BMC Medicine

RESEARCH

Open Access

Effects of fructan and gluten on gut microbiota in individuals with self-reported non-celiac gluten/wheat sensitivity—a randomised controlled crossover trial

Anne Mari Herfindal[1], Morten Nilsen[1], Trude E. Aspholm[1], Gry I. G. Schultz[2], Jørgen Valeur[3,4], Knut Rudi[1], Magne Thoresen[5], Knut E. A. Lundin[6,7], Christine Henriksen[8] and Siv K. Bøhn[1*]

Abstract

Background Individuals with non-celiac gluten/wheat sensitivity (NCGWS) experience improvement in gastrointestinal symptoms following a gluten-free diet. Although previous results have indicated that fructo-oligosaccharides (FOS), a type of short-chain fructans, were more likely to induce symptoms than gluten in self-reported NCGWS patients, the underlying mechanisms are unresolved.

Methods Our main objective was therefore to investigate whether FOS-fructans and gluten affect the composition and diversity of the faecal microbiota (16S rRNA gene sequencing), faecal metabolites of microbial fermentation (short-chain fatty acids [SCFA]; gas chromatography with flame ionization detector), and a faecal biomarker of gut inflammation (neutrophil gelatinase-associated lipocalin, also known as lipocalin 2, NGAL/LCN2; ELISA). In the randomised double-blind placebo-controlled crossover study, 59 participants with self-reported NCGWS underwent three different 7-day diet challenges with gluten (5.7 g/day), FOS-fructans (2.1 g/day), and placebo separately (three periods, six challenge sequences).

Results The relative abundances of certain bacterial taxa were affected differently by the diet challenges. After the FOS-fructan challenge, *Fusicatenibacter* increased, while *Eubacterium* (*E.*) *coprostanoligenes group*, *Anaerotruncus*, and unknown *Ruminococcaceae* genera decreased. The gluten challenge was primarily characterized by increased abundance of *Eubacterium xylanophilum group*. However, no differences were found for bacterial diversity (α-diversity), overall bacterial community structure (β-diversity), faecal metabolites (SCFA), or NGAL/LCN2. Furthermore, gastrointestinal symptoms in response to FOS-fructans were generally not linked to substantial shifts in the gut bacterial community. However, the reduction in *E. coprostanoligenes group* following the FOS-fructan challenge was associated with increased gastrointestinal pain. Finally, correlation analysis revealed that changes in gastrointestinal symptoms following the FOS-fructan and gluten challenges were linked to varying bacterial abundances at baseline.

Conclusions In conclusion, while FOS-fructans induced more gastrointestinal symptoms than gluten in the NCGWS patients, we did not find that substantial shifts in the composition nor function of the faecal microbiota could explain these differences in the current study. However, our results indicate that individual variations in baseline

*Correspondence:
Siv K. Bøhn
sivb@nmbu.no
Full list of author information is available at the end of the article

bacterial composition/function may influence the gastrointestinal symptom response to both FOS-fructans and gluten. Additionally, the change in *E. coprostanoligenes group*, which was associated with increased symptoms, implies that attention should be given to these bacteria in future trials investigating the impact of dietary treatments on gastrointestinal symptoms.

Trial registration Clinicaltrials.gov as NCT02464150.

Keywords Non-celiac gluten/wheat sensitivity (NCGWS), Gluten, FODMAP, Fructan, Fructo-oligosaccharides (FOS), Gut microbiota, Gastrointestinal symptoms, Short-chain fatty acids (SCFA), Neutrophil gelatinase-associated lipocalin (NGAL), Lipocalin-2 (LCN2)

Background

Although the first cases of gluten sensitivity in the absence of celiac disease (CeD) were reported 40 years ago [1], the aetiology and underlying mechanisms remain elusive, leading to ongoing debates regarding its definition, diagnostic criteria, and the estimation of its prevalence [2, 3]. Today, the condition is often labelled non-celiac gluten/wheat sensitivity (NCGWS). Individuals afflicted by NCGWS report a diverse range of gastrointestinal (GI) and extraintestinal symptoms when consuming gluten- and/or wheat-containing foods, without the diagnosis of CeD or wheat allergy (WA) [3, 4]. In contrast to CeD and WA, which are characterized by a well-defined pathophysiology [5, 6], NCGWS lacks clear diagnostic markers [2]. The clinical presentation of NCGWS with respect to GI symptoms shares common features with other disorders of gut–brain interaction, notably irritable bowel syndrome (IBS) [7].

According to the Salerno criteria (2015) [4], the diagnosis of NCGWS is confirmed through the improvement of symptoms upon adherence to a gluten-/wheat-free diet, followed by the reappearance of symptoms during a double-blind placebo-controlled rechallenge with gluten. However, the results from rechallenge studies have shown that the majority of self-reported NCGWS patients did not display gluten-specific symptoms [8]. The challenges in replicating the patients' alleged sensitivity to gluten have raised questions whether other components of gluten-/wheat-containing foods could be a culprit for problems in NCGWS patients [8, 9]. These components include α-amylase/trypsin inhibitors, agglutinin, and importantly, fructans [10].

Fructans are major carbohydrate components of plant foods and are composed of fructose oligomers or polymers [11]. Although several types of fructans exist, inulin-type fructans are the most studied due to their use as prebiotics, serving as substrates for beneficial gut microbes. For instance, inulin-type fructans can stimulate the colonic growth of health-promoting *Bifidobacteria* [12]. However, while generally considered beneficial for gut health, fructan oligosaccharides belong to the broader category of compounds known as fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP) [13], which have been extensively studied for their ability to cause GI symptoms in IBS patients [14].

Depending on their degree of polymerization (DP), i.e. the length of the fructose chain, the inulin-type fructans are often categorized as inulins with DPs between 2 and 60 or as fructo-oligosaccharides (FOS) which exclusively consist of low-DP molecules (DP 2–8) [15]. Low-DP fructans are particularly enriched in certain plant foods, such as wheat, onions, and garlic [13]. Since fructans are nondigestible in the small intestine, they are subject to fermentation by gut microbes, particularly in the colon. Following fructan intake, the ensuing production of gases by gut microbes, such as hydrogen and methane, and short-chain fatty acids (SCFA), in addition to osmotic effects, may cause abdominal discomfort, bloating, and altered bowel habits [16]. However, susceptibility to fructan-induced GI discomforts may depend on the initial state of the gut microbiota, as shown in children with IBS [17].

Based on the results from a randomised double-blind placebo-controlled crossover study, we previously concluded that FOS-fructans were more likely to induce IBS-like GI symptoms than gluten in individuals self-reporting sensitivity to gluten-/wheat-containing foods [18] and the involvement of FODMAP, such as FOS-fructans, as a contributing factor in NCGWS has also been implicated by others [19]. Recent advances into how the gut microbiota can affect the gut–brain axis point to possible microbial involvement in food-induced GI problems in various populations including NCGWS [20–22].

To our knowledge, the effects of FOS-fructan supplementation on the gut microbiota have not previously been investigated in self-reported NCGWS populations, apart from those investigating restrictions in total FODMAPs [23]. The aim of the current work was therefore to explore whether FOS-fructan and gluten affect the faecal microbiota and related variables of gut health differently and to investigate the possible

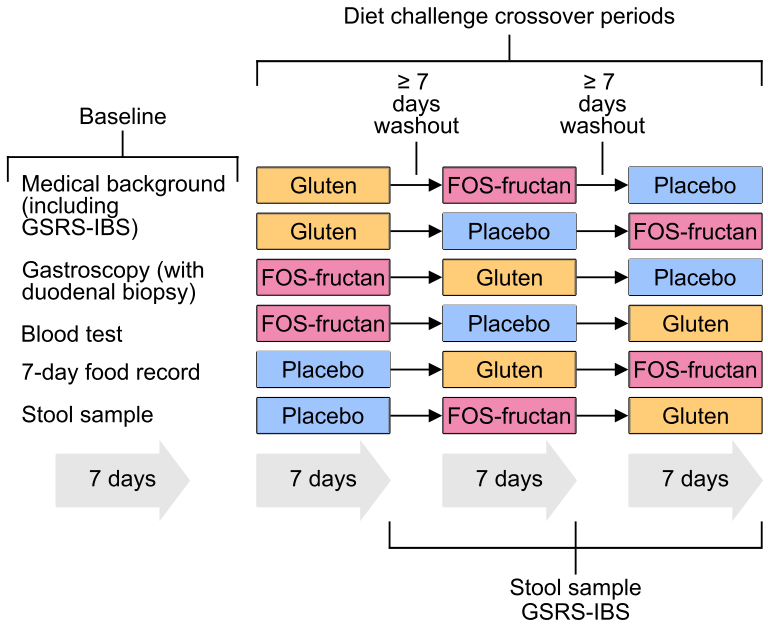

Fig. 1 Timeline and data collection during the randomised double-blind placebo-controlled crossover study. *FOS* fructo-oligosaccharides, *GSRS-IBS* gastrointestinal symptom rating scale IBS version, *IBS* irritable bowel syndrome

involvement of the faecal variables in the GI symptom response to FOS-fructans and gluten in NCGWS.

Methods

Participants

The present study is part of a clinical trial conducted from October 2014 to May 2016 at Oslo University Hospital, Rikshospitalet, Oslo, Norway. The study population has been described previously by Skodje et al. [18]. Briefly, to be eligible for participation, adults (age 18–80 years) were required to have experienced relief of GI symptoms when adhering to a gluten-free diet (GFD). The exclusion criteria were CeD or WA, pregnancy or lactation, use of immunosuppressive agents, inflammatory bowel disease (IBD) or other gastrointestinal comorbidities, significant infection, childbearing potential without adequate contraception, residing a considerable distance from the study site, or allergy to nuts or sesame seeds. IBS was not an exclusion criterion. All participants were required to follow a strict GFD for at least 6 months before the start of the study and to continue with GFD throughout the study period.

Study intervention and design

The study followed a randomised double-blind placebo-controlled crossover design, as previously described [18] (Fig. 1). The diet challenges consisted of three different 50-g muesli bars with added gluten, FOS-fructans, or no additives (placebo). The ingredients for the three different muesli bars are presented in Additional file 1: Table S1. The FOS-fructan muesli bar was added 2.1 g FOS-fructans (Orafti; Oligofructose, Beneo, Tienen, Belgium) while the gluten bar was added 5.7 g gluten (Vital Wheat Gluten; Manildra Group, Gladesville, New South Wales, Australia) which was equivalent to the FOS-fructan and gluten content of four slices of wheat bread, respectively. All three muesli bars were composed to be approximately energy equivalent (~220 kcal per 50 g), with a similar balance between protein, carbohydrates, fat, and fibre [18]. All participants underwent three consecutive 7-day challenge periods consuming one muesli bar daily with gluten, FOS-fructans, or placebo. Each 7-day challenge period was followed by a washout period of at least 1 week or until GI symptoms returned to baseline levels.

To address potential carry-over effects in the crossover study, participants were randomised to follow one of six diet challenge sequences (GFP, GPF, FGP, FPG, PGF, or PFG, where G = gluten, F = FOS-fructan, and P = placebo) (Fig. 1). The randomisation sequences were prepared by a statistician with no clinical involvement in the study (web-based service at http://randomization.com/, second generator, balanced permutations, accessed September 26, 2014). The block size was equal to the trial size. The allocation sequence was concealed for participants and the involved researchers throughout the study (see details in Skodje et al. [18]). The researchers who were responsible for assigning participants to the challenge

sequences were not involved in generating the sequence randomisation.

GI symptom data were recorded and stool samples were collected at baseline and at follow-up after each of the three diet challenges (four stool samples from each participant in total). Other baseline assessments included recording medical background, gastroscopy, blood tests, 7-day food recordings, and evaluation of diet adherence to the GFD by trained dietitians [18]. Adherence during the study was not re-evaluated, but the participants were asked to keep their diet consistent with the baseline diet throughout the study.

Outcomes

The present study reports on the secondary outcomes of a clinical trial that was designed to investigate the effects of gluten and FOS-fructan intake on GI symptoms in individuals with self-reported NCGWS [18]. The results of the primary outcome of the clinical trial (i.e. GI symptoms) have been reported previously [18]. The main aims of the present study were to compare the effects of the gluten, FOS-fructan, and placebo challenges on faecal microbiota (β-diversity, α-diversity, and bacterial abundances), SCFA, and human neutrophil gelatinase-associated lipocalin, also known as lipocalin 2 (NGAL/LCN2). Specifically, we investigated whether the diet challenges induced different changes in the faecal outcomes. Moreover, we investigated whether baseline levels and changes in the faecal outcomes were associated with GI symptom responses following the different diet challenges.

Gastrointestinal symptoms

As previously described [18], GI symptoms were measured using the IBS version of the Gastrointestinal Symptom Rating Scale (GSRS-IBS), a questionnaire developed for self-evaluation of GI symptoms in patients with IBS [24]. The questionnaire consists of 13 items that cover symptoms related to satiety (two items), abdominal pain (two items), diarrhoea (four items), constipation (two items), and bloating (three items). All 13 items have a Likert scale ranging from 1 (no discomfort at all) to 7 (very severe discomfort). Thus, the total GSRS-IBS score ranges from 13 to 91. GSRS-IBS scores were retrospectively recorded to reflect the last 7 days.

Collection and preparation of stool samples

The collection and preparation of stool samples for the different laboratory analyses were carried out as described elsewhere [25]. Briefly, the stool samples were collected in 13-mL tubes by the study participants in their own home and stored in their freezers. On the day of the clinic visits, the samples were transferred to Oslo University Hospital under cold conditions (~4 °C), followed by storage at −80 °C. During transfer between collaborating institutions, the samples were kept on dry ice. Semi-frozen stool samples were divided into smaller aliquots which were subsequently weighed and logged. Aliquots designated for DNA extraction (for assessment of microbiota composition) were added Stool Transport and Recovery Buffer (cat. no. 03335208001, Roche) and vortexed. All aliquots were stored at −80 °C until further processing.

Assessment of faecal microbiota composition

Gene sequencing of 16S rRNA has been described in detail previously [25]. A brief description follows. The faecal aliquots in Stool Transport and Recovery Buffer were homogenized and subjected to mechanical lysis using the FastPrep 96 (MP BioMedicals). Supernatants from processed samples were treated with protease, and DNA was extracted using the Mag Midi LGC kit (cat. No. NAP40420, LGC Genomics) on the KingFisher Flex DNA extraction robot (Thermo Fisher Scientific). 16S rRNA genes (V3–V4 region) were amplified by PCR (prokaryote-targeting primers: forward 5'-CCTACGGGRBGCASCAG-3', reverse 5'-GGACTACYVGGGTATCTAAT-3' [26]). After purification of amplicon PCR products using AMPure XP (Beckman-Coulter), PCR with index primers modified with Illumina adapters (Additional file 1: Table S2) [27] was performed. The resulting products were quantified, normalized, and pooled. After being purified using AMPure XP and diluted to 6 pM, the pooled library was sequenced with the MiSeq Reagent Kit V3 (Illumina, cat. No. MS-102–3003, Illumina) on the Illumina MiSeq. PhiX (20%, Illumina, cat. No. FC-110–3001) served as internal control.

Forward and reverse paired-end reads (300 bp) were assembled, split into their respective samples, and quality-filtered using Quantitative Insights Into Microbial Ecology (QIIME) [28]. The resulting dataset included in total 7,790,257 sequences from the 227 faecal samples, ranging from 12,420 to 115,287 sequences per sample. The mean (SD) sequencing depth was 34,318 (14,255) sequences per sample. The closed-reference USEARCH algorithm (version 8) [29, 30] was used against the SILVA database (release 128) [31] to cluster the sequences into taxonomically assigned operational taxonomic units (OTUs) with ≥ 97% identity. The OTU counts for each sample were normalized by down-sampling (rarefying) to an even library size of 10,000 sequences per sample in QIIME (core_diversity_analyses.py). In total, 1096 OTUs were identified in the normalized dataset. The OTUs were taxonomically binned into 13 phylum (p_)-, 25 class (c_)-, 41 order (o_)-, 68 family (f_)-, and 237 genus (g_)-level taxa when excluding OTUs with no taxonomic assignment below kingdom level (i.e. OTUs classified as 'unassigned' or 'unknown bacteria'). The taxon

abundances are presented as relative abundances (%) where the lowest detectable abundance was 0.01% (1 out of 10,000 sequences).

Bacterial between-sample diversities (β-diversity) and within-sample diversities (α-diversity) were calculated based on the normalized OTU table(s) using the default QIIME script (core_diversity_analyses.py). Three measures of β-diversity (binary Jaccard, Bray–Curtis, and weighted UniFrac) and five indices of α-diversity (observed number of OTUs, Chao1, Shannon–Wiener, Simpson, and phylogenetic diversity [PD] whole tree) were calculated. Rarefaction curves for the α-diversity indices are shown in Additional file 1: Fig. S1. While β-diversity aims to quantify the overall dissimilarity ('distance') between two bacterial communities, α-diversity describes the bacterial diversity within a given community. A higher value indicates less similarity and greater diversity, respectively. The different α-diversity indices are meant to capture the species richness (observed number of OTUs, Chao1), the combination of richness and evenness (Shannon–Wiener, Simpson), and phylogenetic diversity (PD whole tree). Ordination of β-diversities was performed using nonmetric multidimensional scaling. Additional details regarding the α- and β-diversity analyses are provided in Additional file 1: Supplementary method description [32, 33].

Faecal short-chain fatty acid measurements

The method for quantification of faecal SCFA has been described elsewhere [25]. Briefly, faecal aliquots were homogenized in distilled water containing internal standard (2-ethylbutyric acid) and sulfuric acid. The homogenates were vacuum distilled [34, 35] and formic acid was added. The distillates were analysed with gas chromatography (Agilent 6850, Agilent) using an HP-FFAP WAX capillary column (part No. 19091F-112E, serial number USE400345H, Agilent J&W GC columns; Agilent). Concentrations of acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, caproic, and iso-caproic acid were determined using internal standardization employing flame ionization detection. All SCFA results were adjusted for faecal wet weight and are expressed as mmol/kg faeces and proportional levels (% of total SCFA concentration). The SCFA caproic and iso-caproic acid were not included in the statistical analyses due to low detectability.

Faecal neutrophil gelatinase-associated lipocalin measurements

Faecal NGAL/LCN2 was quantified by enzyme-linked immunosorbent assay (ELISA) as described elsewhere [25]. Briefly, faecal aliquots were added Dulbecco's phosphate-buffered saline (L0615, Biowest) with Tween20 (P1379, Sigma-Aldrich) and vigorously mixed using an MM2 mixer mill (Retsch), followed by centrifugation. The supernatants were diluted 1:50 in Dulbecco's phosphate-buffered saline. ELISA was performed using the Human lipocalin-2/NGAL Duoset ELISA (DY1757, R&D Systems). Samples were analysed in duplicate. The results were adjusted for faecal wet weight and are expressed as ng/g faeces.

Statistics

Sample size estimation was based on paired *t*-test of differences in the GSRS-IBS total score (the primary outcome of the clinical trial) between two challenges within the same subject, as previously reported [18]. The total level of significance was set to 0.05 (two-sided), and we used 0.02 for the pairwise comparisons (0.05/3, Bonferroni multiple comparison correction). With a power of 80% and anticipated drop-out of 30%, it was estimated that 66 participants were needed to detect a mean difference in the GSRS-IBS total score of 1.5 points (SD, 3.2).

All the statistical analyses were conducted in the R programming environment (version 4.2.3) [33]. Additional details regarding the statistical analyses, including specific use of packages and functions in R, can be found in Additional file 1: Supplementary method description [33, 36–43]. A P value < 0.05 was considered statistically significant. The Benjamini–Hochberg (BH) method was in some cases (specified in the text) used to control the false discovery rate (FDR level 5%).

The responses to the gluten, FOS-fructan, and placebo challenges were evaluated for the faecal outcomes (α-diversity, taxa abundances, SCFA, and NGAL/LCN2) using linear mixed models in which change values were used as the response variable. The change values for each of the three diet challenge periods were calculated for each participant by subtracting the baseline value from the follow-up value (after gluten, FOS-fructan, or placebo challenge). Hence, each participant had three change values, one for each diet challenge. Baseline values (continuous), period (categorical; period 1, 2, 3), sequence (categorical; GFP, GPF, FGP, FPG, PGF, PFG), and diet challenge (categorical; gluten, FOS-fructan, placebo) were used as fixed effects. A random intercept for participant was included. Interactions between the fixed effects were not included in the models. Significant differences in the mean change across the diet challenges ($P < 0.05$) were followed by post hoc pairwise comparisons of the three diet challenges (FOS-fructan vs. gluten, FOS-fructan vs. placebo, and gluten vs. placebo). The three P values from pairwise comparisons for each faecal response were adjusted using the Bonferroni method. For each faecal outcome, only participants with available data from baseline and at least one of the challenge periods were included in the linear mixed model analysis. As

IBS status, age, gender, duration of gluten-free diet, body mass index, HLA-DQ status nor thyroid disease had no effect on the GI symptom response in the linear mixed model [18], we did not include these variables as explanatory variables in the analyses in the current manuscript. Furthermore, since positive wheat-specific IgE levels were an exclusion criterion in the study, adjustment for wheat allergy status was not applicable.

Differences in the overall bacterial communities between diet challenges based on the β-diversity measures were assessed using global permutation-based multivariate analysis of variance (PERMANOVA).

Spearman's rank correlation coefficients (Rho) and corresponding *P* values were calculated to assess the associations between the changes in GI symptoms (GSRS-IBS scores) and faecal outcomes (taxa abundances, α-diversity, SCFA, and NGAL/LCN2). For the faecal outcomes, both change and baseline values were used in the analyses. Rho values between 0 and (−) 0.3 are described as negligible, between (−) 0.3 and (−) 0.5 as low, between (−) 0.5 and (−) 0.7 as moderate, between (−) 0.7 and (−) 0.9 as high, and between (−) 0.9 and (−) 1 as very high [44]. Rho values for the correlations between GI symptoms and the faecal outcomes were visualized in correlograms with hierarchical clustering (Euclidean distances, complete linkage).

For statistical procedures involving the relative abundances of bacterial taxa, only taxa with a certain average abundance and detectability were included. Specifically, when performing linear mixed model analysis and correlation analysis using changes in relative abundances, a taxon was excluded if the median relative abundance was less than 0.1% for at least one time point (baseline or after a challenge) or if the taxon was detected in less than 50% of the samples from all time points (baseline and after each challenge). When performing correlation analysis using baseline abundances only, a taxon was excluded if it was detected in less than 50% of the baseline samples. Furthermore, in cases where a taxon at a given taxonomic level (e.g. family) included only one lower-level taxon (e.g. family *Bacteroidaceae* and genus *Bacteroides*), the results for the higher-level taxon are not presented. Using the described filtering, 81 taxa were included in the analyses involving change values, and 123 taxa were included in the analyses involving baseline values only. The 0.1% abundance limit was considered a suitable threshold to filter the taxa for statistical analyses, since 0.1% in our dataset equals a read count of 10 (10 out of 10,000 reads equals 0.1%). The 50% presence limits were used to exclude taxa that were present in few samples to assure that the data was more suitable for the linear mixed model and correlation analyses.

Results

Participants, data collection, and baseline characteristics

The study population was described in detail by Skodje et al. [18]. A graphical representation of participant/sample inclusion and exclusion can be found in Fig. 2. Initially, 232 individuals were subjected to eligibility assessment, 164 of whom were deemed ineligible either because they did not meet the specified inclusion criteria or because they fulfilled at least one exclusion criterion (see details in Fig. 2). Consequently, the final cohort comprised 68 eligible participants who were randomised to the 6 diet challenge sequences (11 to each challenge sequence, in addition to 2 surplus subjects). Subsequently, nine participants were excluded: two due to redundancy in participation, one due to loss to follow-up, three due to withdrawal, and three due to protocol violations. This culminated in a subset of 59 participants for whom both GSRS-IBS data and stool samples were available (challenge sequence GFP: $n=10$, GPF: $n=9$, FGP: $n=10$, FPG: $n=9$, PGF: $n=11$, PFG: $n=10$). However, stool samples/faecal outcomes were not obtained from all participants at every designated time point. Specifically, the baseline stool sample was missing for one participant (sequence order PGF), the samples for both gluten and placebo challenges were missing for two participants (challenge sequence FGP and FPG), one participant was missing the gluten challenge sample (challenge sequence GPF), and one participant was missing the samples from all diet challenges (challenge sequence FPG). Additionally, for the SCFA analysis, the amount of stool material obtained from baseline was insufficient for two participants (challenge sequence GFP and FGP). Finally, the NGAL/LCN2 results from the FOS-fructan and placebo challenges for one participant were excluded due to technical issues in the laboratory (challenge sequence FGP). Thus, in cases where the faecal outcome was missing from baseline, the participant had to be excluded from all the statistical analyses, and participants had to be excluded from the statistical analyses involving changes in faecal outcomes in cases where outcomes were missing from all diet challenges (Fig. 2).

Table 1 provides an overview of the baseline characteristics pertaining to the 58 participants whose faecal outcomes were included in the subsequent statistical analyses. The demographic was dominated by females (90%), predominantly within the age range of 35 to 55 years, with a body mass index distribution primarily categorized as normal or overweight. Prior to the intervention, participants had embraced a GFD for an average duration of approximately 2 years, driven by their experiences of GI symptoms such as bloating, diarrhoea, constipation, and/or nausea while on a gluten-containing

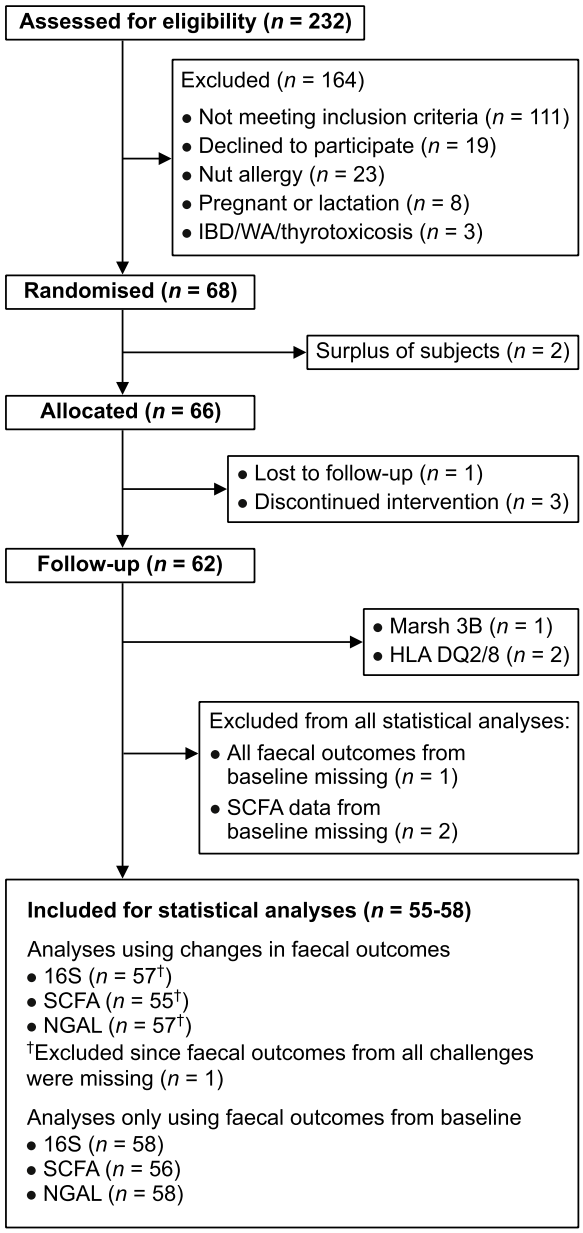

Fig. 2 Flow chart of the population selection process and overview of analysed samples/data. *IBD* Inflammatory bowel disease, *NGAL* Human neutrophil gelatinase-associated lipocalin, *SCFA* Short-chain fatty acids, *WA* Wheat allergy

diet. A notable majority (75%) had also experienced extraintestinal symptoms, including manifestations such as joint and muscle pain, and headaches, prior to their adoption of GFD. Furthermore, it should be acknowledged that the participants' baseline state was not devoid of GI symptoms, as evidenced by the average GSRS-IBS total score of 30. Nevertheless, only 30% of the participants were formally diagnosed with IBS as per the Rome III criteria. It is also worth noting that the average fructan intake at baseline was 2 g per day, a quantity that doubled during the subsequent FOS-fructan challenge period. Additional baseline information has been reported by Skodje et al. [18, 45]. None of the participants experienced severe adverse effects from the diet challenges. Mean (SD) duration of the first and second washout periods were 13 (8.3) days and 16 (13) days, respectively.

Table 1 Participant characteristics at baseline ($n = 58$)

Characteristic	Value
Female/male	52/6 (90/10)
Age (years)	44 ± 12
BMI (kg/m^2)	25 ± 4
BMI category	
Underweight (< 18.5)	1 (2)
Normal weight (18.5–24.9)	31 (53)
Overweight (25.0–29.9)	20 (35)
Obese (> 30.0)	6 (10)
Symptoms before GFD	
Gastrointestinal symptoms	58 (100)
Extraintestinal symptoms	42 (75)[b]
Duration on GFD (months)	22 [10, 49]
IBS by Rome III	17 (30)[c]
Allergy/intolerance	14 (25)[c]
Other food exclusions	37 (64)
Study gastroscopy	47 (81)
Marsh 0	42 (89)
Marsh 1[a]	5 (11)
GSRS-IBS total score	30 [21, 37]
Total FODMAP intake (g/day)	10 [5, 15][c]
Total fructan intake (g/day)	2 [1, 3][c]

Values are presented as the means ± SDs for normally distributed variables, medians [25th,75th percentiles] for nonnormally distributed variables, or *n* (%) for categorical variables. Additional baseline information has been reported by Skodje et al. [18, 45]

BMI Body mass index, *GFD* Gluten-free diet, *IBS* Irritable bowel syndrome, *GSRS-IBS* Gastrointestinal symptom rating scale IBS version, *FODMAP* Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols

[a] > 25 intraepithelial lymphocytes per 100 epithelial cells

[b] $n = 56$ (two missing)

[c] $n = 57$ (one missing)

No differences in overall faecal microbiota profiles (β-diversity) across diet challenges

To evaluate potential differences in the overall bacterial communities after the gluten, FOS-fructan, and placebo challenges, we constructed ordination plots utilizing nonmetric multidimensional scaling based on three distinct measures of between-sample diversity (β-diversity): binary Jaccard, Bray–Curtis, and weighted UniFrac. The three β-diversity measures were selected based on their capacity to capture various types of dissimilarities

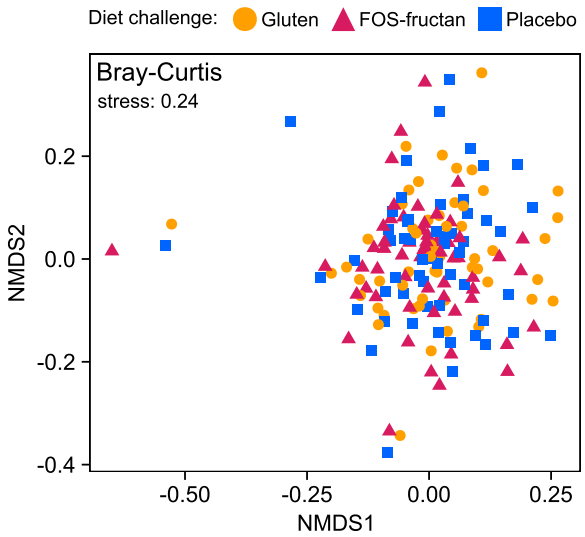

Fig. 3 Ordination plots from nonmetric multidimensional scaling (NMDS) of Bray–Curtis distances. Each symbol represents one follow-up sample from one participant after a given 7-day challenge (gluten, FOS-fructan, or placebo), in total 169 samples. Stress values indicate the goodness-of-fit of the NMDS. *FOS* fructo-oligosaccharides

between bacterial community structures, including presence/absence, abundance, and phylogenetic relationships. However, regardless of β-diversity measures, visual inspection did not reveal any evident clustering based on the diet challenges (Bray–Curtis in Fig. 3, binary Jaccard and weighted UniFrac in Additional file 1: Fig. S2B, Fig. S3B), as confirmed by PERMANOVA ($P \geq 0.99$). This high degree of overlap of samples collected following the gluten, FOS-fructan, and placebo challenges suggests that the diet challenges involving gluten and FOS-fructans did not give rise to substantial and discernible systematic alterations in the faecal microbiota compared to placebo. To corroborate this observation, we extended our analysis by constructing ordination plots encompassing both baseline and challenge follow-up samples, as well as pairwise comparisons. Notably, these additional plots (Additional file 1: Fig. S2, S3, and S4) also failed to reveal distinctive clustering patterns among the samples, as confirmed by PERMANOVA ($P \geq 0.92$).

No differences in faecal bacterial α-diversity across diet challenges

To assess the potential impact of the gluten, FOS-fructan, and placebo challenges on faecal bacterial richness and evenness, we employed five distinct indices of within-sample diversity (α-diversity): observed number of OTUs, Chao1, Shannon–Wiener, Simpson, and PD whole tree. However, through linear mixed model analyses, no significant differences in the mean change in α-diversity across the gluten, FOS-fructan, and placebo challenges were found for any of the α-diversity indices ($P \geq 0.11$, Additional file 1: Table S3). Summary statistics for the five α-diversity indices at baseline and after each diet challenge are given in Additional file 1: Table S4.

Differences in faecal bacterial abundances across diet challenges

Linear mixed model analyses were performed to assess whether the gluten, FOS-fructan, and placebo challenges induced different changes in faecal bacterial abundances (%) at various taxonomic levels (phylum, class, order, family, and genus). Of the 81 taxa included in the analysis (see method Sect. 'Statistics'), significant differences in the mean change across the diet challenges were found for 12 taxa (Table 2). The significant effects of diet challenge were followed by pairwise comparisons (i.e. FOS-fructan vs. gluten, FOS-fructan vs. placebo, and gluten vs. placebo) while applying Bonferroni corrections for multiple testing (Table 2).

Following the FOS-fructan challenge, the mean change for four taxa belonging to the *Clostridiales* order tended to be different or was significantly different compared to the gluten and placebo challenges. These genera included *Fusicatenibacter*, *Eubacterium coprostanoligenes group* (*E. coprostanoligenes*), *Anaerotruncus*, and *unknown Ruminococcaceae* genera (Table 2). While the abundance of *Fusicatenibacter* increased with the FOS-fructan challenge, the abundances of the genera *E. coprostanoligenes*, *Anaerotruncus*, and *unknown Ruminococcaceae* decreased. For these four taxa, no differences in the mean change were found between the gluten and placebo challenges.

Following the gluten challenge, the mean changes for four taxa were significantly different from the mean changes for both the FOS-fructan and placebo challenges: the genera *Eubacterium xylanophilum group*, *Ruminococcus torques group*, and *Bilophila*, as well as the family *Desulfovibrionaceae* (primarily driven by the genus *Bilophila*) (Table 2). The differences in the mean change in the *Eubacterium xylanophilum group* between the diet challenges were driven by increase in abundance with the gluten challenge. For the *Ruminococcus torques group* and *Bilophila*/*Desulfovibrionaceae*, the differences in mean change were due to increased and decreased abundance following the gluten and FOS-fructan/placebo challenges, respectively.

For the *Clostridiales*-families *Christensenellaceae* and *Family XIII*, along with the genera *Ruminococcaceae NK4A214 group* and *Ruminococcaceae UCG-002*, the changes from baseline to follow-up tended to be or were significantly different between the gluten (increased abundance) and FOS-fructan challenge (decreased abundance) (Table 2). However, no significant differences were

Table 2 Changes in faecal bacterial abundances following the 7-day gluten, FOS-fructan, and placebo challenges ($n = 57$)

	Gluten	FOS-fructan	Placebo	Effect of challenge	F vs. G	F vs. P	G vs. P
	EMM (CI)	EMM (CI)	EMM (CI)	*P* value[a]	*P* value[b]	*P* value[b]	*P* value[b]
g_Fusicatenibacter	0.044 (−0.048, 0.14)	0.16 (0.072, 0.25)	0.029 (−0.063, 0.12)	0.02*	0.07	0.03*	> 0.99
g_Eubacterium coprostanoligenes group	−0.014 (−0.085, 0.058)	−0.11 (−0.18, −0.039)	−0.032 (−0.10, 0.039)	0.03*	0.04*	0.13	> 0.99
g_Anaerotruncus	0.020 (−0.028, 0.069)	−0.047 (−0.094, −0.000)	0.030 (−0.018, 0.077)	0.01*	0.05*	0.02*	> 0.99
g_Unknown Ruminococcaceae	0.025 (−0.10, 0.15)	−0.16 (−0.28, −0.035)	0.037 (−0.091, 0.16)	0.02*	0.05	0.03*	> 0.99
g_Eubacterium xylanophilum group	0.100 (0.037, 0.16)	−0.033 (−0.095, 0.028)	0.000 (−0.062, 0.063)	< 0.001*	< 0.001*	> 0.99	0.02*
g_Ruminococcus torques group	0.072 (−0.003, 0.15)	−0.063 (−0.14, 0.010)	−0.067 (−0.14, 0.007)	0.005*	0.02*	> 0.99	0.01*
f_Desulfovibrionaceae	0.025 (−0.018, 0.068)	−0.037 (−0.078, 0.005)	−0.040 (−0.082, 0.003)	0.01*	0.04*	> 0.99	0.03*
g_Bilophila	0.017 (−0.024, 0.058)	−0.043 (−0.084, −0.003)	−0.035 (−0.076, 0.006)	0.009*	0.01*	> 0.99	0.04*
f_Christensenellaceae	0.044 (−0.053, 0.14)	−0.074 (−0.17, 0.021)	0.018 (−0.078, 0.11)	0.04*	0.05	0.19	> 0.99
f_Family XIII	0.008 (−0.023, 0.038)	−0.043 (−0.073, −0.013)	−0.024 (−0.054, 0.006)	0.02*	0.02*	0.90	0.26
g_Ruminococcaceae NK4A214 group	0.061 (−0.025, 0.15)	−0.061 (−0.14, 0.023)	−0.007 (−0.092, 0.078)	0.04*	0.03*	0.73	0.45
g_Ruminococcaceae UCG-002	0.068 (−0.017, 0.15)	−0.050 (−0.13,0.032)	0.015 (−0.069, 0.099)	0.05*	0.04*	0.50	0.80

Only taxa with significant differences across diet challenges are included in the table. Changes in bacterial relative abundances from baseline to diet challenge follow-ups are presented as estimated marginal means (EMM) (95% confidence intervals (CI)) from linear mixed models with change values as the response variable and baseline values, period, sequence, and diet challenge as explanatory variables. The change values for each diet challenge were calculated for each participant by subtracting the baseline value from the follow-up value (i.e. positive value: increased, negative value: decreased). Follow-up and baseline values were sqrt-transformed, i.e. a change value is the sqrt-transformed follow-up value minus the sqrt-transformed baseline value for a given participant/challenge. Among the 57 participants included in the analyses, two participants had abundance data only from the FOS-fructan challenge, and one participant was missing abundance data from the gluten challenge

FOS Fructo-oligosaccharides, *F* FOS-fructan, *G* Gluten, *P* Placebo

[a] *P* value for the main effect of diet challenge from linear the mixed model (not adjusted for the total number of taxa subjected to mixed model analysis)

[b] Bonferroni-adjusted *P* values from pairwise comparisons between diet challenges

* $P < 0.05$

observed between the placebo and gluten challenges or between the placebo and FOS-fructan challenges for these four taxa.

In addition to the 12 taxa described above, 22 taxa displayed notable changes from baseline to follow-up for at least one of the three diet challenges but did not significantly differ across challenges (Additional file 1: Table S5). These included increased abundance of the genera *Bifidobacterium*, *Anaerostipes*, *Faecalibacterium*, and *Sutterella*, and decreased abundance of *Subdoligranulum* following the FOS-fructan challenge. For the gluten challenge, the abundances of the genera *Streptococcus* and *Phascolarctobacterium* were found to be decreased and increased, respectively.

When examining the average (median) relative abundances at the phylum- and family levels across all samples, the phyla *Firmicutes* (60%), *Bacteroidetes* (34%), *Proteobacteria* (2.0%), and *Actinobacteria* (1.3%) exhibited the highest relative abundances, along with the families *Lachnospiraceae* (28%), *Ruminococcaceae* (27%), *Bacteroidaceae* (21%), and *Rikenellaceae* (4.1%). However, substantial variations were observed among the samples, as shown by the abundance distributions in Fig. 4. For instance, the ratio of *Firmicutes* to *Bacteroidetes* (%:%) ranged from 35:60 to 91:4, and the abundances of *Proteobacteria* and *Actinobacteria* ranged from < 0.1% to 26% and 9%, respectively. The variation among samples at the genus level is shown in Additional file 1: Fig. S5. Summary statistics for bacterial relative abundances at baseline and after each diet challenge are given in Additional file 1: Table S6.

It should also be noted that if the statistical tests of the main effects of diet challenge were to be adjusted for the number of taxa subjected to linear mixed model analysis (81 taxa, see method Sect. 'Statistics') using the BH method for multiple adjustments, the 12 taxa in Table 2 would not attain significance. Furthermore, to check whether the 50% presence and 0.1% abundance limit would lead to the exclusion of important taxa influenced by the dietary challenges (see method Sect. 'Statistics'), we also performed a filtration based on a 25% presence limit and a 0.05% abundance limit. Using these limits, 11 additional taxa were included in the mixed model analysis (92 taxa in total instead of the original 81). However,

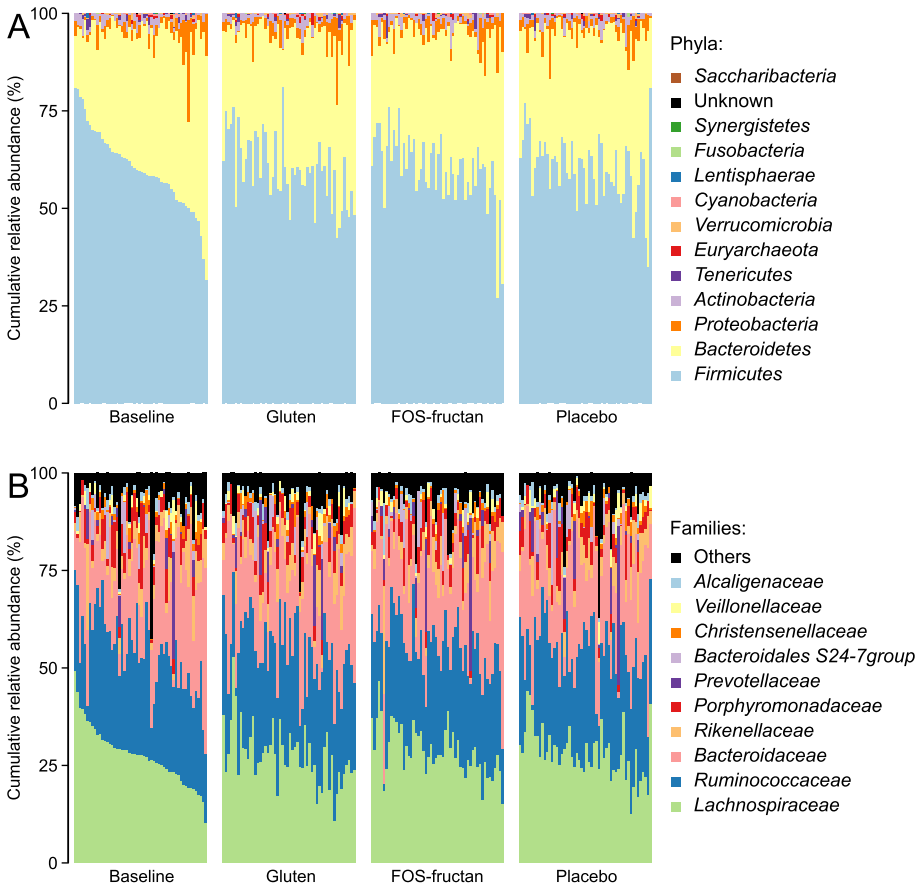

Fig. 4 Faecal bacterial abundances (%) at baseline and following the 7-day gluten, FOS-fructan, and placebo challenges. **A** Bacterial phyla (all detected) and **B** abundant families. Only participants with 16S sequencing data from all four time points are included in the figure ($n=54$). One bar represents one participant at a given time point. Participant bars for baseline and after each challenge are ordered according to the abundance of *Firmicutes* (in A) and *Lachnospiraceae* (in B) at baseline. Taxa are ordered according to mean overall abundance. Families with a mean overall abundance ≤ 1% are displayed as 'Others'. *FOS* fructo-oligosaccharides

no differences across diet challenges were found for any of these 11 additional taxa (data not shown).

No differences in faecal short-chain fatty acids across diet challenges

Faecal SCFA were examined to discern potential differential effects of the gluten, FOS-fructan, and placebo challenges on bacterial fermentation. Utilizing linear mixed model analyses, no differences in the mean change in total SCFA concentration (mmol/kg faeces) or in the concentrations or proportional levels (% of total) of individual SCFA (acetic, propionic, butyric, iso-butyric, valeric, and iso-valeric) were found across the diet challenges (Table 3). However, we observed that the faecal SCFA concentrations generally increased from baseline to follow-ups, regardless of diet challenge (see the positive estimated marginal means in Table 3). Summary statistics for SCFA at baseline and after each diet challenge are given in Additional file 1: Table S7.

No differences in faecal neutrophil gelatinase-associated lipocalin across diet challenges

Faecal NGAL/LCN2 concentrations (ng/g faeces) were quantified to explore potential changes in low-grade intestinal inflammation induced by the gluten, FOS-fructan, and placebo challenges. Using linear mixed model analysis, the estimated marginal mean change (95% confidence interval) for the diet challenges were 17 (−52, 87), 31 (−37, 99), and 25 (−45, 95), respectively, with no significant differences across challenges ($P=0.96$, $n=57$). Among the 57 participants included in the linear mixed model analysis, two participants had only NGAL/LCN2 data from the FOS-fructan challenge, one participant had missing NGAL/LCN2 data from the gluten challenge, and one participant had only NGAL/LCN2 data from the gluten challenge. Summary statistics for NGAL/LCN2 at baseline and after each diet challenge are given in Additional file 1: Table S8.

Table 3 Changes in faecal SCFA following the 7-day gluten, FOS-fructan, and placebo challenges ($n = 55$)

	Gluten	FOS-fructan	Placebo	Effect of challenge
	EMM (CI)	EMM (CI)	EMM (CI)	*P* value[a]
Total SCFA, conc	5.5 (−0.3, 11)	6.0 (0.4, 12)	4.8 (−0.9, 11)	0.95
Acetic acid, conc	2.6 (−0.9, 6.0)	3.0 (−0.4, 6.4)	1.9 (−1.5, 5.4)	0.86
Butyric acid, conc	1.2 (−0.4, 2.7)	1.7 (0.2, 3.2)	1.3 (−0.2, 2.8)	0.86
Propionic acid, conc	1.4 (0.3, 2.5)	1.1 (0.1, 2.2)	1.3 (0.2, 2.4)	0.93
Valeric acid, conc	0.2 (0.0, 0.3)	0.1 (−0.1, 0.2)	0.1 (−0.0, 0.3)	0.67
Iso-valeric acid, conc	0.1 (−0.1, 0.4)	0.0 (−0.2, 0.3)	0.1 (−0.2, 0.4)	0.76
Iso-butyric acid, conc	0.1 (−0.1, 0.2)	0.0 (−0.1, 0.2)	0.1 (−0.1, 0.2)	0.94
Acetic acid, %	−2.0 (−3.8, −0.3)	−1.6 (−3.3, 0.1)	−2.2 (−4.0, −0.5)	0.84
Butyric acid, %	1.1 (−0.1, 2.3)	1.7 (0.6, 2.9)	1.6 (0.4, 2.8)	0.69
Propionic acid, %	0.8 (−0.0, 1.7)	0.4 (−0.4, 1.2)	0.8 (−0.0, 1.6)	0.57
Valeric acid, %	0.2 (0.0, 0.4)	−0.0 (−0.2, 0.1)	0.1 (−0.1, 0.3)	0.09
Iso-valeric acid, %	0.0 (−0.4, 0.4)	−0.3 (−0.7, 0.1)	−0.1 (−0.5, 0.3)	0.37
Iso-butyric acid, %	−0.0 (−0.3, 0.2)	−0.2 (−0.4, 0.1)	−0.1 (−0.3, 0.1)	0.57

Changes in SCFA concentrations ('conc', mmol/kg faeces) and proportional levels ('%', of total SCFA concentration) from baseline to diet challenge follow-ups are presented as estimated marginal means (EMM) (95% confidence intervals (CI)) from linear mixed models with change values as the response variable and baseline values, period, sequence, and diet challenge as explanatory variables. The change values for each diet challenge were calculated for each participant by subtracting the baseline value from the follow-up value (i.e. positive value: increased, negative value: decreased). Among the 55 participants included in the analyses, 2 participants had SCFA data only from the FOS-fructan challenge, and 1 participant was missing SCFA data from the gluten challenge

FOS Fructo-oligosaccharides, *SCFA* Short-chain fatty acids

[a] *P* value for the main effect of diet challenge from the linear mixed model. Since no significant differences were found across diet challenges ($P \geq 0.05$), pairwise comparisons between the three diet challenges were not performed

Associations between changes in gastrointestinal symptoms and faecal outcomes

The principal investigations concerning the faecal outcomes showed that the diet challenges with FOS-fructans and gluten induced different changes in certain bacterial taxa, but that no differences were found for the remaining outcomes (α-diversity, SCFA, NGAL/LCN2). We next examined whether the 12 bacterial taxa that were differentially affected by the diet challenges (see results Sect. 'Differences in faecal bacterial abundances across diet challenges' and Table 2) were associated with the changes in the IBS-like GI symptoms (assessed via GSRS-IBS total, pain, bloating, constipation, diarrhoea, and satiety dimension scores). To examine these associations, we employed Spearman's rank correlation analysis, and the results are graphically depicted in correlograms featuring hierarchical complete linkage clustering (Fig. 5). As shown in Fig. 5, correlation coefficients for the different GSRS-IBS dimensions clustered mostly according to diet challenges, i.e. exacerbation of GI symptoms following the FOS-fructan and gluten challenges was associated with different types of changes in bacterial abundances. However, few significant associations were found (not adjusted for multiple testing), and all were considered negligible or low in strength ($-0.36 < \text{Rho} < 0.32$).

For the three taxa that increased and decreased following the gluten and FOS-fructan challenge, respectively (*Christensenellaceae*, *Ruminococcaceae UCG-002*, and *Ruminococcaceae NK4A214 group*), the changes in bacterial abundance were not associated with increased GI symptoms. There was also no association between increased GI symptoms and the FOS-related decrease in *Anaerotruncus*, unknown *Ruminococcaceae* genera, or *Clostridiales Family XIII*, or for the FOS-fructan-related increase in *Fusicatenibacter*. However, the decreased abundance of the *E. coprostanoligenes* induced by the FOS-fructan challenge was associated with increased GI symptoms, significantly for the GSRS-IBS pain dimension. For the *Eubacterium xylanophilum group* and *Ruminococcus torques group*, which both increased following the gluten challenge, increased abundances were associated with exacerbation in GI symptoms, however not significantly.

Correlograms depicting the correlations between changes in GI symptoms and the remaining faecal outcomes (bacterial taxa not described above, α-diversity, SCFA, and NGAL/LCN2) can be found in Additional file 1: Fig. S6. Interestingly, increased faecal concentrations of SCFA were associated with less exacerbation of the GSRS-IBS constipation dimension following the FOS-fructan challenge (significantly for total SCFA, butyric-, and propionic acid). If adjusted for multiple testing using the BH method, all correlations depicted in Additional file 1: Fig. S6 were considered nonsignificant.

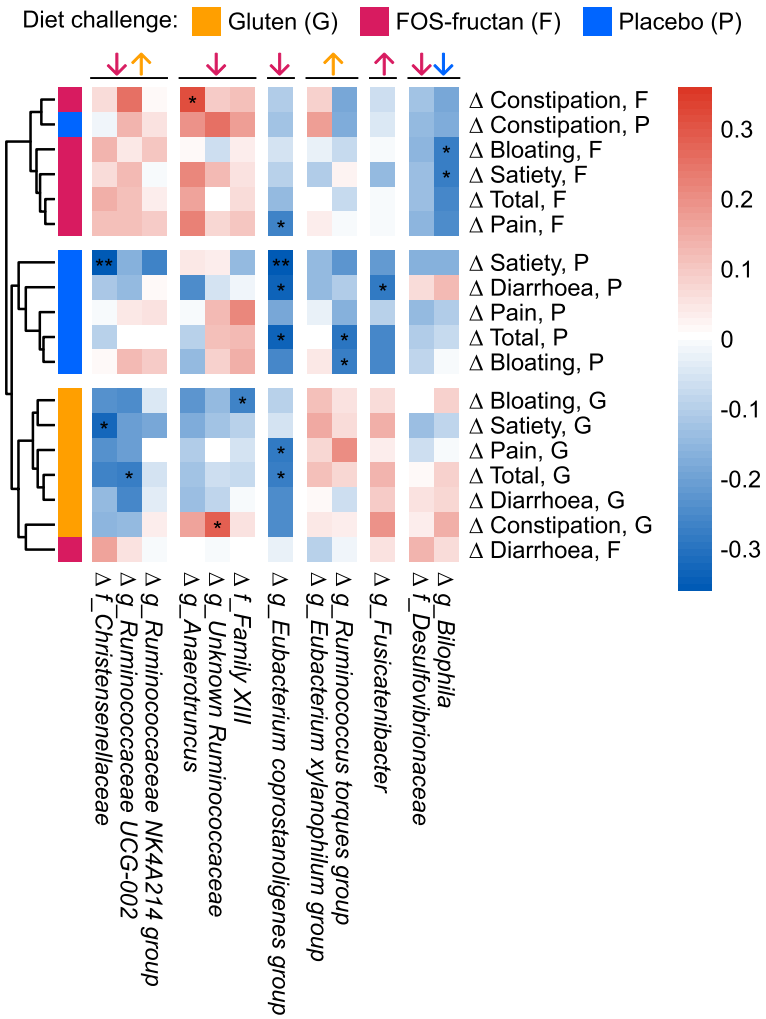

Fig. 5 Correlations between changes in faecal outcomes and changes in GI symptoms. Correlograms illustrating Spearman's rank correlation coefficients (Rho) between changes ('Δ') in gastrointestinal symptoms (GSRS-IBS total, pain, bloating, constipation, diarrhoea, and satiety dimension scores) and changes in faecal taxa abundances following the 7-day gluten, FOS-fructan, and placebo challenges. The change values for each diet challenge were calculated for each participant by subtracting the baseline value from the follow-up value. The colour scale indicates the strength and direction of the correlations (–0.36 < Rho < 0.32). Significant correlations (not adjusted for multiple testing) are marked with asterisks (*$P < 0.05$, **$P < 0.01$). A positive correlation indicates that increased taxon abundances are associated with increased GSRS-IBS scores, while a negative correlation indicates that reduced taxon abundances are associated with increased GSRS-IBS scores. Only the taxa that had changed differently across the diet challenges (see 'Results' Sect. 'Differences in faecal bacterial abundances across diet challenges' and Table 2) were included in the analysis. The arrows indicate the direction of change that was observed for the taxon following the different diet challenges, in accordance with results shown in Table 2 (upwards arrow indicates increased abundance, downwards arrow indicates decreased abundance). *FOS* fructo-oligosaccharides, *GSRS-IBS* gastrointestinal symptom rating scale IBS version, *IBS* irritable bowel syndrome

Associations between baseline gut microbiota and inflammation status, and changes in gastrointestinal symptoms

As previously reported by Skodje et al. [18], it was evident that the participants in the study did not exhibit uniform GI symptom responses to the diet challenges. This led us to postulate that variations in the faecal microbiota and related variables at baseline might underlie the diversity in symptom responses. To explore this proposition, we employed Spearman's rank correlation to evaluate potential associations between faecal outcomes at baseline (i.e. bacterial abundances, bacterial α-diversity, SCFA, and NGAL/LCN2) and changes in IBS-like GI symptoms (assessed via GSRS-IBS pain, bloating, constipation, diarrhoea, and satiety scores) following the diet challenges. The results of this analysis are visually represented using correlograms featuring hierarchical clustering (Fig. 6). It should be noted that only correlations between NGAL/

LCN2 and GI symptoms remained significant if adjusted for multiple testing using the BH method.

Complete linkage clustering of the correlation coefficients between baseline bacterial abundances and changes in GI symptoms revealed that the coefficients were organized into diet challenge clusters to a certain extent (Fig. 6A, −0.37 < Rho < 0.39; negligible or low in strength). Notably, the changes in GSRS-IBS total, bloating, pain, and diarrhoea scores following the FOS-fructan and gluten challenges were found to be linked to varying bacterial abundances at baseline. Following the FOS-fructan challenge, low abundances of several taxa were associated with increased GI symptoms (negative associations). For instance, exacerbation of diarrhoea symptoms was associated with low abundances of the order *Clostridiales* (including the genera *Coprococcus1* and *Lachnospiraceae FCS020 group* and the *Ruminococcaceae* family) and the genera *Dialister* and *Bilophila*. In contrast to these negative associations between taxa and GI response to the FOS-fructan challenge, high abundances of the order *Bacteroidales* (including the abundant genus *Odoribacter*) and the *Lachnospiraceae NK4A136 group* were associated with increased symptoms. For the GSRS-IBS bloating dimension, which was of particular interest [18], negative associations were found only for the genera *Ruminiclostridium9* and *Coprococcus2* and positive associations were found only for the genera *Odoribacter* and *Lachnospiraceae NK4A136 group* following the FOS-fructan challenge. Baseline abundances of several taxa were also associated with symptom exacerbation following the gluten challenge. Among others, these included the family *Coriobacteriaceae*, *Clostridiales*-genus *Eubacterium hallii group*, genus *Streptococcus*, and *Erysipelotrichaceae UCG-003*, for which high abundances were associated with increased GI symptoms. The association patterns described above were mainly present for the specified challenge. See Fig. 6A for details.

When investigating the correlations between α-diversities at baseline and the GI symptom responses (Fig. 6B), we found that increased total and satiety symptoms following the FOS-fructan challenge were associated with low α-diversity, particularly for the indices Shannon, Simpson, and observed number of OTUs. However, the correlation coefficients did not cluster according to diet challenge, and all correlations were negligible or low in strength (−0.32 < Rho < 0.12).

Only baseline concentrations of the low-abundant SCFA valeric, iso-valeric-, and iso-butyric acid were found to be associated with changes in GI symptoms following the gluten and FOS-fructan challenges (Fig. 6C). For the FOS-fructan challenge, low concentrations of valeric acid and proportional levels of iso-valeric acid were associated with exacerbated bloating and satiety symptoms, respectively. Following the gluten challenge, low concentrations of iso-butyric acid were associated with increased constipation. The correlation coefficients did not cluster according to diet challenges, and all correlations were negligible in strength (−0.28 < Rho < 0.27).

No significant associations were found between baseline concentrations of faecal NGAL/LCN2 and symptom responses to the gluten and FOS-fructan challenges (Fig. 6D, −0.05 < Rho < 0.26; negligible or low in strength). However, high NGAL/LCN2 concentrations at baseline were significantly associated with increased GSRS-IBS total, satiety, bloating, and pain scores following the placebo challenge (0.34 < Rho < 0.38; low in strength).

Discussion

While low FODMAP diets have been shown to reduce GI problems in individuals with NCGWS [23, 46], the causal mechanisms remain unclear. Potential explanations include changes in the gut microbiota due to reduced bacterial fermentation and reduction in osmotic active components which may alleviate intestinal distention associated with bloating and abdominal pain [20–22]. The current work adds knowledge as to how the gut microbiota community and biomarkers of gut health respond to changes in the intake of one

(See figure on next page.)

Fig. 6 Correlations between faecal outcomes at baseline and changes in GI symptoms. Correlograms illustrating Spearman's rank correlation coefficients (Rho) between changes ('Δ') in gastrointestinal symptoms (GSRS-IBS total, pain, bloating, constipation, diarrhoea, and satiety dimension scores) following the 7-day gluten, FOS-fructan, and placebo challenges and baseline ('Bas') faecal **A** taxon abundances (−0.37 < Rho < 0.39) **B** α-diversity (−0.32 < Rho < 0.12), **C** SCFA (−0.28 < Rho < 0.27), and **D** NGAL/LCN2 (ng/g faeces; −0.05 < Rho < 0.38). The change values for each diet challenge were calculated for each participant by subtracting the baseline value from the follow-up value. The colour scales indicate the strength and direction of the correlations. Significant correlations (not adjusted for multiple testing) are marked with asterisk (*$P < 0.05$, **$P < 0.01$). A positive correlation indicates that higher baseline values of the faecal outcome are associated with increased GSRS-IBS scores, while a negative correlation indicates that lower baseline values of the faecal outcome are associated with increased GSRS-IBS scores. **A** A taxon was excluded from the correlation analysis if it was detected in less than 50% of the baseline samples. Only taxa with significant correlations are shown in the figure (not adjusted for multiple testing). **C** Both SCFA concentrations ('conc', mmol/kg faeces) and proportional levels ('%', of total SCFA concentration) were used in the analysis. *FOS* fructo-oligosaccharides, *GSRS-IBS* gastrointestinal symptom rating scale IBS version, *IBS* irritable bowel syndrome, *NGAL/LCN2* neutrophil gelatinase-associated lipocalin/lipocalin 2, *SCFA* short-chain fatty acids

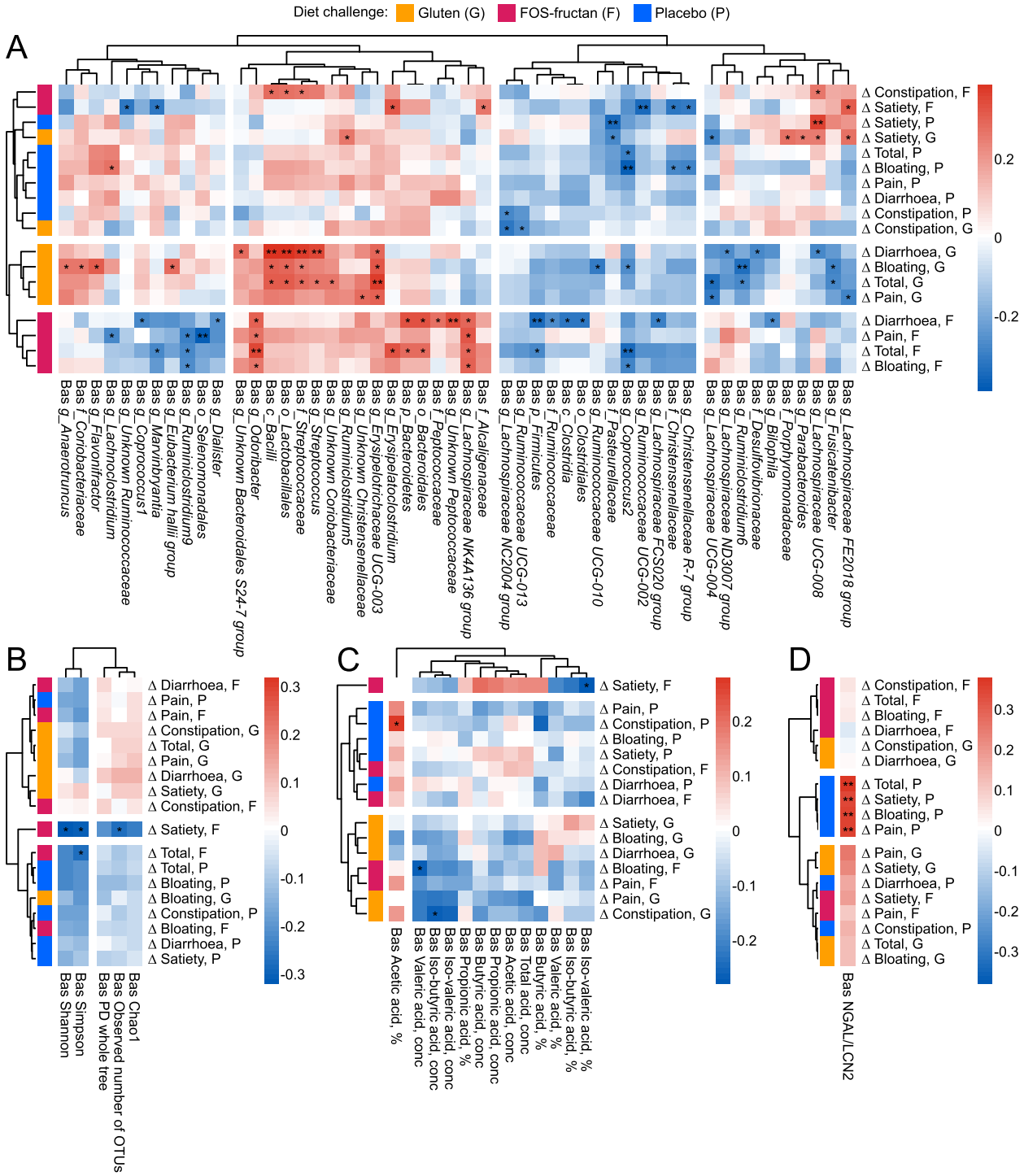

Fig. 6 (See legend on previous page.)

specific FODMAP, namely FOS-fructans, compared to gluten and placebo. The effects of FOS-fructans on the gut microbiota have not previously been studied in self-reported NCGWS. Although we previously showed that the 7-day FOS-fructan challenge was more likely to induce IBS-like symptoms than gluten in the current randomised double-blind placebo-controlled crossover trial [18], we found no prominent differential impacts of gluten versus FOS-fructan on the faecal microbial diversity, SCFA, or NGAL/LCN2. However,

some interesting differences across the diet challenges were found for certain bacterial taxa, which could be relevant to the GI problems experienced by the participants. Additionally, associations were found between the baseline microbial composition and responses to the FOS-fructan and gluten challenges, potentially influencing individual symptom responses.

No significant differences were detected in bacterial richness and diversity (α-diversity) or in overall bacterial community structure (β-diversity) between the diet challenges, suggesting no prominent impact on the microbiota composition. This finding aligns with previous studies using short-term FOS-fructan supplementation [17, 47], gluten supplementation [48], or low/high gluten diets [49, 50]. However, the use of low-dose FOS-fructan supplementation in our study leaves the possibility of more pronounced effects resulting from the use of higher doses or longer intervention periods. Indeed, long-term intervention with FOS-fructan supplementation increased the α-diversity in healthy individuals [51]. Conversely, in a separate study, 2 weeks of supplementation with inulin and FOS-fructans separately led to decreased α-diversity in healthy individuals [52].

Although most bacterial taxa did not exhibit differential effects from the diet challenge, certain changes were observed at the family and genus levels. With respect to the FOS-fructan challenge, the most evident changes were found for the *Firmicutes* genera *Fusicatenibacter* and *E. coprostanoligenes*, which increased and decreased, respectively.

The increase in *Fusicatenibacter* following FOS-fructan intake may be attributed to enhanced bacterial growth due to FOS-fructan availability. *Fusicatenibacter*, a SCFA-producing genus [53, 54], has shown negative associations with diseases such as Parkinson's disease [55], colon cancer [56], non-alcoholic fatty liver disease [57], *Clostridioides difficile* infection [58], and chronic kidney disease [59]. *Fusicatenibacter* has also been shown to decrease with age [60]. Although few studies have explored its role in IBS symptoms, one small clinical trial (18 participants) noted increased *Fusicatenibacter* abundance alongside constipation relief after faecal microbiota transplantation [61]. Conversely, increased *Fusicatenibacter* abundance after a high FODMAP diet was indirectly associated with increased abdominal pain, possibly mediated by an increase in the bacterial metabolite 3-indolepropionic acid (IPA) [62, 63]. Additionally, in overweight young adults, *Fusicatenibacter* was associated with unhealthy eating patterns [64]. In our trial, we observed weak, nonsignificant correlations between *Fusicatenibacter* and GI symptoms post-FOS-fructan challenge, but a significant negative association was noted with diarrhoea symptoms post-placebo challenge, suggesting a potential role in alleviating IBS-like symptoms.

The decrease in *E. coprostanoligenes* after increasing the intake of FOS-fructans aligns with a study in young undergraduate students showing that a high-fibre diet decreased the abundance of *E. coprostanoligenes* [65]. Furthermore, a recent study by Nordin et al. showed a lower abundance of these bacteria with a high FODMAP diet than with low FODMAP [62]. Nordin et al. also reported that lower *E. coprostanoligenes* was weakly associated with increased frequency of abdominal pain [62], which suggests a potential role for *E. coprostanoligenes* in mitigating IBS-like symptoms. In line with these findings, the present study also found a significant negative correlation between changes in pain symptoms and *E. coprostanoligenes* following the FOS-fructan challenge (i.e. reduced abundance associated with increased symptoms). We also found convincing negative correlations between *E. coprostanoligenes* and several GI symptoms during placebo and gluten challenge. In contrast to Nordin et al. and our findings, one study in children with autism found *E. coprostanoligenes* to be positively associated with high GSRS scores [66]. Apart from its cholesterol-reducing abilities, little is known about the role of *E. coprostanoligenes* in health in general [67].

The abundances of some bacteria, including those in the *Eubacterium xylanophilum group*, increased in response to gluten intake compared to the placebo and FOS-fructan challenge, a novel finding with uncertain implications. However, an experimental trial demonstrated increased *Eubacterium xylanophilum group* abundance when culturing human faeces with wheat bran [68], suggesting a possible association with gluten intake. For the other bacteria affected by the gluten challenge compared to FOS-fructan and placebo (*Ruminococcus torques group*, *Desulfovibrionaceae*, *Bilophila*), the impact of gluten was less clear. The minor effects of gluten on the gut microbiota composition are consistent with results from previous studies [48, 62].

No differences were found in faecal SCFA concentrations or proportional levels across the gluten, FOS-fructan, and placebo challenges. The lack of difference between the FOS-fructan and placebo challenge may be due to the low dose and short duration of the FOS-fructan supplementation, consistent with findings from studies using higher doses but similar durations [52, 69, 70]. Longer durations of fructan supplementation have been associated with increased SCFA concentrations [71–73], although one study reported reduced levels [74]. It is worth noting that the studies showing increased SCFA used a mix of FOS-fructans and inulin, unlike our study which used only FOS-fructans. With gluten challenge, earlier studies have yielded mixed results on faecal

SCFA [49, 50, 62, 75]. Despite no significant differences between the diet challenges, we observed a general increase in SCFA concentrations from baseline to follow-up for all challenges, possibly influenced by the fibre-containing ingredients in the muesli bars.

Changes in GSRS-IBS total, bloating, pain, and diarrhoea scores following the FOS-fructan and gluten challenges were associated with certain bacterial abundances at baseline. This finding may explain the variable GI symptom responses previously reported by Skodje et al. [18]. Additionally, lower faecal concentrations of the SCFA valeric and iso-valeric acid were associated with greater increases in GI symptoms following the FOS-fructan challenge, supporting the hypothesis of a potential link between baseline microbial function and the response to FOS-fructans.

Since there are no available biomarkers for NCGWS [76], the response of faecal NGAL/LCN2 to the different diet challenges was investigated due to its potential role in low-grade intestinal inflammation [77–79], which could be relevant in NCGWS [80–82]. However, in accordance with the previously reported plasma cytokine results [83], no differences in faecal NGAL/LCN2 were found across the diet challenges. Nevertheless, the significant correlations found between baseline NGAL/LCN2 concentrations and changes in several GI symptoms following the placebo challenge might indicate that the inflammatory state of the gut is important for fluctuations in GI symptoms in general (regardless of diet challenge), consistent with the tendency for higher faecal NGAL/LCN2 concentrations in IBS patients than in healthy controls [84].

A key strength of the current trial is the strong crossover design with a placebo-controlled diet challenge. To address potential carry-over effects, different challenge sequences were employed, and Skodje et al. also reported no influence of sequence order on the GI symptoms [18]. However, one challenge in interpreting the results is that the participants' background diets were not controlled for FOS-fructans or other types of FODMAP. To improve the protocol, a low FODMAP GFD could have served as the background diet throughout the challenge and wash-out periods, particularly since it previously was shown that FODMAP intake varies significantly between individuals with IBS [85]. Furthermore, because the dietary recordings from baseline were not re-evaluated during the other study periods, we cannot rule out that changes in macro-/micronutrients might have impacted the changes in the microbiota. However, since the participants were asked to keep their diet consistent with the baseline diet throughout the study, we have hopefully avoided dietary changes to influence the microbiota results. Also, as the muesli bars were not analysed for 'background' FOS-fructan/FODMAP content, we cannot rule out that there might be a low level of FOS-fructans present in the placebo and gluten bar. However, as the ingredients used in the muesli bars are reported to contain very low levels of FODMAPs, we argue that these levels will not be clinically relevant compared to the amount of FOS-fructans added to the FOS-fructan bars. Additionally, it is possible that NCGWS is too wide of an umbrella term, forcing different patient cohorts under the same definition [8], potentially limiting the generalizability of our results. This was indicated by the evident heterogeneity in the current study population with respect to which challenge that had the highest GI symptom score [18]. Thus, this heterogeneity can have contributed to heterogeneous microbiota responses, making it challenging to detect uniform effects on the gut microbiota following the different diet challenges. Finally, it is crucial to recognize that the gut microbiota is a complex community influenced by cohabitants and cross-feeding dynamics [86], meaning that the total impact of a dietary intervention on microbial metabolic outcomes depends on individual variability within the microbial ecosystems.

Conclusions

In the first randomised double-blind placebo-controlled crossover study examining the impact of FOS-fructan and gluten on the faecal microbiota in self-reported NCGWS patients adhering to a gluten-free background diet, we found no noteworthy differences in how the diet challenges impacted the gut microbiota composition and related variables of gut health. However, both gluten and FOS-fructans induced changes in certain bacteria. In particular, a reduction in *E. coprostanoligenes* was most prominently associated with an increase in GI symptoms, suggesting that attention should be given to these bacteria in future trials investigating the impact of dietary treatments on GI symptoms. Additionally, while the baseline composition of the microbiota seems to influence the variation in the individual GI symptom responses to the FOS-fructan challenge, the symptoms experienced when consuming FOS-fructans does not seem to be explained by large changes in the microbiota. This study also provides evidence suggesting that the GI symptoms induced by FOS-fructan are not driven by inflammation.

Abbreviations

BH	Benjamini–Hochberg
CeD	Celiac disease
DP	Degree of polymerization
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols
FOS	Fructo-oligosaccharides
GFD	Gluten-free diet
GI	Gastrointestinal

GSRS-IBS	Gastrointestinal symptom rating scale IBS version
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IPA	3-Indolepropionic acid
NCGWS	Non-celiac gluten/wheat sensitivity
NGAL/LCN2	Human neutrophil gelatinase-associated lipocalin/lipocalin 2
NMDS	Nonmetric multidimensional scaling
OTU	Operational taxonomic unit
PD	Phylogenetic diversity
PERMANOVA	Permutation-based multivariate ANOVA
QIIME	Quantitative insights into microbial ecology
SCFA	Short-chain fatty acids
WA	Wheat allergy

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12916-024-03562-1.

Additional file 1: Table S1. Content of muesli bars used for the 7-day gluten, FOS-fructan, and placebo challenges in g per 100 g. Table S2. Primers modified with Illumina adapters used for index PCR during library preparation for gene sequencing of 16S rRNA. Table S3. Changes in faecal α-diversity following the 7-day gluten, FOS-fructan, and placebo challenges. Table S4. Summary statistics for faecal bacterial α-diversity at baseline and after the 7-day FOS-fructan, gluten, and placebo challenges. Table S5. Changes in faecal bacterial abundances following the 7-day gluten, FOS-fructan, and placebo challenges. Table S6. Summary statistics for faecal bacterial abundancesof at baseline and after the 7-day FOS-fructan, gluten, and placebo challenges. Table S7. Summary statistics for faecal SCFA at baseline and after the 7-day FOS-fructan, gluten, and placebo challenges. Table S8. Summary statistics for faecal NGAL/LCN2 concentrationsat baseline and after the 7-day FOS-fructan, gluten, and placebo challenges. Fig. S1. Rarefaction curves. Fig. S2. Ordination plots from non-metric multidimensional scaling of binary Jaccard β-diversity distances. Fig. S3. Ordination plots from non-metric multidimensional scaling of Bray–Curtis β-diversity distances. Fig. S4. Ordination plots from non-metric multidimensional scaling of weighted UniFrac β-diversity distances. Fig. S5. Faecal relative abundancesof bacterial genera at baseline and after the 7-day gluten, FOS-fructan, and placebo challenges. Fig. S6. Correlations between changes in faecal outcomes and changes in GI symptoms.

Acknowledgements
We acknowledge Gunn Helen Malmstrøm and Jennifer T. Fiennes (Lovisenberg Diaconal Hospital) for performing the analysis of the SCFA, Kjersti Langballe Rolfsen and Ingunn Hillestad Minelle (master students, University of Oslo) for contributing to the collection of stool samples, and Inga Leena Angell and Ida Ormaasen (Norwegian University of Life Sciences) for the technical support. Finally, we would like to thank all the participants in the study.

Authors' contributions
AMH conducted the research (microbiota laboratory analysis, performed statistical analyses), interpreted the data, and wrote the paper. MN conducted the research (supervised the microbiota laboratory analysis) and interpreted the data. TEA conducted the research (NGAL laboratory analysis). GS designed the research (conceptualization). JV conducted the research (responsible for SCFA analysis). KR provided essential materials (facilitating the microbiota analysis). MT conducted the research (statistical analysis). KEAL designed the research (conceptualization, funding acquisition). CH designed the research (conceptualization). SKB designed the research (conceptualization, funding acquisition), conducted the research (supervision), analysed and interpreted the data, wrote the paper, and had the primary responsibility for the final content. All authors read, commented, and approved the final manuscript.

Funding
This work was supported by the Norwegian research fund for celiac disease (SKB). The funding sources had no role in the conceptualization, design, data collection, analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials
The datasets analysed during the current study are not publicly available because the participants did not give written consent for their data to be shared publicly but may be available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human patients were approved by the Regional Committee for Medical and Health Research Ethics, South East A on September 16 2014 (identification 2013/1237 REC South East A). Written informed consent was obtained from all participants. The study was also registered at clinicaltrials.gov with the registration number NCT02464150. The manuscript was prepared according to the CONSORT statement (http://www.consort-statement.org).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Faculty of Chemistry, Biotechnology and Food Sciences, Norwegian University of Life Sciences, P. O. Box 5003, N-1432 Ås, Norway. [2]Healthy Life Centre, Municipality of Nes, Norway. [3]Unger-Vetlesen Institute, Lovisenberg Diaconal Hospital, Oslo, Norway. [4]Institute of Clinical Medicine, University of Oslo, Oslo, Norway. [5]Department of Biostatistics, Faculty of Medicine, University of Oslo, Oslo, Norway. [6]Disease Research Centre, Norwegian Coeliac, University of Oslo, Oslo, Norway. [7]Department of Gastroenterology, Oslo University Hospital Rikshospitalet, Oslo, Norway. [8]Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway.

Received: 15 May 2024 Accepted: 14 August 2024
Published online: 04 September 2024

References

1. Cooper BT, Holmes GKT, Ferguson R, Thompson RA, Allan RN, Cooke WT. Gluten-sensitive diarrhea without evidence of celiac disease. Gastroenterology. 1980;79(5):801–6.
2. Cardenas-Torres FI, Cabrera-Chavez F, Figueroa-Salcido OG, Ontiveros N. Non-celiac gluten sensitivity: an update. Medicina (Kaunas). 2021;57(6):526.
3. Henggeler JC, Veríssimo M, Ramos F. Non-coeliac gluten sensitivity: a review of the literature. Trends Food Sci Technol. 2017;66:84–92.
4. Catassi C, Elli L, Bonaz B, Bouma G, Carroccio A, Castillejo G, et al. Diagnosis of non-celiac gluten sensitivity (NCGS): the Salerno experts' criteria. Nutrients. 2015;7(6):4966–77.
5. Cianferoni A. Wheat allergy: diagnosis and management. J Asthma Allergy. 2016;9:13–25.
6. Catassi C, Verdu EF, Bai JC, Lionetti E. Coeliac disease. Lancet. 2022;399(10344):2413–26.
7. Catassi C, Alaedini A, Bojarski C, Bonaz B, Bouma G, Carroccio A, et al. The overlapping area of non-celiac gluten sensitivity (NCGS) and wheat-sensitive irritable bowel syndrome (IBS): an update. Nutrients. 2017;9(11):1268.
8. Molina-Infante J, Carroccio A. Suspected nonceliac gluten sensitivity confirmed in few patients after gluten challenge in double-blind, placebo-controlled trials. Clin Gastroenterol Hepatol. 2017;15(3):339–48.
9. Muir JG, Varney JE, Ajamian M, Gibson PR. Gluten-free and low-FODMAP sourdoughs for patients with coeliac disease and irritable bowel syndrome: a clinical perspective. Int J Food Microbiol. 2019;290:237–46.
10. Mumolo MG, Rettura F, Melissari S, Costa F, Ricchiuti A, Ceccarelli L, et al. Is gluten the only culprit for non-celiac gluten/wheat sensitivity? Nutrients. 2020;12(12):3785.
11. Shi Y, Si D, Zhang X, Chen D, Han Z. Plant fructans: recent advances in metabolism, evolution aspects and applications for human health. Curr Res Food Sci. 2023;7: 100595.

12. Meyer D, Stasse-Wolthuis M. The bifidogenic effect of inulin and oligofructose and its consequences for gut health. Eur J Clin Nutr. 2009;63(11):1277–89.
13. Barrett JS, Gibson PR. Clinical ramifications of malabsorption of fructose and other short-chain carbohydrates. Pract Gastroenterol. 2007;31(8):51–65.
14. van Lanen AS, de Bree A, Greyling A. Efficacy of a low-FODMAP diet in adult irritable bowel syndrome: a systematic review and meta-analysis. Eur J Nutr. 2021;60(6):3505–22.
15. Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, et al. Prebiotic effects: metabolic and health benefits. Br J Nutr. 2010;104(Suppl 2):S1–63.
16. Staudacher HM, Whelan K. The low FODMAP diet: recent advances in understanding its mechanisms and efficacy in IBS. Gut. 2017;66(8):1517–27.
17. Chumpitazi BP, Hoffman KL, Smith DP, McMeans AR, Musaad S, Versalovic J, et al. Fructan-sensitive children with irritable bowel syndrome have distinct gut microbiome signatures. Aliment Pharmacol Ther. 2021;53(4):499–509.
18. Skodje GI, Sarna VK, Minelle IH, Rolfsen KL, Muir JG, Gibson PR, et al. Fructan, rather than gluten, induces symptoms in patients with self-reported non-celiac gluten sensitivity. Gastroenterology. 2018;154(3):529–39.e2.
19. Priyanka P, Gayam S, Kupec JT. The role of a low fermentable oligosaccharides, disaccharides, monosaccharides, and polyol diet in nonceliac gluten sensitivity. Gastroenterol Res Pract. 2018;2018:1561476.
20. De Palma G, Reed DE, Bercik P. Diet-microbial cross-talk underlying increased visceral perception. Gut Microbes. 2023;15(1):2166780.
21. Guo R, Chen LH, Xing C, Liu T. Pain regulation by gut microbiota: molecular mechanisms and therapeutic potential. Br J Anaesth. 2019;123(5):637–54.
22. Wu J, Masuy I, Biesiekierski JR, Fitzke HE, Parikh C, Schofield L, et al. Gut-brain axis dysfunction underlies FODMAP-induced symptom generation in irritable bowel syndrome. Aliment Pharmacol Ther. 2022;55(6):670–82.
23. Dieterich W, Schuppan D, Schink M, Schwappacher R, Wirtz S, Agaimy A, et al. Influence of low FODMAP and gluten-free diets on disease activity and intestinal microbiota in patients with non-celiac gluten sensitivity. Clin Nutr. 2019;38(2):697–707.
24. Wiklund IK, Fullerton S, Hawkey CJ, Jones RH, Longstreth GF, Mayer EA, et al. An irritable bowel syndrome-specific symptom questionnaire: development and validation. Scand J Gastroenterol. 2003;38(9):947–54.
25. Herfindal AM, van Megen F, Gilde MKO, Valeur J, Rudi K, Skodje GI, et al. Effects of a low FODMAP diet on gut microbiota in individuals with treated coeliac disease having persistent gastrointestinal symptoms - a randomised controlled trial. Br J Nutr. 2023;130(12):2061–75.
26. Yu Y, Lee C, Kim J, Hwang S. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol Bioeng. 2005;89(6):670–9.
27. Avershina E, Lundgard K, Sekelja M, Dotterud C, Storro O, Oien T, et al. Transition from infant- to adult-like gut microbiota. Environ Microbiol. 2016;18(7):2226–36.
28. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335–6.
29. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460–1.
30. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013;10(10):996–8.
31. Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glockner FO. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 2007;35(21):7188–96.
32. Oksanen J, Simpson G, Blanchet F, Kindt R, Legendre P, Minchin P, et al. vegan: community ecology package (2022, R package version 2.6–4). https://CRAN.R-project.org/package=vegan
33. R Core Team. R: A language and environment for statistical computing (Version 4.2.3). R Foundation for Statistical Computing, Vienna, Austria. 2023. URL https://www.R-project.org/.
34. Zijlstra JB, Beukema J, Wolthers BG, Byrne BM, Groen A, Dankert J. Pre-treatment methods prior to gaschromatographic analysis of volatile fatty acids from faecal samples. Clin Chim Acta. 1977;78(2):243–50.
35. Hoverstad T, Fausa O, Bjorneklett A, Bohmer T. Short-chain fatty acids in the normal human feces. Scand J Gastroenterol. 1984;19(3):375–81.
36. Kuznetsova A, Brockhoff PB, Christensen RHB. lmerTest package: tests in linear mixed effects models. J Stat Softw. 2017;82(13):1–26.
37. Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J Stat Softw. 2015;67(1):1–48.
38. Kassambara A. ggpubr: 'ggplot2' based publication ready plots (2023, R package version 0.6.0). https://CRAN.R-project.org/package=ggpubr
39. Mangiafico S. rcompanion: functions to support extension education program evaluation (2023, R package version 2.4.21). https://CRAN.R-project.org/package=rcompanion
40. Kassambara A. rstatix: pipe-friendly framework for basic statistical tests (2023, R package version 0.7.2). https://CRAN.R-project.org/package=rstatix
41. Nieuwenhuis R, te Grotenhuis M, Pelzer B. Influence.ME: tools for detecting influential data in mixed effects models. The R Journal. 2012;4(2):38–47.
42. Revelle W. psych: procedures for psychological, psychometric, and personality research (2023, R package version 2.3.6). https://CRAN.R-project.org/package=psych
43. Kolde R. pheatmap: pretty heatmaps (2019, R package version 1.0.12). https://CRAN.R-project.org/package=pheatmap
44. Mukaka MM. Statistics corner: a guide to appropriate use of correlation coefficient in medical research. Malawi Med J. 2012;24(3):69–71.
45. Skodje GI, Minelle IH, Rolfsen KL, Iacovou M, Lundin KEA, Veierod MB, Henriksen C. Dietary and symptom assessment in adults with self-reported non-coeliac gluten sensitivity. Clin Nutr ESPEN. 2019;31:88–94.
46. Biesiekierski JR, Peters SL, Newnham ED, Rosella O, Muir JG, Gibson PR. No effects of gluten in patients with self-reported non-celiac gluten sensitivity after dietary reduction of fermentable, poorly absorbed, short-chain carbohydrates. Gastroenterology. 2013;145(2):320-8.e3.
47. Liu F, Li P, Chen M, Luo Y, Prabhakar M, Zheng H, et al. Fructooligosaccharide (FOS) and galactooligosaccharide (GOS) increase bifidobacterium but reduce butyrate producing bacteria with adverse glycemic metabolism in healthy young population. Sci Rep. 2017;7(1):11789.
48. Nobel YR, Rozenberg F, Park H, Freedberg DE, Blaser MJ, Green PHR, et al. Lack of effect of gluten challenge on fecal microbiome in patients with celiac disease and non-celiac gluten sensitivity. Clin Transl Gastroenterol. 2021;12(12): e00441.
49. Bonder MJ, Tigchelaar EF, Cai X, Trynka G, Cenit MC, Hrdlickova B, et al. The influence of a short-term gluten-free diet on the human gut microbiome. Genome Med. 2016;8(1):45.
50. Hansen LBS, Roager HM, Sondertoft NB, Gobel RJ, Kristensen M, Valles-Colomer M, et al. A low-gluten diet induces changes in the intestinal microbiome of healthy Danish adults. Nat Commun. 2018;9(1):4630.
51. Tandon D, Haque MM, Gote M, Jain M, Bhaduri A, Dubey AK, Mande SS. A prospective randomized, double-blind, placebo-controlled, dose-response relationship study to investigate efficacy of fructo-oligosaccharides (FOS) on human gut microflora. Sci Rep. 2019;9(1):5473.
52. Yin P, Du T, Yi S, Zhang C, Yu L, Tian F, et al. Response differences of gut microbiota in oligofructose and inulin are determined by the initial gut Bacteroides/Bifidobacterium ratios. Food Res Int. 2023;174(Pt 1): 113598.
53. Takada T, Kurakawa T, Tsuji H, Nomoto K. Fusicatenibacter saccharivorans gen. nov., sp. nov., isolated from human faeces. Int J Syst Evol Microbiol. 2013;63(Pt 10):3691–6.
54. O'Riordan KJ, Collins MK, Moloney GM, Knox EG, Aburto MR, Fulling C, et al. Short chain fatty acids: microbial metabolites for gut-brain axis signalling. Mol Cell Endocrinol. 2022;546: 111572.
55. Nishiwaki H, Ito M, Hamaguchi T, Maeda T, Kashihara K, Tsuboi Y, et al. Short chain fatty acids-producing and mucin-degrading intestinal bacteria predict the progression of early Parkinson's disease. NPJ Parkinsons Dis. 2022;8(1):65.
56. He T, Cheng X, Xing C. The gut microbial diversity of colon cancer patients and the clinical significance. Bioengineered. 2021;12(1):7046–60.
57. Rodriguez-Diaz C, Taminiau B, Garcia-Garcia A, Cueto A, Robles-Diaz M, Ortega-Alonso A, et al. Microbiota diversity in nonalcoholic fatty liver disease and in drug-induced liver injury. Pharmacol Res. 2022;182: 106348.
58. Crobach MJT, Ducarmon QR, Terveer EM, Harmanus C, Sanders I, Verduin KM, et al. The bacterial gut microbiota of adult patients infected, colonized or noncolonized by Clostridioides difficile. Microorganisms. 2020;8(5):677.

59. Tran NTD, Chaidee A, Surapinit A, Yingklang M, Roytrakul S, Charoenlappanit S, et al. Strongyloides stercoralis infection reduces Fusicatenibacter and Anaerostipes in the gut and increases bacterial amino-acid metabolism in early-stage chronic kidney disease. Heliyon. 2023;9(9): e19859.
60. Yoshida N, Watanabe S, Yamasaki H, Sakuma H, Takeda AK, Yamashita T, Hirata KI. Average gut flora in healthy Japanese subjects stratified by age and body mass index. Biosci Microbiota Food Health. 2022;41(2):45–53.
61. Zhang X, Li N, Chen Q, Qin H. Fecal microbiota transplantation modulates the gut flora favoring patients with functional constipation. Front Microbiol. 2021;12: 700718.
62. Nordin E, Hellstrom PM, Dicksved J, Pelve E, Landberg R, Brunius C. Effects of FODMAPs and gluten on gut microbiota and their association with the metabolome in irritable bowel syndrome: a double-blind, randomized, cross-over intervention study. Nutrients. 2023;15(13):3045.
63. Nordin E, Hellstrom PM, Vuong E, Ribbenstedt A, Brunius C, Landberg R. IBS randomized study: FODMAPs alter bile acids, phenolic- and tryptophan metabolites, while gluten modifies lipids. Am J Physiol Regul Integr Comp Physiol. 2023;325(3):R248–59.
64. Medawar E, Haange SB, Rolle-Kampczyk U, Engelmann B, Dietrich A, Thieleking R, et al. Gut microbiota link dietary fiber intake and short-chain fatty acid metabolism with eating behavior. Transl Psychiatry. 2021;11(1):500.
65. Lin Z, Zhang X, Wu M, Ming Y, Wang X, Li H, et al. High-fiber diet and rope-skipping benefit cardiometabolic health and modulate gut microbiota in young adults: a randomized controlled trial. Food Res Int. 2023;173(Pt 2): 113421.
66. Li N, Chen H, Cheng Y, Xu F, Ruan G, Ying S, et al. Fecal microbiota transplantation relieves gastrointestinal and autism symptoms by improving the gut microbiota in an open-label study. Front Cell Infect Microbiol. 2021;11: 759435.
67. Juste C, Gerard P. Cholesterol-to-coprostanol conversion by the gut microbiota: what we know, suspect, and ignore. Microorganisms. 2021;9(9):1881.
68. Duncan SH, Russell WR, Quartieri A, Rossi M, Parkhill J, Walker AW, Flint HJ. Wheat bran promotes enrichment within the human colonic microbiota of butyrate-producing bacteria that release ferulic acid. Environ Microbiol. 2016;18(7):2214–25.
69. Gibson GR, Beatty ER, Wang X, Cummings JH. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology. 1995;108(4):975–82.
70. Hustoft TN, Hausken T, Ystad SO, Valeur J, Brokstad K, Hatlebakk JG, Lied GA. Effects of varying dietary content of fermentable short-chain carbohydrates on symptoms, fecal microenvironment, and cytokine profiles in patients with irritable bowel syndrome. Neurogastroenterol Motil. 2017;29(4): e12969.
71. Birkeland E, Gharagozlian S, Birkeland KI, Valeur J, Mage I, Rud I, Aas AM. Prebiotic effect of inulin-type fructans on faecal microbiota and short-chain fatty acids in type 2 diabetes: a randomised controlled trial. Eur J Nutr. 2020;59(7):3325–38.
72. Clarke ST, Green-Johnson JM, Brooks SP, Ramdath DD, Bercik P, Avila C, et al. beta2-1 Fructan supplementation alters host immune responses in a manner consistent with increased exposure to microbial components: results from a double-blinded, randomised, cross-over study in healthy adults. Br J Nutr. 2016;115(10):1748–59.
73. Valcheva R, Koleva P, Martinez I, Walter J, Ganzle MG, Dieleman LA. Inulin-type fructans improve active ulcerative colitis associated with microbiota changes and increased short-chain fatty acids levels. Gut Microbes. 2019;10(3):334–57.
74. Salazar N, Dewulf EM, Neyrinck AM, Bindels LB, Cani PD, Mahillon J, et al. Inulin-type fructans modulate intestinal Bifidobacterium species populations and decrease fecal short-chain fatty acids in obese women. Clin Nutr. 2015;34(3):501–7.
75. Caminero A, Nistal E, Arias L, Vivas S, Comino I, Real A, et al. A gluten metabolism study in healthy individuals shows the presence of faecal glutenasic activity. Eur J Nutr. 2012;51(3):293–9.
76. Ierardi E, Losurdo G, Piscitelli D, Giorgio F, Amoruso A, Iannone A, et al. Biological markers for non-celiac gluten sensitivity: a question awaiting for a convincing answer. Gastroenterol Hepatol Bed Bench. 2018;11(3):203–8.
77. Chakraborty S, Kaur S, Guha S, Batra SK. The multifaceted roles of neutrophil gelatinase associated lipocalin (NGAL) in inflammation and cancer. Biochim Biophys Acta. 2012;1826(1):129–69.
78. Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol Cell. 2002;10(5):1033–43.
79. Abella V, Scotece M, Conde J, Gomez R, Lois A, Pino J, et al. The potential of lipocalin-2/NGAL as biomarker for inflammatory and metabolic diseases. Biomarkers. 2015;20(8):565–71.
80. Carroccio A, Giannone G, Mansueto P, Soresi M, La Blasca F, Fayer F, et al. Duodenal and rectal mucosa inflammation in patients with non-celiac wheat sensitivity. Clin Gastroenterol Hepatol. 2019;17(4):682–90.e3.
81. Losurdo G, Piscitelli D, Pezzuto F, Fortarezza F, Covelli C, Marra A, et al. T Helper lymphocyte and mast cell immunohistochemical pattern in nonceliac gluten sensitivity. Gastroenterol Res Pract. 2017;2017:5023680.
82. Uhde M, Ajamian M, Caio G, De Giorgio R, Indart A, Green PH, et al. Intestinal cell damage and systemic immune activation in individuals reporting sensitivity to wheat in the absence of coeliac disease. Gut. 2016;65(12):1930–7.
83. Tye-Din JA, Skodje GI, Sarna VK, Dzuris JL, Russell AK, Goel G, et al. Cytokine release after gluten ingestion differentiates coeliac disease from self-reported gluten sensitivity. United European Gastroenterol J. 2020;8(1):108–18.
84. Akiho H, Ihara E, Nakamura K. Low-grade inflammation plays a pivotal role in gastrointestinal dysfunction in irritable bowel syndrome. World J Gastrointest Pathophysiol. 2010;1(3):97–105.
85. Nybacka S, Storsrud S, Liljebo T, Le Neve B, Tornblom H, Simren M, Winkvist A. Within- and between-subject variation in dietary intake of fermentable oligo-, di-, monosaccharides, and polyols among patients with irritable bowel syndrome. Curr Dev Nutr. 2019;3(2):nzy101.
86. Smith NW, Shorten PR, Altermann EH, Roy NC, McNabb WC. Hydrogen cross-feeders of the human gastrointestinal tract. Gut Microbes. 2019;10(3):270–88.

Publisher's Note