) persist in the lumen of the small intestine and some of them are capable of triggering the inflammatory process associate with CD [12–14].

Some studies have reported the presence of unbalanced gut microbiota in CD patients, in both those treated or untreated with a GFD, which could play a pathogenic role or be a risk factor for CD [15–17]. Moreover, differences between the metabolic activity of the gut microbiota of CD patients and healthy people have been shown [18]. These differences could be partially due to the diet. The metabolic effect of a GFD compared with a gluten-containing diet on the gut ecosystem is largely unknown.

The objective of this study was to evaluate the activity of intestinal microbiota in healthy people consuming different amounts of gluten in the diet because part of the gluten ingested in the diet is expected to be malabsobed in the small intestine being then a substrate for colonic bacteria.

Experimental methods

Subjects

Eleven healthy subjects were enroled in this study: seven women (mean age 30.5 years; range 24–42 years) and four men (mean age 31.3 years; range: 24–42). All of the participants were members of our staff at the hospital and the university. The exclusion criteria comprised symptoms of digestive disease, use of medications, antibiotics or probiotics in the previous 2 months and a family history of CD. All subjects were screened for CD; they showed normal serum tTGA levels, and their HLA-DQ phenotype was not DQ2 or DQ8. The participants' haemoglobin levels were normal, and serum biochemistry analysis for renal and hepatic function gave results that were within normal laboratory values. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the local ethics committee of our hospital. Written informed consent was obtained from all of the subjects.

In order to avoid differences caused by ingesting gluten from different sources, the same wheat gluten (without prior heat treatment) was administered in all cases. The commercially available, non-processed, powdered gluten was obtained from el Granero Integral S.L. (Paracuellos del Jarama, Madrid). The subjects were instructed to ingest the entire dose.

Diet

The diet was controlled during 15 days (Fig. 1). It was initiated by a GFD during 7 days, and then it was followed by 4 days in GFD with addition of 9 g of non-processed

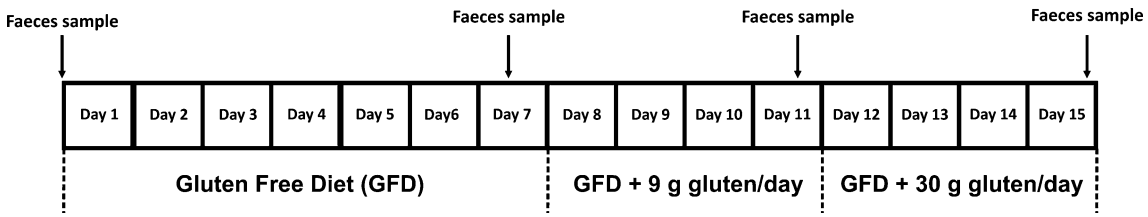

Fig. 1 A diagram of the different modifications in the diet of the subjects and the time of faecal sample collections

gluten ingested over the three meals of the day and then another 4 days in GFD with addition of 30 g of non-processed gluten ingested over the three meals of the day. The subjects were instructed to fully comply with a GFD.

Faecal sampling

Fresh stools were obtained from the 11 healthy subjects under the different diet conditions (Fig. 1). Four samples were collected: (i) before the GFD was initiated, on the patient's normal gluten-containing diet; (ii) on the seventh day of the GFD; (iii) on the fourth day of consuming 9 g of gluten; and (iv) on the fourth day of consuming 30 g per day of gluten. All samples were homogenised and aliquoted within 3 h of defecation. Depending on the analysis performed, some of the aliquots were freshly processed and others were stored at −80 °C.

Faecal SCFAs identification and quantification

Sample preparations were performed following the protocol described by Whelan et al. [19] using 4-methyvaleric as internal standard. Briefly, the faecal samples were frozen immediately after collection and stored at −20 °C. One gram of faeces was thawed, suspended in at least 2 mL of water and homogenised in a vortex mixer for approximately 2 min. The suspension was centrifuged for 10 min at 12,000 rpm, giving a clear supernatant. The pH of the supernatant was decreased by addition of HCl (80 mM final concentration). The internal standard 4-methylvaleric acid solution was spiked into the supernatant at a final concentration of 258 μM, and then the supernatant was injected into a GC for analysis.

The short-chain fatty acids (SCFAs) were identified according to the procedure described by Zhao et al. [20]. The SCFAs were identified on chromatograms by their specific retention times using a Perkin Elmer Autosystem XL GC equipped with a flame ionisation detector (FID). A fused silica capillary column (TRB-FFAP, Teknokroma SL), coated with 1-μm-thick film, was used. It was 30 cm long and had an inner diameter of 0.53 mm. Helium was supplied as the carrier gas at a pressure rate of 13.8 psi. The initial oven temperature was 130 °C and was maintained at that value for 3 min. The temperature was then raised to 170 °C at a rate of 40 °C/min and held at that value for 2.0 min. The temperature of the FID and the injection port was 250 °C. The flow rates of hydrogen and air (used as makeup gases) were 50 and 500 mL/min, respectively. The injected sample volume for the GC analysis was 2 mL. Volatile fatty acid standards were obtained from Sigma Co (USA). To quantify the peak area in terms of concentration, the relative response factor was used, following the methodology described by Ranilla et al. [21].

Faecal tryptic activity

Assays of the faecal tryptic activity (FTA) were performed following the protocol described by Ramare et al. and Midtvedt et al. [22, 23], with modifications. The faecal samples were thoroughly mixed with saline (1:5), held at 4 °C for 90 min and centrifuged (35,000×*g*, 4 °C, 30 min). The supernatants were kept at −20 °C before the assays were performed. A 0.1 mL supernatant fraction was added to 2.9 mL of Tris buffer (pH 8.2) containing 4.4 g of calcium chloride per litre. The reaction was initiated by adding 0.6 mL of 3 mM BAPNA (N-benzoyl-DL-arginine-4-nitroanilide hydrochloride). The reaction took place at 20 °C and was stopped after 20 min by adding 0.6 mL of 5 M acetic acid. Bovine pancreas trypsin type III diluted in 2 mM hydrochloric acid was used for the construction of a standard curve. All of the samples and standards were analysed spectrophotometrically in parallel with blanks at 405 nm. After corrections for blank values, the FTA was calculated and expressed as milligrams of trypsin per kilogram of faeces.

Faecal glutenasic activity

The faecal samples were homogenised after addition of saline (1:5), maintained at 4 °C for 90 min and centrifuged (35,000 *g*, 4 °C, 30 min). The supernatant was filtered through a 0.2-μm sterile filter. The glutenasic activity was measured using a bioassay, on agar plates containing gluten (3%); the reaction was incubated at 37 °C for 24 h. The plates were evaluated by measuring the diameter of the halo formed. Trypsin diluted in saline was used for construction

of the standard curve. The glutenasic activity was expressed as milligrams of trypsin per kilogram of faeces.

Gluten in faeces

Gliadin extraction: The gliadin was prepared in 60% (v/v) ethanol at a concentration of 1 mg/mL. The suspension was centrifuged at 13,000×*g* for 10 min, and the supernatant recovered. The concentration of gliadin was then measured by the Bradford method [24].

Extraction of prolamines from faeces: Prolamines were extracted by mixing 1 g of faeces with 10 mL of 60% (v/v) ethanol in a rotary shaker for 1 h at room temperature. The suspension was then centrifuged at 13,000×*g* for 10 min, and the supernatant separated.

G12 MoAb inmunochromatographic test for detection of gluten: The G12 monoclonal antibody (MoAb) was developed based on the toxicity of the 33-mer peptide that is recalcitrant to gluten digestion [25]. The sensitivity with which this MoAb effectively detects the toxic peptides of gluten in food is higher than that of equivalent methods recognising other gluten epitopes [25]. The assay was a modification of the manufacturer's instructions (GlutenTox stick, Biomedal S.L. Sevilla, Spain). The positive control (gliadin) and the samples were diluted (1:10–1:20,000) in the buffer solution provided by the manufacturer. The test sticks were dipped into the solution (300 μL) for 10 min before being removed and allowed to air dry.

Statistics

All the variables were analysed with SPSS v 17.0. Categorical variables were expressed as numbers and percentages, and quantitative variables as means and standard deviations (SDs). Because of the small sample size, our data did not show a normal curve and non-parametric tests were employed to compare the data. The Friedman test was performed to evaluate differences between various related samples. A p value < 0.05 was selected to reject the null hypothesis by two-tailed tests. The Wilcoxon test was performed as a post hoc test to evaluate the differences between two related samples. A Bonferroni correction was applied to the Wilcoxon test, and we used 0.05/number of comparisons as our critical level of significance: $p < 0.01$ (0.05/5 = 0.01).

Results

FTA in healthy individuals is high when gluten is present in the diet

The lowest FTA was obtained under a GFD; this activity increased with the incorporation of gluten into the GFD.

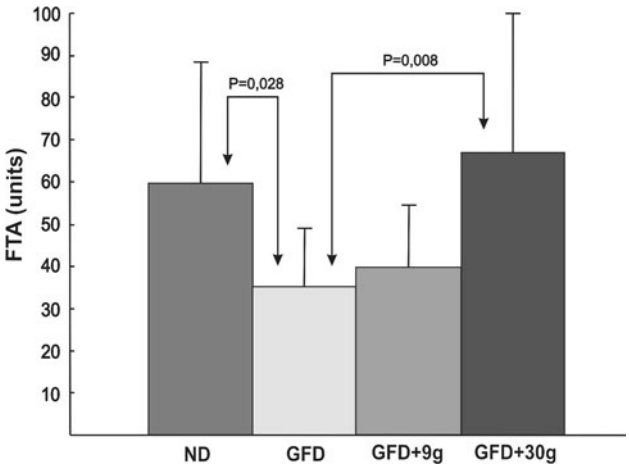

Fig. 2 FTA changes related to gluten intake in the diet. *ND* normal subjects diet, *GFD* gluten-free diet, *GFD+9* gluten-free diet with 9 g of gluten per day, *GFD+30* gluten-free diet with 30 g of gluten per day

The highest FTA was obtained both with GFD + 30 g of gluten/day and under the subject's normal daily gluten diet. The differences were statistically significant between the groups when gluten increased in the diet (Fig. 2). Paired comparisons between the GFD period and the other gluten periods were also statistically significant, except for GFD versus GFD + 9 g of gluten/day. There were no statistically significant FTA differences between men and women.

SCFA pattern related to gluten intake

In all faecal samples, acetic acid was the main SCFA, followed by propionic and butyric acids. Longer SCFAs such as valeric and hexanoic acid were present in much lower amounts. Branched SCFAs such as isobutyric and isovaleric acids were also present in lower concentrations than acetic, propionic and butyric acids. It seems that all SCFA concentrations (Table 1) increased when gluten was incorporated into the GFD and also under their normal gluten-containing diet. However, for all the SCFAs analysed, significant differences were only found between the GFD and the GFD + 30 g of gluten/day (Fig. 3).

Faecal glutenasic activity is proportional to gluten intake in the diet

The results show (Fig. 4) that the faecal glutenasic activity (FGA) under the GFD was of 993 units and increased to 2,063 units in the GFD + 9 g of gluten/day, to 6,091 units in the GFD + 30 g of gluten/day and to 3,551 units when subjects were on their normal daily gluten diet. A statistical analysis demonstrated that these differences were all significant, showing that faecal glutenasic activity increased

Table 1 Comparison of faecal SCFAs in faecal samples of 11 healthy individual under different gluten intakes

Type of metabolite	Normal diet	GFD	GFD + 9 g of gluten/day	GFD + 30 g of gluten/day
Acetic acid*	24.60 ± 9.18	20.60 ± 10.46	31.21 ± 25.15	43.67 ± 21.88**
Propionic acid*	7.05 ± 3.21	5.82 ± 2.27	8.66 ± 5.72	11.7 ± 3.07**
i-Butyric acid*	0.98 ± 0.38	0.90 ± 0.49	1.01 ± 0.48	1.43 ± 0.34**
n-Butyric acid*	5.33 ± 2.84	4.22 ± 2.15	6.17 ± 4.89	9.70 ± 4.77**
i-Valeric acid*	1.36 ± 0.62	1.18 ± 0.55	1.25 ± 0.66	1.72 ± 0.65
n-Valeric acid*	0.97 ± 0,46	0.77 ± 0.42	1.03 ± 0.49	1.59 ± 0.38**
Hexanoic acid*	0.44 ± 0.34	0,36 ± 0.33	0.35 ± 0.32	0.7 ± 0.55**

ND normal subjects diet, *GFD* gluten-free diet, *GFD + 9* gluten-free diet with 9 g of gluten per day, *GFD + 30* gluten-free diet with 30 g of gluten per day

* Mean of 11 samples (mmol/kg faeces)

** Significant differences versus GFD: $p < 0.01$

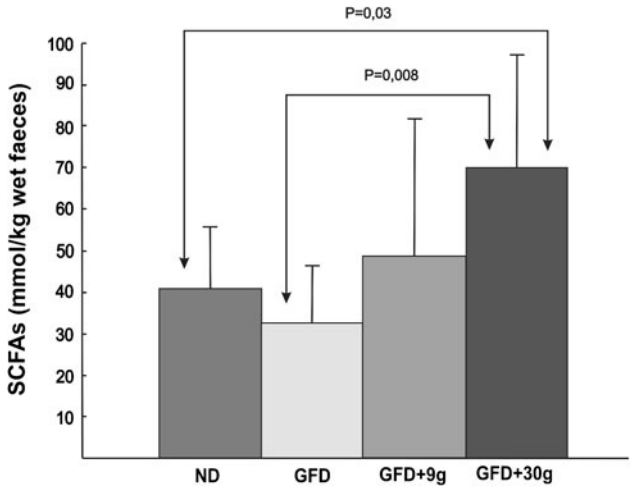

Fig. 3 SCFAs increase when diet is supplemented with higher doses of gluten. *ND* normal subjects diet, *GFD* gluten-free diet, *GFD+9* gluten-free diet with 9 g of gluten per day, *GFD+30* gluten-free diet with 30 g of gluten per day

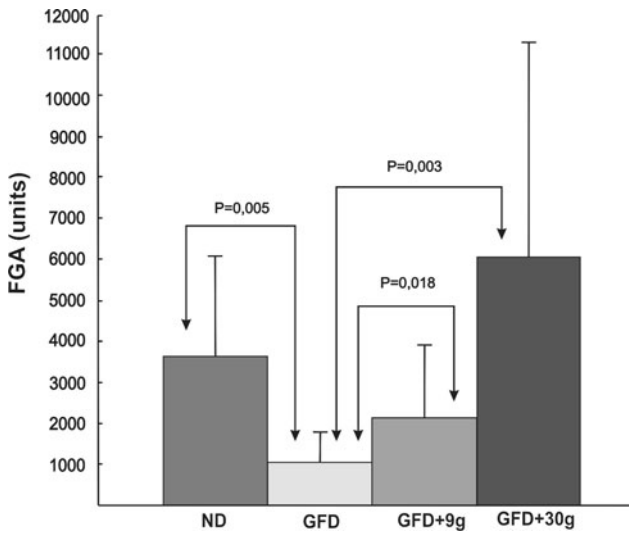

Fig. 4 Faecal glutenasic activity increases proportionally as gluten is incorporated into the diet. *ND* normal subjects diet, *GFD* gluten-free diet, *GFD+9* gluten-free diet with 9 g of gluten per day, *GFD+30* gluten-free diet with 30 g of gluten per day

proportionally as gluten was incorporated into the diet. There were no gender-related differences in the FGA.

Determination of the presence of gluten in faeces

As shown above, the FGA is proportional to gluten intake. If this enzymatic activity is present in faeces, it is also possible that there is a proportional presence of gluten or different gluten fragments that could act as substrates for this enzyme or other enzymes in the faeces. In order to investigate this possibility, we used the G12 monoclonal antibody to screen for the presence of gluten in the faeces of 11 healthy individuals under a controlled gluten-containing diet. The results (Table 2) showed that all the subjects presented an excretion of gluten above 250 ppm under a normal gluten-containing diet and a GFD + 30 g of gluten/day. After a week-long GFD, none of the cases presented detectable gluten levels. The consumption of 9 g of gluten daily for a period of 4 days led to the detection of faecal gluten levels greater than 250 ppm (except in three subjects). There were no differences in faecal gluten levels between men and women.

These results show that at least a portion of the gluten ingested was eliminated in the large intestine, where it would be able to provide a substrate for microbial proteases.

Discussion

Wheat is a staple food for most of the world's population. However, as far as we know, there are no studies on gluten

Table 2 Semiquantitative detection of gluten eliminated in the faeces

Subjects	GFD	GFD + 9 g of gluten/day	GFD + 30 g of gluten/day	Normal diet
CB1	–	++	++	++
CB2	–	++	++	++
CB3	–*	++	++	++
CB4	–	++	++	++
CB5	–	+	++	++
CB6	–*	+	++	++
CB7	–	++	++	++
CB8	–	++	++	++
CB9	–	+	++	++
CB10	–	++	++	++
CB11	–	++	++	++

CB1–CB11: Eleven healthy volunteer subjects. *GFD* Gluten-free diet, *GFD+9 g* GFD plus 3 g of gluten every 8 h, *GFD+30 g* GFD plus 10 g of gluten every 8 h

++ ppm gluten >250, + ppm gluten >6, –ppm gluten <6. –* Traces of gluten were detected

metabolism in vivo in healthy individuals or in CD patients. In this study, we have focused on the effect of gluten intake on gut microbial metabolism in healthy individuals following a normal diet, a gluten-free diet (GFD) and a GFD with a supplemental intake of 9 g and 30 g of gluten/day. The metabolic activity of the intestinal microbiota may be modified by gluten intake in the diet because part of the gluten proteins could be malabsorbed in the small intestine. Previous in vitro studies report [12–14] that gluten proteins are only partially hydrolysed in the small intestine suggesting that gluten peptides are excreted in healthy people [3]. We showed the presence of gluten or gluten peptides in faeces of healthy individual consuming different amounts of gluten, suggesting that gluten is not completely assimilated in the small intestine. Incomplete assimilation of proteins in the small intestine has been previously demonstrated with other dietary proteins even in those that are known as easily digestive like the egg protein [7, 8].

In the large bowel, bacterial metabolism of protein that escapes digestion and absorption in the small bowel will produce SCFAs and branched fatty acids [26]. In our study, we did not observe significant differences in the SCFA pattern between the GFD and the subjects' normal gluten-containing daily diet, suggesting that there is no marked effect of the GFD on the SCFA pattern; only significant changes in the SCFAs were in diet with 30 g of gluten/day compared to GFD. This agrees with previous data showing that carbohydrates fermented by bacteria in the proximal colon are quantitatively the most important substrates in the formation of linear SCFAs [27–30]. Gluten comprises mostly proteins rather than carbohydrates, and fermentation of proteins has been reported to yield mainly branched SCFAs and other products. However, significant differences were observed only in the amount of isobutyric acid between the GFD and GFD with 30 g of gluten/day. Similarly, Tjellström et al. [18] found no differences in SCFAs between CD patients on a GFD and on a normal diet. However, gluten is not only composed of protein but also a low proportion of non-absorbable starch could be present [4]. It has been described that starch present in the wheat flour is incompletely absorbed and partially hydrolysed in the small intestine and contributes to colonic SCFA formation [31]. This starch could be responsible for the changes in the linear SCFAs seen for the high gluten dose.

Non-absorbed gluten peptides that escape small intestine enter to the large intestine where the lumen of the distal bowel has been described as an intensely proteolytic environment [32, 33]. In this study, we have detected (for the first time as far as we know) an enzymatic activity, called glutenase, that increases in faeces as gluten is incorporated into the diet. Moreover, it is detected in high levels under a normal gluten-containing diet. The presence of this enzyme correlates with the presence of gluten in the faeces; the more gluten is included in the diet, the more gluten is present in the faeces and the more faecal glutenasic activity is detected. However, although we detected this activity in the faeces, this metabolic activity could occur higher up in the intestine as happens in CD patients. It has been suggested recently that specific CD mucosal bacteria could develop gliadinase activities that would be able to degrade gliadins not present in healthy people [34]. This hypothesis is in supported by the presence of different small intestine microbiota in CD patients than in healthy people [16, 17]. If CD patients can digest gliadins in the small intestine and they have an altered mucosal permeability [35], does this mean that they do not excrete gluten in the faeces? Do they have a lower faecal glutenase activity or a different one than healthy people? Future research will be directed at purifying and measuring this enzymatic activity in healthy people and in CD patients so as to compare its proteolytic properties in each population.

In conclusion, the metabolic activity of the intestinal microbiota may be modified by gluten intake in the diet. Gluten can be detected in the faeces and provides a substrate to intestinal microbial proteases. A new high faecal glutenasic activity (FGA) has been detected, which suggests that bacterial protease activity plays a role in gluten digestion. More studies are needed to investigate this behavioural pattern in CD.

Acknowledgments The authors are grateful to Eva Vallejo, Ph.D., for her valuable suggestions on the statistical methods. This study was supported by a grant from la Obra Social Caja Burgos and by a grant

from la Junta de Castilla y León, Consejería de Sanidad (Ref 318/A/08). Esther Nistal received a fellowship from La Junta de Castilla y León. I. C., A. R., and C. S. were responsible for the gluten analysis in faeces. M. A. F. and L. B. R.-A. were responsible for the SCFA studies. A. C. and E. N. were responsible for the FTA, FGA and data analysis. L. A, J., M. R. M. and S. V. were responsible for volunteer recruitment and statistics, and they also contributed to the design of the study. J. C. wrote the paper and contributed to the design of the study. All of the co-authors commented on the paper and approved the final version. None of the authors had a financial conflict of interest.

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