

No Effects of Gluten in Patients With Self-Reported Non-Celiac Gluten Sensitivity After Dietary Reduction of Fermentable, Poorly Absorbed, Short-Chain Carbohydrates

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BACKGROUND & AIMS: Patients with non-celiac gluten sensitivity (NCGS) do not have celiac disease but their symptoms improve when they are placed on gluten-free diets. We investigated the specific effects of gluten after dietary reduction of fermentable, poorly absorbed, short-chain carbohydrates (fermentable, oligo-, di-, monosaccharides, and polyols [FODMAPs]) in subjects believed to have NCGS. **METHODS:** We performed a double-blind cross-over trial of 37 subjects (aged 24–61 y, 6 men) with NCGS and irritable bowel syndrome (based on Rome III criteria), but not celiac disease. Participants were randomly assigned to groups given a 2-week diet of reduced FODMAPs, and were then placed on high-gluten (16 g gluten/d), low-gluten (2 g gluten/d and 14 g whey protein/d), or control (16 g whey protein/d) diets for 1 week, followed by a washout period of at least 2 weeks. We assessed serum and fecal markers of intestinal inflammation/injury and immune activation, and indices of fatigue. Twenty-two participants then crossed over to groups given gluten (16 g/d), whey (16 g/d), or control (no additional protein) diets for 3 days. Symptoms were evaluated by visual analogue scales. **RESULTS:** In all participants, gastrointestinal symptoms consistently and significantly improved during reduced FODMAP intake, but significantly worsened to a similar degree when their diets included gluten or whey protein. Gluten-specific effects were observed in only 8% of participants. There were no diet-specific changes in any biomarker. During the 3-day rechallenge, participants' symptoms increased by similar levels among groups. Gluten-specific gastrointestinal effects were not reproduced. An order effect was observed. **CONCLUSIONS:** In a placebo-controlled, cross-over rechallenge study, we found no evidence of specific or dose-dependent effects of gluten in patients with NCGS placed diets low in FODMAPs. www.anzctr.org.au. ACTRN1261000524099

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gluten-containing products continues to increase worldwide.² The clinical entity of non-celiac gluten sensitivity (NCGS) has been defined as those without celiac disease but whose gastrointestinal symptoms improve on a gluten-free diet (GFD).^{3,4} Since its original description in 1980,⁵ reports of NCGS have not taken into account the presence of other components of wheat, particularly fructans, that might have been pathogenically responsible for the symptoms. The first evidence that gluten might specifically induce symptoms in patients with IBS derived from a randomized, placebo-controlled trial of a single dose of carbohydrate-deplete gluten in 36 patients remaining on their habitual GFD in parallel groups.⁶ Although there is some evidence of the effects of gluten in animal models or cancer cell lines,^{7–9} little else is known about this entity. For example, mechanisms have not been identified and dose dependence has not been demonstrated.

To further evaluate this concept of NCGS, the current study aimed to examine the hypotheses that, in subjects who report to have NCGS, gluten induces dose-dependent, reproducible gastrointestinal and systemic symptoms. To do this, a randomized, double-blind, cross-over controlled feeding trial of 3 diets differing in gluten content was conducted in patients with IBS fulfilling the definition of NCGS, followed by a rechallenge trial in the same patient cohort. In order to control other potential triggers of gut symptoms, all diets had reduced content of fermentable, poorly absorbed short-chain carbohydrates (ie, fermentable, oligo-, di-, monosaccharides, and polyols [FODMAPs])¹⁰ and, in the second, dairy products and food chemicals were additionally controlled.

Patients and Methods

Patients

Patients were recruited between January 2010 and January 2011 via advertisements in e-newsletters and community newspapers in metropolitan Melbourne, Australia and by referrals from private dietetics practice or gastroenterology clinics. The inclusion

Abbreviations used in this paper: D-FIS, Daily-Fatigue Impact Scale; FODMAP, fermentable, oligo-, di-, monosaccharides, and polyols; GFD, gluten-free diet; IBS, irritable bowel syndrome; NCGS, non-celiac gluten sensitivity; VAS, visual analogue scale.

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There is an emerging belief that gluten can mediate the symptoms of at least some patients with irritable bowel syndrome (IBS),¹ and the avoidance of wheat- and

criteria were age older than 16 years; symptoms of IBS fulfilling Rome III criteria that self reportedly improved with a GFD; symptoms well controlled on a GFD; and adherence to the GFD for at least 6 weeks immediately before screening as assessed at an interview by a trained nutritionist (JRB). Celiac disease was excluded either by absence of the HLA-DQ2 and HLA-DQ8 haplotype or by a normal duodenal biopsy (Marsh 0) performed at endoscopy while on a gluten-containing diet in individuals expressing the HLA-DQ2 or HLA-DQ8 haplotype. Patients with significant gastrointestinal disease (such as cirrhosis or inflammatory bowel disease), excessive alcohol intake, intake of nonsteroidal anti-inflammatory agents, use of systemic immunosuppressant medication, poorly controlled psychiatric disease, and those unable to give written informed consent were excluded.

Study Protocol

The first study was a randomized, placebo-controlled, double-blinded cross-over trial. After an initial 1-week baseline period where the subjects recorded their usual diet and symptoms, participants entered a 2-week run-in period, at the beginning of which all were educated on a diet low in FODMAPs.¹⁰ They were continued on a GFD low in FODMAPs throughout. Patients then received 1 of 3 diet treatments (high-gluten, low-gluten, or placebo) for one week, followed by a washout period of at least 2 weeks and until symptoms induced during the previous dietary challenge resolved, before crossing over to the next diet. Patients were randomized at recruitment according to a computer-generated order, held by an independent observer. Patients unable to continue a treatment due to intolerable symptoms were permitted to cease the study food of that particular arm, but continue to collect data as per day 6 (ie, symptom assessment, physical activity studies, blood and stool samples collected) and collect symptom and food diaries when not on the study diet. Patients then resumed any remaining treatment arms after the allocated washout period.

All participants were invited to return to take part in a rechallenge trial. This was designed and conducted after the initial trial was analyzed. A 3-day challenge period was chosen on the basis of the kinetics of symptom induction in the first trial and a stricter background control of potential triggers of gut symptoms was employed (see Study Food Preparation section). As the time between participation of the 2 trials varied from 8 to 17 months, inclusion/exclusion criteria (as mentioned) were confirmed. Participants were randomly allocated (as for the first study) to receive 1 of the 3 dietary treatments (see Study Food Preparation section) for 3 days, followed by a washout period of minimum 3 days (or until symptoms induced during the previous dietary challenge resolved), before crossing over to the next diet. Patients unable to continue a treatment due to intolerable symptoms were permitted to cease the study food of that particular arm, but continue to collect data as per day 3 (symptom assessment) and go on to resume any remaining treatment arms after the allocated washout period.

Both trials were approved by Eastern Health Research and Ethics Committee and the 7-day protocol also registered with Australia and New Zealand Clinical Trials Register (ANZCTR): ACTRN12610000524099. All authors had access to the study data, and had reviewed and approved the final manuscript.

End Points

The primary end point was the change in overall symptom score measured on a visual analogue scale (VAS) from the

run-in period to that at the end of the treatment period. Secondary end points comprised the proportions of participants demonstrating an increase of at least 20 mm on the VAS in overall and individual symptom scores; the change in individual symptom scores compared with run-in; changes in biomarkers and byproducts of protein metabolism; the magnitude of gluten-specific T-cell responses after gluten challenge; change and comparison in scores on fatigue scales and activity levels; and the reproducibility of gastrointestinal symptom scores between the 7-day trial and 3-day rechallenge.

Study Food Preparation

For the initial 7-day trial, the background diet was gluten-free and low in FODMAPs, a major trigger of gut symptoms. During the 3 treatment periods, the background diet had the following incorporated: 16 g/d whole-wheat gluten (high-gluten arm), 2 g/d whole-wheat gluten/d, and 14 g/d whey protein isolate (low-gluten arm) or 16 g/d whey protein isolate (placebo arm).

For the 3-day rechallenge trial, the background diet was gluten-free, and not only reduced in FODMAPs, but also dairy-free and low in naturally occurring and artificially added food chemicals (ie, salicylates, amines, monosodium glutamate, as well as preservatives benzoates, propionate, sulfites, nitrites, sorbic acid, plus added antioxidants and colors), which are all putatively capable of triggering symptoms in some patients.^{10,11} During the 3 treatment periods, the study diets had the following incorporated: 16 g/d whole-wheat gluten (gluten arm), 16 g/d whey protein isolate (whey arm), or no additional protein (placebo arm).

All main meals were supplied to the subjects. Detailed food lists of low FODMAP fruit and vegetables were supplied to the participants so they were able to purchase fresh perishable items themselves. The meal plan was adequate in macronutrients, micronutrients, and provided 8 MJ energy daily. Volunteers with smaller energy requirements were given smaller portions, but the same proportion of gluten was added. Volunteers with larger energy requirements were provided with additional low FODMAP, gluten-free meals, and snacks.

Meals in each trial were similar across the 3 diets in texture, taste, and appearance, confirmed with preliminary testing in 5 healthy people where the food containing the gluten could not be differentiated from those that did not. The gluten used was commercially available, carbohydrate-depleted wheat gluten (Vital Wheat Gluten; Penford Australia Ltd, Tamworth, Australia) and contained 75% protein, 1.8% crude fiber, 6.9% lipid, 15.6% starch, and 0.6% ash, as shown on reversed-phase high-performance liquid chromatography. On the basis of size-exclusion high-performance liquid chromatography, the protein content had a distribution of 6.6% non-gluten protein (albumin/globulin), 53.4% glutenin, and 40.0% gliadin. The whey protein isolate (RESOURCE Beneprotein Instant Protein Powder; Nestle Healthcare Nutrition, Inc., Minneapolis, MN) was lactose-free and low FODMAP, as measured following methodologies described previously.^{12,13}

The investigator (JRB) and University research chef, assisted by 2 hospitality students, prepared all food in commercial kitchens. Meals were provided as frozen complete meals with instructions to thaw and warm either via microwave or oven. They were free of charge and delivered to participants' homes weekly.

Measurements

Medical history, examination and, if not already done, HLA genotyping were completed at baseline. For the 7-day trial,

dietary adherence was assessed by entries into a tick-box diary completed during the week and by an unused food count at the end of each treatment. A description of any additional food consumed was written in the diary and discussed with one of the investigating team (JRB). Adherence to the GFD was assessed at entry by specific questioning and using a flow chart to give a numerical score.¹⁴ This was cross checked with assessment of participants' baseline 7-day food diary. Gastrointestinal symptoms were assessed by the participant completing daily diary cards via a 100-mm VAS to score the presence and severity of overall abdominal symptoms, abdominal pain, bloating, wind, satisfaction with stool consistency, tiredness, and nausea, as applied previously.^{6,15} Gastrointestinal symptom cards were completed daily throughout both trials. Clinically significant change of symptoms was defined as a change of at least 20 mm. Severity of fatigue was evaluated by the Daily-Fatigue Impact Scale (D-FIS),¹⁶ a questionnaire containing 8 items that evaluates the impact of fatigue on cognition, physical functioning, and daily activities. Accelerometry was used to objectively assess physical activity and sleep patterns.^{17,18} The participants were asked to wear the accelerometer (ActiGraph GT3X Accelerometer, LLC, Fort Walton Beach, FL) for 7 consecutive days, at all times during the baseline week and during each treatment arm.

Glutadin-specific T cells in the peripheral blood were assessed by an enzyme-linked immunospot assay in which the immunological readout is interferon gamma, as described previously,¹⁹ using commercially available kits (Mabtech, Nacka Strand, Sweden). Blood was taken from patients on day 0 and day 6 of each treatment week.

Sera from baseline and on day 6 of each treatment week were examined for antibodies to whole gliadin (IgA and IgG) and deamidated gliadin (IgA and IgG) by enzyme-linked immunosorbent assay using commercially available assays (INOVA Diagnostics, San Diego, CA). All tests were performed in conjunction with total IgA level. Serum from day 6 of each treatment week was analyzed for human eosinophil cationic protein by enzyme-linked immunosorbent assay (Cuasbio Biotech Co, Ltd, Newark, NJ) and for IgE antibodies to wheat by radioallergosorbent test (Phadia AB, Uppsala, Sweden). Assays were performed in duplicate according to manufacturer's instructions.

All feces passed from days 5–7 were collected during every randomized dietary arm. Volunteers were asked to collect all output during this 3-day period, avoiding urine contamination. The date and time of collection was noted on each container, which was then placed immediately into a –20°C portable freezer that was supplied. The fecal samples from each patient were thawed, combined, and weighed. The pH of an approximately 20-g aliquot warmed to room temperature was measured using a pH electrode probe and portable meter (Mettler Toledo InLab pH Combination Electrode, and AG FiveGo Duo reader, Schwerzenbach, Switzerland). The remainder of the feces was freeze dried to obtain a dry weight. The concentration of ammonia was measured enzymatically (Megazyme Ammonia Rapid Kit; Megazyme International Ireland Ltd, Wicklow, Ireland), and human β -defensin-2 (Immundiagnostik AG, Bensheim, Germany) and calprotectin (Bühlmann Laboratories AG, Schönenbuch, Switzerland) were analyzed by enzyme-linked immunosorbent assay.

For the 3-day trial, only gastrointestinal symptoms (via the VAS as mentioned) and severity of fatigue by the D-FIS were measured.

Statistical Analyses

Power calculations were based on previous data⁶ and allowed for dropout, missing data, and error rate, and assumed a measure of variance from that score (0.29). This indicated that 37 patients were required to achieve a power of 80%, at a 2-sided 5% significance level (if the true difference is 0.2).

Per-protocol analyses were performed. Comparisons of symptom severity scores and measured parameters across treatment periods were assessed by repeated measures analysis of variance or Friedman test, as appropriate. Paired *t* tests were used to compare the normally distributed data and Wilcoxon signed rank test to compare the nonparametric data. Spearman's correlations were used for associations between symptom severity and biomarkers. The reproducibility was assessed by the test-retest reliability by calculating the correlation between measured symptoms using the Pearson's correlation coefficient. High test-retest correlations indicate a more reliable sale. Two-tailed *P* values $\leq .05$ were considered statistically significant.

Results

Study Population

Subject flow is shown in [Supplementary Figure 1](#). After randomization for the 7-day trial, 3 patients were withdrawn due to poor symptom control during the run-in period. Thirty-seven patients completed the 7-day trial as per protocol. Twenty-two subjects returned to complete the 3-day rechallenge. The details of those patients are shown in [Table 1](#).

Dietary Adherence

For the 7-day trial, all 37 patients adhered to the GFD during the study and undertook all 3 treatment arms. Nearly all (98%) of the main meals during the interventional periods were consumed. Two patients ceased a study diet treatment arm prematurely because of intolerable symptoms. One patient was in the high-gluten arm and withdrew after 4 days, and the other was in the

Table 1. Study Subject Characteristics at Baseline

Characteristics	7-Day trial	3-Day rechallenge
No. of patients	37	22
Sex	6 male	5 male
Age, y, median (range)	45 (24–61)	48 (24–62)
Body mass index, median (range)	23 (17–39)	23 (17–32)
Predominant bowel habit, %		
Diarrhea	43	36
Constipation	35	46
Mixed/alternating	22	18
HLA type, %		
DQ2- or DQ8-positive	57	55
Celiac serology concentrations, U/mL, mean $\pm$ SEM (% elevated)		Not tested
Whole-gliadin (IgA)	27 $\pm$ 3 (37)	
Whole gliadin (IgG)	8 $\pm$ 1 (5)	
Deamidated gliadin (IgA)	17 $\pm$ 1 (13)	
Deamidated gliadin (IgG)	9 $\pm$ 2 (5)	

NOTE. Reference ranges for celiac antibody assays: negative <20 U/mL; weak positive 20–30 U/mL; strong positive >30 U/mL.

placebo arm and withdrew after 3 days. Serum and stool samples were collected from these patients upon cessation of the diet as per day 6. Mean consumption of each diet is detailed in [Supplementary Table 1](#). Five participants continued to consume their usual milk products (containing lactose) as they had previous negative lactose breath hydrogen tests. There was a significant decrease in dietary fiber and FODMAP intake during the run-in and also a mean decrease in energy content from 7.9 MJ per day during baseline to 7.3 MJ per day during the run-in.

For the 3-day rechallenge, all 22 volunteers undertook the 3 randomized treatment arms. One patient ceased the whey arm prematurely because of intolerable symptoms after lunch on the second day. Data continued to be collected as per day 3. Nearly all meals (96%–99%) were consumed in the dietary arms. All patients adhered to the gluten-free, low-FODMAP diet during the study. There were 7 participants who consumed snacks high in natural food chemicals (eg, 1 banana per day), but this did not differ across the treatment arms within participants.

Effect on Gastrointestinal Symptoms

Seven-day trial. Gastrointestinal symptoms during the baseline period varied across the patients with a median (range) of mean overall symptom scores of 12.1 mm (range, 0–55.7 mm). The average of symptoms from the second week of the low FODMAP run-in period generally improved compared with the baseline. This included overall symptoms ([Figure 1](#)), abdominal pain, bloating, satisfaction with stool consistency, wind, and tiredness (all $P < .0001$; Wilcoxon signed rank test), but not nausea ($P = .149$). Eight participants (22% of total cohort) had a mean improvement on the VAS for overall abdominal symptoms of >20 mm during the low FODMAP run-in period from their baseline level.

Overall symptoms and pain significantly worsened compared with mean scores during the last week of each dietary treatment period, irrespective of the diet, as

detailed in [Figure 2](#). Bloating and tiredness significantly worsened during low-gluten and placebo treatment arms only.

Only 6 participants (16% of total cohort) had a mean increase in overall abdominal symptoms of >20 mm on the high-gluten arm compared with those during the run-in period. Three of these patients were HLA-DQ2 positive and 3 were HLA-DQ2/8 negative. Only 1 of these patients also had a positive response to the low-gluten arm. Three patients also had a positive response to the placebo arm. One patient responded in all 3 arms. A dose effect of gluten was not observed and gluten specificity of symptomatic responses was observed in only 3 subjects (8% of the total cohort). Eleven participants (30%) had a positive response in overall symptom severity in the placebo arm, 8 of whom also reacted in the low-gluten arm. Only 1 of these 8 responded to the high-gluten arm. Seven subjects (19% of the total cohort) had whey-specific symptomatic responses.

Three-day rechallenge trial. There were no differences across the dietary treatment arms for change in overall symptoms on day 3 compared with the average during the baseline period. Changes in individual symptoms (eg, bloating, satisfaction with stool consistency, wind, pain, tiredness, and nausea) were similar across the 3 dietary periods (all $P > .209$; data not shown).

The reproducibility of participants' responses to gluten (16 g/d) and whey (16 g/d) between the 7-day challenge and the 3-day rechallenge was evaluated by comparing the change in severity of overall symptoms. There were no significant differences (shown in [Figure 3](#)) and those identified with a positive symptomatic response to gluten and whey differed between the 2 trials. The 2 participants who had a mean increase on the VAS for overall abdominal symptoms of >20 mm on the gluten (16 g/d) arm in the 7-day trial were not the same 2 participants who had a positive response to the gluten (16 g/d) arm in the 3-day rechallenge ([Figure 3A](#)). Gluten specificity was not reproduced in any subject. Six participants had a positive response in overall symptom severity in the whey (16 g/d) arm in the 7-day trial, one of whom also reacted to the whey (16 g/d) arm in the 3-day rechallenge. Three different participants also had a positive whey response in the 3-day rechallenge ([Figure 3B](#)). Only 1 subject reproduced their whey-specific symptomatic response.

Re-test reliability in mean change in overall symptom severity score (mm) between the 2 challenges showed no correlation for either gluten (Pearson $r = -0.04$; $P = .858$) or whey (Pearson $r = 0.08$; $P = .748$) treatment arms.

For both studies, several patient-related factors were examined in terms of their association with symptomatic responses to the diets in the 7- and 3-day dietary challenges. The predominant bowel habits, body mass index, age, sex, duration of GFD and HLA-DQ status did not predict the responses to any of the diets (Spearman's correlation and χ^2 analysis, data not shown).

In both studies, the order of the dietary interventions was associated with the degree of symptomatic response. In the 7-day study, the first intervention significantly

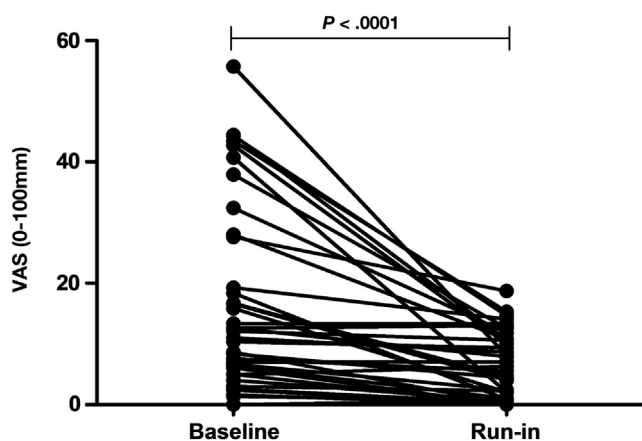


Figure 1. Individual responses in mean overall symptom severity score during the run-in period, where low FODMAP diet was commenced, compared with the baseline period, where participant's usual gluten-free diet was consumed during 7-day trial. Scores were significantly greater during the baseline period ($P < .0001$, Wilcoxon signed rank test).

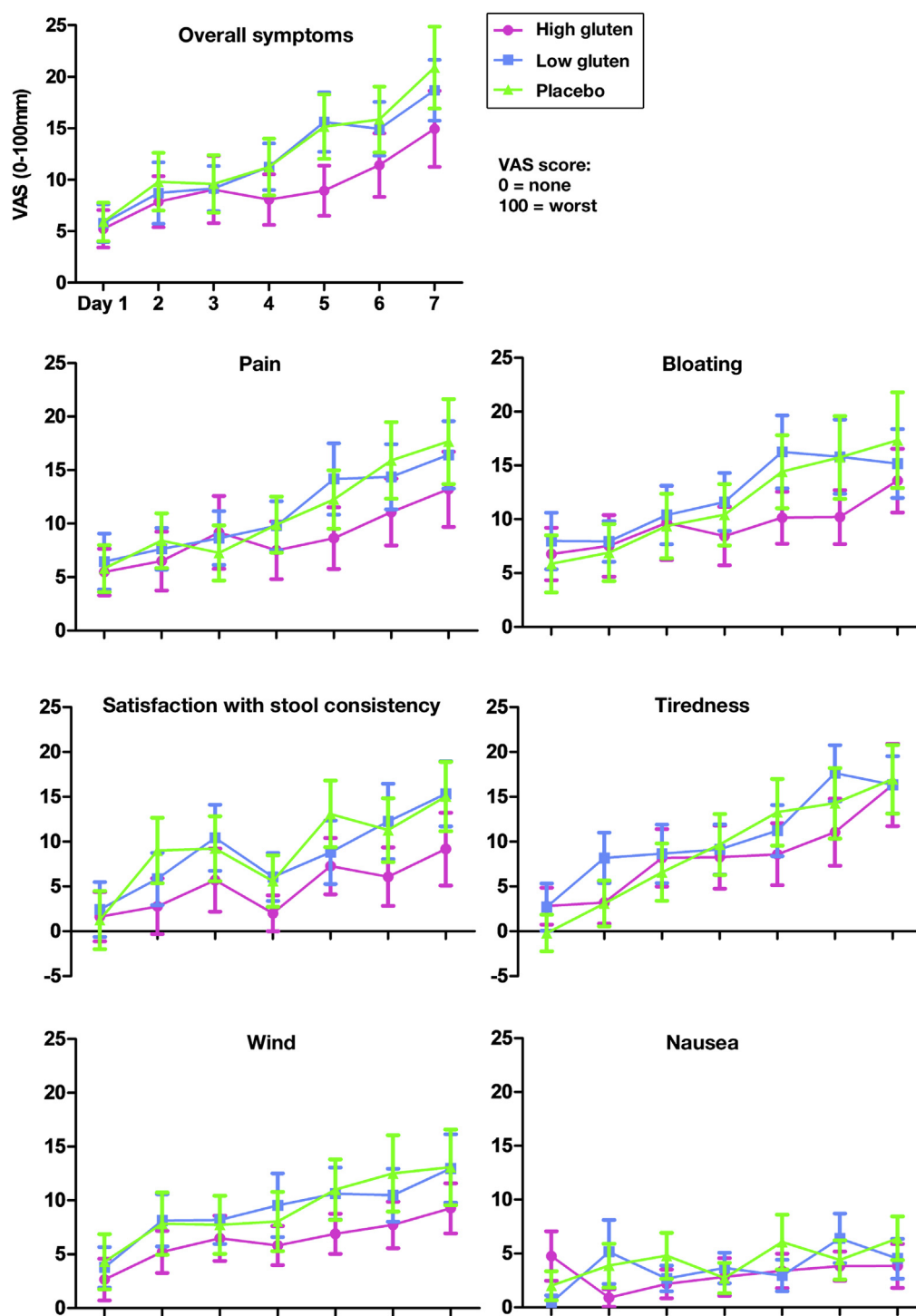


Figure 2. Change in symptom severity from run-in for each dietary treatment over 7-day study period. Data shown represent mean $\pm$ SEM. Differences across the treatment arms were compared by Friedman test, in which overall symptoms ($P = .001$), bloating ($P = .016$), satisfaction with stool consistency ($P = .008$), and wind ($P = .003$) were statistically significant, but abdominal pain ($P = .085$), tiredness ($P = .305$), and nausea ($P = .486$) were not.

induced greater symptomatic changes than subsequent challenges, regardless of what it contained (Figure 4A). Likewise, there was a significant difference across the 3 groups ($P = .044$; repeated measures analysis of variance) in the 3-day rechallenge (Figure 4B), with the first intervention being associated with greater symptomatic changes (mean, 15.5 mm) than the second (mean, 5.3 mm) or third (mean, 4.0 mm) challenges, regardless of its content.

Effect on fatigue, physical activity, and sleep. For the 7-day trial, the low FODMAP run-in period was associated with the lowest mean D-FIS score (mean $\pm$ SEM, 1.95 ± 0.53), which was significantly less than that in the baseline period (5.04 ± 0.87 ; $P = .0006$, paired t test). There were no differences in levels of fatigue across or during the dietary treatment arms, but there was a significant increase compared with the run-in period for high gluten (2.19 ± 0.76 ; $P = .005$), low gluten ($2.87 \pm$

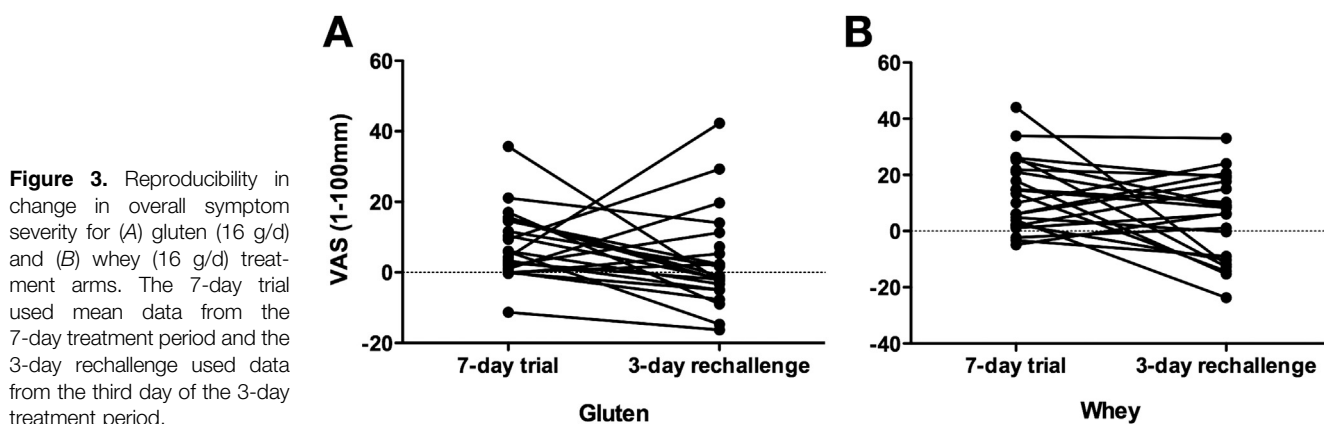


Figure 3. Reproducibility in change in overall symptom severity for (A) gluten (16 g/d) and (B) whey (16 g/d) treatment arms. The 7-day trial used mean data from the 7-day treatment period and the 3-day rechallenge used data from the third day of the 3-day treatment period.

0.77; $P = .004$), and placebo (2.41 ± 0.81 ; $P = .003$) for the 7-day trial. There were no differences for gluten (2.05 ± 1.44), whey (1.85 ± 1.03), and placebo (2.42 ± 1.45) for the 3-day rechallenge. There were no apparent effects of dietary treatment on activity levels or any sleep measure analyzed by accelerometry (data shown in [Supplementary Table 2](#)).

Effect on gliadin-specific T-cell responses. All subjects responded to one or both of the positive controls (tetanus toxoid and phytohemagglutinin; [Supplementary Figure 2](#)). Only 1 participant elicited a positive T-cell response after the high-gluten (16 g/d) challenge, and her day-6 response was a >3 -fold change from day 0 ([Supplementary Figure 2A](#)), a response similar to those reported in patients with celiac disease.¹⁹

Effect on other biomarkers. There were no significant differences across the treatment periods for serological or other blood markers, eosinophil cationic protein, or radioallergosorbent test, for the whole sample ($N = 37$), the gluten responders ($n = 6$) or the placebo responders ($n = 11$) ([Table 2](#)). Likewise, fecal wet and dry weight, pH, and concentrations of human β -defensin-2, calprotectin, and ammonia levels were similar across the treatment groups. No correlation existed between mean overall symptom score on high-gluten and any of the markers. There was no apparent trend for those patients

who had elevated scores on any biomarker with those who demonstrated a gluten- or whey-specific symptom response. No differences in the response of biomarkers to high-gluten exposure were noted according to HLA-D status.

Discussion

Generally, NCGS is viewed as a defined illness, much like celiac disease, where gluten is the cause and trigger for symptoms. In such a case, it would be anticipated that removal of gluten from the diet would lead to minimal symptoms and subsequent exposure to gluten would lead to specific triggering of symptoms. The results of the current study have not supported this concept. First, some of the patients were not minimally symptomatic, despite apparent adherence to and previous considerable improvement on a GFD. Reduction of FODMAPs in their diets uniformly reduced gastrointestinal symptoms and fatigue in the run-in period, after which they were minimally symptomatic. Secondly, in 2 double-blind, randomized, placebo-controlled, cross-over trials, specific and reproducible induction of symptoms with gluten could not be demonstrated.

Such findings must be reconciled with the results of our recent double-blind, randomized trial, in which gluten

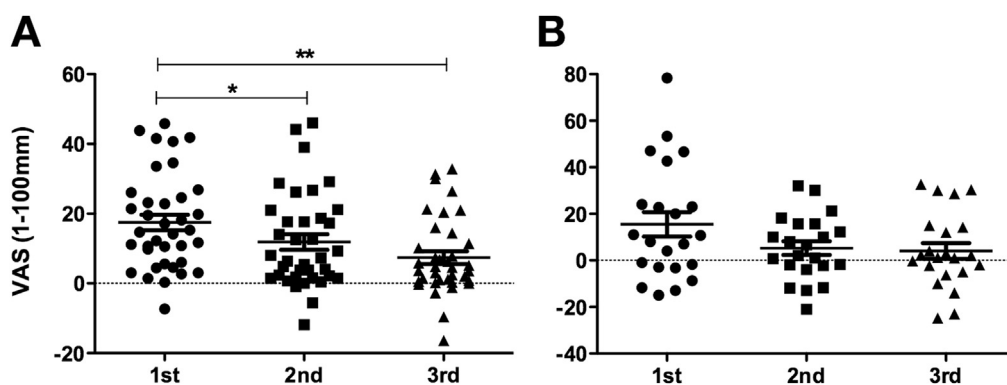


Figure 4. Mean change in overall symptom severity grouped in order of treatment arm received during (A) 7-day trial and (B) 3-day rechallenge. Differences were compared by repeated-measures analysis of variance (7-day trial: $P = .001$; 3-day rechallenge: $P = .044$). Differences were also compared between each group by a paired t test (7-day trial: $*P = .026$; $**P = .001$; 3-day rechallenge: all, $P > .058$).

Table 2. Celiac Serology, Biomarker, and Fecal Characteristic Results During Treatment Periods Biomarker

	High gluten	Low gluten	Placebo
Celiac serology, U/mL			
Whole gliadin (IgA)	19 ± 3.6 (21)	19 ± 3.4 (13)	17 ± 1.6 (13)
Whole gliadin (IgG)	11 ± 2.9 (8)	9.4 ± 1.8 (5)	11 ± 2.1 (8)
Deamidated gliadin (IgA)	16 ± 1.4 (8)	15 ± 1.4 (3)	14 ± 1.2 (5)
Deamidated gliadin (IgG)	8.7 ± 1.5 (8)	8.8 ± 1.6 (11)	9.3 ± 1.5 (11)
Human β -defensin-2, ng/mL	35 ± 4.9 (21)	33 ± 4.8 (24)	34 ± 5.6 (29)
Eosinophil cationic protein, ng/mL	3.6 ± 0.6 (3)	3.5 ± 0.6 (3)	3.4 ± 0.5 (3)
Radioallergosorbent test, kU/L	0.09 ± 0.05 (0)	0.07 ± 0.02 (0)	0.07 ± 0.02 (0)
Frequency, times/d, median (range)	1 (0–4)	1 (0–3)	1 (0–4)
Output, g wet wt/d	127 ± 14	113 ± 11.5	124 ± 13
Dry wt/d, %	25 ± 1.3	25 ± 1.1	26 ± 1.1
Fecal pH	6.9 ± 0.06	6.9 ± 0.07	6.9 ± 0.06
Fecal ammonia, μ g/L	316 ± 23	328 ± 24	336 ± 25
Calprotectin, μ g/g	31 ± 9.1	33 ± 7.8	26 ± 7.7

NOTE. Data shown as mean $\pm$ SEM (% elevated) unless otherwise indicated. There were no significant differences for diet difference on any measure (compared by repeated measures analysis of variance or Friedman test as appropriate). Healthy reference ranges: human β -defensin-2 (<46.4 ng/mL), eosinophil cationic protein (2.45–14.12 ng/mL), radioallergosorbent test (<50.01–100 kU/L), fecal calprotectin (<50 μ g/g). Reference ranges for celiac antibody assays: negative <20 U/mL; weak positive 20–30 U/mL; strong positive >30 U/mL.

induced greater gastrointestinal symptoms and fatigue than did placebo in an identically selected population of patients who fulfilled the criteria for NCGS.⁶ Several key differences in study design might have potentially influenced the results. First, in contrast to the previous use of supplements with the habitual diet, food intake was carefully controlled. All food provided was low in FODMAPs and gluten-free to reduce “background noise” and control for changes in participants’ usual diet, particularly intake of other potential dietary triggers. FODMAPs were especially important because they are well-documented inducers of gastrointestinal symptoms¹⁰ and some of the patients in the current study reported intolerance to one (38%) or multiple (27%) foods containing FODMAPs (unpublished observations). Symptoms uniformly improved after instruction on restricting FODMAPs in the run-in period. Although the fiber intake might have altered with this dietary change, education was given to alternative low FODMAP sources of fiber, and fiber alteration is not a reliable means of improving symptoms in IBS.

The restriction of all dairy products and food chemicals was also employed in the rechallenge trial in order to control other putative triggers of gut and other symptoms.¹⁰ This ensured that known potential dietary confounders capable of inducing symptoms were minimized and that the only difference between the treatments was the nature of the protein intake. It is possible, however, that provision of foods not normally consumed as part of some participants’ diets might have led to negative associations of these foods with symptom induction and obscured their actual response to the challenges.

The third difference was the utilization of a crossover design to reduce the influence of confounders and increase power. Adequate washout and run-in periods were employed (confirmed with checking of symptom diaries) to minimize carry-over and order effects.^{10,20} Although there has been previous reserved criticism for the use of a

cross-over design within the IBS population,²¹ they have been used successfully in rechallenge dietary studies with IBS subjects.^{15,22} However, in the current patient population, an order effect was apparent in both the 7-day and 3-day studies indicating that, in this patient group, a strong anticipatory symptomatic (ie, nocebo) response was present independently of the nature of the challenge protein.

Fourthly, the duration of treatment was reduced from 6 weeks to 1 week on the basis that symptoms were uniformly induced within the first week of the original study. It is unlikely that a longer time frame of challenge would capture any delayed responses to gluten, as the 3 gluten responders in the current study reached their highest symptom level at day 3. This also formed the rationale for the 3-day rechallenge study duration.

Fifthly, the high participant burden and rigorous demands of the 7-day trial included frequent visits to clinic for blood taking, fecal collection, wearing accelerometers, and completion of daily questionnaires, all while following a restrictive diet. This might have been perceived as stressful and might have contributed to the nocebo effect and, therefore, positive symptomatic responses across all treatment arms. This was at least partly addressed in the subsequent study by considerable simplification of the 3-day rechallenge, which was purposely designed to be of a short-duration, highly controlled and with less participant effort and application. Regardless, a nocebo response was again found.

Finally, pure lactose-free whey protein isolate was used as the placebo in the 7-day trial to balance overall protein levels. It was chosen for its rapid digestibility²³ and the minimal effects it had on the study food’s texture and flavor. The results from the 7-day trial suggested that whey protein itself might have triggered symptoms in some patients. However, the effects of whey protein independent of gluten were not reproduced in the 3-day rechallenge.

Although such methodological criticisms can be waged against the current studies, most patients did not

exacerbate their symptoms when exposed to gluten. In addition, multiple potential biomarkers that are associated with food-related gut disorders were performed as objective end points. The serological pattern was mostly negative, but there were a lower proportion of cases with positive IgG AGA compared with recent data on gluten sensitivity.²⁴ Concordant with symptomatic responses, no biomarker-specific changes were shown in the patients who had gluten- or whey-specific symptoms induced, nor were there any trends among participants who had inconsistent or elevated biomarker results. In addition, given the apparently specific effect of gluten on fatigue when measured by a simple VAS in the previous study,⁶ the use of validated tools (D-FIS and accelerometry) for assessing fatigue also failed to show any gluten-specific effects.

A key feature of the current study was the care taken in selection of participants for the study. They were sought by advertising and underwent careful screening to ensure celiac disease was not present. This included HLA-DQ assessment (non-celiac haplotype in 47%) and assurance that those with an at-risk haplotype had duodenal biopsies that were performed while taking adequate gluten (assessed historically) with normal histopathology. Other studies of NCGS have often included patients with increased density of intraepithelial lymphocytes,²⁵ increasing the risk that patients with latent celiac disease are being included. None had evidence of wheat allergy (negative radioallergosorbent test). In addition, all patients were assessed for gliadin-specific T cells using the enzyme-linked immunospot assay, performed using the methodology that identifies celiac-specific responses.¹⁹ Only 1 patient demonstrated a positive response, but follow-up testing was negative and her duodenal biopsy on gluten was normal. The other essential inclusion criterion was that the participants had well-controlled symptoms on a GFD pre-enrollment. When assessed by a VAS, 11 patients rated their gastrointestinal symptoms >20 mm for overall abdominal symptoms and 22% experienced clinically significant improvement (change >20 mm) in overall symptoms. However, all patients indicated that they were markedly improved on the GFD and their symptoms were well controlled as per the entry criteria. This same feature was noted in the previous gluten-challenge study.⁶ In order to participate in the study, the patients were carefully selected to fulfill current criteria for NCGS. It is likely then that these criteria need modification so that the issue of whether gluten is indeed a trigger for gut and other symptoms in the broader IBS population can be addressed.

It is possible that the gluten used in the current study was different from that in the first (suppliers were different). The gluten content was similar, but the non-gluten proteins were not characterized. For instance, α -amylase/trypsin inhibitors²⁶ might have been present in the previously reported study only. However, evidence points to inflammatory mechanisms by which they might

induce symptoms²⁷ and no evidence of such a process was found in that study.

Alternatively, gluten might induce symptoms only in the presence of a moderate content of FODMAPs. Many gluten-containing cereals are high in fructans, which are a problem in patients with IBS¹⁵ and their concomitant reduction with the introduction of the GFD might lead to improved gut symptoms, wrongly perceived to be due to a reduction in gluten intake. Gluten is hypothesized to have direct effects on the brain leading to depression and other neurological maladies.²⁸ Although fatigue did not change in the current study with exposure to gluten, it was a prominent effect in the initial study. More focused attention to anxiety and depression rather than fatigue might provide additional clues to why patients who follow a GFD feel better. One mechanism by which this interaction might work is that FODMAPs are predominant triggers of gut symptoms and gluten is the predominant trigger for a loss of wellness. This intriguing potential interaction deserves additional investigation.

In conclusion, these consecutive double-blind, randomized, placebo-controlled, cross-over rechallenge studies showed no evidence of specific or dose-dependent effects of gluten in patients with NCGS placed on a low FODMAP diet. A high nocebo response was found regardless of known background dietary triggers being controlled and reproducibility of symptom induction to a specific protein was poor. These data suggest that NCGS, as currently defined, might not be a discrete entity or that this entity might be confounded by FODMAP restriction, and that, at least in this highly selected cohort, gluten might not be a specific trigger of functional gut symptoms once dietary FODMAPs are reduced.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2013.04.051>.

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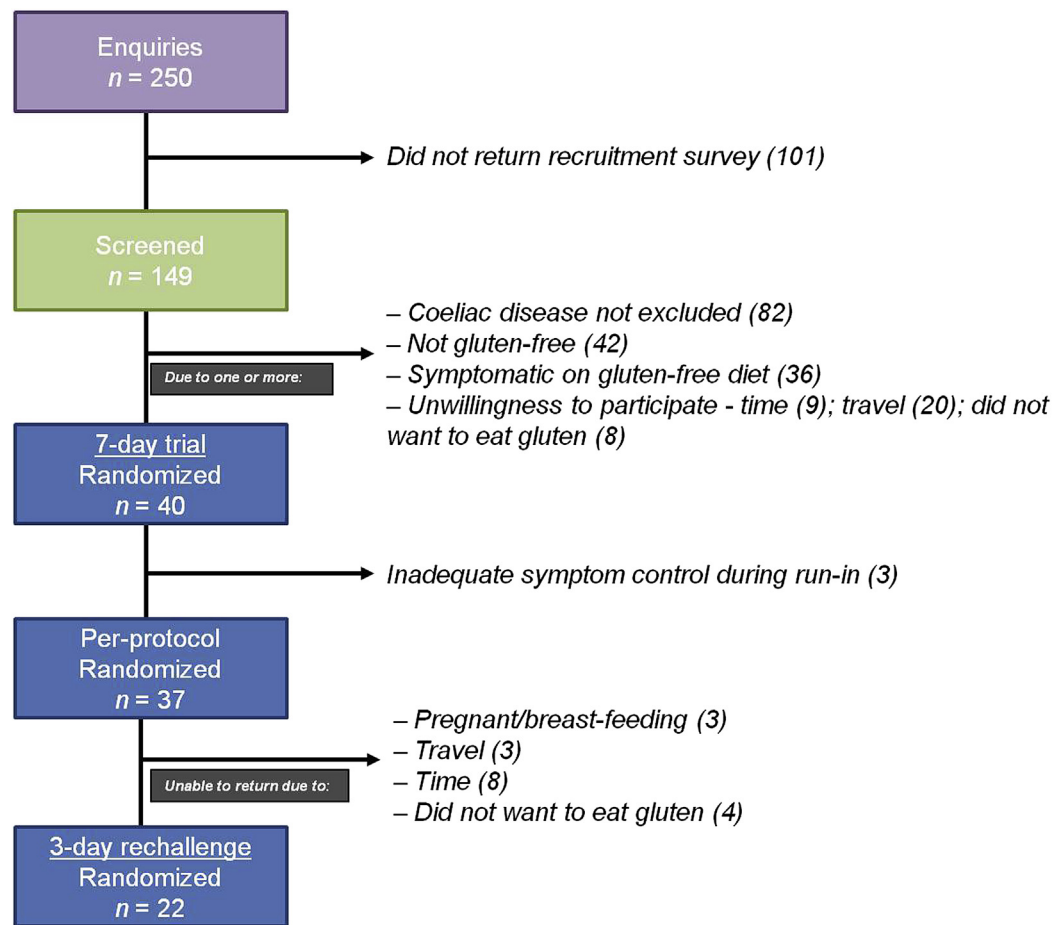
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Conflicts of interest

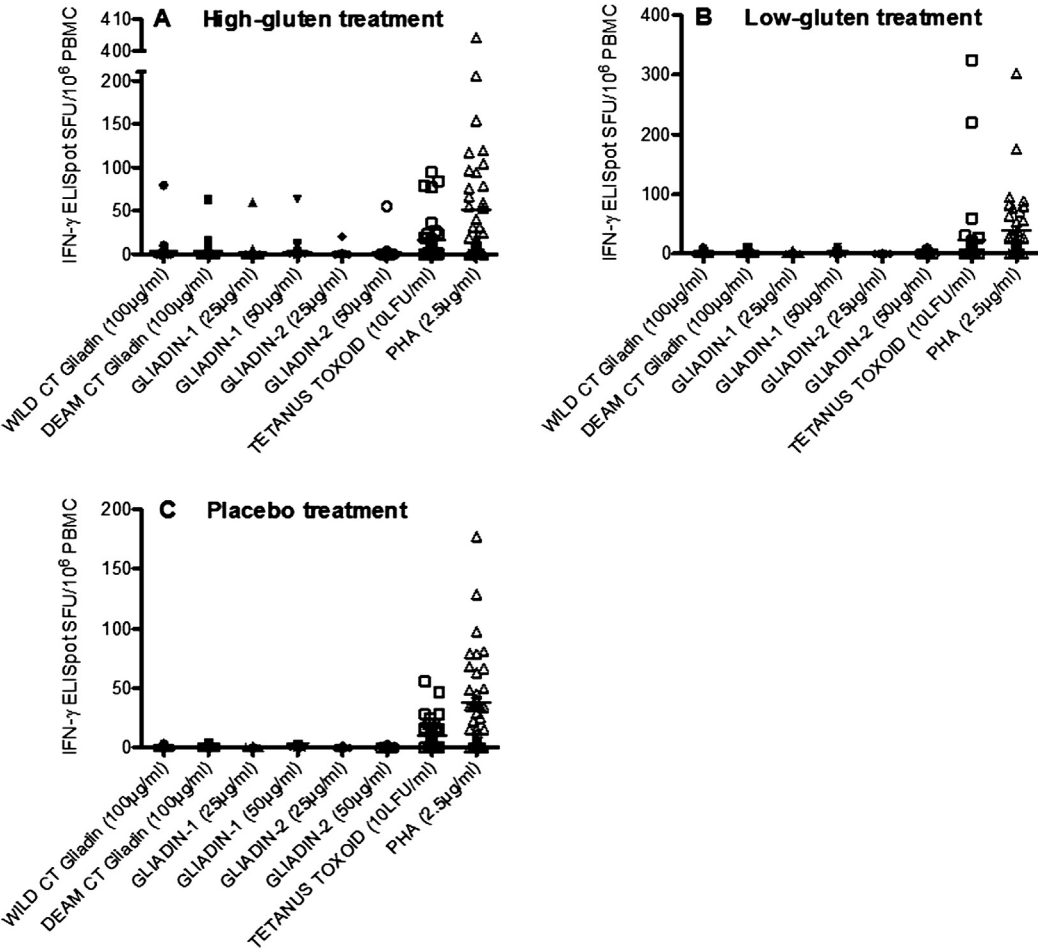
Peter R. Gibson discloses the following: He has published a book on a diet for irritable bowel syndrome. The remaining authors disclose no conflicts.

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Supplementary Figure 1. Recruitment pathway and reasons for screen failure. Recruitment survey was a 23-item questionnaire about symptoms, diet, and investigations for celiac disease described previously.²⁰



Supplementary Figure 2. Interferon-γ (IFN-γ) ELISpot responses of peripheral blood mononuclear cells (PBMC) from study participants after a gluten-free diet for ≥2 weeks in all study participants (n=37) on day 6 after commencing a 7-day treatment period in a random order of (A) high-gluten (16 g/d), (B) low-gluten (2 g/d), and (C) placebo (0 g/d). SFU, spot forming units.

Supplementary Table 1. Actual Daily Dietary Intake During Each Phase of the 7-Day Trial

Dietary component	Baseline	Run-in	<i>P</i> value	High gluten	Low gluten	Placebo	<i>P</i> value
Energy, <i>MJ</i>	7.9 ± 0.3	7.3 ± 0.3	.003	7.9 ± 0.2	8.1 ± 0.2	8.0 ± 1.8	NS
Protein, <i>g</i>	83 ± 3.2	84 ± 3.9	NS	76 ± 1.8	78 ± 2.1	77 ± 1.9	NS
Total fat, <i>g</i>	69 ± 3.0	67 ± 3.6	NS	75 ± 1.8	76 ± 1.9	75 ± 1.9	NS
Total starch, <i>g</i>	118 ± 5.9	113 ± 6.3	NS	134 ± 3.4	135 ± 2.6	135 ± 3.1	NS
Dietary fiber, <i>g</i>	23 ± 2.3	19 ± 1.9	<.0001	26 ± 0.6	26 ± 0.6	26 ± 0.6	NS
Carbohydrates	210 ± 8.7	183 ± 9.2	.001	215 ± 5.3	221 ± 5.5	220 ± 5.3	NS
Monosaccharides, <i>g</i>							
Glucose	18 ± 1.6	15 ± 1.1	.017	21 ± 1.0	23 ± 1.5	21 ± 1.2	NS
Fructose	15 ± 1.5	9.5 ± 0.7	.001	12 ± 0.7	13 ± 0.9	12 ± 0.7	NS
Disaccharides, <i>g</i>							
Sucrose	28 ± 3.3	21 ± 2.7	.001	24 ± 1.2	26 ± 1.6	25 ± 1.4	NS
Lactose	14 ± 2.0	9.8 ± 1.1	.030	2.4 ± 0.7	3.3 ± 1.1	3.5 ± 1.2	NS
Sugar polyols, <i>g</i>							
Sorbitol	1.1 ± 0.2	0.4 ± 0.07	<.0001	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.02	NS
Mannitol	0.4 ± 0.05	0.2 ± 0.03	.011	0.4 ± 0.02	0.4 ± 0.02	0.4 ± 0.02	NS
Oligosaccharides, <i>g</i>							
Fructans	1.5 ± 0.1	1.2 ± 0.1	.011	0.9 ± 0.03	0.9 ± 0.03	0.8 ± 0.03	NS
GOS	1.1 ± 0.2	1.0 ± 0.1	NS	0.5 ± 0.1	0.5 ± 0.09	0.4 ± 0.08	NS
Total FODMAPs, <i>g</i>	19 ± 2.0	12 ± 1.1	.003	4.3 ± 0.7	5.2 ± 1.1	5.4 ± 1.2	NS
Alcohol, <i>g</i>	12 ± 5.8	6.7 ± 1.4	NS	2.6 ± 0.8	3.7 ± 1.1	2.9 ± 0.9	NS

NOTE. Total FODMAPs was calculated as the sum of excess fructose (fructose minus glucose), lactose, sorbitol, mannitol, fructans, and galacto-oligosaccharides (GOS). Foods were analysed directly as described previously.^{12,13} Results from laboratory analysis were added to the Foodworks database and are expressed as mean ± SEM. Comparisons were made using Wilcoxon signed rank or Friedman test.

NS, not significant.

Supplementary Table 2. Physical Activity and Sleep Characteristics of Study Participants for 7-Day Trial

Characteristic	Baseline	High gluten	Low gluten	Placebo
Activity, <i>kJ/d</i>	1732 ± 146	1766 ± 134	1678 ± 121	1640 ± 121
% of Time spent at each activity level while accelerometer worn				
Sedentary	76 ± 1.0	75 ± 0.9	76 ± 1.0	76 ± 1.0
Light intensity	16 ± 0.7	17 ± 0.7	16 ± 0.6	16 ± 0.8
Lifestyle	5.7 ± 0.4	5.6 ± 0.2	5.8 ± 0.4	5.6 ± 0.4
Moderate intensity	2.2 ± 0.3	2.3 ± 0.2	2.1 ± 0.2	2.3 ± 0.2
Vigorous intensity	0.1 ± 0.06	0.1 ± 0.05	0.1 ± 0.04	0.1 ± 0.06
Bout				
No. of bouts	4.4 ± 0.8	4.5 ± 0.7	4.5 ± 0.8	3.8 ± 0.7
Time in bout, <i>min</i>	13 ± 1.2	13 ± 1.3	12 ± 1.3	12 ± 1.3
Sleep characteristics				
Latency, ^a <i>min</i>	3.6 ± 0.5	3.8 ± 0.6	3.5 ± 0.5	3.2 ± 0.6
Efficiency	95 ± 0.5	95 ± 0.5	95 ± 0.5	94 ± 0.5
Time in bed, <i>min</i>	499 ± 10	488 ± 7.7	491 ± 9.0	498 ± 8.6
Total sleep time, <i>min</i>	471 ± 9.3	461 ± 7.8	466 ± 8.5	469 ± 8.1
No. of awakenings	8.1 ± 0.9	7.5 ± 0.7	7.4 ± 0.8	7.4 ± 0.7
Mean awakening length, <i>min</i>	3.8 ± 0.2	3.9 ± 0.3	3.8 ± 0.3	4.1 ± 0.3

NOTE. Values are mean ± SEM. There were no significant differences for diet difference on any measure, compared by repeated-measures analysis of variance.

^aLatency is the time taken to fall sleep.