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Original article

Influence of low FODMAP and gluten-free diets on disease activity and intestinal microbiota in patients with non-celiac gluten sensitivity

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SUMMARY

Background & aims: Non-celiac gluten sensitivity (NCGS) is characterized by intestinal and extra-intestinal symptoms triggered by ingestion of gluten. However, non-gluten triggers have recently been implicated, and a FODMAP (fermentable oligo-, di-, monosaccharides and polyols)-reduced diet can partially improve symptoms in NCGS. Our aim was to analyze the effect of a low FODMAP versus a gluten-free diet (GFD) on clinical symptoms, psychological well-being, intestinal inflammation and integrity, and stool microbiota.

Methods: Nineteen patients with NCGS and ten healthy controls consumed a gluten-containing standard diet before starting a two-week low FODMAP diet; after a five day transition period, participants ingested a GFD for another two weeks. The primary outcome measure was the improvement of clinical symptoms in NCGS patients under the different diets. Secondary outcomes were the determination of dietary effects on intestinal inflammation, psychological well-being, and differences in stool microbiota between NCGS patients and controls.

Results: The low FODMAP diet and especially the GFD led to a significant improvement of clinical and psychological symptoms in NCGS. A clear reduction in duodenal intraepithelial lymphocytes and mucin-producing Goblet cells was found after the GFD in these patients. Significant microbial differences between NCGS patients and controls were noticed in stool samples at every time point. Both diets caused microbial shifts in all participants, with a greater variability on genus level and metabolisms groups in NCGS patients.

Conclusions: Our findings suggest a multifactorial etiology of NCGS, due to a functional effect caused by FODMAPs, combined with a mild gluten-triggered immune reaction, and a microbiota dysbalance.

ClinicalTrial.gov ID: : NCT03268720.

1. Introduction

Cereals and especially wheat products are the main carbohydrate sources in Western diets. Interestingly, during the last years an increasing number of patients have reported gastrointestinal as well as extra-intestinal symptoms after eating gluten-containing cereal products. Celiac disease is well characterized by a destructive intestinal immune response against immunogenic gluten peptides in genetically predisposed individuals, affecting about 1% of most populations worldwide [1]. Non-celiac gluten sensitivity

Abbreviations: ANOVA, analysis of variance; ATI, amylase-trypsin-inhibitor; BMI, body mass index; FODMAP, fermentable oligo-, di- and monosaccharides and polyols; IBS, irritable bowel syndrome; IEL, intraepithelial lymphocyte; MWU, Mann-Whitney-U test; NCGS, non-celiac gluten sensitivity; PAS, periodic acid-Schiff's reagent; PGWB, psychological general well-being index; GSRS, gastrointestinal symptom rating scale; RAST, radioallergosorbent test.

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(NCGS) is now considered as a distinct clinical entity, which is caused by wheat consumption [2,3]. In contrast to patients with celiac disease, there are no specific serological markers for NCGS. Increased serum IgG antibodies against gliadin were described in approximately half of the patients [3,4], but other studies questioned these antibodies as low prognostic markers [5,6]. No mucosal damage can be observed in patients with NCGS, and only some patients display moderately increased numbers of small intestinal intraepithelial lymphocytes (IEL) [3,7]. The symptom complex that is dominated by abdominal pain, irregular bowel movements, muscle, head and body aches, and severe fatigue, improves under a gluten-free diet (GFD) and symptoms rapidly recur after ingestion of gluten-containing cereals. Nevertheless, the causative role of gluten is still unclear, even after double-blind placebo controlled studies [7,8]. Probably other wheat components, e.g. carbohydrates or amylase-trypsin-inhibitors (ATI) are in part responsible for symptoms in NCGS. In this context, it was shown that gluten-containing cereals possess high amounts of ATIs which are highly resistant to food processing and digestion. ATIs are able to stimulate the naive immune system by activating gut myeloid cells and thus might trigger or sustain intestinal inflammation [9,10].

Recently, a FODMAP-reduced diet (fermentable oligo-, di- and monosaccharides and polyols) has received increasing interest and this diet has shown a distinct clinical improvement, especially in patients with irritable bowel syndrome (IBS) [11,12]. Interestingly, also patients with NCGS often complain about disorders that correlate with FODMAP consumption, and some patients with NCGS seem to benefit from a low FODMAP diet with improvement of gastrointestinal symptoms [8,13].

In addition, the intestinal microbiota gained increasing interest, since they are considered as basis for a healthy gut, and a dysbiosis was assumed to be responsible for various metabolic and inflammatory diseases. Beside genetics, ethnicity, age, infections and drugs, the nutrition has a significant influence on the intestinal microbiota pattern [14–16].

We are not aware of any study that has addressed the influence of different sequential diets on the intestinal microbiota and their correlation with disease-relevant scores and signs of intestinal immune activation, such as IELs and Goblet cells in NCGS patients. Therefore, we studied the effect of a low FODMAP diet followed by a GFD on the gastrointestinal and psychological symptoms of NCGS patients, the mucosal inflammation and diet-dependent changes in the microbiota of NCGS patients compared to healthy controls.

2. Methods

2.1. Subjects

Patients were recruited in the specialized outpatient clinic of the Medical Clinic 1, University Hospital Erlangen, between 2014 and 2016. Patients with autoimmune disorders like Hashimoto thyroiditis were excluded, to rule out primary or secondary effects of a deranged immune system. Eighty seven patients reported self-diagnosed wheat intolerance with fast resolution on a GFD. 19 patients with NCGS were enrolled in the study. Patients with wheat allergy, celiac disease, unclear endoscopic results, doubts that wheat was the main trigger, not accepting a wheat challenge, or non-compliant with diets were excluded. All our patients complained about intestinal symptoms, and with the exception of two patients also about extra-intestinal symptoms. The symptoms appeared shortly (within one and 12 h) after ingestion of wheat products. None of the NCGS patients had first-degree relatives with celiac disease. Ten age- and gender-matched healthy controls without any diseases and complaints on standard diets were included. At the beginning of the study, age, sex, medications, complaints and underlying diagnoses were recorded, and sera were analyzed for routine parameters, vitamin levels and micronutrients, signs of infectious diseases, and a complete blood count (Table 1).

2.2. Sugar intolerance

Two of our NCGS patients reported an intolerance to fructose, one patient to lactose, three to sorbitol, and two patients to lactose and sorbitol, or lactose and fructose (data not shown). All these patients avoided the relevant sugars already before the beginning of the study but still had clinical complaints that resolved on the gluten (wheat)-free diet and thus were characterized as patients with NCGS.

2.3. Exclusion of wheat allergy

To exclude wheat allergy, total and wheat-specific immunoglobulin E antibodies were determined from all participants before the start of the study (ImmunoCAP™250, ThermoFisher Scientific, Germany). In addition, a prick test was used to exclude cutaneous wheat sensitization.

2.4. Exclusion of celiac disease

At baseline, celiac specific serum IgA and IgG titers against tissue transglutaminase and deamidated gliadin peptides were determined from all participants of the study using the Eu-tTG IgA, Eu-tTG IgG, α-GliaPep IgA, and α-GliaPep IgG test (Eurospital S.p.A., Italy) to exclude celiac disease. In addition, patients with NCGS underwent a gastroduodenoscopy and celiac disease was ruled out by histological mucosal analysis of four to five duodenal biopsies according to Marsh criteria.

2.5. Follow-up gastroduodenoscopy from patients with NCGS

At the end of the study, patients with NCGS were offered a follow-up gastroduodenoscopy in case of clinical indication or persisting symptoms (IEL >40/100 enterocytes, n = 2; reflux oesophagitis, n = 6; type C gastritis, n = 3; stomach or duodenal irritation, n = 6) and sampling of one duodenal biopsy was performed (17 patients). Due to ethical constrains, no gastroduodenoscopy was requested from healthy controls, and no follow-up gastroduodenoscopy was offered to two NCGS patients without any complaints at the end of the study.

2.6. Immunohistochemistry

When NCGS patients underwent gastroduodenoscopy an additional biopsy to determine intraepithelial lymphocytes (IEL) and Goblet cells was taken. IEL were quantified on paraformaldehyde-fixed 4 μm sections using anti-human CD3 antibody (BioRad MCA1477, Germany). Detection was done with diaminobenzidine (DAB; ImmPACT™ DAB Substrate Kit, Vector Laboratories, USA) and slides counterstained with hemalaun. Goblet cells were labeled with periodic acid-Schiff's reagent (Sigma–Aldrich, Germany). Goblet cells were also determined on duodenal tissue sections derived from 8 healthy individuals who had gastroduodenoscopy for medical checkup. Numbers of CD3-positive IELs and Goblet cells were counted per 100 epithelial cells in two (for CD3-IELs) or three (for Goblet cells) different areas by the same investigator.

Table 1
Demographic, serologic and clinical characteristics of study participants. Statistical significances were calculated with the t-test for independent scaled values, and the Chi-square-test.

Demographic characteristics	all values in mean ± standard deviation or % (n)		
	Controls	NCGS	p-value
Number [n]	10	19	–
Age [years]	32.8 ± 10.9	33.8 ± 11.9	0.820
Sex [% female (w/m)]	70.0 (7/3)	78.9 (15/4)	0.593
Body mass index [kg/m^2]	20.9 ± 2.8	22.9 ± 4.7	0.214
Blood parameters			
Liver function			
Aspartate transaminase (AST) [U/ml]	21.3 ± 3.1	26.9 ± 10.5	**0.040***
Alanine transaminase (ALT) [U/ml]	18.9 ± 6.2	27.1 ± 21.4	0.134
Gamma-glutamyl transferase (γ-GT) [U/ml]	24.5 ± 12.4	17.8 ± 9.9	0.126
Alkaline phosphatase (ALP) [U/ml]	52.9 ± 14.4	64.9 ± 11.8	**0.023***
Vitamins and micronutrients			
Iron [μg/dl]	113.3 ± 41.4	104.4 ± 41.5	0.589
Folate [ng/ml]	10.9 ± 3.4	10.8 ± 2.6	0.925
Vitamin B6 [ng/ml]	18.2 ± 7.9	13.2 ± 6.8	0.217
Vitamin B12 [pg/ml]	464.6 ± 178.6	377.8 ± 130.7	0.152
Zinc [μg/dl]	79.1 ± 11.2	88.8 ± 16.2	0.102
Selenium [μg/dl]	94.2 ± 16.7	96.8 ± 22.6	0.764
Differential blood count			
Neutrophils [%]	57.9 ± 11.6	59.0 ± 9.1	0.782
Eosinophils [%]	1.2 ± 0.4	1.8 ± 1.1	0.066
Basophils [%]	0.7 ± 0.5	0.8 ± 0.4	0.657
Lymphocytes [%]	33.8 ± 10.1	30.8 ± 8.5	0.413
Monocytes [%]	6.3 ± 2.2	7.3 ± 2.5	0.297
Allergy testing			
IgE [U/ml]	41.7 ± 30.7	36.0 ± 46.1	0.736
Wheat flour IgE [kU/ml]	0.0 ± 0.0	0.0 ± 0.0	–
IgE RAST/Skin Pricktest Wheat flour	negative	negative	–
Infections			
C-reactive protein [mg/l]	1.29 ± 1.3	1.3 ± 1.3	0.941
Epstein Barr virus IgG-positive [% (n)]	66.6 (6)	72.2 (13)	0.766
Symptoms			
Abdominal pain	10.0 (1)	68.4 (13)	**0.005****
Diarrhea	0.0 (0)	57.9 (11)	**0.003****
Flatulence/meteorism	0.0 (0)	89.5 (17)	<**0.0001*****
Constipation	0.0 (0)	21.1 (4)	0.268
Headache	10.0 (1)	63.2 (12)	**0.008****
Pain in the limbs	10.0 (1)	21.1 (4)	0.633
Hearing disorder	0.0 (0)	15.8 (3)	0.532
Muscle pain	20.0 (2)	47.4 (9)	0.234
Fatigue	80.0 (8)	94.7 (18)	0.267
Depression	0.0 (0)	47.4 (9)	**0.011***

IgE, immunoglobulin E; RAST, radioallergosorbent test; *p < 0.05; **p < 0.01; ***p < 0.001.

2.7. Microbiota analysis

Stool samples were taken at the beginning of the study (Western standard diet), at the end of the low FODMAP diet, and after the gluten-free diet, and stored at −20 °C until DNA isolation. The genomic bacterial DNA was isolated from faecal samples with the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) according manufacturer's recommendations. Afterwards, the V3–V4 region of bacterial 16sRNA genes was amplified with the NEBNext Q5 Hot Start HiFi PCR Master Mix (NEB, USA). The amplified genes were isolated with AMPure XP Beads (Beckmann Coulter Genomics, Germany), and DNA content measured by fluorometric quantitation using the Qubit® dsDNA-Kit (ThermoFisher Scientific, Germany). DNA was combined and analyzed by "2 × 300 bp paired-end" sequencing on the Illumina MiSeq platform [18]. Quality control was done with the UPARSE pipeline [19] and the "Ribosomal database projects" (RDP, release 11) was used for classification. Bioinformatics analysis and metabolomic grouping was done with METAGENassist. The operational taxonomic units were kept separated and normalization of data was done on sum [20].

To compare microbial patterns all data were converted to percentages of whole counts. When microbial families were studied, families with overall frequencies below 0.01% were neglected. Inter-assay variances were determined in advance by repeated analysis of six different stool samples for microbial families and genus, and paired t-tests never revealed significant differences (data not shown).

2.8. Study design

The study protocol was approved by ethics committee of University of Erlangen (AZ 90_14 B) and informed consent was obtained from all individuals. Clinical trial no NCT 03268720. The study diagram is shown in Supplementary Fig. 1.

To ensure that no specific elimination diet was ingested, all participants were requested to eat a Western standard diet with at least two gluten-containing meals per day corresponding to 10 g of gluten daily for at least four weeks before starting of the study [17]. The diets were introduced after an initial detailed nutritional consultation and supervised by a professional dietitian. First, the participants were instructed to consume a low FODMAP diet for two weeks. After a five days transition period, when participants were asked to eat normal diet, the individuals consumed a GFD for another two weeks. The guidelines of the diets were summarized

and given in writing form to the participants. Gluten-free products were kindly delivered from Dr. Schär AG/SPA, Italy, the instructions for a low FODMAP diet are shown in Supplementary Table 1.

Stool samples were collected at baseline, at the end of the low FODMAP and after the gluten-free diet.

All participants completed questionnaires concerning gastrointestinal symptoms (gastrointestinal symptom rating scale, GSRS) [21], and psychological general well-being (psychological general well-being index, PGWB) [22], before the beginning of the study and at the end of each diet. The participants were asked to record the consistency of the stool daily, according the Bristol stool chart [23]. Daily data were available from 10 patients with NCGS and 5 healthy controls, while from the others some single data missed (on average 1–5 data missed, mainly under normal diet). The data on stool consistency were averaged for diets. Furthermore, a 3-day food diary was filled in on every diet according to Freiburger nutrition protocol. A detailed nutritional analysis was performed for analysis of proteins, fats and carbohydrates, with differentiated data for lactose, fructose, sorbitol, mannitol and xylitol using the nutrition software PRODI® (Nutri-Science GmbH, Germany).

The primary outcome measure was the recovery from gastrointestinal symptoms in NCGS patients under the diets. Secondary outcome measure was the improvement of psychological symptoms, the normalization of numbers of intraepithelial lymphocytes and Goblet cells, and the dietary effect on microbiota in NCGS patients compared to healthy controls.

2.9. Statistics

Statistics were performed using IBM SPSS Statistics 21 (IBM, USA) and GraphPad Prism 6 (GraphPad software Inc., USA). Data were analyzed for normal distribution with Chi-squared test. Parametric t-test and non-parametric Mann–Whitney U or ANOVA were used to determine statistical power. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Study participants

We included 19 well-characterized patients with NCGS and ten healthy controls in our study. Patients did not differ from controls in routine laboratory parameters, including serum levels of vitamins or micronutrients, C-reactive protein, or IgG titers against Epstein–Barr virus, and showed no nutritional deficiencies. The BMI of patients was in the normal range (22.9 ± 4.7), and only slightly increased in patients with NCGS compared to controls (20.9 ± 2.8). Some liver parameters were elevated in NCGS patients reaching significance for aspartate transaminase and alkaline phosphatase (Table 1).

3.2. Impact of diets on nutritional parameters

Patients and controls were advised to consume a comparable amount of nutrients over the whole study period. However, under the low FODMAP diet the participants showed a reduced daily caloric intake (controls 2163 ± 547 vs 1680 ± 430 kcal/d, $p < 0.05$; NCGS 2368 ± 643 vs 1759 ± 738 kcal/d, $p < 0.01$), and NCGS patients consumed fewer calories under the GFD (2368 ± 643 vs 1828 ± 589 kcal/d, $p < 0.05$). As expected, the low FODMAP diet resulted in significant reductions in ingested lactose, fructose, maltose, and sorbitol. (For detailed analysis see Supplementary Table 2).

3.3. Effect of diets on gastrointestinal symptoms

At baseline, all patients with NCGS reported gastrointestinal symptoms when consuming a gluten-containing standard diet. All but two patients also suffered from extra-intestinal symptoms. Main complaints were flatulence (89.5%), abdominal pain (68.4%), diarrhea (57.9%), fatigue and tiredness (94.7%), headache (63.2%), muscle pain (47.4%) and depression (47.4%), and less frequently constipation (21.1%), and limb pain (21.1%) (Table 1). The gastrointestinal symptom rating scale (GSRS) revealed that at beginning of the study the severity of gastrointestinal symptoms was significantly different between NCGS patients and non-symptomatic healthy controls (13.8 ± 6.2 vs 3.5 ± 2.4; $p < 0.001$). (Fig. 1a).

After consuming the low FODMAP diet, the total GSRS of the NCGS patients improved significantly to 8.7 ± 5.2 ($p = 0.001$), with significant recovery for reflux, abdominal pain and indigestion. Notably, the symptom complex of NCGS patients further improved under the GFD (4.6 ± 4.3; $p < 0.05$). Interestingly, abdominal pain, diarrhea and constipation improved significantly more on the GFD than on low FODMAP diet (Fig. 1b, and Supplementary Table 3).

At the beginning of the study, patients with NCGS reported lose stool consistency significantly more often compared to healthy controls (4.5 ± 1.4 vs 3.44 ± 0.8). However, nutritional changes had a positive influence on the stool consistency of NCGS patients, and as measured by the Bristol stool chart, previously pasty or liquid stools (values >4) normalized especially under the GFD (score 4.5 ± 1.4 vs 3.6 ± 0.7; $p < 0.05$). Scores remained unchanged in the controls (3.44 ± 0.8 vs 3.38 ± 0.8) (Fig. 1c).

3.4. Effect of dietary regimens on psychological well being

At the beginning of the study, NCGS patients reported a significantly compromised overall psychological well-being compared to healthy controls (63.1 ± 18.5 vs 80.6 ± 11.8, resp.; $p < 0.01$). While consuming the low FODMAP diet, the psychological parameters from patients with NCGS improved significantly (74.2 ± 18; $p = 0.001$), and further improved on the GFD, reaching values comparable to the healthy controls (84.5 ± 17.3). In contrast, the healthy controls showed a reduced psychological well-being under the low FODMAP diet, which was likely due to the imposed dietary restrictions (Fig. 2, and Supplementary Table 4).

3.5. Effect of dietary interventions on numbers of intraepithelial lymphocytes and goblet cells in patients with NCGS

Paired biopsies before and at the end of the study were available from 17 patients with NCGS. Noteworthy, none of them showed mucosal lesions characteristic of celiac disease (Marsh 2–3). At the beginning of the study, i.e. under gluten-containing standard diet, three patients presented increased numbers of CD3-positive IELs (Marsh 1, >25/100 enterocytes), while most patients expressed moderately elevated IEL numbers with a mean of 18.4 ± 8.2 indicating an ongoing inflammation in the mucosa. Importantly, after the GFD, these IEL numbers were significantly decreased in all but one patient (mean 8.4 ± 8.7; $p < 0.01$) (Fig. 3).

In addition, the number of Goblet cells significantly declined on the GFD, and displayed similar numbers as in healthy controls (19.1 ± 3.3 vs 14.2 ± 3.1; $p < 0.01$ vs 13.4 ± 1.5, $p < 0.001$) (Fig. 4).

3.6. Differences in intestinal microbiota between NCGS patients and controls

On the phylum level Bacteroidetes were most abundantly present in both groups, with higher values in healthy controls compared to NCGS patients (58.7 ± 14% and 48 ± 17.3%,

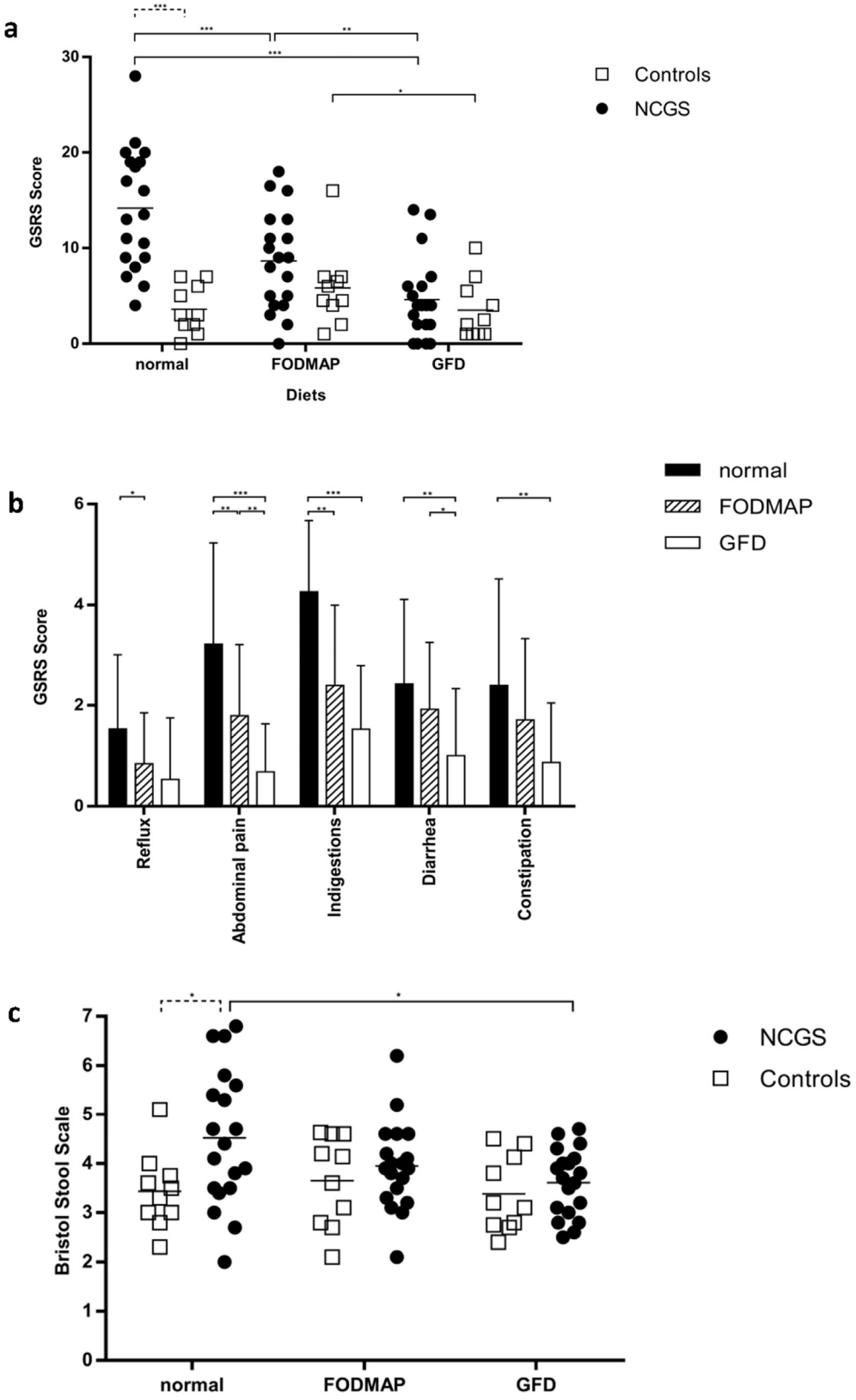

Fig. 1. Clinical symptoms in NCGS and healthy controls. a, b) Gastrointestinal symptom rating scale (GSRS) to record gastrointestinal complaints. a) Gastrointestinal symptoms of NCGS patients and healthy individuals under normal diet, low FODMAP diet or GFD. b) Detailed analysis of gastrointestinal complaints of NCGS patients. For item explanation see Supplementary Table 3a. c) Analysis of stool consistency according to the Bristol stool chart. NCGS patients demonstrated a significantly worse consistency of the stool under normal diet, which normalized under diets. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

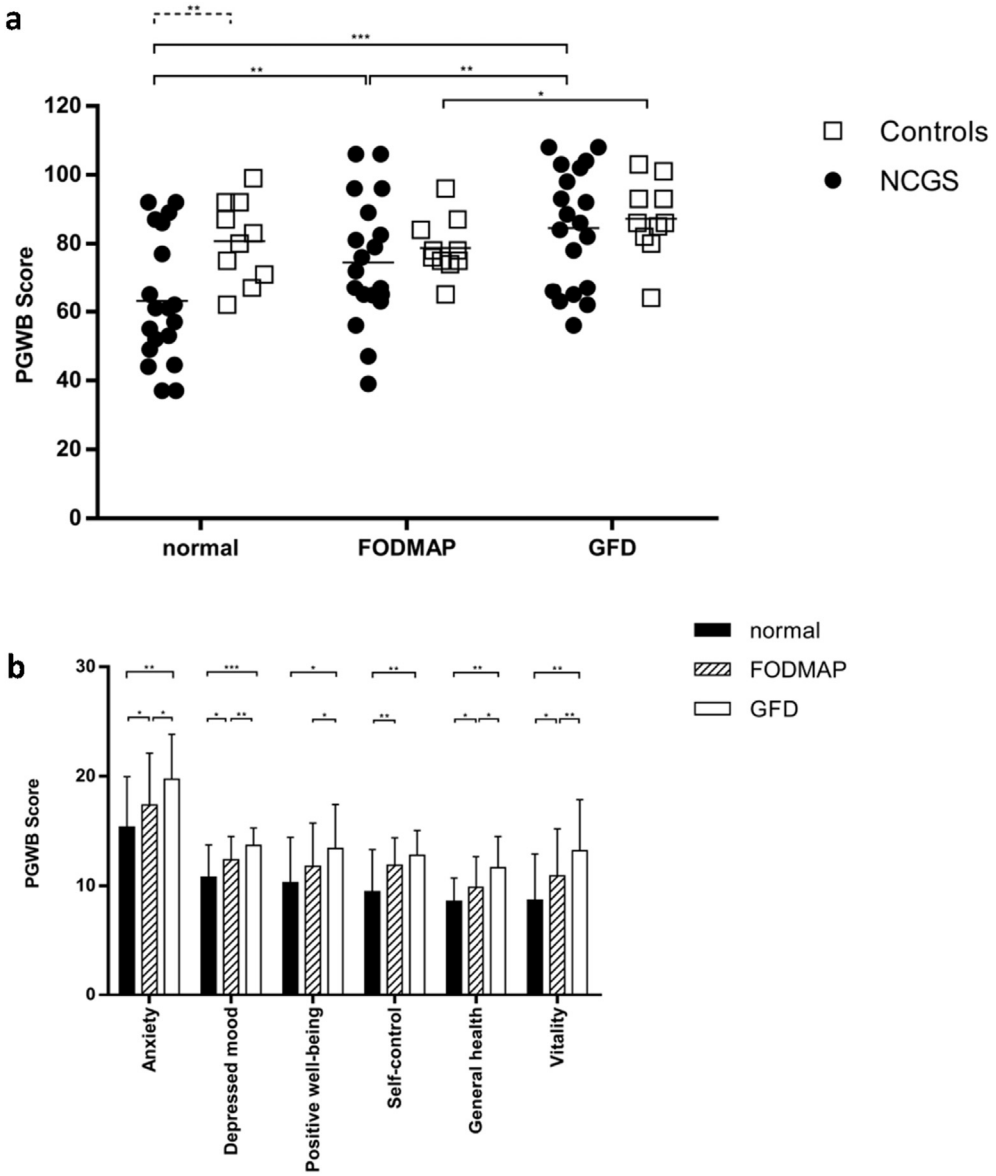

Fig. 2. Psychological general well-being index (PGWB) of the dietary intervention groups. a) Comparison of PGWB between NCGS patients and healthy controls. b) Improvement of PGWB in NCGS patients under the different diets. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

respectively), while the numbers of Firmicutes were lower in healthy controls compared to NCGS patients (32.2 ± 13.6% vs 43 ± 16.2%). In the healthy controls, the diets had only a minor effect on phyla. In contrast, in NCGS patients the GFD caused a significant increase in Bacteroidetes compared to the low FODMAP diet (FODMAP 44.6 ± 19% vs GFD 54 ± 14.5%; $p < 0.01$) and a concomitant drop in Firmicutes (FODMAP 47 ± 17.1% vs GFD 39 ± 16.3%; $p < 0.05$), thus reaching phylum counts with high similarity to healthy controls at the end of the study (Table 2).

When microbial families were analyzed (abundances below 0.01% were neglected), the gluten-containing standard diet displayed significantly increased abundances of Ruminococcaceae and Peptostreptococcaceae (both Firmicutes; $p < 0.05$) but significantly reduced numbers of Porphyromonadaceae (Bacteroidetes; $p < 0.05$) in NCGS patients compared to healthy controls. After the low FODMAP diet differences in the microbial pattern were maintained, with significantly increased Peptococcaceae (Firmicutes; $p < 0.05$) but reduced Rikenellaceae (Bacteroidetes; $p < 0.05$) and Sutterellaceae (Proteobacteria; $p < 0.05$) in NCGS patients. Two weeks of the GFD revealed still significantly reduced Porphyromonadaceae (Bacteroidetes; $p < 0.01$) in NCGS patients, as already noticed under the normal diet. Acidaminococcaceae (Firmicutes; $p < 0.01$) displayed also decreased numbers whereas Eubacteriacea (Firmicutes; $p < 0.01$) were enriched in patients with NCGS on the GFD. Although the differences did not reach overall statistical significance, the Ruminococcaceae, Peptococcaceae and Peptostreptococcaceae (all Firmicutes) were always enriched in NCGS patients compared to controls, whereas Porphyromonadaceae, Rikenellaceae (both Bacteroidetes), and Sutterellaceae (Proteobacteria) were always diminished (Table 3a, Supplementary Fig. 4).

3.7. Diet-dependent microbial alterations in study groups

Already the two-week nutritional modifications caused significant alterations in certain intestinal bacterial families in our study participants. Within the group of NCGS patients, the GFD resulted

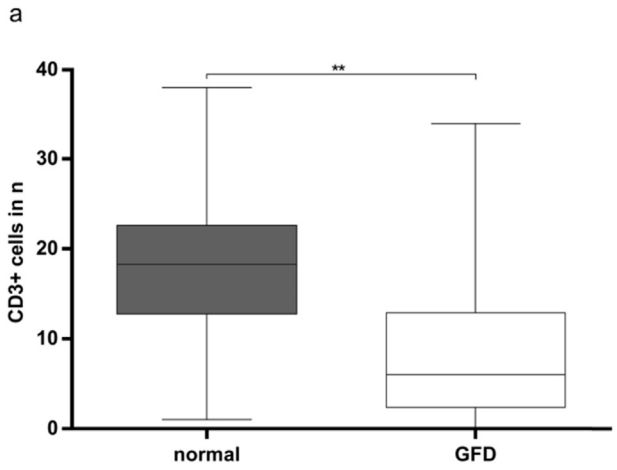

Fig. 3. Numbers of intraepithelial CD3+ cells per 100 enterocytes from 17 NCGS patients before and after the GFD. A representative immunohistochemistry of CD3+ cells is shown in Supplementary Fig. 2.

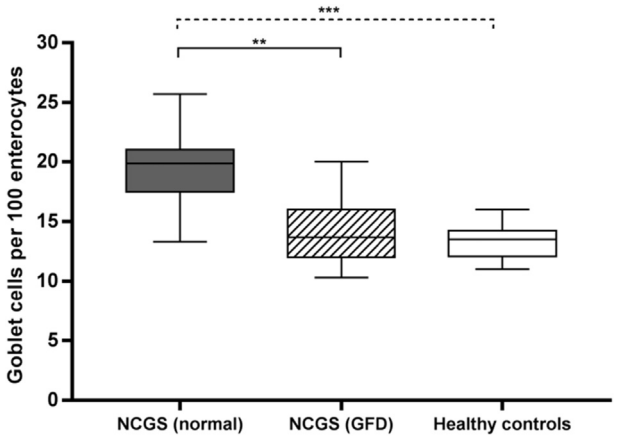

Fig. 4. Numbers of intestinal Goblet cells per 100 enterocytes. A representative PAS staining of mucin-producing Goblet cells is shown in Supplementary Fig. 3.

in significantly enriched families of Bacteroidaceae (Bacteroidetes; normal vs GFD $p < 0.05$; FODMAP vs GFD $p < 0.01$) and the low FODMAP diet caused diminished Bifidobacteriaceae (Actinobacteria; normal vs FODMAP $p < 0.05$). The Lachnospiraceae (Firmicutes) were enriched under a low FODMAP diet but dropped under the GFD ($p < 0.05$). Healthy controls presented reduced Hyphomicrobiaceae (Proteobacteria; $p < 0.01$) and Clostridiaceae (Firmicutes; $p < 0.05$) but significantly increased numbers of Enterobacteriaceae (Proteobacteria; $p < 0.05$) on the GFD (Table 3b).

Interestingly, distinctly more significant variations were detected in NCGS compared to the healthy controls on the genus level (Supplementary Table 5). Not unexpectedly, the differences in microbiota composition had an impact on predicted bacterial metabolism. Whereas no significant variations in metabolic signatures were detected in healthy controls, NCGS patients showed significant changes in bacteria able to dehalogenation, ammonia oxidation, xylan and cellulose degradation, sulfate reduction, and nitrogen fixation, especially under the GFD (Table 4).

4. Discussion

NCGS was defined as activation of intestinal innate immunity in the absence of celiac disease and classical wheat allergy [2]. Though gluten is well-known as main trigger in celiac disease, its role in NCGS is increasingly debated. A meta-analysis showed that only 16% of patients diagnosed with NCGS developed symptoms after re-challenge with pure gluten, and double-blind studies demonstrated a significant improvement in gastrointestinal symptoms in NCGS patients under a FODMAP-restricted diet [8,24]. Furthermore, a recent study showed that NCGS patients developed more severe gastrointestinal symptoms after ingestion of fructans than after consuming gluten [25]. All these studies question the role of gluten as the main or sole responsible agent in NCGS, and suggest that other components in wheat and even other foods may be (partly) responsible for the pathogenesis of NCGS. This is further underlined by recent data showing that NCGS patients on a long term gluten-free diet still complain of persistent clinical symptoms of mild severity [26].

In contrast to patients with celiac disease, no mucosal damage could be detected in NCGS patients, and only 42% of subjects showed moderately increased numbers of IELs (Marsh stage 1) [3], with higher densities than healthy controls but lower numbers than celiac patients [27]. Our NCGS patients had elevated IELs under the standard gluten-containing diet, and densities were comparable to those reported by Brottveit et al. [27]. We could show for the first time that a 14-day GFD resulted in significantly reduced numbers of IEL in these patients. Thus, our data strongly underline the involvement of the innate immune system in NCGS and clearly point to a nutrient-dependent modification of the immune response in NCGS and the benefit of an appropriate diet in NCGS. However, as discussed, other wheat components besides gluten may be the causative agents for the observed increase in IEL. We did not assess histology after the low FODMAP diet and thus cannot completely exclude some effect on histological changes caused by FODMAPs. However, there are no prior studies that detected histological changes on a normal versus a low FODMAP diet.

Interestingly, the gastrointestinal and psychological symptoms of all our patients with NCGS improved after consuming the low FODMAP diet, thus confirming the recent observations from others that FODMAPs at least partially account for the clinical symptoms in NCGS patients [13]. However, a gluten-free diet further resolved the clinical symptoms in all our patients, especially for abdominal pain, diarrhea, and constipation, going along with a significantly improved subjective well-being.

The dietary compliance of our participants was high, except for the transition period, when patients rather consumed a “minor strict FODMAP diet” than a normal standard diet, because they feared gastrointestinal symptoms. In any case, this interim period is of no major relevance for our study that compared the low FODMAP diet to the gluten-free diet, and which assessed a broad spectrum of patient related clinical outcomes and differences in intestinal microbiota. Our study is limited by the open design but this is a general problem of similar nutritional interventional studies. Only recently, a blinded low FODMAP diet study was described [28]. However, we have our doubts in blinding, since patients are well-educated and one short look into the internet allows them to identify the arm of the study (low FODMAP vs normal diet) to which they were assigned. For this reason we did not intend to perform a blinded study but rather based our design on the sequential exposure of patients with clinically proven NCGS vs healthy controls to either a low FODMAP or a gluten-free diet, and studying also (likely unbiased) histological and microbial endpoints. Our study design included dietary interventions that lasted for only two-weeks. This is reasonable, since patients with NCGS develop clinical symptoms rapidly after ingestion of gluten-containing food, and the duration of the symptoms is of short time (hours up to 1–2 days). In accordance with other studies, we noticed a clear improvement of the clinical symptoms already after

Table 2
Comparison of differences in microbial phylum from healthy controls and NCGS patients. Values are microbial percentages. Analysis of variance (ANOVA) was used to demonstrate significant differences caused by varying diets.

Phylum	Diet	Controls (n = 10)	NCGS (n = 19)	p value
Bacteroidetes	Normal	58.69 ± 13.95	47.95 ± 17.32	0.103
	FODMAP	53.32 ± 11.18	44.56 ± 18.99	0.193
	GFD	52.64 ± 15.66	53.95 ± 14.45	0.844
	FODMAP vs GFD		**0.003****	
Firmicutes	Normal	32.21 ± 13.62	43.01 ± 16.18	0.083
	FODMAP	39.36 ± 11.24	47.00 ± 17.08	0.214
	GFD	38.73 ± 14.92	39.04 ± 16.33	0.961
	FODMAP vs GFD		**0.015***	
Proteobacteria	Normal	7.48 ± 3.40	7.43 ± 4.54	0.979
	FODMAP	5.69 ± 2.75	6.58 ± 4.46	0.571
	GFD	7.65 ± 5.59	6.13 ± 4.50	0.436
Actinobacteria	Normal	1.00 ± 1.27	0.66 ± 0.61	0.441
	FODMAP	0.86 ± 1.38	0.34 ± 0.31	0.271
	GFD	0.44 ± 0.44	0.39 ± 0.65	0.82
Verrucomicrobia	Normal	0.21 ± 0.25	0.55 ± 1.13	0.227
	FODMAP	0.57 ± 1.37	1.30 ± 2.72	0.435
	GFD	0.26 ± 0.33	0.22 ± 0.59	0.831
Acidobacteria	Normal	0.35 ± 1.04	0.26 ± 1.05	0.836
	FODMAP	0.09 ± 0.24	0.02 ± 0.07	0.39
	GFD	0.13 ± 0.35	0.20 ± 0.57	0.709
Tenericutes	Normal	0.018 ± 0.033	0.10 ± 0.22	0.268
	FODMAP	0.02 ± 0.04	0.12 ± 0.36	0.402
	GFD	0.07 ± 0.22	0.03 ± 0.08	0.485
Lentisphaerae	Normal	0.04 ± 0.08	0.02 ± 0.05	0.41
	FODMAP	0.04 ± 0.12	0.01 ± 0.04	0.34
	GFD	0.03 ± 0.06	0.005 ± 0.014	0.257
Synergistetes	Normal	0.009 ± 0.012	0.02 ± 0.04	0.35
	FODMAP	0.04 ± 0.07	0.05 ± 0.09	0.841
	GFD	0.05 ± 0.08	0.03 ± 0.07	0.493
Fusobacteria	Normal	0.00 ± 0.00	0.0008 ± 0.0015	0.061
	FODMAP	0.003 ± 0.006	0.008 ± 0.021	0.492
	GFD	0.007 ± 0.021	0.002 ± 0.007	0.331

Significant differences between diets are typed in bold.
*p < 0.05; **p < 0.01.

such short periods following elimination of FODMAPS or gluten. There is some overlap between a low FODMAP and a gluten-free diet, since wheat products contain considerable amounts of bloating carbohydrates including polyfructans. Although our nutritional analysis of the GFD gave us detailed data about lactose, fructose, and sorbitol, and showed overall increased percentages of carbohydrates in the GFD, we cannot completely exclude that some polysaccharides belonging to FODMAPs are also reduced under the GFD. The benefit of a low FODMAP diet was shown for patients suffering from IBS [11,29–31], but the mechanisms how a FODMAP-restricted diet may affect IBS symptoms is still unclear [29,32].

An important role of intestinal bacteria and bacterial dysbiosis was suggested in IBS [33]. When we compared the microbiota on the phylum level, there was a clear tendency to decreased Bacteroidetes but increased Firmicutes in NCGS patients compared to controls. Interestingly, these data resemble findings from other studies, where a similar dysbalance was described in most IBS patients [34,35]. Our data show a trend towards increased unclassified bacterial families of Ruminococcaceae, Peptococcaceae, and Peptostreptococcaceae (Firmicutes), but diminished populations of unclassified Porphyromonadaceae (Bacteroidetes), Rikenellaceae (Bacteroidetes), and Sutterella (Proteobacteria) in NCGS patients under all diets. This is important, since the elimination diets clearly improved symptoms, whereas the differences in the microbiota still persisted, suggesting that these microorganisms per se may not be responsible for the clinical symptoms. The low FODMAP and the GFD induced a decrease in Bifidobacteriaceae (Actinobacteria) in both groups, although statistical significance was only reached in NCGS patients. The reduced proportion of Bifidobacteriaceae confirmed former data on the low FODMAP diet [31,32,36]. The fact, that a low FODMAP diet improved clinical symptoms in NCGS but caused reduced amounts of Bifidobacteriaceae is of interest, because *Bifidobacteria* are considered valuable probiotic agents for gastrointestinal disorders [37]. A recent study confirmed the positive effect of a low FODMAP diet in IBS but additional supply with probiotic bacteria had no effect on clinical symptoms [28]. Other data indicated that the supply with probiotic species didn't strongly influence the microbiota composition, but rather changed the activity of resident bacteria and their interaction with the host [38], challenging conventional interpretations of selected bacteria species. Thus, the complex interactions of the overall intestinal microbiota, their variable metabolome and the interplay with the intestinal immune system appear to be relevant for health and disease.

Interestingly, the dietary effect on the microbial pattern was distinctly more pronounced in our NCGS patients than in the controls. In addition, greater metabolomics variability was noted in NCGS patients, suggesting that the microbiota in NCGS patients may be more susceptible to nutrient changes. In line, prior studies proposed a higher microbial fluctuation in patients with IBS and dietary challenges resulted in more variations in microbial composition, whereas microbiota of healthy controls showed higher stability [39,40]. However, a direct causal role of these over- or under-represented microorganisms in NCGS remains unclear and more detailed functional analyses are required.

Table 3
Summary of bacterial families in percentages. a) Statistically significant microbial abundancies between healthy controls and NCGS patients. b) Significant variations under different diets within the study groups. Statistics was done with Mann–Whitney U test.

a) Family	Diet	Controls (n = 10)	NCGS (n = 19)	p value
Ruminococcaceae (Firmicutes)	Normal	9.76 ± 5.21	14.80 ± 6.39	**0.041***
	FODMAP	12.34 ± 4.65	13.95 ± 6.99	0.519
	GFD	10.73 ± 5.88	12.73 ± 6.13	0.403
Porphyromonadaceae (Bacteroidetes)	Normal	10.19 ± 4.95	6.29 ± 3.85	**0.026***
	FODMAP	10.78 ± 8.61	6.07 ± 4.13	0.055
	GFD	11.84 ± 4.75	5.30 ± 2.85	**0.002****
Rikenellaceae (Bacteroidetes)	Normal	10.24 ± 8.31	6.05 ± 4.51	0.164
	FODMAP	11.91 ± 7.56	5.87 ± 5.32	**0.027***
	GFD	8.76 ± 5.88	6.30 ± 5.55	0.275
	FODMAP vs GFD	**p<0.05***		
Acidaminococcaceae (Firmicutes)	Normal	3.38 ± 2.83	3.43 ± 6.60	0.980
	FODMAP	4.29 ± 3.91	1.96 ± 4.24	0.160
	GFD	7.26 ± 9.32	1.16 ± 2.04	**0.002****
Sutterellaceae (Proteobacteria)	Normal	2.62 ± 3.24	1.08 ± 1.30	0.178
	FODMAP	2.18 ± 1.45	0.78 ± 1.13	**0.014***
	GFD	2.29 ± 2.04	1.23 ± 1.50	0.123
Eubacteriaceae (Firmicutes)	Normal	0.99 ± 0.59	1.75 ± 1.95	0.132
	FODMAP	2.26 ± 2.88	2.23 ± 2.31	0.979
	GFD	0.68 ± 0.67	1.76 ± 1.76	**0.026***
Peptococcaceae (Firmicutes)	Normal	0.15 ± 0.16	0.30 ± 0.55	0.419
	FODMAP	0.17 ± 0.13	0.41 ± 0.41	**0.032***
	GFD	0.14 ± 0.12	0.33 ± 0.46	0.206
Peptostreptococcaceae (Firmicutes)	Normal	0.10 ± 0.10	0.27 ± 0.27	**0.025***
	FODMAP	0.14 ± 0.11	0.23 ± 0.45	0.535
	GFD	0.18 ± 0.18	0.20 ± 0.31	0.848

b) Family	Diet	Controls (n=10)	NCGS	p value
Bacteroidaceae (Bacteroidetes)	Normal	37.21 ± 18.43	29.81 ± 18.93	0.322
	FODMAP	28.34 ± 18.29	28.52 ± 16.31	0.979
	GFD	30.70 ± 14.71	37.12 ± 18.47	0.351
	Normal vs GFD		**p<0.05***	
	FODMAP vs GFD		**p<0.01****	
Lachnospiraceae (Firmicutes)	Normal	10.91 ± 6.71	13.58 ± 7.16	0.339
	FODMAP	11.93 ± 7.44	17.45 ± 11.90	0.195
	GFD	12.65 ± 10.46	12.85 ± 10.26	0.962
	FODMAP vs GFD		**p<0.05***	
Bifidobacteriaceae (Actinobacteria)	Normal	0.72 ± 1.07	0.44 ± 0.60	0.376
	FODMAP	0.50 ± 1.07	0.12 ± 0.18	0.293
	GFD	0.21 ± 0.31	0.21 ± 0.63	0.994
	Normal vs FODMAP		**p<0.05***	
Hyphomicrobiaceae (Proteobacteria)	Normal	2.23 ± 2.46	2.82 ± 2.84	0.585
	FODMAP	1.67 ± 1.22	2.77 ± 3.35	0.213
	GFD	1.10 ± 0.95	2.65 ± 3.67	0.203
	FODMAP vs GFD	**p<0.01****		
Enterobacteriaceae (Proteobacteria)	Normal	0.43 ± 0.73	2.24 ± 4.23	0.083
	FODMAP	0.28 ± 0.39	1.24 ± 2.92	0.318
	GFD	1.44 ± 1.62	0.35 ± 0.53	0.065
	FODMAP vs GFD	**p<0.05***		
Clostridiaceae (Firmicutes)	Normal	1.28 ± 1.22	0.44 ± 0.52	0.064
	FODMAP	0.71 ± 1.04	0.87 ± 1.30	0.732
	GFD	0.24 ± 0.19	0.90 ± 1.70	0.109
	Normal vs GFD	**p<0.05***		

GFD, gluten-free diet; *p < 0.05; **p < 0.01.

Goblet cells are the main producers of mucin in the small intestine. Since the mucus serves as a mechanical barrier and sequesters antibacterial peptides, Goblet cells are the key players in the first defense against intestinal microorganisms and their short life span with only about 6 days allows a rapid adaptation on microbial changes. Modifications of mucin expression or structure were shown after bacterial, viral or nematode infections. (For review refer to [41,42]). Our finding of decreased numbers of Goblet cells in NCGS patients under the GFD is of special interest. An increased mucus production under the normal gluten-containing diet, as shown in our patients with NCGS, might be an adaptation to microbial dysbalance and/or nutrient stimulation and is worth of further research.

In summary, our study suggests a multifactorial etiology of NCGS, due to a functional effect caused by FODMAPs, combined with a mild gluten-triggered immune reaction, and a microbial dysbalance.

Table 4
Classification of microbiota according to metabolism in percentages. No major differences were found in controls, but several significant variations in bacterial metabolisms groups were present in NCGS. Statistics was done with ANOVA.

Controls (n = 10)	Normal	FODMAP	GFD	ANOVA p value	N vs G p value	N vs G p value	F vs G p value
Dehalogenation	27.31 ± 3.1	24.59 ± 6.5	26.63 ± 2.8	0.317	0.299	0.208	0.352
Ammonia oxidizer	27.49 ± 3.3	23.62 ± 5.6	27.11 ± 3.5	0.100	0.100	0.575	0.101
Xylan degrader	25.79 ± 4.2	21.86 ± 8.5	23.33 ± 4.5	0.201	0.197	0.147	0.348
Cellulose degrader	8.43 ± 6.6	15.76 ± 19.4	10.28 ± 8.3	0.335	0.315	0.413	0.343
Sulfate reducer	2.34 ± 1.7	4.49 ± 4.5	3.13 ± 1.8	0.178	0.139	0.236	0.310
Sulfide oxidizer	3.36 ± 2.0	3.93 ± 3.3	3.48 ± 2.8	0.686	0.510	0.777	0.558
Nitrite reducer	1.26 ± 1.1	2.16 ± 3.2	1.45 ± 1.4	0.450	0.333	0.722	0.379
Dinitrogen-fixing	2.55 ± 1.6	2.10 ± 1.9	2.80 ± 2.6	0.101	0.084	0.504	0.056
Nitrogen fixation	1.02 ± 0.9	1.03 ± 0.8	1.11 ± 0.9	0.851	0.926	0.666	0.688
Sulfur oxidizer	0.24 ± 0.4	0.13 ± 0.2	0.40 ± 0.8	0.370	0.352	0.425	0.285
Chitin degradation	0.03 ± 0.0	0.05 ± 0.1	0.04 ± 0.1	0.475	0.230	0.575	0.521
Degrades aromatic hydrocarbons	0.14 ± 0.2	0.16 ± 0.2	0.20 ± 0.3	0.254	0.588	0.120	0.279
NCGS (n=19)	Normal	FODMAP	GFD	ANOVA p value	N vs F p value	N vs G p value	F vs G p value
Dehalogenation	25.32 ± 3.4	25.66 ± 3.8	27.32 ± 3.4	0.068	0.728	0.053	**0.033***
Ammonia oxidizer	24.91 ± 5.3	24.09 ± 6.3	26.23 ± 4.3	0.123	0.491	0.175	**0.037***
Xylan degrader	20.50 ± 7.3	20.94 ± 6.6	23.79 ± 6.4	**0.015***	0.709	0.020	**0.012***
Cellulose degrader	12.52 ± 10.7	13.39 ± 12.3	9.08 ± 9.4	0.130	0.735	0.111	**0.040***
Sulfate reducer	6.59 ± 4.4	6.19 ± 5.2	4.43 ± 4.2	**0.039***	0.685	**0.007****	0.064
Sulfide oxidizer	4.41 ± 4.9	3.55 ± 4.0	3.72 ± 4.0	0.375	0.084	0.297	0.832
Nitrite reducer	3.63 ± 4.6	3.25 ± 3.5	2.74 ± 3.9	0.382	0.483	0.191	0.480
Dinitrogen-fixing	1.29 ± 1.2	1.37 ± 1.6	1.51 ± 1.2	0.675	0.700	0.430	0.713
Nitrogen fixation	0.53 ± 0.5	0.90 ± 0.7	0.67 ± 0.6	**0.032***	**0.001*****	0.343	0.104
Sulfur oxidizer	0.10 ± 0.2	0.41 ± 1.0	0.38 ± 1.1	0.214	0.176	0.248	0.540
Chitin degradation	0.07 ± 0.1	0.12 ± 0.1	0.07 ± 0.1	0.067	0.085	0.839	**0.038***
Degrades aromatic hydrocarbons	0.07 ± 0.2	0.09 ± 0.2	0.06 ± 0.1	0.535	0.461	0.675	0.261

N = normal diet; F = low FODMAP; G = gluten-free diet (GFD); *p < 0.05; **p < 0.01; ***p < 0.001.

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Statement of authors

WD, MS, and YZ designed and performed the study, and analyzed the data. SW performed microbial analysis. SW, DS, RS, AA, and MFN, provided critical revision and intellectual impact. All authors wrote and approved the final draft submitted.

Conflict of interest

WD and DS were holder of a patent for ELISA determination of transglutaminase antibodies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.03.017

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