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The Influence of Various Food Components and Psychological Factors

Marlijne de Graaf

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CHAPTER 1

General introduction

Role of diet in gastrointestinal disorders

The Western diet is characterised by a high intake of processed and sugar-rich foods, fat, and red meat, and a low intake of fibre-rich foods such as fruits, vegetables, and wholegrains.[1] The resulting low-quality diet negatively affects intestinal health and has been associated with symptoms like bloating, altered bowel habits, and abdominal pain.[2,3] Epidemiological data shows that in line with Westernisation, the prevalence of several diseases, including gastrointestinal (GI) disorders, is increasing.[4,5] Diet is considered to play an important role in the onset and disease course of a wide range of GI disorders, comprising both inflammatory and non-inflammatory conditions.

One of the most prevalent GI disorders is irritable bowel syndrome (IBS), a disorder of gut-brain interaction (DGBI) affecting 5-10% of the Western population.[6] As defined by the Rome IV criteria, published in 2016, IBS is characterised by recurrent abdominal pain (on average) at least one day per week in the last three months, combined with at least two of the following criteria: (1) related to defecation; (2) associated with a change in stool frequency; and/or (3) associated with a change in stool consistency. Symptom onset should be at least six months prior to diagnosis. Based on the predominant stool pattern, IBS can be subtyped as constipation-predominant (IBS-C), diarrhoea-predominant (IBS-D), mixed bowel habits (IBS-M), or unclassified (IBS-U).[7] Other common symptoms include bloating, abdominal distension, flatulence, and faecal urgency.[8] Currently, no objective biomarkers are available for diagnosis as the exact underlying mechanisms are not clear. Alterations in intestinal motility, barrier function, visceral perception and brain-gut interaction, microbiome perturbations, and low-grade inflammation have been reported as possible causes.[9] Low overall diet quality, for example the typical Western diet, and various food components, such as intake of (rapidly) fermentable carbohydrates, spicy, and fatty foods, are factors associated with these potential mechanisms,[2,3,10,11] and together with psychological distress[12] are well-recognised triggers of symptom occurrence in IBS.

IBS-like symptoms are also reported in about 35% of inflammatory bowel disease (IBD) patients in remission.[13] IBD is a chronic inflammatory disease characterised by alternating sequences of active inflammation and remission, and comprises Crohn's disease (CD) and ulcerative colitis (UC). CD is characterised by transmural inflammation with a patchy distribution. It can present throughout the entire GI tract, but most often involves the ileum and colon. Common presenting symptoms of CD include chronic diarrhoea, rectal bleeding, abdominal pain, fatigue, and weight loss. In UC, the inflammation is limited to the mucosal layer, affecting the rectum and to a variable extent the colon in a continuous distribution. Symptoms of UC commonly include bloody diarrhoea, urgency, faecal incontinence, and abdominal pain. Both CD and UC are diagnosed by clinical evaluation and a combination of endoscopic, histological, radiological, and/or biochemical investigations as defined by the European Crohn's and Colitis Organisation (ECCO) guidelines, with phenotyping according to the Montréal classification.[14,15] Although the exact pathogenesis is unclear, IBD is

generally considered to arise from a complex interaction between host genetics, the intestinal microbiome, and immune factors, as well as environmental factors, including diet.[16,17] Epidemiological studies have linked various dietary components to the onset and relapses of IBD.[18] Together with the global trend,[19] an increase in IBD incidence has been noted in our South Limburg area.[20] The link between the Western lifestyle, including the Western diet, is further supported by the increased incidence along with industrialisation in developed countries,[21] as well as in second-generation immigrants from Asia to Western countries.[22-26] The overall prevalence of IBD is 0.003% in Western countries and up to 0.001% in Asian and South American countries.[27]

Up to 90% of IBS patients, 58-68% of IBD patients with active disease and 29-39% of IBD patients in remission indicate that meals and/or certain food products induce GI symptoms like abdominal pain, bloating and diarrhoea.[28,29] Dairy products, spicy foods, wheat products, and 'gas-producing' foods, including some fruits and vegetables, are reported as the main culprit foods causing intestinal distress by both IBS and IBD patients.[29,30]

Particularly wheat-based products, and other gluten-containing foods, received more and more negative attention over the last years, accompanied by an increasing popularity of the gluten-free diet (GFD) on social media, though without clear scientific evidence.[31] Nevertheless, it is well known that wheat can elicit adverse reactions (*i.e.* coeliac disease or wheat allergy) in susceptible individuals. Coeliac disease is a chronic small intestinal immune-mediated enteropathy initiated by exposure to dietary gluten in genetically predisposed individuals (HLA-DQ2 or HLA-DQ8 positive), with a prevalence of 0.6-1.0% in the Western population.[32,33] Wheat allergy is an immunoglobulin-E (IgE) or non-IgE mediated allergic response (*i.e.* characterised by chronic eosinophilic and lymphocytic infiltration in the GI tract) to gluten, with a prevalence of 0.2-1.0%.[34]

In addition, a substantial proportion of the general population, with estimates ranging from 0.5 to 30%, is avoiding or reducing its consumption of wheat products because of symptoms, despite the fact that coeliac disease and wheat allergy have been ruled out.[35-39] Initially, this was defined as non-coeliac gluten sensitivity (NCGS) due to gluten being the presumed cause.[40] However, as other wheat-components are also considered potential triggers, the term non-coeliac wheat sensitivity (NCWS) has emerged.[41] Whereas earlier studies mainly focused on NCGS, nowadays the term NCWS in increasingly used, although a clear distinction is not always made. NCGS/NCWS individuals often present with IBS-like symptoms and improve on a gluten- or wheat-free diet.[40] The estimated prevalence is up to 15%, but the true prevalence remains unclear, in part also due to lack of biomarkers.[42-44] At the moment, NCGS diagnosis is defined by the Salerno Experts' Criteria. These include a double-blind, placebo-controlled gluten challenge, which is not always feasible in clinical practice.[45] No such criteria have been established for NCWS, *i.e.* addressing components other than gluten. Accordingly, in many individuals the diagnosis of NCGS or NCWS is self-reported.[46]

Due to the associations between food and symptoms in GI disorders, treatment options include dietary intervention, which often involves targeted restrictions of specific dietary components.[47,48] However, eliminating foods from the diet is not always without risks, as high food avoidance is associated with lower diet quality, nutritional deficiencies, decreased quality of life, and increased risk of eating disorders.[49-53] Therefore, proper identification of trigger foods or components is important, as well as understanding potential underlying mechanisms.

Trigger food products & components

Several surveys have been conducted in both IBS and IBD patients assessing food groups and products that patients associate with GI symptoms. Frequently reported foods include grains, dairy, fatty foods, spicy foods, gas-producing foods including some fruits and vegetables, alcohol, and caffeine.[29,30]

A generally accepted first-line dietary treatment for IBS symptoms is based on guidelines by the National Institute for Health and Care Excellence (NICE) in the UK, which is being applied, albeit in a modified way, worldwide. These guidelines include general advice like eating small, regular meals and taking time to eat, drinking enough fluids, and restriction of commonly identified trigger foods like coffee, alcohol, fizzy drinks, high-fibre foods, and fresh fruit.[54]

Others focus on a diet low in fermentable oligo-, di-, monosaccharides and polyols (FODMAPs). FODMAPs are present in a variety of dietary sources, including fruit, vegetables, grain, legumes, dairy products, and sugar alcohols. The low-FODMAP diet consists of three phases: (1) a 4-6 week period of FODMAP restriction; (2) re-introduction of individual food items to determine tolerance to each; and (3) personalisation to create a modified FODMAP-containing diet based on the individual's tolerance of FODMAPs identified in the second phase.[55,56]

A recent meta-analysis showed the low-FODMAP diet to be the most effective dietary treatment for IBS.[57] Additionally, also IBD patients with functional GI symptoms, such as abdominal pain and bloating, in the absence of active inflammation, may benefit from the low-FODMAP diet.[58] Nevertheless, with a symptom reduction in 50-80% of IBS patients, there also remains a large proportion of non-responders to the low-FODMAP diet.[57] Furthermore, the long-term efficacy needs further study in addition to awareness for potential negative consequences on total fibre intake and the intestinal microbiome.[59] Finally, hypnotherapy was shown to have a similar effectiveness,[60] further questioning the need for the low-FODMAP diet.

Wheat-containing products are among the top five trigger foods for IBS and IBD patients,[29,30] and are considered the main culprit food for NCGS/NCWS. Nevertheless, the exact wheat component responsible for symptom induction is still under debate. Besides fructans (being a type of FODMAP), gluten and non-gluten proteins like amylase trypsin inhibitors (ATIs) are often hypothesised as triggers.[40]

Gluten is a complex protein mixture composed of glutenin and gliadin, each with their own unique features and important for dough quality of bread. Glutenin proteins are particularly important for the elasticity of the dough, while gliadin proteins ensure the

viscosity.[61] Gliadins are commonly known to be involved in coeliac disease and wheat allergy.[33,34] However, studies investigating their effect in IBS and NCGS/NCWS show conflicting results. Whereas some studies show that a GFD is effective in reducing symptoms[62,63] or that a gluten-containing intervention triggers symptoms,[64-69] others show no effect of gluten,[70,71] or individual differences in the dosage of gluten that is tolerated.[72]
Furthermore, these studies should be carefully interpreted as the isolated wheat gluten fractions used generally contain significant amounts of ATIs.[73] ATIs are known triggers of wheat allergy[34,74] and baker's asthma,[34,74-76] and have been hypothesised to have a synergistic effect with gliadins in coeliac disease.[77,78] Based on animal and *in vitro* studies, they were also suggested to play a role in NCGS/NCWS, but so far human studies are limited.[77,79-83] Additionally, eliciting the contribution of these components is further complicated by biochemical differences between wheat species and varieties, and the effect of bread processing methods.[40,84,85]

Studying specific individual food compounds is complicated by the fact that they are always ingested as part of a habitual diet. The food matrix and interactions between food compounds, affected also by processing, may impact their effect.[86] This complexity is for example illustrated by the inflammatory potential of the diet. Various food products and nutrients have been associated with pro- or anti-inflammatory properties. Whereas the Western diet has been associated with increased levels of inflammatory markers,[87] the Mediterranean diet, rich in olive oil, fatty fish, fruits, vegetables, and wholegrains, is associated with a reduction of these markers.[88] Additionally, nutrients such as animal-based protein, saturated fatty acids, and salt, can activate pro-inflammatory pathways, but on the other hand, components like omega-3 fatty acids, polyphenols, and fibres are reported to have anti-inflammatory properties.[89]
Moreover, the Western diet is high in processed and ultra-processed foods. Especially the intake of ultra-processed foods, which also contain food additives like emulsifiers, thickeners, colorants, and artificial sweeteners, has been associated with an increased risk of IBS and IBD.[90,91] Furthermore, processing, especially heating, of foods containing proteins and reduced sugars induces the Maillard reaction. During this complex network of many thousands of individual non-enzymatic reactions, many different classes of Maillard reaction products (MRPs) are formed. On one hand, MRPs contribute to browning and palatability of foods, while on the other hand, MRPs have been identified as potentially harmful compounds.[92] A class of end products of the Maillard reaction, the advanced glycation endproducts (AGEs), has been associated with detrimental health outcomes like low-grade inflammation, endothelial dysfunction, and insulin resistance.[93] For their major precursors, the highly reactive dicarbonyls, both pro- and anti-inflammatory effects have been reported.[94,95] Dicarbonyls and AGEs are not completely digested and may directly impact the mucosal layer of the small and large intestine.[96-101]

Mechanisms underlying food-related GI symptoms

Although exact pathways underlying food-related GI symptoms, especially as part of the habitual diet, remain to be identified, various potential mechanisms have been described.

Food allergies are probably the most well-known and clearly defined mechanism related to food-related symptoms. They typically present with respiratory and dermatologic symptoms, as well as GI symptoms like abdominal pain, diarrhoea, nausea, and vomiting. Allergic responses can either be IgE mediated or non-IgE mediated.[102]

IgE-mediated allergic responses typically occur rapidly after exposure to the food allergen by activation of T helper 2 (Th2) and T follicular helper (Tfh) cells, resulting in stimulation of B cell differentiation into IgE-secreting plasma cells. IgE binds to the high-affinity Fc receptor on mast cells, and subsequently activates the mast cell to secrete various mediators responsible for immediate hypersensitivity reactions, as well as cytokines resulting in late-phase reactions.[103] Severe IgE-mediated allergic responses, for example to peanuts, can quickly trigger life-threatening anaphylaxis.[104] Nevertheless, true IgE-mediated food allergies are rather rare in the general population and have not been found more commonly in gastroenterology patients,[105-107] and thereby do not explain the majority of food-related GI symptoms.

Additionally, antibodies other than IgE, typically IgG, may induce local inflammation, phagocytosis and destruction of cells, or interference with normal cellular function by binding to their target antigens in different tissues.[103] Nevertheless, allergen-specific IgG tests have a high false positive rate and are therefore not routinely applied in clinical practice.[108] Non-IgE mediated entities include coeliac disease, eosinophil oesophagitis, eosinophil gastritis, and dermatitis herpetiformis.[109] Also, a recent rodent study, supported by analyses in human samples, showed that local activation of gut mast cells may contribute to abnormal pain signalling in IBS.[110]

Together with the intestinal microbiota, the immune system plays an important role in maintaining the intestinal barrier. The intestinal barrier protects the host against the external environment and consists of multiple components, including digestive juices, antimicrobial peptides and secretory immunoglobulin-A (sIgA), the commensal intestinal microbiota providing colonisation resistance, the mucus layer, and the intestinal epithelial layer.[111]

Impairment of the intestinal barrier function, for example by altered expression of intercellular tight junction proteins or epithelial cell damage, may lead to increased (paracellular) permeation of microbes and toxins and subsequent activation of the mucosal immune system, inducing various pathways resulting in the production of pro-inflammatory cytokines.[112] Several studies have reported that food components like alcohol, gluten, and emulsifiers can increase intestinal permeability.[113]

The intestinal microbiota is a complex ecosystem, with up to 10^{12} cells/gram of luminal content present in the colon, and plays an important role in maintaining intestinal homeostasis. The microbiota has a large metabolic capacity, involved *e.g.* in metabolism of bile salts, xenobiotics, and production of vitamins.[114,115] Bacterial fermentation of undigested carbohydrates (such as FODMAPs) results in the production of short-chain fatty acids (SCFAs) including acetate, propionate, and butyrate. They are important for intestinal health, for example by serving as energy substrate for the epithelium, reinforcement of the epithelial barrier, as well as having anti-inflammatory and anti-oxidative effects.[116] Protein fermentation on the other hand mainly results in the production of toxic metabolites such as ammonia, indoles, phenols, and hydrogen sulphide. As the intestinal microbiota has a preference for carbohydrate over protein fermentation, SCFA production is generally more prominent in the proximal colon.[117] Pronounced dietary changes can impact both the microbiota composition and activity.[118] Altered gut microbiota composition and activity has been observed in IBS and IBD patients.[115] How this may contribute to symptom development and/or flare occurrence, especially in relation to perceived food intolerances, is however not yet clear. Recent studies also point to possible involvement of the microbiota perturbations in NCGS/NCWS.[63,119-121]

Lactose, fructose, but also other FODMAPs, *i.e.* fructans, polyols, and galacto-oligosaccharides, when not (completely) digested and/or absorbed in the small intestine, will trigger an influx of fluids, potentially resulting in diarrhoea. Additionally, fermentation of these FODMAPs by the gut microbiota results in gas production, leading to colonic distention, which is associated with *e.g.* bloating and abdominal pain.[122,123] Patients with a DGBI can experience symptoms due to visceral hypersensitivity[124,125] and altered gut-brain interactions.[126]

The bidirectional interaction between the GI tract and the central nervous system, including the brain and spinal cord, is referred to as the gut-brain axis. The gut-brain axis involves multiple pathways, such as the autonomic and enteric nervous system, endocrine system, hypothalamic-pituitary-adrenal (HPA) axis, immune system, and the microbiota and its metabolites.[127] Part of these may be affected by diet. The gut-brain axis is especially important to consider in food sensitivities as psychological factors can influence GI symptoms, and vice versa.[128] Psychological distress is a common factor associated with symptom occurrence in GI diseases, with anxiety and depression being more prevalent in IBD,[129] IBS[130] and NCGS[131] as compared to healthy controls. A recent meta-analysis showed a high placebo response in IBS patients.[132] The opposite, a nocebo response, occurs when the expectation of experiencing negative effects from a treatment leads to the actual manifestation of those symptoms, even if the treatment itself is inert.[133] A pooled analysis found that 40% of NCGS/NCWS individuals showed a nocebo response when confronted with a double-blind placebo-controlled gluten challenge.[134] This was elegantly illustrated by Biesiekierski *et al.* in a double-blind, placebo-controlled, cross-over study in IBS patients with self-reported gluten sensitivity. They showed a significant worsening of

overall symptoms and pain irrespective of the diet (*i.e.* placebo, low-gluten, or high-gluten). Interestingly, symptom scores were highest with the first treatment the patients received, regardless of the actual intervention, suggesting a nocebo effect.[70]

Aims & outline of this thesis

Food plays an important role in symptom generation in GI disorders like IBS, IBD, and NCGS/NCWS. Although a variety of potential trigger foods, food components, and underlying mechanisms are suggested to be involved, clear evidence is often limited. Further insight into trigger compounds and contributing factors is necessary to improve dietary treatment and overall diet quality in these patients. Therefore, the overall aim of this thesis was to investigate the role of food in GI symptoms. To this end, we evaluated the role of various food products and components, with special focus on their effect on biological mechanisms such as intestinal inflammation, and the impact of psychological factors. We used a combined approach of human observational and intervention studies. Figure 1 presents an overview of the topics included in this thesis and the corresponding chapters.

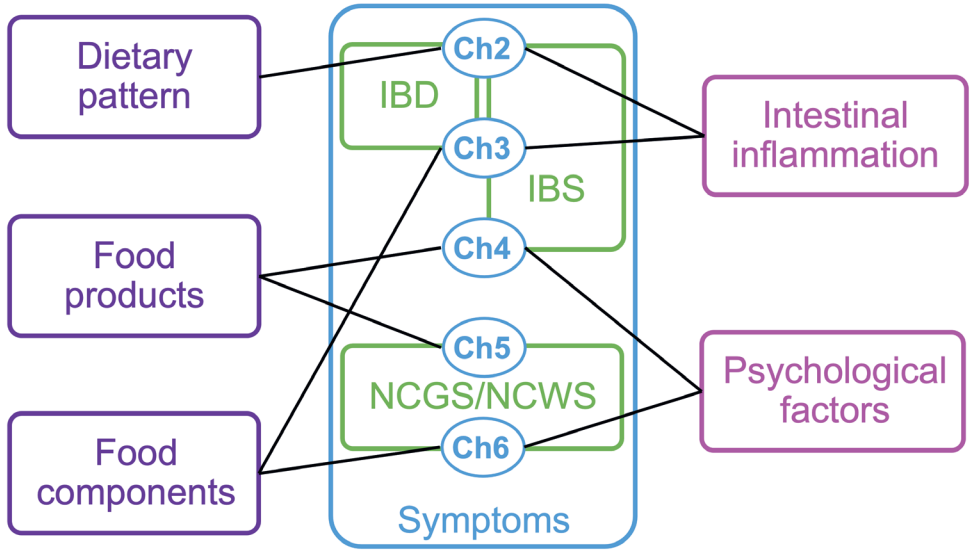

Figure 1. Overview of topics presented in this thesis and the corresponding chapters. Ch = Chapter; IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; NCGS/NCWS = non-coeliac gluten/wheat sensitivity.

Several food components have been associated with pro- or anti-inflammatory properties. However, food products and components are often studied individually, but are generally consumed as part of the habitual diet. Furthermore, patients often adjust their diet without guidance, resulting in a decreased diet quality and an increased risk of nutritional deficiencies. Therefore, in **Chapter 2** we investigated the relationship of diet quality, assessed by adherence to the Dutch dietary guidelines, and the

inflammatory potential of the diet with intestinal inflammation and GI symptoms in both IBD and IBS patients.
Besides the dietary composition, also the processing of food may have an important impact on diet quality and related health effects. In **Chapter 3** we investigated the intake of dietary dicarbonyls and AGEs as part of the habitual diet in both IBD and IBS patients, and their association with intestinal inflammation.

To avoid symptoms, patients often adjust their dietary intake. Therefore, in **Chapter 4** we used an extensive questionnaire to explore the extent and nature of food intolerance and avoidance due to GI symptoms in IBS. In addition, we aimed to investigate the association of food avoidance behaviour with type of symptoms and psychological comorbidities.

Wheat-containing products are often identified as culprit food by both IBS and IBD patients, and are considered the main trigger food for NCGS/NCWS. Nevertheless, the exact trigger component(s) of NCGS/NCWS as well as underlying mechanisms are still unclear. Therefore, in **Chapter 5** we investigated the effects of well-characterised yeast- or sourdough fermented bread made from bread wheat, spelt, or emmer on GI and extra-intestinal symptoms in individuals with self-reported NCWS in two parallel studies. Furthermore, in **Chapter 6**, we investigated the effects of expectancy about gluten intake versus actual gluten intake on GI and extra-intestinal symptoms in individuals with self-reported NCGS.

Finally, **Chapter 7** integrates the key findings of the studies presented in this thesis and discusses the outcomes in terms of potential implications for dietary treatment and future research.

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CHAPTER 2

Diet quality and dietary inflammatory index in Dutch inflammatory bowel disease and irritable bowel syndrome patients

Marlijne C. G. de Graaf, Corinne E. G. M. Spooren, Evelien M. B. Hendrix, Martine A.M. Hesselink, Edith J. M. Feskens, Agnieszka Smolinska, Daniel Keszthelyi, Marieke J. Pierik, Zlatan Mujagic, Daisy M. A. E. Jonkers

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Abstract

Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) share common culprit foods and potential pathophysiological factors. However, how diet may contribute to disease course and whether this differs between both entities is unclear. We therefore investigated the association of dietary indices with intestinal inflammation and gastrointestinal symptoms in both IBD and IBS patients. Food frequency questionnaires from 238 IBD, 261 IBS, and 195 healthy controls (HC) were available to calculate the overall diet quality by the Dutch Healthy Diet index 2015 (DHD-2015) and its inflammatory potential by the Adapted Dietary Inflammatory Index (ADII). Intestinal

inflammation and symptoms were evaluated by faecal calprotectin and the Gastrointestinal Symptom Rating Scale, respectively. The DHD-2015 was lower in IBD and IBS versus HC ($p<0.001$), being associated with calprotectin levels in IBD ($b=-4.009$, $p=0.006$), and with abdominal pain ($b=-0.012$, $p=0.023$) and reflux syndrome ($b=-0.016$, $p=0.004$) in IBS. ADII scores were comparable between groups and were only associated with abdominal pain in IBD ($b=0.194$, $p=0.004$). In this side-by-side comparison, we found a lower diet quality that was differentially associated with disease characteristics in IBD versus IBS patients. Longitudinal studies are needed to further investigate the role of dietary factors in the development of flares and predominant symptoms.

Introduction

Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) are both multifactorial and heterogeneous intestinal disorders. IBD is a chronic inflammatory disease, comprising Crohn's disease (CD) and ulcerative colitis (UC), and is characterised by alternating sequences of active inflammation and remission. IBD is generally considered to arise from a complex interaction between host genetics, the intestinal microbiome, and immune factors, as well as environmental factors.[1,2] The latter is supported amongst others by the rising incidence in line with Westernisation.[3] IBS is found to be present in 5-10% of the Western population,[4] and is characterised by recurrent abdominal pain in combination with altered bowel habits. In addition to microbiome perturbations, alterations in intestinal motility, barrier function, visceral perception, and brain-gut interaction, a low-grade inflammation is reported in subgroups of IBS patients. Although the exact underlying mechanisms are not clear, symptoms can also be triggered by environmental factors.[5] IBS-like symptoms are also reported in about 35% of IBD patients in remission.[6]

One of the environmental factors associated with both IBD and IBS is the Western diet, characterised by, for example, high fat, high sugar, and low fruit and vegetable intake.[7,8] Furthermore, 58-68% of IBD patients with active disease, 29-39% of IBD patients in remission,[9] and up to 90% of IBS patients[10] indicate that meals and/or certain food products exacerbate flares and/or gastrointestinal (GI) symptoms. Dairy products, spicy foods, wheat products, and gas-producing foods including some fruits and vegetables, are reported to be the main culprits by both patient groups.[9,10] Diet can influence both disease onset and disease course, for example, through interaction with the immune system, but also by modulating the intestinal microbiota composition and activity, and/or intestinal barrier function.[7,8]

As a consequence, interest is increasing in nutrients or foods that have an (anti-) inflammatory potential or can contribute to GI symptoms, for example, by increased gas production and osmotic effects.[7,8] As foods are generally not consumed in isolation, but as part of the total diet, this further adds to the complexity. Although various dietary intervention strategies are currently being investigated, it is not completely clear how overall diet quality in IBD and IBS relates to inflammation markers and symptom occurrence.

Various indices have been developed to assess diet quality. Overall diet quality can be defined by adherence to the Dutch dietary guidelines[11] by calculating the Dutch Healthy Diet index 2015 (DHD-2015).[12] Furthermore, a diet can be defined by its pro- or anti-inflammatory potential, and by calculating indices based on the (anti-) inflammatory properties of certain nutrients and food items. Examples of these indices include the Adapted Dietary Inflammatory Index (ADII), based on nutrients,[13] and the Empirical Dietary Inflammatory Index (EDII), based on food products.[14]

IBD and IBS share common culprit foods as well as underlying mechanisms, but the magnitude of these factors differs between the diseases, for example, with inflammation being more prominent in IBD. Therefore, a side-by-side comparison of IBD and IBS can provide further insight into the association of overall diet quality with

markers for inflammation and symptom occurrence. This may identify leads for further mechanistic studies and will aid in providing patients with adequate advice. Therefore, we aim to investigate the relationship of the adherence to the Dutch dietary guidelines (using the DHD-2015) and the inflammatory potential of the diet (using the ADII) with inflammatory markers and GI symptoms in both IBD and IBS patients.

Methods

Study population

For this study, cross-sectional data on habitual dietary intake and clinical data were collected from two large cohorts from the same geographical region in the Netherlands. All participants provided written informed consent prior to participation.

IBD South Limburg Cohort

The IBD South Limburg (IBDSL) cohort is a well-characterised population-based inception cohort in the South Limburg area in the Netherlands and has been used to study IBD epidemiology and disease course since 1991.[15] Patients included were at least 18 years old and were diagnosed with either CD or UC according to the Lennard-Jones criteria[16] and proven by endoscopic, radiological and/or histological findings. Relevant demographical and clinical data were retrieved from the IBDSL data warehouse.[15] Data on habitual dietary intake were collected using a validated food frequency questionnaire (FFQ) as part of a sub-study within the IBDSL cohort. Both the IBDSL cohort and the sub-study have been approved by the medical research ethics committee of the Maastricht University Medical Center+ (MUMC+) (NL31636.068.10 and NL42101.068.12, respectively), and have been registered at the US National Library of Medicine (NCT02130349 and NCT0176963, respectively).

Maastricht IBS Cohort

The Maastricht IBS (MIBS) cohort has been used to study the phenotypical and genotypical characterisation of patients with IBS at the MUMC+ since 2009. All patients included were at least 18 years old and complied with the Rome III criteria for IBS.[17] Furthermore, healthy controls (HC) were included as described previously.[18] The MIBS cohort was approved by the medical research ethics committee of the MUMC+ (NL24160.068.08) and has been registered at the US National Library of Medicine (NCT00775060). Participants with dietary intake data as part of a previous study[19] were re-analysed for the current study.

Demographic and Clinical Data Collection

In both cohorts, demographic and clinical characteristics were collected including age, sex, body mass index (BMI), smoking, medication use, and disease phenotype. Faecal calprotectin was used as the marker for intestinal inflammation. Faecal samples were collected at home, stored in a fridge, and brought to the hospital within 24 h after defecation for routine analysis of faecal calprotectin by the clinical chemistry department using a fluorescent enzyme immune assay (FEIA) (IBDSL cohort), or using

a commercial enzyme-linked immunosorbent assay (ELISA, Bühlmann Laboratories, Schönenbuch, Switzerland) (MIBS cohort). The presence of GI symptoms was assessed using the Gastrointestinal Symptom Rating Scale (GSRS), consisting of 16 items clustered into five major GI syndromes: abdominal pain, reflux syndrome, diarrhoea syndrome, indigestion syndrome, and constipation syndrome.[20]

For IBD patients, disease phenotype at time of inclusion was defined by the Montreal classification, including age of onset, disease location and behaviour (for CD), or extent (for UC).[21] Furthermore, disease duration, clinical activity indices (*i.e.*, Harvey Bradshaw Index (HBI) for CD[22] and Simple Clinical Colitis Activity Index (SCCAI) [23] for UC) and time since last flare were retrieved from the IBDSL data warehouse. A flare was defined by the following criteria, in line with clinical practice and previous studies:[24,25] (1) presence of active disease confirmed by a physician based on endoscopy and/or radiological imaging; (2) faecal calprotectin ≥250 μg/g; (3) faecal calprotectin ≥100 μg/g with at least a fivefold increase from previous visit; (4) clinical symptoms indicative for active disease or increased HBI (≥5) or SCCAI (≥3) accompanied by dose escalation or initiation of a new drug; or (5) dose escalation or initiation of a new drug accompanied by C-reactive protein (CRP) ≥10 mg/L. Active disease at inclusion was defined as having a flare at inclusion or during the three months prior to inclusion. In addition, when data were incompletely registered in patients' records in the period before inclusion, IBD-related hospitalisation due to disease activity and IBD-related surgery were examined to be able to evaluate disease activity.

For IBS patients, subtypes — diarrhoea (IBS-D), constipation (IBS-C), mixed stool pattern (IBS-M), and unspecified stool pattern (IBS-U) — were defined according to the Rome III criteria.[17]

Dietary Data Collection

Habitual dietary intake was evaluated by using the same self-administered FFQ in both cohorts, with a recall period of a month, which has been developed and validated by the division of Human Nutrition of Wageningen University.[26,27] The intake was assessed by scoring the frequency of consumption and by estimating portion sizes using natural portions and commonly used household measures. The intake of nutritional supplements was not included in the FFQ; it was recorded separately. Data were linked to the Dutch food composition table (NEVO 2010, RIVM, Bilthoven, the Netherlands), resulting in a calculated individual mean consumption of 45 nutrients and 148 food items.

Only participants with complete dietary intake, clinical, and demographic data were eligible for inclusion in the current study. Participants were excluded if they were on tube feeding or if FFQ data were incomplete or considered implausible, *i.e.*, an overall intake for males <800 or >4000 kcal/day and for females <500 or >3500 kcal/day.[28]

Dutch Healthy Diet index 2015 (DHD-2015)

To assess the adherence to the Dutch healthy diet guidelines,[11] the DHD-2015 was computed as described previously by Looman *et al.*[12] Based on our FFQ data, the difference between filtered and unfiltered coffee could not be made, and salt intake could not be calculated, finally resulting in 13 components available for our calculation (Appendix A, Tables A1 and A2). Briefly, for each component a minimum, maximum, or optimum intake was defined. Based on these criteria, each component received 0-10 points, resulting in a total score ranging from 0 to 130 points. A higher score indicates a better adherence to the dietary guidelines.

Adapted Dietary Inflammatory Index (ADII)

To assess the inflammatory potential of the diet, the ADII was computed as described previously by Van Woudenbergh *et al.*[13] The ADII is a literature-derived index that summarises an individual's diet on the continuum from maximally anti-inflammatory to maximally pro-inflammatory. The score was defined by the pro- or anti-inflammatory properties of various macro- and micronutrients based on a literature search for their effect on inflammatory markers (*i.e.*, IL-1β, IL-4, IL-6, IL-10, TNF-α and CRP). This resulted in a (weighed) positive (pro-inflammatory) or a negative (anti-inflammatory) value for each component. The sum finally indicates the overall diet score, which has been validated in healthy individuals, elderly, and those at risk of type 2 diabetes and cardiovascular disease,[13,29,30] and used in various patient groups.[31-34]

Based on our FFQ data, the exact intake of caffeine, quercetin, and garlic could not be calculated, resulting in 26 components available for our calculation (Appendix A, Table A3). First, the intake of each component was adjusted for energy intake using the residual method. As energy intake was significantly different between groups, the ADII was computed separately for IBD, IBS, and HC. Next, this calculated standardised energy-adjusted intake was multiplied by the inflammatory weight. Then, these values were summed to obtain the final score. A higher (positive) score points to a more pro-inflammatory diet, whereas a lower (negative) score indicates a more anti-inflammatory diet.

Statistical Analysis

A statistical analysis was performed using IBM SPSS Statistics version 26.0.[35] Normality of data was checked using a normal probability plot. Baseline characteristics were presented as mean with corresponding standard deviation (SD) for continuous parametric variables, and as percentages for categorical variables. Differences in baseline characteristics between IBD patients, IBS patients and HC were tested with an analysis of variance (ANOVA) and post-hoc Bonferroni correction (for continuous data), and the Chi-square test with Fisher exact when necessary (for categorical data). A linear regression analysis was used to assess the association between the dietary indices (DHD-2015 or ADII) and intestinal inflammation (using faecal calprotectin as marker) or GSRS domains. Analyses were performed for each subgroup (IBD, IBS, HC) separately. The following parameters were included in the analyses: age, sex, smoking, BMI, medication, subtype (IBS) or phenotype (IBD), and for IBD patients,

additionally, disease duration (in years) and age at diagnosis (defined by the Montreal classification). Missing values were excluded listwise. A two-sided p-value<0.05 was considered to be statistically significant.

In addition to using predefined indices (*i.e.*, DHD-2015 and ADII), an explorative unsupervised random forest (URF) analysis[36] was performed to identify possible combinations of food items or nutrients of relevance to distinguish IBD, IBS and HC. More details can be found in Appendix B.

Results

Baseline characteristics

Complete FFQ data were available for 239 IBD patients, 274 IBS patients, and 207 HC. Because of implausibly low or high intake, 1 IBD patient, 13 IBS patients, and 12 HC were excluded, resulting in 238 IBD patients, 261 IBS patients, and 195 HC being included in the present study.

Demographic and clinical characteristics are displayed in Table 1. Age was comparable between IBD patients (45.7±14.8 years), IBS patients (43.3±17.0 years), and HC (44.4±18.9 years). In the IBS group, significantly more women (74%) were included as compared to IBD (52.9%, p<0.001) and HC (63.1%, p=0.007). BMI was significantly lower in HC (23.9±3.8 kg/m^2) compared to IBD (25.5±4.2 kg/m^2, p<0.001) and IBS patients (25.0 ±4.6 kg/m^2, p=0.021). Smoking behaviour was also significantly different between groups, with more active smokers in IBD (20.4%, p<0.001) and IBS patients (23.6%, p<0.001) as compared to HCs (6.7%), and more former smokers among the IBD patients (41.7%) compared to IBS (24.4%, p<0.001) and HC (31.8%, p=0.035).

The IBD patients comprised of 156 CD (65.5%) and 82 UC (34.5%) patients, with 61.5% of all patients (36.5% and 28.0%, respectively) being in remission at the time of inclusion. In IBS patients, the IBS-M subtype was predominant (39.5%), followed by IBS-D (35.6%), IBS-C (21.5%), and IBS-U (3.4%).

Table 1. Baseline characteristics in inflammatory bowel disease (IBD) patients, irritable bowel syndrome (IBS) patients, and healthy controls (HC).

	IBD patients (n = 238)	IBS patients (n = 261)	HC (n = 195)	p-value
Age (years)	45.7 ± 14.8	43.3 ± 17.0	44.4 ± 18.9	0.285
Sex				< 0.001
Male	47.1%	25.3%	36.9%	
Female	52.9%	74.7%	63.1%	
BMI (kg/m^2) *	25.5 ± 4.2	25.0 ± 4.6	23.9 ± 3.8	< 0.001
Smoking **				< 0.001
Active smoker	20.4%	23.6%	6.7%	
Former smoker	41.7%	24.4%	31.8%	
Never smoker	37.9%	52.0%	61.5%	
IBD Phenotype				
Crohn's disease	65.5%	n/a	n/a	n/a
Ulcerative colitis	34.5%	n/a	n/a	n/a
Age of onset **				
A1 - below 17 years old	5.9%	n/a	n/a	n/a
A2 - 17-40 years old	64.0%	n/a	n/a	n/a
A3 - above 40 years old	30.1%	n/a	n/a	n/a
Behaviour of Crohn's disease at inclusion (n=156)				
B1 - non-stricturing, non-penetrating	57.1%	n/a	n/a	n/a
B2 - stricturing	17.9%	n/a	n/a	n/a
B3 - penetrating	25.0%	n/a	n/a	n/a
Location of Crohn's disease at inclusion (n=82)				
L1 - ileal	23.7%	n/a	n/a	n/a
L2 - colonic	16.7%	n/a	n/a	n/a
L3 - ileocolonic	59.6%	n/a	n/a	n/a
L4 - upper-GI modifier	10.3%	n/a	n/a	n/a
Extent of ulcerative colitis (UC) at inclusion **				
E1 - ulcerative proctitis	11.1%	n/a	n/a	n/a
E2 - left sided UC (distal UC)	39.5%	n/a	n/a	n/a
E3 - extensive UC (pancolitis)	49.4%	n/a	n/a	n/a
Disease activity at inclusion				
Active disease	34.9%	n/a	n/a	n/a
Remission	61.5%	n/a	n/a	n/a
Disease duration (years) **	11.5 ± 10.1	n/a	n/a	n/a
Time to last flare (months)	37.7 ± 67.7	n/a	n/a	n/a
Bowel resection at inclusion				
Yes	23.1%	n/a	n/a	n/a
No	76.9%	n/a	n/a	n/a
Symptom score *				
Harvey Bradshaw Index	2.9 ± 3.4	n/a	n/a	n/a
Simple Clinical Colitis Activity Index	1.2 ± 1.8	n/a	n/a	n/a
IBS Subtype				
Constipation predominant IBS	n/a	21.5%	n/a	n/a
Diarrhoea predominant IBS	n/a	35.6%	n/a	n/a
Mixed stool pattern IBS	n/a	39.5%	n/a	n/a
Unspecified subtype IBS	n/a	3.4%	n/a	n/a
Medication ***				
No medication	14.3%	26.8%	52.8%	< 0.001

Table 1 (Continued).

	IBD patients (n = 238)	IBS patients (n = 261)	HC (n = 195)	p-value
Medication *** (continued)				
5-ASA, local immunosuppressants, or local corticosteroids	17.6%	n/a	n/a	n/a
Systemic corticosteroids	0.4%	n/a	n/a	n/a
Immunomodulators	22.7%	n/a	n/a	n/a
Biologicals	45.0%	n/a	n/a	n/a
PPIs	n/a	20.7%	3.1%	< 0.001
NSAIDs	n/a	24.9%	20.0%	0.217
Laxatives	n/a	18.4%	0.0%	n/a
Spasmolytic drugs	n/a	14.2%	0.0%	n/a
Antihypertensive drugs	n/a	15.3%	13.3%	0.550
Statins	n/a	10.0%	7.7%	0.402
Antidepressant drugs	n/a	10.0%	3.6%	0.009

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; BMI = body mass index; 5-ASA = 5-aminosalicylic acid; PPIs = proton pump inhibitors; NSAIDs = non-steroidal anti-inflammatory drugs; n/a = not applicable or not available.

* Missing data from max. 25 participants per subgroup. ** Missing data from max. 3 participants per subgroup. *** Missing data from 4 IBS patients.

Medication for IBD patients was classified as the highest category of use. For IBS medication, only the most important medications are displayed. Other medication included prokinetics, anti-diarrhoeal drugs, oral contraceptives, antipsychotic drugs, and antibiotics.

Continuous data are expressed as mean ± standard deviation (SD). Categorical data are expressed as percentages of total group (IBD, IBS or HC). The differences between IBD, IBS, and HC were tested with ANOVA and post-hoc Bonferroni correction for continuous data, and the Chi-square test with Fisher for categorical data.

Dietary Intake, Diet Quality, and Inflammatory Potential of the Diet

Mean total energy intake was significantly lower in IBS (1939.6±604.9 kcal) when compared to IBD (2180.0±634.3 kcal, $p<0.001$) and HC (2180.4±622.9, $p<0.001$). Full details on the intake of specific food items and nutrients are given in Appendix A, and Tables A2 and A3, respectively.

The DHD-2015 (Figure 1A) ranged from 24.64 to 115.58 in IBD, 21.57 to 111.34 in IBS and 32.47 to 119.10 in HC, with a significantly lower mean in IBD (69.00±16.53) and IBS (71.61±16.58) as compared to HC (77.34±17.43; IBD vs. HC: $p<0.001$; IBS vs. HC: $p=0.001$; IBD vs. IBS: $p=0.251$).

For all groups, adherence to the Dutch dietary guidelines was highest for alcohol, wholegrain, and red meat. However, the absolute intake of vegetables, fruit, wholegrain products and the DHD-2015 score for these components were significantly lower in IBD and IBS as compared to HC. Furthermore, in both IBD and IBS, the absolute intake for dairy was significantly lower as compared to HC, but this did not reflect in a significantly lower DHD-2015 score. In IBD only, absolute intake of red meat was significantly higher compared to IBS and HC; this reflected in a significantly lower DHD-2015 score for this component. The lowest mean component scores were observed for refined grain, nuts, and processed meat (for IBD and IBS) or tea (for HC). The exact order of highest and lowest component scores was slightly different per subgroup (Appendix A, Table A2).

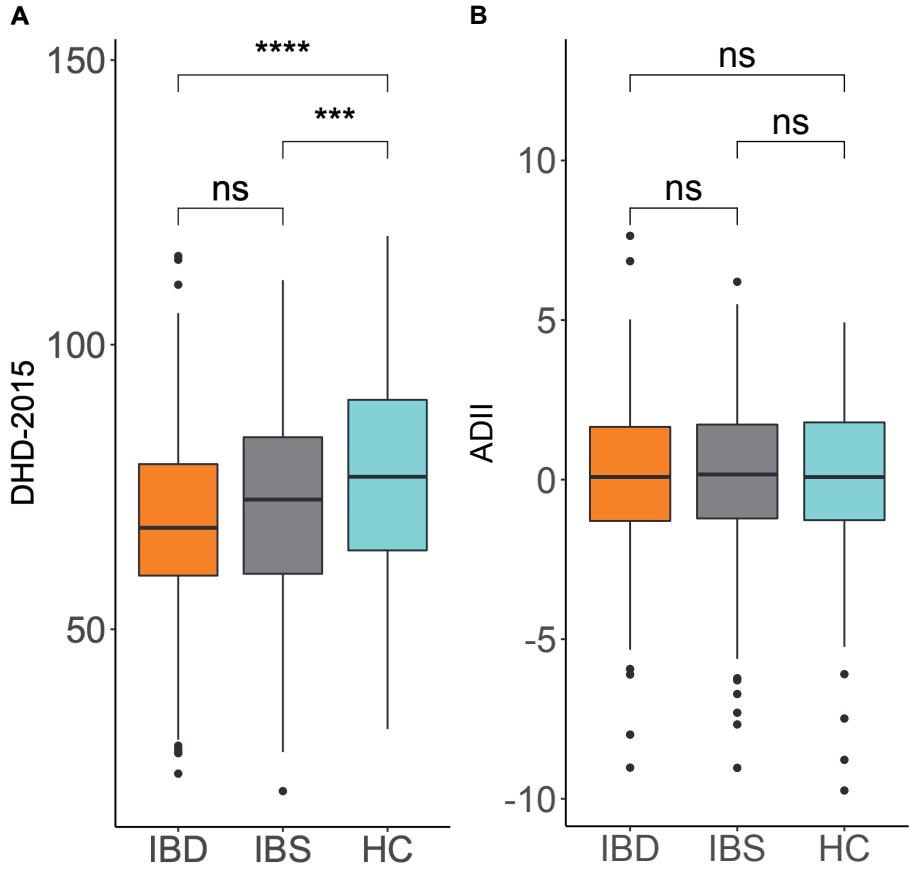

Figure 1. Dietary indices for inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and healthy controls (HC). (A) Dutch Healthy Diet index 2015 (DHD-2015), (B) Adapted Dietary Inflammatory Index (ADII). The difference between subgroups was tested with analysis of variance (ANOVA) and post-hoc Bonferroni correction. ns = not significant, *** = $p<0.001$ and **** = $p<0.0001$.

The ADII scores (Figure 1B) ranged from -9.02 to 7.64 in IBD, from -9.03 to 6.20 in IBS and -9.74 to 4.93 in HC, with a mean score that did not differ between IBD (0.052±2.41), IBS (0.055±2.47) and HC (0.054±2.33). The mean ADII was above zero in all groups, indicating a slightly pro-inflammatory diet. The differences in scores for vitamins and minerals varied per micronutrient. Further details are given in Appendix A, Table A3.

The explorative URF resulted in principal coordinate analysis (PCoA) score plots, which showed no relevant grouping based on either food items or nutrients (Appendix B, Figures A1 and A2) when considering PCo1 and PCo2. Only PCo4 and PCo7 of nutrient intake data (Figure A3) showed a separation of IBS as compared to IBD and HC, explaining only 3.8% of the total variance. More details are given in Appendix B.

Table 2. Intestinal inflammation and gastrointestinal symptoms.

	IBD (n = 238)		IBS (n = 261)		HC (n = 195)		p-value
Calprotectin (μg/g)	197.3 ± 426.3	(n = 209)	64.4 ± 87.1	(n = 90)	39.3 ± 63.6	(n = 148)	< 0.001
GSRS							
Abdominal pain	2.1 ± 1.0	(n = 80)	3.3 ± 1.2	(n = 258)	1.6 ± 0.7	(n = 194)	< 0.001
Constipation syndrome	1.9 ± 1.1	(n = 70)	3.4 ± 1.3	(n = 257)	1.6 ± 0.8	(n = 193)	< 0.001
Diarrhoea syndrome	2.7 ± 1.5	(n = 77)	3.3 ± 1.5	(n = 258)	1.4 ± 0.6	(n = 194)	< 0.001
Indigestion syndrome	2.7 ± 1.2	(n = 80)	4.1 ± 1.3	(n = 256)	2.0 ± 0.8	(n = 193)	< 0.001
Reflux syndrome	1.4 ± 0.8	(n = 80)	2.2 ± 1.4	(n = 258)	1.2 ± 0.5	(n = 195)	< 0.001

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; GSRS = Gastrointestinal Symptom Rating Scale.
Continuous data are expressed as mean ± standard deviation (SD). The differences between IBD, IBS, and HC were tested with ANOVA and post-hoc Bonferroni correction.

Table 3. Results of multivariable linear regression analysis (after adjustment for possible confounders) of dietary indices for disease parameters.

	IBD			IBS			HC		
	β	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
Faecal calprotectin									
DHD-2015	-4.009	-6.875; -1.143	0.006	0.006	-1.105; 1.117	0.991	-0.506	-1.186; 0.175	0.144
ADII	11.259	-7.157; 29.675	0.229	-2.880	-10.853; 5.093	0.474	3.036	-2.349; 8.421	0.267
Abdominal pain									
DHD-2015	-0.006	-0.024; 0.011	0.460	-0.012	-0.022; -0.002	0.023	-0.001	-0.006; 0.005	0.784
ADII	0.194	0.065; 0.323	0.004	0.005	-0.065; 0.074	0.895	0.014	-0.028; 0.056	0.510
Constipation syndrome									
DHD-2015	-0.007	-0.025; 0.011	0.454	0.008	-0.001; 0.017	0.075	0.001	-0.006; 0.008	0.724
ADII	-0.015	-0.161; 0.132	0.843	-0.027	-0.090; 0.036	0.402	-0.030	-0.081; 0.020	0.235
Diarrhoea syndrome									
DHD-2015	-0.017	-0.042; 0.008	0.168	0.000	-0.011; 0.011	0.989	0.000	-0.005; 0.005	0.997
ADII	0.173	-0.021; 0.367	0.079	0.023	-0.052; 0.097	0.545	-0.019	-0.060; 0.022	0.358
Indigestion syndrome									
DHD-2015	-0.016	-0.035; 0.003	0.101	-0.001	-0.012; 0.011	0.906	0.001	-0.006; 0.009	0.740
ADII	0.107	-0.049; 0.262	0.174	-0.007	-0.083; 0.070	0.857	-0.030	-0.086; 0.026	0.291
Reflux syndrome									
DHD-2015	-0.000	-0.014; 0.013	0.970	-0.016	-0.027; -0.005	0.004	0.002	-0.003; 0.007	0.395
ADII	-0.064	-0.173; 0.044	0.240	0.058	-0.018; 0.134	0.133	-0.014	-0.050; 0.022	0.430

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; β = regression coefficient; 95% CI = 95% confidence interval; DHD-2015 = Dutch Healthy Diet index 2015; ADII = Adapted Dietary Inflammatory Index.

Faecal calprotectin was measured in μg/g (marker for intestinal inflammation). Abdominal pain, constipation syndrome, diarrhoea syndrome, indigestion syndrome and reflux syndrome were defined using the Gastrointestinal Symptom Rating Scale.

Analyses were performed using multivariable linear regression, and were corrected for: age, sex, smoking, body mass index, disease specific medication (all subgroups), plus phenotype, disease duration (years), and age of onset according to the Montreal classification for IBD, or plus subtype for IBS.

Disease Phenotypes

Separate explorative analyses on disease phenotypes showed that the DHD-2015 was significantly lower in active as compared to remissive IBD patients (64.77±15.38 vs. 71.15±16.72, p=0.004) and also in CD compared to UC (65.47±15.94 vs. 75.71±15.61, p<0.001). No significant differences were found for the DHD-2015 between IBS subtypes, nor did the ADII differ between disease phenotypes. Further details are given in the Supplementary Tables S1-S3.

Intestinal Inflammation

Mean faecal calprotectin levels (Table 2) were significantly higher in IBD patients (197.3±426.3 μg/g) as compared to IBS (64.6±87.1 μg/g, p=0.001) and HC (39.3±63.6 μg/g, p<0.001), but no differences were found between IBS and HC (p>0.999).

Based on the multivariable linear regression analysis (Table 3), the DHD-2015 was associated with faecal calprotectin in IBD patients (b=-4.009, p=0.006), but not in IBS patients or HC (IBS: p=0.991; HC: p=0.144). Faecal calprotectin levels were not associated with the ADII in either of the groups (IBD: p=0.229; IBS: p=0.474; HC: p=0.267).

GI Symptoms

IBS patients scored significantly higher on all GSRS subdomains as compared to IBD and HC individuals (p<0.001 for all comparisons, Table 2). In addition, IBD patients scored significantly higher than HC on subdomains abdominal pain (p=0.002), diarrhoea syndrome (p<0.001) and indigestion syndrome (p<0.001), but not for other subdomains.

Using a multivariable linear regression analysis (Table 3), abdominal pain was significantly associated with the ADII in IBD patients (b=0.194, p=0.004), and with the DHD-2015 in IBS patients (b=-0.012, p=0.023). Furthermore, in IBS patients, reflux syndrome was significantly associated with the DHD-2015 (b=-0.016, p=0.004). No significant associations were found for the GSRS subdomains constipation syndrome, diarrhoea syndrome, and indigestion syndrome. In HC, none of the associations were significant.

Discussion

We found that diet quality was significantly lower in IBD and IBS patients as compared to HC. However, there was no difference in the dietary inflammatory potential between groups based on the ADII. Furthermore, our results showed that a lower diet quality was associated with more intestinal inflammation in IBD, while it was associated with higher symptom scores in IBS patients. A more pro-inflammatory diet was only associated with higher abdominal pain scores in IBD patients.

Overall diet quality was lower in both IBD and IBS patients compared to HC, being especially lower for dairy and high-fibre foods such as wholegrain products, fruit and vegetables, and legumes. This is in line with previous studies reporting these food

groups as perceived food culprits in both patient groups,[9,10] and with studies indicating that IBD and IBS patients are at increased risk for nutritional deficiencies and malnutrition.[37-39] This emphasises the importance of good dietary advice when avoiding certain food products.

Whereas overall diet composition cannot be used to differentiate between IBD and IBS, it should be noted that some differences can be found, such as the lower intake of wholegrain products and red meat in IBS. Additionally, it is important to note that the DHD-2015 was validated in healthy subjects, while IBD and IBS patients may need other recommendations. For example, IBD patients with active disease have been reported to require a higher protein intake than those in remission or healthy individuals.[40] Further, patients may need higher intakes due to more loss (diarrhoea) and less absorption of nutrients.[37,38] This further stresses the relevance of adequate dietary advice, using a tailored approach and taking into account disease characteristics and nutritional status.

Diet in general, and specific food items in particular, can impact mechanisms that may contribute to disease course in IBD and IBS directly by impacting host immune function or indirectly via the intestinal microbiome and barrier disruptive effects.[7,29] We therefore evaluated the ADII as an indicator for the inflammatory potential of the overall diet, and found a wide range with on average a slightly pro-inflammatory index (*i.e.*, above 0) in all groups, which did, however, not differ between the groups. In future studies, it would be interesting to further investigate whether this could impact intestinal health differently in susceptible patients as compared to healthy control subjects. Additionally, the ADII takes into account that foods are generally not consumed in isolation, but may miss over- or underconsumption of specific nutrients. In line with this, the standardised energy-corrected intake of nutrients used for this score is important to avoid overestimation of the effect of certain nutrients; however, this may also partially explain why we found no differences between groups, despite some differences in the absolute intake of several pro- and anti-inflammatory components. A limited group difference was also illustrated by our explorative URF analyses, which, based on PCo4 and PCo7 (explaining <4% of variance), indicated a minor but clear distinction between the nutrient intake of IBS patients compared to IBD and HC (see Appendix B). The URF was added to identify any relevant unknown dietary patterns, but findings should be interpreted with care as no distinction was found by PCo1 and PCo2. This further illustrates the complexity of interpreting dietary data, and the need for longitudinal studies on the exact role of both dietary patterns and specific nutrients and product groups in the development of intestinal inflammation and symptoms, studied separately for these patient groups because of potential differences.

In line with our results, a previous study using the Dietary Inflammatory Index (DII) in IBD patients also pointed towards a slightly pro-inflammatory diet.[41] The (A)DII was not previously assessed in IBS, but a previous study using the EDII found a pro-inflammatory diet being associated with higher odds of having IBS.[42] The EDII[14] is based on food groups rather than nutrients. We chose not to incorporate the EDII in our analyses because the defined food groups were not representative for the Dutch dietary intake.

In our study, no association was found between the ADII and faecal calprotectin as a marker for intestinal inflammation in IBD nor in IBS. In addition, no difference was observed in the ADII score between remissive versus active IBD. These findings are in line with a study by Mirmiran *et al.* that found no association between the inflammatory potential of diet and disease severity, as defined by the CDAI and Mayo score.[43] In contrast, Lamers *et al.* found that the DII was significantly lower in IBD patients in remission, compared to IBD patients with mild or moderate active disease, and that a more pro-inflammatory diet was associated with higher Clinical Disease Activity Index (sCDAI) in CD patients.[41] It should, however, be considered that clinical activity indices do not necessarily correlate with active inflammation.[41]

Although a more pro-inflammatory diet did not correlate significantly with low diet quality in either of our groups, a lower diet quality was significantly associated with more intestinal inflammation in IBD, but not in IBS. Diet quality as scored by the DHD-2015 was also significantly lower in active IBD patients compared to IBD patients in remission. We cannot exclude that the observation (in part) was due to related symptoms, but we do not have sufficient power to draw firm conclusions on this. In addition, it is important to note the limitation of the cross-sectional design and that the relation between diet quality and intestinal inflammation could be bidirectional. A low intake of favourable nutrients, such as antioxidants and fibres — the latter of which leads to enhanced production of short-chain fatty acids — can increase the risk of a flare.[44] On the other hand, patients with active disease (*i.e.*, more inflammation) often change their diet in an attempt to mitigate symptom burden, which can result in poorer diet quality.[45] Thus, longitudinal studies are necessary to gain more insight in the causality of such associations.

As diet can also play a role in symptom onset via, for example, osmotic effects and distension, we investigated the association with symptom domains associated with IBS that are also common in IBD. We found a more pro-inflammatory diet, but not an overall diet quality to be associated with more abdominal pain in IBD patients. Although abdominal pain scores were not different in active versus quiescent IBD patients, diarrhoea was more common.

Based on our results, the inflammatory potential of the diet does not seem to be the driving factor for symptom severity in IBS, which is in line with a previous study.[42] However, in IBS, a lower diet quality was associated with more GI symptoms. Again, these associations could be bidirectional. Multiple previous studies reported both IBD and IBS patients adjusting their diet because of food-related symptoms, resulting in a less healthy diet.[10,46-51] Although data on individual dietary advice were not available for the current study, a recent national Dutch survey showed that 71% of IBS patients indicated having changed their diet because of symptoms, of which only 30% were supervised by a dietitian.[52] Notwithstanding, in the current study, symptom scores were still increased as compared to controls and a lower diet quality can also (further) exacerbate symptoms. This again stresses the importance of further investigating the causality of such associations using longitudinal studies. Hereby, it would be interesting to add further markers for malnutrition and potential underlying mechanisms related to, *e.g.*, the immune system and the microbiome.

A strength of our study was the assessment of the overall dietary patterns in different patient populations and HC, rather than just single foods or nutrients in homogeneous study populations. A limitation was that the FFQ was not validated for the calculation of micronutrients intake, and that use of nutritional supplements was not incorporated into the analysis. Furthermore, some anti-inflammatory components, such as caffeine, quercetin, and garlic, could not be calculated. Therefore, the ADII might slightly overestimate the pro-inflammatory potential of the diet.

Conclusions

In this study, we investigated the relationship of the adherence to the Dutch dietary guidelines (using the DHD-2015) and the inflammatory potential of the diet (using the ADII) with inflammatory markers and GI symptoms in both IBD and IBS patients that share culprit foods.

A low overall diet quality and a slightly pro-inflammatory diet was observed in both IBD and IBS patients, indicating the need of improving diet quality with adequate nutritional guidance. Furthermore, diet quality was associated with faecal calprotectin in IBD and with several GI symptoms in IBS, whereas the inflammatory potential of the diet was only associated with GI symptoms in IBD. These differences between the studied patient groups may point to differential roles in the pathophysiology. However, due to the cross-sectional design, we cannot draw firm conclusions on the direction or presence of causality between diet, intestinal inflammation, and GI symptoms. Our findings support the need for longitudinal studies to further investigate the role of dietary factors in the development of flares and predominant symptoms.

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Appendix A

Table A1. Categorization of food items derived from the food frequency questionnaire (FFQ) into the Dutch Healthy Diet index 2015 (DHD-2015).

Component DHD-2015	Included FFQ food items Dutch item name	English item name
1. Vegetables	Gekookte bloemkool en broccoli	Boiled cauliflower or broccoli
	Gekookte koolsoorten (witte-, rode-, spits-, groene-, savooie-, Chinese-, boeren- en zuurkool)	Boiled cabbage varieties (white-, red-, oxheart-, green-, savoy-, Chinese cabbage, kale, sauer-kraut)
	Gekookte ui en prei	Boiled onion and leak
	Overige gekookte groente	Other boiled vegetables
	Rauwe groente	Raw vegetables
2. Fruit	Appels (vers)	Apples (fresh)
	Banaan (vers)	Bananas (fresh)
	Citrusfruit (vers)	Citrus fruits (fresh)
	Overig vers fruit	Other fruits (fresh)
3a. Wholegrain products	All Bran	All bran cereal
	Bruin brood	Brown bread
	Meergranen brood	Multigrain bread
	Papgranen (Brinta, havermout, enz.)	Porridge grains (Brinta, oatmeal, etc.)
	Roggebrood	Rye bread
	Volkoren brood	Wholegrain bread
3b. Refined grain products	Beschuit, knäckebröd en crackers	Plain rusk, Swedish crispbread and crackers
	Cornflakes	Cornflakes
	Croissants	Croissants
	Muesli, cruesli	Muesli and granola
	Overige ontbijtproducten	Other breakfast cereals, breads, etc.
	Pasta	Pasta
	Rijst	Rice
	Rozijnen-, krenten- of mueslibrood	Raisin bread, plum loaf, muesli bread
	Wit brood	Plain white bread
4. Legumes	Peulvruchten	Legumes
5. Nuts	Noten, notenmix, studenten-haver	Nuts, nut mixes, trail mix
6a. Dairy	Crème fraîche en andere bereidingsroom	Crème fraiche and other cooking creams
	Halfvolle melk	Reduced-fat milk
	Halfvolle (vruchten)yoghurt	Reduced-fat (fruit) yoghurt
	Karnemelk	Buttermilk
	Koffiemelk en -creamer	Coffee milk and coffee creamer
	Kwark en vruchtenkwark	Quark or curd
	Magere (vruchten)yoghurt	Low-fat (fruit) yoghurt
	Magere melk	Low-fat milk
	Pappen	Porridge
	Roomijs en ijs(jes) op melkbasis	Milk-based ice-cream
	Slagroom en topping	Whipped cream and toppings
	Vla en pudding	Custard and pudding
	Volle melk	Whole milk
	Volle (vruchten)yoghurt	Whole yoghurt
6b. Cheese	Kaas	Cheese
	Roomkaas en buitenlandse kaas	Cream cheese or foreign cheese
	Smeerkaas en zuivelspread	Cheese spread or dairy spread

Table A1 (Continued).

Component DHD-2015	Included FFQ food items Dutch item name	English item name
7a. Fish - Oily	Forel, tonijn (vers, diepvries, in blik)	Trout or tuna (fresh, frozen, or canned)
	Gerookte en gestoomde vis (bv. zalm, makreel, bokking)	Smoked or steamed fish (salmon, mackerel, herring, etc.)
	Haring en sardines	Herring and sardines
	Zalm, makreel, paling, pan-haring, enz. (vers, diepvries, in blik)	Fatty fish (salmon, mackerel, eel, etc.)
7b. Fish - Lean	Kabeljauw, schol, schelvis, kool-vis, tong, enz.	Low-fat white fish (cod, plaice, haddock, pollock, sole, etc.)
	Lekkerbekje of kibbeling	Fried fillet of haddock
	Schaal- en schelpdieren	Crustaceans and shellfish
	Vissticks	Fish fingers
8. Tea	Thee	Tea
9a. Solid cooking fats	Bak en braadproduct (vast)	Solid baking and roasting product
	Frituurvet (vast)	Solid frying product
	Halfvolle roomboter	Reduced-fat butter
	Margarine in pakje	Margarine (foil)
	Roomboter	Butter
	Spekvet of rundervet	Bacon fat or beef fat
9b. Liquid cooking fats	Dieethalvarine	Diet low-fat margarine
	Dieetmargarine	Diet margarine
	Halvarine	Low-fat margarine
	Halvarine met plantensterolen/ stanolen	Low-fat margarine with plant sterols/stanols
	Laagvet halvarine product	Low-fat margarine product
	Margarine in kuipje	Margarine (tub)
	Margarine met plantensterolen/ stanolen	Margarine with plant sterols/stanols
	Olijfolie	Olive oil
	Vloeibaar bak en braadproduct	Liquid baking and roasting product
	Vloeibaar frituurproduct	Liquid frying product
	Vloeibare margarine	Liquid margarine
	Zonnebloemolie, sojaolie, slaolie, enz. (geen olijfolie)	Sunflower oil, salad oil, etc.
10. Coffee	No data available on filtered vs unfiltered	
11. Red meat	Gehakt	Minced meat
	Lamsvlees of schapenvlees	Lamb, hogget, mutton
	Orgaanvlees	Organ meats, giblets
	Overig varkensvlees	Other types of pork meat
	Overige soorten vlees en wild	Other types of game meat
	Runderbiefstuk, rundertartaar, runder-baklap, runderbraadlap, runderrosbief	Beef steak, roast, casserole, tartare, etc.
	Runderentrecote, runder-braadworst, rundersukadelap, runderriblap, doorregen runder-lap	Beef entrecote, bratwurst, sirloin steak, etc.
	Varkenshaas, varkensschnitzel, varkensfricandeau, varkens-hamlap	Pork tenderloin, cutlet, fillet, ham steak, etc.
	Varkenskarbonade (schouder-, rib- en haas karbonade)	Pork chops (shoulder, rib or fillet chops)

Table A1 (Continued).

Component DHD-2015	Included FFQ food items Dutch item name	English item name
12. Processed meat	(Smeer)leverworst, paté, leverpastei, leverkaas, berliner	Liverwurst spread, paté, liver pate, liver cheese, Berliner liver sausage
	Boterhamworst, gekookte worst, palingworst, gebraden gehakt	Cold cut sausages (pork)
	Cervelaatworst, snijworst, metworst, salami	Cold cut sausages (beef)
	Gekookte lever	Cooked liver
	Ham	Ham
	Hamburger	Hamburger
	Overige soorten vleeswaar	Other types of cold cuts
	Rookvlees, fricandeau, rosbief, casselerrib, kipfilet, kiprollade	Cold cuts varieties (including poultry)
	Rookworst of knakworst	Smoked sausage, frankfurters
	Speklappen en spekjes	Bacon, pork belly
	Varkensbraadworst en slavink	Pork sausages
13. Sweetened beverages and fruit juices	(Light) vruchtendrank (met zoetstof), dubbeldrank, multi-vruchtendrank	Light fruit drinks
	Chocolademelk	Chocolate milk
	Drinkontbijt	Breakfast drink
	Drinkyoghurt en andere zuivel-dranken	Sweetened dairy drinks
	Frisdrank, vruchtenlimonade, sportdrank en energiedrank	Soda, lemonade, sport drinks, energy drinks
	Milkshake	Milkshake
	Vruchtensap uit pak of fles of versgeperst	Fruit juice (fresh or bottle)
14. Alcohol	Alcohol (nutrient), met behulp van producten: - Bier - Breezer - Sherry, port, vermout, enz. - Sterke drank - Wijn	Alcohol (nutrient), assessed using products: - Beer - Breezer - Fortified wines: Sherry, Port, Vermouth, etc. - Spirits - Wine
15. Salt	No data available	

DHD-2015 = Dutch Healthy Diet index 2015; FFQ = food frequency questionnaire.

More details on the components used to calculate the DHD-2015 can be found in Looman *et al.*[12]

Table A2. Absolute intake and Dutch Healthy Diet index (DHD-2015) score per component.

	IBD patients (n = 238)		IBS patients (n = 261)		HC (n = 195)		
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	p-value
Absolute intake							
1. Vegetables (g/day)	108.52 ± 68.50	0.00 - 344.95	118.64 ± 87.91	0.00 - 705.74	133.72 ± 103.42	7.57 - 888.62	0.011
2. Fruit (g/day)	134.42 ± 113.59	0.00 - 579.35	148.13 ± 108.91	0.00 - 495.42	185.20 ± 143.85	0.00 - 882.64	<0.001
3a. Wholegrain products (g/day)	113.36 ± 73.58	0.00 - 431.56	95.83 ± 69.42	0.00 - 420.00	117.89 ± 71.34	0.00 - 334.00	0.002
3b. Refined grain products (g/day)	90.89 ± 56.87	0.00 - 361.15	85.52 ± 60.89	1.60 - 341.67	103.98 ± 77.77	0.00 - 534.50	0.010
3c. Wholegrain / Refined grain ratio	3.31 ± 10.20	0.00 - 115.28	2.44 ± 9.15	0.00 - 138.91	2.07 ± 3.38	0.00 - 33.39	0.278
4. Legumes (g/day)	17.30 ± 36.96	0.00 - 305.08	14.71 ± 24.13	0.00 - 209.90	20.22 ± 28.65	0.00 - 173.29	0.158
5. Nuts (g/day)	5.27 ± 12.81	0.00 - 154.27	5.95 ± 11.99	0.00 - 92.69	7.17 ± 12.96	0.00 - 121.58	0.291
6a. Milk and yoghurt (g/day)	189.95 ± 173.10	0.00 - 1344.04	191.65 ± 169.38	0.00 - 1243.13	232.28 ± 195.19	0.00 - 1432.13	0.023
6b. Cheese (g/day)	25.88 ± 23.01	0.00 - 129.14	24.15 ± 23.08	0.00 - 120.42	31.02 ± 30.16	0.00 - 164.72	0.014
6c. Dairy (g/day)	211.49 ± 174.18	0.00 - 1344.64	211.48 ± 171.75	0.00 - 1283.13	255.60 ± 194.37	0.00 - 1438.58	0.015
7a. Fish - Oily (g/day)	8.79 ± 11.54	0.00 - 69.32	10.65 ± 13.82	0.00 - 94.44	11.11 ± 12.02	0.00 - 73.51	0.114
7b. Fish - Lean (g/day)	12.59 ± 15.15	0.00 - 113.86	10.65 ± 12.88	0.00 - 114.34	10.34 ± 10.39	0.00 - 51.78	0.136
7c. Fish total (g/day)	11.80 ± 12.05	0.00 - 73.32	13.59 ± 14.29	0.00 - 98.44	14.07 ± 12.50	0.00 - 77.51	0.151
8. Tea (g/day)	248.06 ± 318.31	0.00 - 1625.00	270.67 ± 319.29	0.00 - 1950.00	260.62 ± 319.02	0.00 - 1950.00	0.731
9a. Solid cooking fats (g/day)	3.94 ± 5.99	0.00 - 37.77	3.98 ± 7.18	0.00 - 61.93	3.83 ± 6.96	0.00 - 48.71	0.971
9b. Liquid cooking fats (g/day)	26.14 ± 15.10	0.00 - 89.31	21.92 ± 13.05	0.00 - 75.26	25.91 ± 15.03	0.00 - 67.44	0.001
9c. Solid/liquid cooking fats ratio	48.60 ± 133.27	0.00 - 1144.50	35.69 ± 98.58	0.00 - 840.50	62.05 ± 295.26	0.00 - 3123.50	0.446
10a. Coffee, unfiltered	Unknown		Unknown		Unknown		
10b. Coffee, filtered	Unknown		Unknown		Unknown		
11. Red meat (g/day)	55.72 ± 36.14	0.00 - 183.73	47.93 ± 30.26	0.00 - 139.86	46.11 ± 38.50	0.00 - 220.16	0.008
12. Processed meat (g/day)	44.34 ± 34.46	0.00 - 208.84	37.95 ± 35.06	0.00 - 265.38	29.12 ± 26.58	0.00 - 141.43	<0.001
13. Sweetened beverages and fruit juices (g/day)	188.10 ± 235.71	0.00 - 1528.62	195.43 ± 235.17	0.00 - 1535.54	166.02 ± 213.45	0.00 - 1569.09	0.384
14. Alcohol (g/day)	8.73 ± 11.68	0.00 - 72.76	8.30 ± 14.94	0.00 - 147.87	11.59 ± 12.86	0.00 - 95.08	0.022
15. Salt (g/day)	Unknown		Unknown		Unknown		

Table A2 (Continued).

	IBD patients (n = 238)		IBS patients (n = 261)		HC (n = 195)		
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	p-value
DHD-2015 score per component							
1. Vegetables	5.20 ± 2.94	0.00 - 10.00	5.38 ± 3.03	0.00 - 10.00	5.90 ± 2.96	0.38 - 10.00	0.043
2. Fruit	5.54 ± 3.74	0.00 - 10.00	6.19 ± 3.64	0.00 - 10.00	6.88 ± 3.47	0.00 - 10.00	0.001
3a. Wholegrain products	3.96 ± 1.53	0.00 - 5.00	3.62 ± 1.75	0.00 - 5.00	4.10 ± 1.49	0.00 - 5.00	0.004
3b. Refined grain products	0.74 ± 1.24	0.00 - 5.00	0.58 ± 0.93	0.00 - 5.00	0.64 ± 1.02	0.00 - 5.00	0.236
3c. Wholegrain / Refined grain ratio	4.71 ± 2.28	0.00 - 10.00	4.21 ± 2.28	0.00 - 10.00	4.74 ± 2.07	0.00 - 10.00	0.013
4. Legumes	5.40 ± 4.57	0.00 - 10.00	5.39 ± 4.61	0.00 - 10.00	6.33 ± 4.52	0.00 - 10.00	0.056
5. Nuts	2.42 ± 3.46	0.00 - 10.00	2.63 ± 3.43	0.00 - 10.00	3.39 ± 3.59	0.00 - 10.00	0.011
6. Dairy	5.48 ± 3.11	0.00 - 10.00	5.52 ± 3.11	0.00 - 10.00	6.10 ± 3.06	0.00 - 10.00	0.076
7. Fish	5.80 ± 3.64	0.00 - 10.00	6.25 ± 3.51	0.00 - 10.00	6.72 ± 3.56	0.00 - 10.00	0.029
8. Tea	4.04 ± 3.91	0.00 - 10.00	4.46 ± 3.92	0.00 - 10.00	4.29 ± 3.82	0.00 - 10.00	0.490
9. Fats and oils	6.72 ± 3.89	0.00 - 10.00	6.52 ± 3.97	0.00 - 10.00	7.24 ± 3.87	0.00 - 10.00	0.141
10. Coffee	Unknown		Unknown		Unknown		
11. Red meat	7.09 ± 3.58	0.00 - 10.00	7.61 ± 3.14	0.00 - 10.00	7.92 ± 3.34	0.00 - 10.00	0.032
12. Processed meat	3.36 ± 3.43	0.00 - 10.00	4.11 ± 3.58	0.00 - 10.00	5.04 ± 3.51	0.00 - 10.00	<0.001
13. Sweetened beverages and fruit juices	5.01 ± 3.89	0.00 - 10.00	4.87 ± 3.84	0.00 - 10.00	5.33 ± 3.81	0.00 - 10.00	0.442
14. Alcohol	8.23 ± 3.31	0.00 - 10.00	8.47 ± 3.25	0.00 - 10.00	7.47 ± 3.75	0.00 - 10.00	0.006
15. Salt	Unknown		Unknown		Unknown		
DHD-2015	68.998 ± 16.53	24.64 - 115.58	71.608 ± 16.58	21.57 - 111.34	77.347 ± 17.43	32.47 - 119.10	<0.001

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; DHD-2015 = Dutch Healthy Diet index 2015.
Continuous data are expressed as mean ± standard deviation (SD). Differences between IBD, IBS and HC were tested exploratively with ANOVA and post-hoc Bonferroni correction, without correction for multiple testing.
More details on the calculation of the DHD-2015 components can be found in Looman *et al.*[12]

Table A3. Absolute intake per component of the Adapted Dietary Inflammatory Index (ADII).

	IBD patients (n = 238)		IBS patients (n = 261)		HC (n = 195)		
	Mean ± SD	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**	**p-value**
Energy (kcal/day)	2179.95 ± 634.33	878.90 - 3962.92	1939.59 ± 603.87	642.72 - 3834.86	2180.41 ± 622.89	821.61 - 3868.48	<0.001
Protein (g/d)	78.69 ± 23.58	29.87 - 169.96	72.33 ± 21.24	19.36 - 148.76	79.18 ± 23.87	29.17 - 153.79	0.001
Saturated fatty acids (g/d)	31.40 ± 11.52	7.15 - 66.65	27.59 ± 10.62	5.64 - 70.69	30.00 ± 10.52	9.06 - 61.22	<0.001
Mono-unsaturated fatty acids (g/d)	31.78 ± 12.19	8.89 - 75.79	28.05 ± 10.86	5.80 - 68.61	30.41 ± 10.80	9.23 - 80.93	0.001
Trans fatty acids (g/d)	1.33 ± 0.53	0.29 - 3.01	1.21 ± 0.53	0.17 - 3.64	1.33 ± 0.51	0.35 - 2.64	0.014
n-3 poly-unsaturated fatty acids (g/d)	2.29 ± 0.85	0.58 - 5.38	2.09 ± 0.80	0.38 - 5.34	2.34 ± 0.92	0.83 - 5.07	0.003
n-6 poly-unsaturated fatty acids (g/d)	15.87 ± 6.58	3.78 - 49.71	13.68 ± 6.30	2.62 - 51.47	15.44 ± 6.81	4.91 - 52.63	<0.001
Cholesterol (mg/d)	207.22 ± 86.19	46.51 - 631.17	195.11 ± 86.47	8.34 - 685.68	198.52 ± 82.95	34.40 - 628.82	0.272
Carbohydrate (g/d)	237.02 ± 73.11	77.75 - 477.29	208.81 ± 71.70	45.49 - 464.17	237.22 ± 72.12	84.69 - 471.34	<0.001
Fibre (g/d)	22.33 ± 8.06	4.81 - 56.04	20.52 ± 7.13	2.17 - 45.53	24.67 ± 8.47	9.10 - 55.26	<0.001
Ethanol (g/d)	8.73 ± 11.68	0.00 - 72.76	8.30 ± 14.94	0.00 - 147.87	11.59 ± 12.86	0.00 - 95.08	0.022
Vitamin A (µg/d)	729.09 ± 616.38	138.87 - 6409.44	613.61 ± 624.87	54.08 - 6897.98	620.11 ± 510.58	88.33 - 5034.54	0.059
b-Carotene (µg/d)	1232.07 ± 615.54	137.83 - 3812.99	1300.97 ± 762.26	126.20 - 6014.49	1421.19 ± 811.94	176.36 - 5889.25	0.027
Thiamine (vitamin B1) (mg/d)	1.06 ± 0.35	0.37 - 2.36	0.99 ± 0.35	0.28 - 3.08	1.05 ± 0.36	0.30 - 2.24	0.053
Riboflavin (vitamin B2) (mg/d)	1.31 ± 0.43	0.50 - 3.15	1.24 ± 0.48	0.29 - 3.27	1.36 ± 0.51	0.31 - 3.46	0.018
Niacin (vitamin B3) (mg/d)	19.18 ± 6.34	5.70 - 39.98	17.58 ± 5.95	3.83 - 42.51	18.81 ± 6.10	3.93 - 37.39	0.010
Vitamin B6 (mg/d)	1.75 ± 0.59	0.44 - 3.66	1.67 ± 0.55	0.49 - 4.34	1.78 ± 0.61	0.63 - 3.51	0.096
Folate (µg/d)	209.16 ± 64.94	49.56 - 443.19	199.87 ± 65.40	59.57 - 409.63	230.98 ± 77.57	73.80 - 499.30	<0.001
Vitamin B12 (µg/d)	4.64 ± 2.30	1.30 - 15.72	4.26 ± 1.95	0.10 - 14.45	4.49 ± 2.08	0.79 - 13.55	0.133
Vitamin C (mg/d)	85.10 ± 41.11	13.44 - 282.00	88.09 ± 44.93	11.08 - 303.30	92.54 ± 45.99	11.23 - 401.16	0.215
Vitamin D (µg/d)	4.13 ± 1.75	1.32 - 11.83	3.73 ± 1.60	0.53 - 9.43	3.85 ± 1.66	0.49 - 10.06	0.025
Vitamin E (mg/d)	14.82 ± 5.72	5.15 - 41.35	13.38 ± 5.07	3.36 - 33.46	14.85 ± 5.50	6.16 - 40.28	0.003
Iron (mg/d)	11.03 ± 3.26	4.11 - 26.03	10.05 ± 3.13	3.38 - 19.88	11.14 ± 3.34	3.78 - 21.15	<0.001
Magnesium (mg/d)	331.02 ± 100.86	99.65 - 713.50	308.30 ± 98.16	118.17 - 710.15	357.36 ± 118.98	117.56 - 829.09	<0.001
Selenium (mg/d)	0.05 ± 0.02	0.02 - 0.11	0.04 ± 0.02	0.01 - 0.10	0.05 ± 0.02	0.01 - 0.10	0.107
Zinc (mg/d)	10.13 ± 3.23	3.85 - 23.60	9.35 ± 2.85	2.51 - 20.63	10.40 ± 3.20	3.59 - 20.03	0.001
Tea (g/d)	248.06 ± 318.31	0.00 - 1625.00	270.67 ± 319.29	0.00 - 1950.00	260.62 ± 319.02	0.00 - 1950.00	0.731

Table A3 (Continued).

	IBD patients (n = 238)		IBS patients (n = 261)		HC (n = 195)		
	Mean ± SD	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**	
ADII	0.052 ± 2.41	-9.02 - 7.64	0.055 ± 2.47	-9.03 - 6.20	0.054 ± 2.33	-9.74 - 4.93	>0.999

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; ADII = Adapted Dietary Inflammatory Index.

Continuous data are expressed as mean ± standard deviation (SD). Differences between IBD, IBS, and HC were tested with ANOVA and post-hoc Bonferroni correction.

More details on the ADII can be found in Van Woudenbergh *et al.*[13]

Appendix B

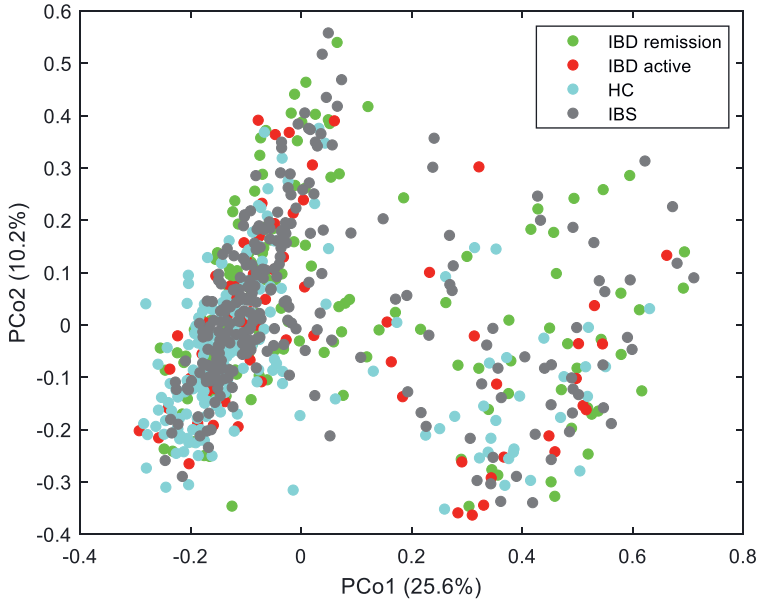
Methods

In addition to using predefined indices (*i.e.* Dutch Healthy Diet index 2015 and Adapted Dietary Inflammatory Index), an explorative unsupervised random forest (URF) analysis[36] was performed to investigate the combined effects of sets of food items and nutrients as potential differentiating factors between inflammatory bowel disease (IBD) patients, irritable bowel syndrome (IBS) patients and healthy controls (HC). This unsupervised machine learning technique allows investigation of the natural grouping that occurs in the data based on the input variables. Consequently, the outcome can be visualized using a principal coordinate analysis (PCoA) score plot. In this plot, each point represents a single participant. Individuals are colour-coded according to the investigated groups, *i.e.* patients in remission, IBD patients with active disease, IBS patients and HC. In this study URF was performed on two sets of variables (food items and nutrients) derived from the food frequency questionnaires.

Results

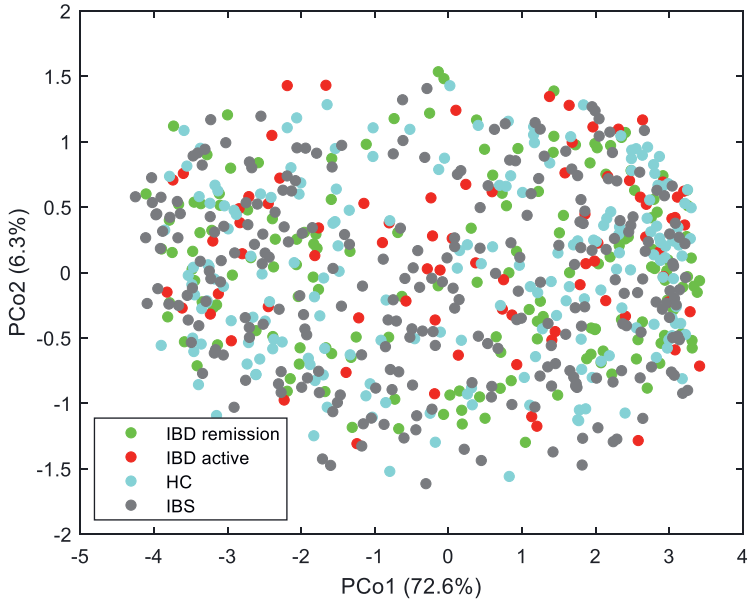
In the PCoA score plot based on food products (Figure B1), no clear separation was observed for the investigated groups. Similarly, the PCoA score plot based on the nutrients (Figure B2) did not show any groupings when the first PCo's were considered. However, when PCo4 and PCo7 were considered (Figure B3), IBS patients were clearly separated from HC and IBD along PCo4. It is relevant to mention that PCo4 and PCo7 describe only a small portion of the total variance (less than 4%). This suggests that although the differences between the groups of interest are there and they correspond to the differences with nutrients level, the small amount of variance describing those differences indicate minor alterations between IBS and the rest based on the nutrient variables. The most important set of variables that cause those differences were in majority reduced in IBS individuals.

Figure B1. Principle coordinate analysis (PCoA) score plot based on food products.



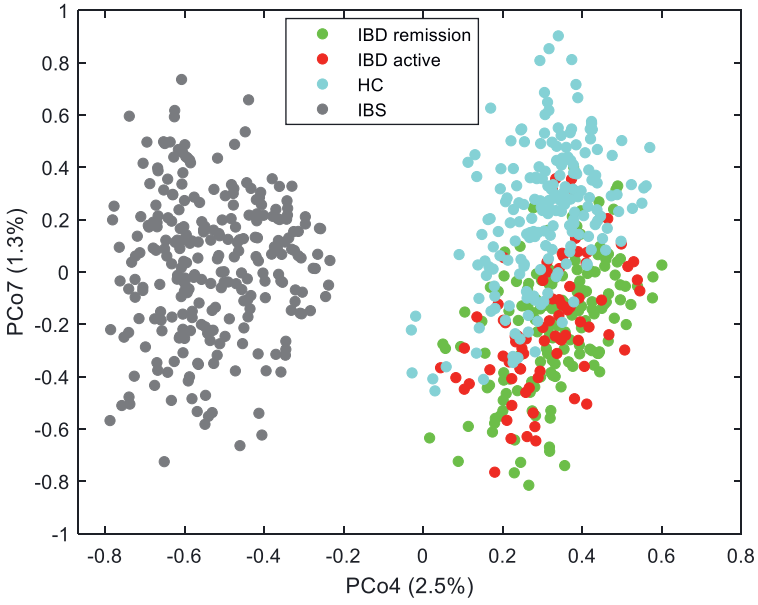
IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; PCo = principle coordinate.

Figure B2. Principle coordinate analysis (PCoA) score plot based on nutrients – PCo1 & PCo2.



IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; PCo = principle coordinate.

Figure B3. Principle coordinate analysis (PCoA) score plot based on nutrients – PCo4 and PCo7.



IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; PCo = principle coordinate.

The most important components that cause the distinction between IBS and the rest are visible along PCo4. It is characterised by among others a combination of a lower intake of zinc, caloric intake, selenium, vitamin B12, magnesium and iron in IBS compared to IBD and HC.

Supplementary Materials

Supplementary Table S1. Dietary indices, intestinal inflammation and gastrointestinal symptoms per disease activity status for inflammatory bowel disease.

	Remission (n = 158)		Active disease (n = 80)		p-value
DHD-2015	71.15 ± 16.72	(n = 158)	64.74 ± 15.38	(n = 80)	0.004
ADII	-0.055 ± 2.107	(n = 158)	0.263 ± 2.932	(n = 80)	0.390
Calprotectin (μg/g)	42.6 ± 50.7	(n = 137)	491.5 ± 627.4	(n = 72)	<0.001
GSRS					
Abdominal pain	2.0 ± 0.9	(n = 55)	2.3 ± 1.1	(n = 25)	0.097
Constipation syndrome	1.7 ± 1.0	(n = 52)	2.2 ± 1.1	(n = 25)	0.102
Diarrhoea syndrome	2.4 ± 1.4	(n = 53)	3.3 ± 1.5	(n = 24)	0.013
Indigestion syndrome	2.6 ± 1.0	(n = 55)	3.1 ± 1.5	(n = 25)	0.193
Reflux syndrome	1.4 ± 0.7	(n = 55)	1.5 ± 0.8	(n = 25)	0.525

DHD-2015 = Dutch Healthy Diet index 2015; ADII = Adapted Dietary Inflammatory Index; GSRS = Gastrointestinal Symptom Rating Scale.
Continuous data are expressed as mean ± standard deviation (SD). Differences between phenotypes were tested with two-sample t-test.

Supplementary Table S2. Dietary indices, intestinal inflammation and gastrointestinal symptoms per inflammatory bowel disease phenotype.

	Crohn's disease (n = 156)		Ulcerative colitis (n = 82)		p-value
DHD-2015	65.47 ± 15.94	(n = 156)	75.71 ± 15.61	(n = 82)	<0.001
ADII	0.193 ± 2.53	(n = 156)	-0.217 ± 2.18	(n = 82)	0.214
Calprotectin (μg/g)	199.3 ± 411.5	(n = 136)	193.4 ± 455.6	(n = 73)	0.924
GSRS					
Abdominal pain	2.1 ± 0.9	(n = 51)	2.0 ± 1.0	(n = 29)	0.507
Constipation syndrome	2.0 ± 1.2	(n = 48)	1.6 ± 0.8	(n = 29)	0.053
Diarrhoea syndrome	2.9 ± 1.6	(n = 48)	2.3 ± 1.2	(n = 29)	0.047
Indigestion syndrome	2.8 ± 1.2	(n = 51)	2.6 ± 1.2	(n = 29)	0.487
Reflux syndrome	1.5 ± 0.9	(n = 51)	1.3 ± 0.6	(n = 29)	0.272

DHD-2015 = Dutch Healthy Diet index 2015; ADII = Adapted Dietary Inflammatory Index; GSRS = Gastrointestinal Symptom Rating Scale.
Continuous data are expressed as mean ± standard deviation (SD). Differences between phenotypes were tested with two-sample t-test.

Supplementary Table S3. Intestinal inflammation and gastrointestinal symptoms per irritable bowel syndrome subtype.

	IBS-C (n = 56)		IBS-D (n = 93)		IBS-M (n = 103)		p-value
DHD-2015	73.39 ± 15.41	(n = 56)	72.41 ± 17.30	(n = 93)	70.53 ± 16.76	(n = 103)	0.541
ADII	-0.133 ± 2.483	(n = 56)	0.142 ± 2.770	(n = 93)	0.113 ± 2.221	(n = 103)	0.786
Calprotectin (μg/g)	59.0 ± 56.5	(n = 17)	46.3 ± 61.8	(n = 33)	85.9 ± 114.1	(n = 37)	0.163
GSRS							
Abdominal pain	3.4 ± 1.1	(n = 56)	3.3 ± 1.3	(n = 92)	3.4 ± 1.3	(n = 101)	0.801
Constipation syndrome	4.4 ± 1.3	(n = 56)	2.6 ± 1.0	(n = 91)	3.5 ± 1.2	(n = 101)	<0.001
Diarrhoea syndrome	2.2 ± 1.1	(n = 56)	3.9 ± 1.5	(n = 91)	3.5 ± 1.4	(n = 102)	<0.001
Indigestion syndrome	4.2 ± 1.2	(n = 55)	4.1 ± 1.4	(n = 92)	4.2 ± 1.3	(n = 101)	0.994
Reflux syndrome	2.4 ± 1.5	(n = 56)	2.1 ± 1.3	(n = 92)	2.2 ± 1.4	(n = 101)	0.321

IBS = irritable bowel syndrome; IBS-C = constipation predominant IBS; IBS-D = diarrhoea predominant IBS; IBS-M = mixed stool pattern IBS; DHD-2015 = Dutch Healthy Diet index 2015; ADII = Adapted Dietary Inflammatory Index; GSRS = Gastrointestinal Symptom Rating Scale.
Difference between subtypes was tested with ANOVA and post-hoc Bonferroni correction. Unspecified subtype IBS (IBS-U) was not included in this comparison due to the small sample size (n = 9).

CHAPTER 3

The intake of dicarbonyls and advanced glycation endproducts as part of the habitual diet is not associated with intestinal inflammation in inflammatory bowel disease and irritable bowel syndrome patients

Marlijne C. G. de Graaf, Jean L. J. M. Scheijen, Corinne E. G. M. Spooren, Zlatan Mujagic, Marieke J. Pierik, Edith J. M. Feskens, Daniel Keszthelyi, Casper G. Schalkwijk, Daisy M. A. E. Jonkers

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Abstract

A Western diet comprises high levels of dicarbonyls and advanced glycation endproducts (AGEs), which may contribute to flares and symptoms in inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). We therefore investigated the intake of dietary dicarbonyls and AGEs in IBD and IBS patients as part of the habitual diet, and their association with intestinal inflammation. Food frequency questionnaires from 238 IBD, 261 IBS as well as 195 healthy control (HC) subjects were used to calculate the intake of dicarbonyls methylglyoxal, glyoxal, and 3-deoxyglucosone, and of the AGEs Nε-(carboxymethyl)lysine, Nε-(1-carboxyethyl)lysine and methylglyoxal-derived hydroimidazolone-1. Intestinal inflammation was assessed using faecal calprotectin. The absolute dietary intake of all dicarbonyls and AGEs was higher in IBD and HC as compared to IBS (all $p<0.05$). However, after energy-adjustment, only glyoxal was lower in IBD versus IBS and HC ($p<0.05$). Faecal calprotectin was not significantly associated with dietary dicarbonyls and AGEs in either of the subgroups. The absolute intake of methylglyoxal was significantly higher in patients with low (<15 μg/g) compared to moderate calprotectin levels (15-<50 μg/g, $p=0.031$). The concentrations of dietary dicarbonyls and AGEs generally present in the diet of Dutch patients with IBD or IBS are not associated with intestinal inflammation, although potential harmful effects might be counteracted by anti-inflammatory components in the food matrix.

Introduction

The Maillard reaction is a biochemical reaction between proteins and reduced sugars that occurs during food processing, especially under conditions of heating. During this complex network of many thousands of individual non-enzymatic reactions, many different classes of Maillard reaction products (MRPs) are formed. Especially baking, grilling, and roasting of food products increases the MRP content of these foods. On one hand, this contributes to browning and organoleptic properties such as aroma, taste, and texture, while on the other hand, MRPs are often reported as potentially harmful and, among others, are associated with impaired metabolic and gut health.[1]

One of the endproducts of the Maillard reaction, namely the advanced glycation endproducts (AGEs), received considerable attention lately due to their potential negative effects on human health. The most well studied AGEs include Nε-(carboxymethyl)lysine (CML), Nε-(1-carboxyethyl)lysine (CEL) and methylglyoxal-derived hydroimidazolone-1 (MG-H1). *In vitro* and *in vivo* studies show that ingested AGEs can induce an inflammatory response[2-5] and affect microbial growth.[5-10] Furthermore, previous human studies showed that a diet high in dietary AGEs is associated with low-grade inflammation, endothelial dysfunction, and insulin resistance.[11] In addition to AGEs, also their precursors may affect health. The dicarbonyls methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG) are major precursors in the formation of AGEs. They are highly reactive intermediate metabolites and potent glycating agents, and have been associated with age-related diseases such as type 2 diabetes, cardiovascular diseases, and cancer.[12,13] Both pro-inflammatory[14] and anti-inflammatory effects[15,16] of the intake of dietary dicarbonyls have been reported.

Several *in vitro* and *in vivo* studies have shown that dietary dicarbonyls and AGEs are not completely digested and absorbed, with an absorption of 0.1-15% of consumed dicarbonyls[17,18] and 10-30% of consumed AGEs,[19] depending on their chemical structure. The remaining dietary dicarbonyls and AGEs may therefore directly impact the mucosal layer of the small and large intestine,[20-22] and/or may be metabolised by intestinal microbes.[23] Some animal and *in vitro* studies suggest that AGEs can infiltrate enterocytes and accumulate there.[20,24]

It is now well known that a Western diet, being rich in processed food and thus in MRPs, is associated with common gastrointestinal diseases such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). IBD and IBS are both multifactorial and very heterogeneous entities in which diet likely plays a pathophysiological role.[25,26] IBD is a chronic inflammatory disease characterised by alternating sequences of active inflammation and remission.[27,28] IBS is characterised by abdominal pain and altered bowel habits, but in a subgroup of IBS a low-grade inflammation is reported.[29] Previous studies found an elevated expression of the receptor for AGEs (RAGE) in inflamed intestinal tissue from IBD patients,[30-32] which may contribute to the production of pro-inflammatory cytokines and reactive oxygen species.[33] However, overall evidence on the role of dietary dicarbonyls and AGEs in IBD and IBS is limited.

Therefore, the aim of this study is to investigate the intake of dietary dicarbonyls and AGEs as part of the habitual diet in both IBD and IBS patients, and their association with intestinal inflammation. We hypothesise that dietary intake of dicarbonyls and AGEs is associated with intestinal inflammation in IBD and IBS.

Methods

Study population

For this study, we used cross-sectional data on habitual dietary intake and clinical data from the IBD South Limburg (IBDSL) cohort and the Maastricht IBS (MIBS) cohort as described previously.[34-36] Prior to participation, all participants provided written informed consent.

IBD South Limburg Cohort

Since 1991, the IBDSL cohort has been used to study IBD epidemiology and disease in the South Limburg area in the Netherlands.[35] This well-characterised population-based inception cohort comprised all newly diagnosed patients with ulcerative colitis (UC) and Crohn's disease (CD) of at least 18 years old living in South Limburg at time of diagnosis. Diagnosis was done according to the Lennard-Jones criteria[37] and proven by endoscopic, radiological and/or histological findings. The IBDSL data warehouse was used to retrieve relevant demographical and clinical data.[35] The IBDSL cohort was approved by the medical research ethics committee of the Maastricht University Medical Center+ (MUMC+) (NL31636.068.10) and registered at the US National Library of Medicine (NCT02130349). The collection of data on habitual dietary intake was done as part of a sub-study within the IBDSL cohort, also approved by the medical research ethics committee of the MUMC+ (NL42101.068.12) and registered at the US National Library of Medicine (NCT0176963).

Maastricht IBS Cohort

Since 2009, the MIBS cohort has been used to study the phenotypical and genotypical characterisation of patients with IBS in the South Limburg area of the Netherlands. This cohort included IBS patients recruited via primary, secondary, and tertiary care and from the general population that fulfilled the Rome III criteria and were at least 18 years old.[38] Additionally, healthy controls (HC) in the same age category were included as described previously.[36] The medical research ethics committee of the MUMC+ approved the MIBS cohort (NL24160.068.08) and the study was registered at the US National Library of Medicine (NCT00775060).

Demographic and Clinical Data Collection

Standardised registration forms were used in both cohorts to collect demographic and clinical data, including sex, age, smoking status, body mass index (BMI), medication use, and disease characteristics.

IBD disease phenotype at time of inclusion was defined by the Montreal classification, including age of onset, disease location, extent (for UC), and behaviour (for CD).[39]

Disease duration was also registered. The Simple Clinical Colitis Activity Index (SCCAI)[40] and Harvey Bradshaw Index (HBI)[41] were used as clinical activity indices for UC and CD, respectively. In line with clinical practice and previous studies,[42,43] a flare was defined as: (1) presence of active disease based on endoscopy and/or radiological imaging, confirmed by a physician; (2) faecal calprotectin ≥250 μg/g; (3) faecal calprotectin ≥100 μg/g with at least a five-fold increase compared to the previous visit; (4) clinical symptoms indicative for active disease, or an increased SCCAI (≥3) or HBI (≥5) together with a dose escalation or initiation of a new drug; or (5) a dose escalation or initiation of a new drug along with C-Reactive Protein (CRP) ≥10 mg/l. Time since last flare was also recorded, and active disease at inclusion was defined as having a flare at inclusion, or during the three months prior to inclusion.
IBS subtypes were defined according to the Rome III criteria, *i.e.*, diarrhoea (IBS-D) or constipation predominant (IBS-C), having a mixed stool pattern (IBS-M) or unspecified stool pattern (IBS-U).[38]
In both cohorts, intestinal inflammation was assessed by analysing calprotectin levels in faecal samples. Participants collected these faecal samples at home, stored them in a fridge, and brought them to the hospital within 24 h after defecation. For the IBDSL cohort, samples were routinely analysed by the clinical chemistry department using a fluorescent enzyme immune assay (FEIA, Thermo Fisher Scientific,Waltham, MA, USA), whereas for the MIBS cohort samples were analysed using a commercial enzyme-linked immunosorbent assay (ELISA, Bühlmann Laboratories, Schönenbuch, Switzerland).

Dietary Data Collection

In both cohorts, the same self-administered food frequency questionnaire (FFQ) was used to assess the habitual dietary intake over the previous month. Frequency of consumption was scored per food product and portion sizes were estimated using natural portions and commonly used household measures. These data were linked to the Dutch food composition table (NEVO online version 2010/2.0, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands) to calculate the individual mean consumption of 45 nutrients and 148 food items. This FFQ was previously developed and validated by the division of Human Nutrition of Wageningen University.[44,45] Intake of nutritional supplements was recorded separately.
If the FFQ data were incomplete or considered implausible; *i.e.*, defined as an overall intake for females <500 or >3500 kcal/day and for males <800 or >4000 kcal/day,[46] or if the participant was on tube feeding, they were excluded from the analyses.
Foods and drinks were categorised in 25 food groups: (1) bread; (2) breakfast cereals; (3) cookies and bakery products; (4) potatoes, rice, and pasta; (5) bread condiments; (6) vegetables and legumes; (7) fruits; (8) meat; (9) fish; (10) vegetarian and soy products; (11) milk and dairy products including cheese; (12) egg; (13) ready-made meals; (14) nuts and snacks; (15) fats and oils; (16) savoury sauces; (17) sweets and chocolate; (18) tea; (19) coffee; (20) soft drinks; (21) fruit juice; (22) vegetable juice; (23) beer; (24) wine; and (25) liqueur.

Additionally, data were used to calculate the Dutch Healthy Diet index 2015 (DHD-2015)[47] and the Adapted Dietary Inflammatory Index (ADII),[48] as also described previously.[34] The DHD-2015, developed to assess the adherence to the Dutch healthy diet guidelines,[49] was based on 13 components with a maximum score (indicating high adherence) of 130. The ADII was used to evaluate the inflammatory potential of the diet, with a more pro-inflammatory diet indicated by a higher (positive) score, whereas a more anti-inflammatory diet is indicated by a lower (negative) score.

Dietary Dicarbonyls & AGEs

The FFQ data were combined with available databases of dietary dicarbonyls MGO, GO, and 3-DG,[50] and dietary AGEs CML, CEL, and MG-H1.[51] For these databases the dicarbonyl and AGEs content of more than 200 foods and drinks were measured using ultra high-performance liquid chromatography tandem mass spectrometry (UHPLCMS/MS) analysis (Acquity UPLC and Xexo TQ-MS,Waters, Milford, KS, USA) as described previously by Maasen *et al.*[50] and Scheijen *et al.*,[51] respectively.

The average intake of each food product estimated by the FFQ (g/day) was multiplied by the amount of MGO, GO, 3-DG, CML, CEL, and MG-H1 (mg/g) according to these databases, to calculate the daily dicarbonyl and AGE intake. For FFQ items that were not in the database, the average dicarbonyl or AGE concentration of comparable food products from the same food group was used as an estimate. Concentrations based on food items consumed were used to calculate the total intake of dietary dicarbonyls (MGO + GO + 3-DG) and dietary AGEs (CML + CEL + MG-H1). Furthermore, to correct for the impact of the amount of food consumed, the energy-adjusted intake (intake per 1000 kcal per day) was also calculated.[46]

To calculate daily intake of dicarbonyls and AGEs for each food group, the concentration of a food product (mg/g) was multiplied by the individual's daily intake of that food product (g/day), and subsequently all food products in a particular food group were summed. The relative contribution (as percentage of total intake) of each food group was determined.

Statistical Analyses

Statistical analyses were performed with IBM SPSS Statistics version 26.0.[52] Data normality was confirmed by normal probability plots. For continuous parametric variables, baseline characteristics were presented as mean with corresponding standard deviation (SD), and differences between subgroups (*i.e.*, IBD patients, IBS patients and HC) were assessed with analysis of variance (ANOVA) and post-hoc Bonferroni correction. For categorical variables, baseline characteristics were presented as percentages and differences between subgroups were assessed with the Chi-square test with Fisher exact when necessary.

To assess the association of dietary intake of dicarbonyls and AGEs with faecal calprotectin (as marker for intestinal inflammation), linear regression analysis was used, including the following parameters: age, sex, smoking, BMI, medication use, subtype (IBS) or phenotype (IBD), and for IBD patients additionally disease duration (in years) and age at diagnosis (defined by the Montreal classification). Analyses were

performed for each subgroup (IBD, IBS, and HC) separately and missing values were excluded listwise. A two-sided p-value<0.05 was considered to be statistically significant.

In addition, clinically relevant cut-off points for faecal calprotectin[53] were used to define subgroups to further explore possible differences in dicarbonyls and AGEs intake with ANOVA and post-hoc Bonferroni correction. Furthermore, correlations between dicarbonyls/AGEs and dietary indices (ADII and DHD-2015) were assessed using Spearman's Rank-Order Correlation.

Results

Baseline Characteristics

FFQ data were available for 239 IBD patients, 274 IBS patients, and 207 HC, of which 1 IBD patient, 13 IBS patients, and 12 HC were excluded because of implausibly high or low energy intake. This resulted in a final inclusion of 238 IBD patients, 261 IBS patients, and 195 HC in the current study.

The IBD group comprised 82 UC (34.5%) and 156 CD (65.5%) patients. At time of inclusion, 61.5% of these patients (36.5% and 28.0%, respectively) were in remission. Among IBS patients, the main subtype was IBS-M (39.5%), followed by IBS-D (35.6%), IBS-C (21.5%), and IBS-U (3.4%).

Demographic and clinical data are shown in Table 1. The percentage of women was higher in the IBS group (74%) as compared to the IBD (52.9%, p<0.001) and HC group (63.1%, p=0.007). BMI was higher in IBD (25.5±4.2 kg/m^2) as well as IBS patients (25.0±4.6 kg/m^2) compared to HC (23.9±3.8 kg/m^2, with p<0.001 and p=0.021, respectively), and more active smokers were present among both IBD (20.4%, p<0.001) and IBS patients (23.6%, p<0.001) as compared to HCs (6.7%). The mean energy intake was lower in IBS patients (1939.6±604.9 kcal) as compared to IBD patients (2180.0±634.3 kcal, p<0.001) and HC (2180.4±622.9 kcal, p<0.001). Further details on the intake of food items and specific nutrients were reported previously.[34]

Table 1. Baseline characteristics in inflammatory bowel disease (IBD) patients, irritable bowel syndrome (IBS) patients, and healthy controls (HC).

	IBD patients (n = 238)	IBS patients (n = 261)	HC (n = 195)	p-value
Age (years)	45.7 ± 14.8	43.3 ± 17.0	44.4 ± 18.9	0.285
Sex				< 0.001
Male	47.1%	25.3%	36.9%	
Female	52.9%	74.7%	63.1%	
BMI (kg/m^2) *	25.5 ± 4.2	25.0 ± 4.6	23.9 ± 3.8	< 0.001
Smoking **				< 0.001
Active smoker	20.4%	23.6%	6.7%	
Former smoker	41.7%	24.4%	31.8%	
Never smoker	37.9%	52.0%	61.5%	
IBD Phenotype				
Crohn's disease	65.5%	n/a	n/a	n/a
Ulcerative colitis	34.5%	n/a	n/a	n/a
Age of onset **				
A1 - below 17 years old	5.9%	n/a	n/a	n/a
A2 - 17-40 years old	64.0%	n/a	n/a	n/a
A3 - above 40 years old	30.1%	n/a	n/a	n/a
Behaviour of Crohn's disease at inclusion (n=156)				
B1 - non-stricturing, non-penetrating	57.1%	n/a	n/a	n/a
B2 - stricturing	17.9%	n/a	n/a	n/a
B3 - penetrating	25.0%	n/a	n/a	n/a
Location of Crohn's disease at inclusion (n=82)				
L1 - ileal	23.7%	n/a	n/a	n/a
L2 - colonic	16.7%	n/a	n/a	n/a
L3 - ileocolonic	59.6%	n/a	n/a	n/a
L4 - upper-GI modifier	10.3%	n/a	n/a	n/a
Extent of ulcerative colitis (UC) at inclusion **				
E1 - ulcerative proctitis	11.1%	n/a	n/a	n/a
E2 - left sided UC (distal UC)	39.5%	n/a	n/a	n/a
E3 - extensive UC (pancolitis)	49.4%	n/a	n/a	n/a
Disease activity at inclusion				
Active disease	34.9%	n/a	n/a	n/a
Remission	61.5%	n/a	n/a	n/a
Disease duration (years) **	11.5 ± 10.1	n/a	n/a	n/a
Time to last flare (months)	37.7 ± 67.7	n/a	n/a	n/a
Bowel resection at inclusion				
Yes	23.1%	n/a	n/a	n/a
No	76.9%	n/a	n/a	n/a
Symptom score *				
Harvey Bradshaw Index	2.9 ± 3.4	n/a	n/a	n/a
Simple Clinical Colitis Activity Index	1.2 ± 1.8	n/a	n/a	n/a
IBS Subtype				
Constipation predominant IBS	n/a	21.5%	n/a	n/a
Diarrhoea predominant IBS	n/a	35.6%	n/a	n/a
Mixed stool pattern IBS	n/a	39.5%	n/a	n/a
Unspecified subtype IBS	n/a	3.4%	n/a	n/a
Faecal calprotectin (μg/g) ***	197.3 ± 426.3	64.4 ± 87.1	39.3 ± 63.6	<0.001

Table 1 (Continued).

	IBD patients (n = 238)	IBS patients (n = 261)	HC (n = 195)	p-value
Medication ****				
No medication	14.3%	26.8%	52.8%	< 0.001
5-ASA, local immunosuppressants, or local corticosteroids	17.6%	n/a	n/a	n/a
Systemic corticosteroids	0.4%	n/a	n/a	n/a
Immunomodulators	22.7%	n/a	n/a	n/a
Biologicals	45.0%	n/a	n/a	n/a
PPIs	n/a	20.7%	3.1%	< 0.001
NSAIDs	n/a	24.9%	20.0%	0.217
Laxatives	n/a	18.4%	0.0%	n/a
Spasmolytic drugs	n/a	14.2%	0.0%	n/a
Antihypertensive drugs	n/a	15.3%	13.3%	0.550
Statins	n/a	10.0%	7.7%	0.402
Antidepressant drugs	n/a	10.0%	3.6%	0.009
Energy intake (kcal/day)	2180.0±634.4	1939.6±604.9	2180.4±622.9	< 0.001

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; BMI = body mass index; 5-ASA = 5-aminosalicylic acid; PPIs = proton pump inhibitors; NSAIDs = non-steroidal anti-inflammatory drugs; n/a = not applicable or not available.

* Missing data from max. 25 participants per subgroup. ** Missing data from max. 3 participants per subgroup. *** Missing data from 29 IBD patients, 171 IBS patients and 47 HC. **** Missing data from 4 IBS patients.

Medication for IBD patients was classified as the highest category of use. For IBS, only medication frequently used in IBS were presented. Other medication included prokinetics, anti-diarrhoeal drugs, oral contraceptives, antipsychotic drugs, and antibiotics.

Continuous data are expressed as mean ± standard deviation (SD), and differences between IBD, IBS, and HC were tested with analysis of variance (ANOVA) and post-hoc Bonferroni correction. Categorical data are expressed as percentages of total group (IBD, IBS, or HC) and differences between IBD, IBS, and HC were assessed with the Chi-square test with Fisher for categorical data.

Intake of Dietary Dicarbonyls

Food groups with the highest contribution to the amount of MGO, GO, and 3-DG were bread, cookies and bakery products, and vegetables and legumes. Furthermore, coffee was an important contributor for MGO and 3-DG, meat for MGO, fruit and ready-made meals for GO, and sweets and chocolate for 3-DG. For details, see Supplementary Figure S1. The main contributing products were comparable between subgroups.

The absolute intake of the dicarbonyls MGO, GO, and 3-DG was lower in IBS as compared to IBD (all $p<0.05$) and HC (all $p<0.05$), but did not differ between IBD and HC (Table 2 and Supplementary Figure S2). When adjusted for the total energy intake (Supplementary Table S1), dietary GO levels were lower in IBD compared to IBS ($p=0.021$) and HC ($p=0.040$). The energy-adjusted intake of MGO and 3-DG was not significantly different between the groups.

Table 2. Absolute dietary intake of individual dicarbonyls and advanced glycation endproducts.

Absolute intake (mg/day, mean ± SD)	IBD patients (n = 238)	IBS patients (n = 261)	HC (n = 195)	p-value
MGO	4.04 ± 1.59	3.53 ± 1.46	3.94 ± 1.45	< 0.001
GO	3.32 ± 1.04	3.09 ± 0.96	3.49 ± 1.06	< 0.001
3-DG	15.55 ± 6.44	13.76 ± 5.85	15.83 ± 5.75	< 0.001
Dicarbonyls	22.91 ± 8.23	20.38 ± 7.50	23.26 ± 7.54	< 0.001
CML	3.35 ± 1.16	2.91 ± 1.07	3.27 ± 1.16	< 0.001
CEL	2.70 ± 0.93	2.40 ± 0.83	2.64 ± 0.94	< 0.001
MG-H1	22.61 ± 7.97	19.97 ± 7.32	23.06 ± 7.84	< 0.001
AGEs	28.67 ± 9.79	25.28 ± 9.02	28.97 ± 9.69	< 0.001

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; SD = standard deviation; MGO = methylglyoxal; GO = glyoxal; 3-DG = 3-deoxyglucosone; CML = Nε-(carboxymethyl)lysine; CEL = Nε-(1-carboxyethyl)lysine; MG-H1 = methylglyoxal-derived hydroimidazolone-1; AGEs = advanced glycation endproducts. The differences between IBD, IBS, and HC were tested with analysis of variance (ANOVA) and post-hoc Bonferroni correction.

Intake of Dietary AGEs

Food groups with the highest contribution to the amount of CML, CEL, and MG-H1 were bread, cookies and bakery products and meat. Furthermore, dairy was an important contributor for CML, bread condiments for CEL, potatoes, rice and pasta for CML and MG-H1, and nuts and savoury snacks for CEL and MG-H1. For details, see Supplementary Figure S1. The main contributing products were comparable between subgroups.

The absolute intake of dietary AGEs CML, CEL, and MG-H1 was lower in IBS compared to IBD (all $p<0.001$) and HC (all $p<0.05$), but was not significantly different between IBD and HC (Table 2 and Supplementary Figure S2). After adjustment for total energy intake (Supplementary Table S1), there were no longer any significant differences between the groups.

Intestinal Inflammation

Faecal calprotectin levels were available for 209 patients with IBD, 90 patients with IBS and 148 HC. Mean faecal calprotectin levels (Table 1) were significantly higher in IBD patients (197.3±426.3 μg/g) versus IBS (64.6±87.1 μg/g, $p=0.001$) and HC (39.3±63.6 μg/g, $p<0.001$), but did not differ between IBS and HC ($p>0.999$).

Based on the multivariable linear regression analysis (Table 3) faecal calprotectin was associated with GO in HC (β=-11.21, $p=0.045$), but was not significantly associated with any of the other individual dietary dicarbonyls or AGEs, nor with the total amount of dicarbonyls or AGEs. The energy-adjusted intake of any of these compounds was also not associated with calprotectin (Supplementary Table S2).

As we only found a significant association in HCs, which was the group with the lowest calprotectin levels, we decided to also explore subgroups based on calprotectin levels rather than disease. Clinically relevant cut-offs for calprotectin were used to divide the total population in subgroups based on low (<15 μg/g), moderate (15-<50 μg/g) or high (50 μg/g or higher) faecal calprotectin levels (Supplementary Table S3). Assessment of significant differences in dietary dicarbonyls and AGEs intake between these calprotectin-based subgroups showed that the absolute intake of MGO was

significantly higher in individuals with low calprotectin levels as compared to moderate calprotectin levels (p=0.031). None of the other comparisons were significantly different between these subgroups.

Inflammatory Potential of Diet and Overall Diet Quality

We found food groups such as bread, vegetables and legumes, nuts, and fruits were among the food groups contributing most to the intake of dietary dicarbonyls and AGEs in all three groups. As these food groups are generally considered healthy because of their high content in components such as vitamins, minerals, and anti-oxidants, which might counteract the potential effects of dicarbonyls and AGEs, we also investigated whether the absolute intake of dicarbonyls and AGEs showed a correlation with the inflammatory potential of the diet, and/or with overall diet quality.

When evaluating the anti-inflammatory potential of the diet by the ADII (Supplementary Table S4), a higher absolute intake of MGO was correlated with a lower ADII in IBS (r=-0.169, p=0.006) and HC (r=-0.195, p=0.006). Furthermore, a higher intake of GO was correlated with a lower ADII in all groups (all p<0.01). The intake of 3-DG was not significantly correlated with the ADII in either of the groups. A higher intake of CML was significantly correlated with a higher ADII (r=0.216, p=0.002) in HC only, while no correlations were found for CEL and MG-H1 in either of the groups. Furthermore, none of the summed intakes correlated significantly with the ADII in either of the subgroups.

With regard to overall diet quality (Supplementary Table S5), a higher absolute intake of the dicarbonyl GO and the AGE MG-H1, but not the others, were correlated with a higher DHD-2015 in all groups (all p<0.05). The summed intake of dietary dicarbonyls was also not significantly associated with the DHD-2015 in either of the subgroups. Additionally, a higher intake of summed dietary AGEs but not total dicarbonyls was correlated with a higher DHD-2015 in IBS (r=0.141 and p=0.022) and HC (r=0.178 and p=0.013).

Table 3. Multivariable linear regression of absolute dietary intake of dicarbonyls and advanced glycation endproducts with faecal calprotectin.

	IBD patients (n = 209)			IBS patients (n = 90)			HC (n = 148)		
	β	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**	**β**	**95% CI**	**p-value**
MGO	-19.01	-48.57; 10.59	0.206	6.44	-7.25; 20.12	0.352	-4.77	-13.71; 4.18	0.294
GO	-20.80	-65.04; 23.45	0.355	-0.50	-21.42; 20.41	0.962	-11.21	-22.17; -0.25	0.045
3-DG	-1.28	-8.61; 6.04	0.730	-1.38	-4.59; 1.82	0.393	-0.86	-2.77; 1.05	0.374
Dicarbonyls	-1.83	-7.54; 3.88	0.528	-0.67	-3.25; 1.91	0.606	-.085	-2.33; 0.63	0.258
CML	-35.48	-75.31; 4.35	0.080	-0.87	-19.78; 17.44	0.925	-1.99	-12.02; 8.04	0.696
CEL	-30.29	-81.11; 20.54	0.241	-1.97	-24.03; 20.09	0.859	-0.87	-13.26; 11.54	0.891
MG-H1	-1.67	-7.74; 4.42	0.590	-0.22	-2.70; 2.27	0.863	-0.51	-1.98; 0.96	0.491
AGEs	-1.91	-6.84; 3.02	0.445	-0.17	-2.21; 1.86	0.867	-0.37	-1.56; 0.82	0.538

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; β = regression coefficient; 95% CI = 95% confidence interval; MGO = methylglyoxal; GO = glyoxal; 3-DG = 3-deoxyglucosone; CML = Nε-(carboxymethyl)lysine; CEL = Nε-(1-carboxyethyl)lysine; MG-H1 = methylglyoxal-derived hydroimidazolone-1; AGEs = advanced glycation endproducts.

Faecal calprotectin was measured in μg/g.

Analyses were performed using multivariable linear regression with faecal calprotectin levels as dependent variable, and were corrected for: age, sex, smoking, body mass index, disease specific medication (all subgroups), plus phenotype, disease duration (years) and age of onset according to the Montreal classification for IBD, or plus subtype for IBS.

Discussion

To our best knowledge, this is the first study investigating the intake of dietary dicarbonyls and AGEs in IBD and IBS patients. We found that the absolute intake of both was lower in patients with IBS as compared to IBD and HC, but not after adjustment for energy intake. The intake of dietary dicarbonyls and AGEs was not significantly associated with faecal calprotectin in IBD and IBS patients, apart from a higher MGO intake in individuals with low as compared to moderate calprotectin levels, indicating a potential protective effect. Furthermore, a higher intake of dicarbonyls and AGEs was not associated with a lower diet quality or higher inflammatory potential of the diet, except for a significant positive correlation between CML intake and the ADII in HC.

In the current study, overall intake of dicarbonyls and AGEs was not higher in IBD and IBS as compared to controls and intakes were largely in line with previous findings in other Dutch cohorts including healthy individuals, and those at increased risk of or with type 2 diabetes.[54-56] In contrast, even lower absolute but not energy-adjusted levels of all dicarbonyls and AGEs were found in IBS, and lower energy-adjusted concentration, but not absolute intake of GO was found in IBD patients.

Although several studies found an association of dietary intake of MRPs with plasma and tissue levels of dicarbonyls[17] and AGEs,[57,58] there is also evidence that AGEs are only partially digested and absorbed,[18,59] indicating that a large proportion reaches the colon. Therefore, MRPs may have a local inflammatory effect in the intestine. However, we found no association between higher intake levels and higher faecal calprotectin levels in either of the subgroups studied. On the contrary, we found a higher absolute MGO intake in individuals with low as compared to moderate calprotectin levels. This is in line with a recent study that found a higher habitual intake of MGO to be associated with less low-grade inflammation as measured in plasma.[16] Nonetheless, no differences were found when comparing the other dietary dicarbonyls and AGEs in those with low, moderate, or high calprotectin levels.

Furthermore, we found that a higher intake of dicarbonyls and AGEs was generally associated with a better diet quality and a more anti-inflammatory diet. Thereby, in the current study, we find no evidence for a higher intake of dicarbonyls and AGEs being associated with intestinal inflammation in IBD or IBS patients as compared to HC, nor for an association with diet.

In line with previous studies,[50,60,61] we found the main food products contributing to the intake of dietary dicarbonyls and AGEs in IBD and IBS patients were not only processed foods such as cookies and bakery products, sweets/chocolate and savoury snacks, but also products generally considered to be healthy such as bread, vegetables, legumes, fruit, potatoes, rice and pasta, and coffee. This food matrix is important to consider when investigating the health effects of any food components, as they contain anti-oxidants, fibres, and micronutrients that may protect against the dicarbonyls and AGEs.[62-64] Therefore, we cannot rule out that any potential detrimental effects from the dicarbonyls and AGEs are counteracted by the anti-inflammatory components of these healthy foods. It should also be emphasised that some studies

even indicate a hormetic effect of dicarbonyls[65,66] and AGEs,[67] and animal studies showing harmful effects are mostly based on supraphysiologic levels of intake.[3,68]
Additionally, the food matrix should be considered for its effect on digestion and absorption. A study using the standardised TNO *in vitro* gastrointestinal digestion model (TIM-1), showed that the protein-bound form of AGEs can survive gastric and small intestinal digestive secretions, and stays intact during upper GI tract passage.[59] Additionally, *in vitro* evidence indicates that dietary dicarbonyls reach the colon largely unaltered by digestion.[69] With these undigested MRPs being present in the GI tract together with proteins from the food matrix or the intestinal environment, the Maillard reaction can also occur endogenously in the GI tract, involving a bidirectional interaction with the intestinal microbiome. Several animal studies have shown that a heat-treated chow diet, high in dietary AGEs, can lead to the gut microbiota composition perturbations.[5,6,8,68,70,71] On the other hand, studies with mice on a lactose or fructo-oligosaccharide-diet resulted in an increased colonic epithelial RAGE expression, increased mucosal mast cells numbers and activity, abdominal hypersensitivity,[72] and a dysregulation of the colonic mucus barrier.[73] As this was accompanied by increased CML levels in the colonic epithelium, and was prevented by co-treatment with pyridoxamine, a known anti-glycation agent, this points towards microbial involvement in glycation processes.[73] As the intestinal microbiota displays large inter-individual variation and moreover differences in composition have been shown in IBD and IBS as compared to controls,[74] further studies are needed to study the impact of the endogenous dicarbonyl and AGEs generation and the involvement of the individual's microbiota composition and activity.
The databases used in our study were both based on UHPLC-MS/MS analysis, which is considered to be the best analytical method to quantify dicarbonyls[50] and AGEs[51] in food. Nevertheless, it is important to mention the limitation that only six components, *i.e.* three dicarbonyls and three AGEs, were included in these databases, whereas foods contain many more MRPs. Furthermore, an important limitation from our FFQ is that it does not include detailed information about food preparation methods for all food items. Several studies showed cooking techniques and heating are fundamental in the formation of MRPs.[50,51,55,61,75] However, the effect of this missing information is considered to be limited because the databases contained mostly uncooked or pre-processed foods, and cooked foods were prepared according to the manufacturer's label or using the most common preparation technique.

Conclusions

Dietary intake of dicarbonyls and AGEs was not higher in IBD and IBS patients as compared to healthy controls, when adjusted for overall energy intake. Furthermore, in this study we found no leads that the concentrations of dicarbonyls and AGEs generally present in the diet of Dutch patients with IBD or IBS are associated with intestinal inflammation. However, we cannot rule out potential harmful effects might be counteracted by anti-inflammatory components in the food matrix, so further studies investigating this are needed.

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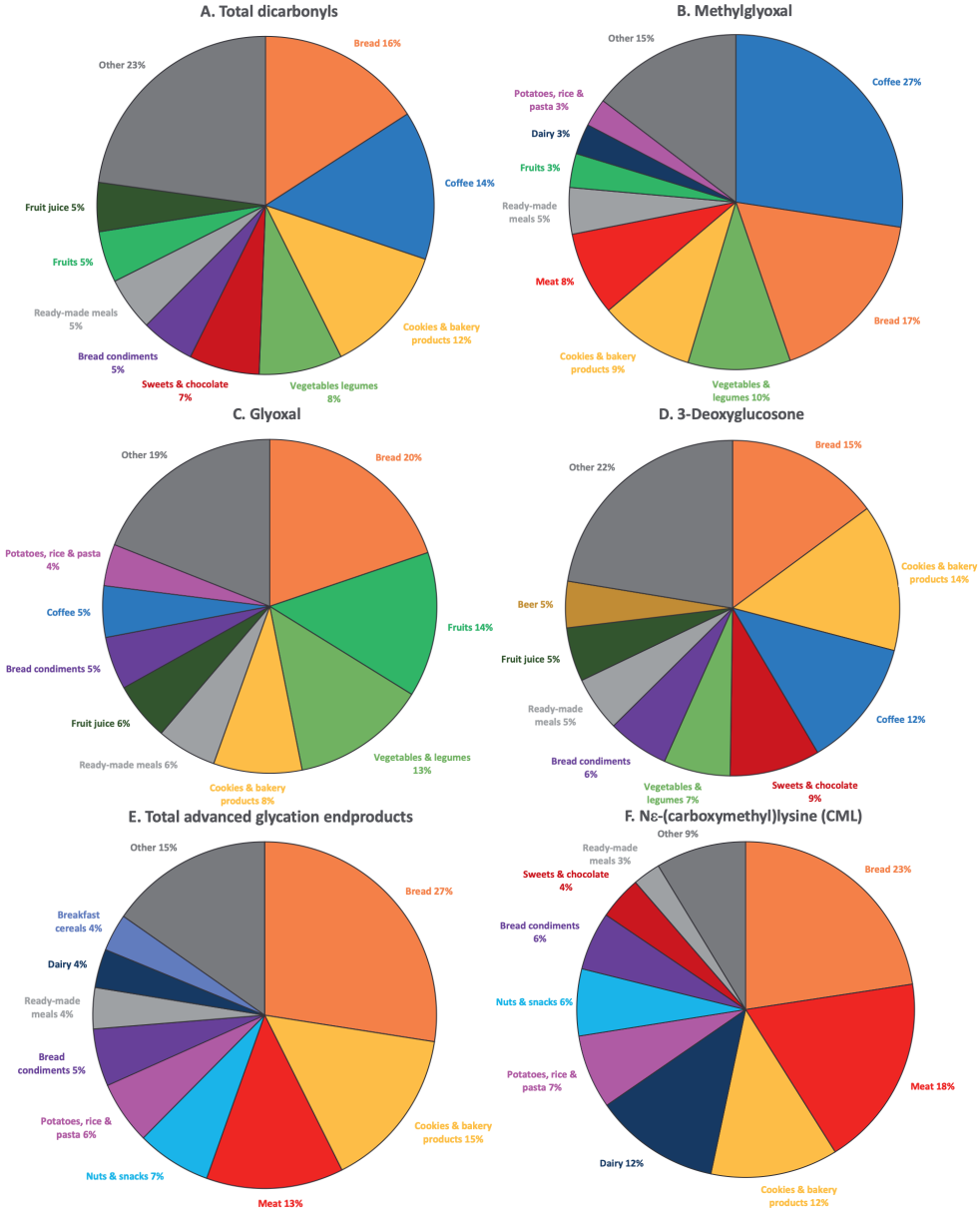
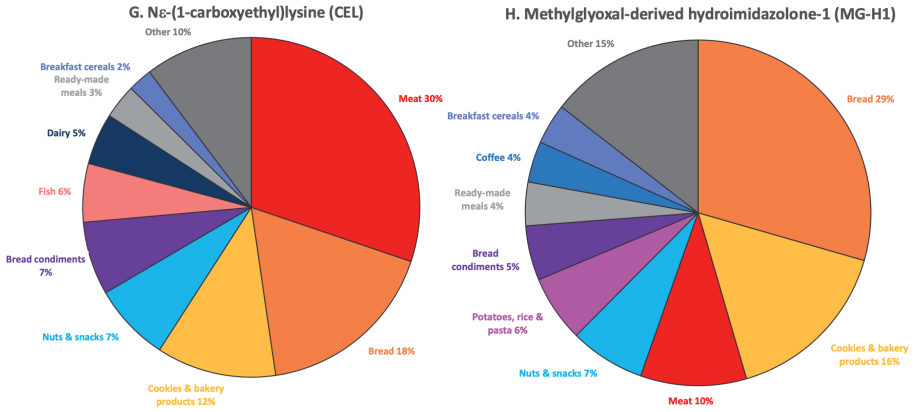
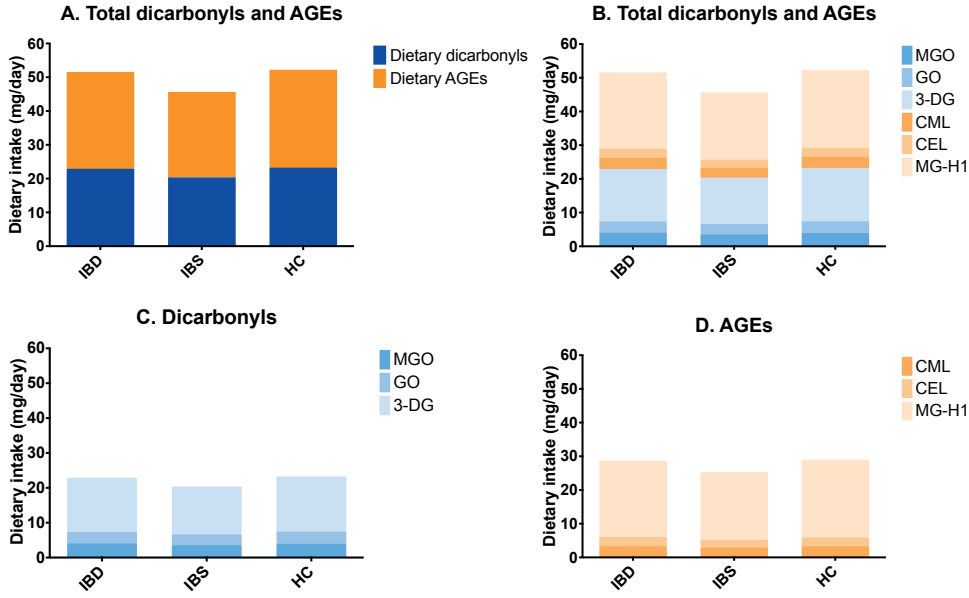
Supplementary Results





Supplementary Figure S1. Main contributing food group (%) for absolute dietary intake of individual dicarbonyls and dietary advanced glycation endproducts (for inflammatory bowel disease, irritable bowel syndrome, and healthy controls combined).



Supplementary Figure S2. Stacked bar chart of sum scores for absolute dietary intake of dicarbonyls methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG), and advanced glycation endproducts (AGEs) Nε-(carboxymethyl)lysine (CML), Nε-(1-carboxyethyl)lysine (CEL), and methylglyoxal-derived hydroimidazolone-1 (MG-H1) for inflammatory bowel disease (IBD) patients, irritable bowel syndrome (IBS) patients, and healthy controls (HC).

Supplementary Table S1. Energy-adjusted dietary intake of dicarbonyls and advanced glycation endproducts.

Energy-adjusted intake (mg/day, mean ± SD)	IBD patients (n = 238)	IBS patients (n = 261)	HC (n = 195)	p-value
MGO	1.91 ± 0.75	1.87 ± 0.72	1.85 ± 0.61	0.636
GO	1.55 ± 0.32	1.62 ± 0.35	1.62 ± 0.30	0.011
3-DG	7.23 ± 2.44	7.16 ± 2.22	7.32 ± 1.86	0.756
Dicarbonyls	10.69 ± 3.07	10.66 ± 2.78	10.80 ± 2.35	0.861
CML	1.54 ± 0.30	1.50 ± 0.31	1.50 ± 0.29	0.364
CEL	1.24 ± 0.24	1.25 ± 0.28	1.21 ± 0.26	0.259
MG-H1	10.38 ± 2.02	10.38 ± 2.39	10.63 ± 2.07	0.401
AGEs	13.16 ± 2.32	13.14 ± 2.77	13.35 ± 2.44	0.655

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; SD = standard deviation; MGO = methylglyoxal; GO = glyoxal; 3-DG = 3-deoxyglucosone; CML = Nε-(carboxymethyl)lysine; CEL = Nε-(1-carboxyethyl)lysine; MG-H1 = methylglyoxal-derived hydroimidazolone-1; AGEs = advanced glycation endproducts. The differences between IBD, IBS, and HC were tested with analysis of variance (ANOVA) and post-hoc Bonferroni correction.

Supplementary Table S2. Multivariable linear regression of energy-adjusted dietary intake of dicarbonyls and advanced glycation endproducts with faecal calprotectin.

	IBD patients (n = 209)			IBS patients (n = 90)			HC (n = 148)		
	β	95% CI	p-value	β	95% CI	p-value	β	95% CI	p-value
MGO	6.82	-52.32; 65.97	0.820	9.39	-19.96; 38.74	0.526	-3.27	-24.74; 18.21	0.764
GO	34.14	-106.18; 174.45	0.632	-15.08	-59.44; 29.27	0.500	-28.23	-64.61; 8.16	0.127
3-DG	9.52	-9.84; 28.89	0.333	-5.77	-13.70; 2.18	0.151	0.72	-5.41; 6.85	0.816
Dicarbonyls	6.58	-8.47; 21.63	0.389	-3.65	-10.11; 2.81	0.264	-0.22	-5.15; 4.72	0.932
CML	-68.92	-214.78; 76.94	0.352	-30.91	-89.88; 28.06	0.300	26.03	-12.94; 64.99	0.189
CEL	-18.05	-197.98; 161.88	0.843	-44.10	-108.02; 19.82	0.173	37.20	-6.25; 80.65	0.093
MG-H1	6.84	-14.41; 28.08	0.526	-4.20	-10.28; 1.89	0.173	1.09	-4.36; 6.54	0.693
AGEs	3.86	-14.59; 22.30	0.680	-3.72	-9.00; 1.56	0.165	1.56	-3.06; 6.18	0.506

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; β = regression coefficient; 95% CI = 95% confidence interval; MGO = methylglyoxal; GO = glyoxal; 3-DG = 3-deoxyglucosone; CML = Nε-(carboxymethyl)lysine; CEL = Nε-(1-carboxyethyl)lysine; MG-H1 = methylglyoxal-derived hydroimidazolone-1; AGEs = advanced glycation endproducts.

Faecal calprotectin was measured in μg/g.

Analyses were performed using multivariable linear regression with faecal calprotectin levels as dependent variable, and were corrected for: age, sex, smoking, body mass index, disease specific medication (all subgroups), plus phenotype, disease duration (years), and age of onset according to the Montreal classification for IBD, or plus subtype for IBS.

Supplementary Table S3. Comparison of absolute dietary intake of dicarbonyls and advanced glycation endproducts (individual values and sum scores) for subgroups based on clinically relevant cut-off points.

Absolute intake (mg/day, ean ± SD)	< 15 μg/g (n = 153)	15 - <50 μg/g (n = 136)	≥ 50 μg/g (n = 158)	p-value
MGO	4.20 ± 1.66	3.75 ± 1.36	3.86 ± 1.52	0.031*
GO	3.48 ± 1.01	3.34 ± 0.99	3.28 ± 1.08	0.220
3-DG	15.50 ± 6.09	15.89 ± 6.36	15.11 ± 5.80	0.547
Dicarbonyls	23.18 ± 8.05	22.97 ± 7.96	22.26 ± 7.51	0.551
CML	3.27 ± 1.07	3.36 ± 1.23	3.18 ± 1.16	0.436
CEL	2.58 ± 0.88	2.71 ± 0.95	2.64 ± 1.01	0.557
MG-H1	22.04 ± 7.42	22.52 ± 7.69	22.76 ± 8.73	0.720
AGEs	27.89 ± 9.11	28.58 ± 9.60	28.58 ± 10.71	0.777

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; SD = standard deviation; MGO = methylglyoxal; GO = glyoxal; 3-DG = 3-deoxyglucosone; CML = Nε-(carboxymethyl) lysine; CEL = Nε-(1-carboxyethyl)lysine; MG-H1 = methylglyoxal-derived hydroimidazolone-1; AGEs = advanced glycation endproducts. The differences between IBD, IBS and HC were tested with analysis of variance (ANOVA) and post-hoc Bonferroni correction.

* Post-hoc Bonferroni showed p=0.036 for <15 μg/g vs. 15 - <50 μg/g, other comparisons not significant.

Supplementary Table S4. Spearman's Rank-Order Correlation of dietary intake of dicarbonyls and advanced glycation endproducts with the Adapted Dietary Inflammatory Index.

	IBD (n = 238)		IBS (n = 261)		HC (n = 195)	
	r	*p*-value	r	*p*-value	r	*p*-value
MGO	-0.115	0.075	-0.169	0.006	-0.195	0.006
GO	-0.244	<0.001	-0.277	<0.001	-0.197	0.006
3-DG	0.034	0.600	0.019	0.760	0.091	0.205
Dicarbonyls	-0.025	0.699	-0.049	0.434	0.001	0.992
CML	0.111	0.089	0.101	0.102	0.216	0.002
CEL	-0.005	0.934	0.024	0.695	0.131	0.067
MG-H1	-0.044	0.496	0.017	0.783	0.097	0.178
AGEs	-0.021	0.747	0.029	0.645	0.116	0.105

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; r = correlation coefficient; MGO = methylglyoxal; GO = glyoxal; 3-DG = 3-deoxyglucosone; CML = Nε-(carboxymethyl) lysine; CEL = Nε-(1-carboxyethyl)lysine; MG-H1 = methylglyoxal-derived hydroimidazolone-1; AGEs = advanced glycation endproducts.

Supplementary Table S5. Spearman's Rank-Order Correlation of dietary intake of dicarbonyls and advanced glycation endproducts with the Dutch Healthy Diet index 2015.

	IBD (n = 238)		IBS (n = 261)		HC (n = 195)	
	r	*p*-value	r	*p*-value	r	*p*-value
MGO	-0.008	0.899	0.089	0.150	0.140	0.052
GO	0.202	0.002	0.166	0.007	0.480	<0.001
3-DG	-0.018	0.787	-0.070	0.261	0.035	0.627
Dicarbonyls	0.014	0.835	-0.020	0.743	0.091	0.206
CML	-0.040	0.543	0.022	0.725	0.078	0.281
CEL	-0.038	0.561	-0.011	0.856	0.066	0.359
MG-H1	0.129	0.047	0.176	0.004	0.204	0.004
AGEs	0.093	0.153	0.141	0.022	0.178	0.013

IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; HC = healthy controls; r = correlation coefficient; MGO = methylglyoxal; GO = glyoxal; 3-DG = 3-deoxyglucosone; CML = Nε-(carboxymethyl) lysine; CEL = Nε-(1-carboxyethyl)lysine; MG-H1 = methylglyoxal-derived hydroimidazolone-1; AGEs = advanced glycation endproducts.

CHAPTER 4

Evaluation of food intolerance and food avoidance in irritable bowel syndrome patients

Marlène C.G. de Graaf*, Johanna T.W. Snijkers*, Bjorn Winkens, Fleur A. Zijlstra, Daniel Keszthelyi[$], Daisy M.A.E. Jonkers[$]

* Shared first author
[$] Shared last author

CHAPTER 5

Two randomised crossover multicentre studies investigating gastrointestinal symptoms after bread consumption in individuals with non-coeliac wheat sensitivity: do wheat species and fermentation type matter?

Marlijne C.G. de Graaf, Emma Timmers, Bo Bonekamp, Gonny van Rooy, Ben J.M. Witteman, Peter R. Shewry, Alison Lovegrove, Antoine H.P. America, Luud J.W.J. Gilissen, Daniel Keszthelyi, Fred J.P.H. Brouns, Daisy M.A.E. Jonkers

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Abstract

Background: Many individuals reduce their bread intake because they believe wheat causes their gastrointestinal (GI) symptoms. Different wheat species and processing methods may affect these responses.

Objective: We investigated the effects of six different bread types (prepared from three wheat species and two fermentation conditions) on GI symptoms in individuals with self-reported non-coeliac wheat sensitivity (NCWS).

Methods: Two parallel, randomised, double-blind, crossover, multicentre studies were conducted. NCWS individuals, in whom coeliac disease and wheat allergy were ruled out, received five slices of yeast fermented (YF) (study A, n=20) or sourdough fermented (SF) (study B, n=20) bread made of bread wheat, spelt, or emmer in a randomised order on three separate test days. Each test day was preceded by a run-in period of 3 days of a symptom-free diet and separated by a wash-out period of ≥7 days. GI symptoms were evaluated by change in symptom score (test day minus average of the 3-day run-in period) on a 0-100mm visual analogue scale (∆VAS), comparing medians using the Friedman test. Responders were defined as an increase in ∆VAS of ≥15mm for overall GI symptoms, abdominal discomfort, abdominal pain, bloating and/or flatulence.

Results: GI symptoms did not differ significantly between breads of different grains (YF bread wheat median ∆VAS 10.4mm [interquartile range 0.0-17.8mm], spelt 4.9mm [-7.6-9.4mm], emmer 11.0mm [0.0-21.3mm], p=0.267; SF bread wheat 10.5mm [-3.1-31.5mm], spelt 11.3mm [0.0-15.3mm], emmer 4.0mm [-2.9-9.3mm], p=0.144). The number of responders was also comparable for both YF (6 to wheat, 5 to spelt, and 7 to emmer, p=0.761) and SF breads (9 to wheat, 7 to spelt, and 8 to emmer, p=0.761).

Conclusions: The majority of NCWS individuals experienced some GI symptoms for at least one of the breads, but on a group level, no differences were found between different grains for either YF or SF breads.

Clinical Trial Registry: ClinicalTrials.gov, NCT04084470

Introduction

Wholegrain wheat products provide a substantial source of nutrients, making an important contribution to energy intake and a healthy diet.[1,2] Accordingly, their consumption has been associated with reduced risks of type 2 diabetes, cardiovascular disease, cancer, and mortality.[3-6] Nevertheless, wheat-based foods can elicit adverse reactions in susceptible individuals, such as those with coeliac disease (CD) and wheat allergy (WA).[7-9] Additionally, some people avoid or reduce wheat intake because of symptoms, even though CD and WA have been excluded. Initially, this was defined as non-coeliac gluten sensitivity (NCGS) due to gluten as presumed cause.[10] As amylase-trypsin inhibitors (ATIs) and fermentable carbohydrates (*i.e.* FODMAPs) are also potential triggers, the term non-coeliac wheat sensitivity (NCWS) is increasingly used,[11,12] and the Salerno Experts' Criteria,[10] including a gluten elimination and challenge, may need reconsideration. NCWS has an estimated self-reported prevalence of up to 15%,[13-15] generally manifesting with gastrointestinal (GI) symptoms like abdominal discomfort or pain, bloating, and diarrhoea, and sometimes extra-intestinal symptoms.[16-18] Symptoms mostly occur within 12 hours after wheat intake and ameliorate within a few hours.[19]

Evidence on the role of gluten is inconsistent.[20-28] Gluten preparations used in previous human studies also contain ATIs,[29] potential activators of innate immune responses, although evidence is mostly based on *in vitro* and animal studies.[30-35] FODMAPs like fructans may lead to osmotic effects and gas production.[36,37] Eliciting the contributions of these components is complicated by the biochemical composition differing between wheat species and varieties, environmental, cultivation, and processing conditions.[11,38,39]

NCWS individuals claim experiencing less GI symptoms from consuming "ancient" grains, including spelt and emmer, compared to modern wheat varieties. [19,40-43] Spelt and emmer contain about 20% more gluten than bread wheat,[44] whereas FODMAP concentrations are comparable between spelt and bread wheat.[38] Furthermore, there is conflicting evidence on hexaploid (AABBDD) wheats, including bread wheat and spelt, inducing more immune reactivity than tetraploid species (AABB) such as emmer.[45,46] Previous double-blinded intervention studies found inconsistent effects of bread from different wheat types on GI symptoms.[40,47]

Whereas yeast fermentation (YF) is the major practice in modern bread baking, sourdough fermentation (SF) has gained renewed interest because of presumed fructan degradation and improved digestive tolerance.[48-50] However, a pilot study in irritable bowel syndrome (IBS) patients did not confirm this.[51]

Currently, the impact of fully characterised breads made with different wheat species and processing systems, and their effects on symptoms in NCWS has not been well investigated. Therefore, we aimed to investigate the effects of YF and SF bread made from bread wheat, spelt, or emmer on overall GI symptoms in individuals with self-reported NCWS in two parallel studies. Secondarily, we investigated their effects on individual GI and extra-intestinal symptoms. We hypothesised that consumption of YF

and SF bread made from emmer would cause less symptoms than bread wheat and spelt.

Methods

Two parallel, randomised, double-blind, crossover, multicentre studies were conducted at Maastricht University and Wageningen University & Research, both in the Netherlands. Participants were recruited between 11 September 2020 and 4 November 2022, and measurements were completed on 29 November 2022. The studies were approved by the Medical Ethics Committee of Academic Hospital Maastricht/Maastricht University, and by the Board of Directors of Wageningen University & Research, and were performed in accordance with the Declaration of Helsinki and Dutch Regulations on Medical Research involving Human Subjects. All participants gave their written informed consent prior to participation. The studies were registered at ClinicalTrials.gov (NCT04084470).

Participants

Participants were recruited via advertisements on social media, patient association websites, notice boards at the university campus and local public areas, and in local newspapers. After being informed via written and verbal information, interested participants were invited for a screening visit to assess eligibility.

Males and females aged 18-70 years who experience self-reported GI symptoms within 12 hours after a single intake of bread, *i.e.* 1-2 slices of bread (NCWS) were included. Medication had to be stable for at least one month prior to and during the study. Participants were excluded if they had been diagnosed with CD, WA, or other organic GI diseases, any malignancies, or any other disease interfering with GI function, or if they previously had major abdominal surgery or radiotherapy interfering with GI function (uncomplicated appendectomy, cholecystectomy and hysterectomy were allowed if more than six months ago). If CD was not excluded by previous serology or upper GI endoscopy, and participants still consumed gluten or were willing to re-introduce gluten into their diet for at least six weeks, an additional visit was scheduled for serological testing to rule out CD by total immunoglobulin A (IgA) and anti-tissue transglutaminase IgA. Furthermore, use of antibiotics, probiotics or prebiotics, participation in other studies 14 days prior to and during the study, excessive use of alcohol (>15 standard serving quantity per week) or any use of illicit drugs, and intentional weight loss during the study period were not allowed. Females could not be pregnant or lactating. Participants had to have sufficient understanding of the Dutch language.

Participants were requested to adhere to a “symptom-free diet”, *i.e.* to replace or avoid food products that they considered to induce GI symptoms. Practical application of this diet varied from replacing their usual bread to following a completely gluten-free diet, depending on what was necessary for the individual participant to obtain a low GI symptom score at baseline. After following the symptom-free diet for at least one week

prior to the screening visit, overall GI symptoms had to be minimal, *i.e.* ≤30mm on a 100mm visual analogue scale (VAS).[52] The individual's symptom-free diet was maintained throughout the study period.
Medical history and Rome IV criteria for irritable bowel syndrome (IBS)[53] and functional dyspepsia (FD)[54] were assessed by the researcher during the screening visit. Smoking behaviour (current, former- or non-smoker) and alcohol intake were self-reported using pre-defined categories (none, <1 unit per week, 1-5 units per week, or 8-15 units per week). Height and weight were self-reported or measured at the screening visit if unknown, and used to calculate body mass index (BMI). After inclusion into the study, but prior to starting the study period, participants completed the Generalized Anxiety Disorder assessment (GAD-7),[55] Patient Health Questionnaire-9 (PHQ-9),[56] and the Patient Health Questionnaire-15 (PHQ-15)[57] to assess anxiety, depression, and somatic symptoms, respectively.

Study design

Two parallel, randomised, double-blind, crossover, multicentre studies were conducted (see Figure 1). Study A tested YF bread made of bread wheat, spelt, or emmer, whereas study B tested SF bread, also made of bread wheat, spelt, or emmer. Within each study, participants received five slices (125-150 gram in total) of these breads in a randomised order on three separate test days.

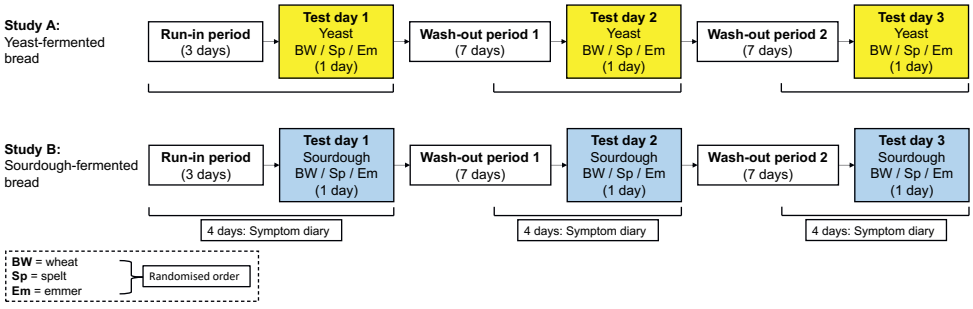

Figure 1. Study design.

Randomisation and blinding

The randomisation list was generated by a colleague unconnected with the trial using a publicly available procedure (https://www.sealedenvelope.com/simple-randomiser/v1/lists). Separate lists were made for study A and B. Per study, the randomisation list ensured an equal number of participants per treatment order (*i.e.* randomised order of bread wheat, spelt, and emmer). The colleague provided the researcher with a randomisation number, which corresponded with the labelled packages of the study breads.
Frozen packages of bread portions per test day (five slices) were provided in sealed non-transparent plastic sachets so participants could not compare the appearance of

the study breads. The sachets were labelled with a randomisation number and a test day number according to the randomisation list.
Participants were unaware of the different bread types under investigation, and the researchers were blinded to the randomisation order. Data analysis was executed before unblinding of the researcher.

Study period
Participants received all three study breads (either YF or SF) at the end of the screening visit. As the full test period was completed at home, the order of consumption was indicated on the package (*i.e.* test day 1, 2, or 3). Participants were instructed to consume the breads for breakfast and lunch, with the choice of consuming 2-3 slices per mealtime. The chosen quantity per mealtime was repeated on all subsequent test days. Each test day was preceded by a 3-day run-in period and separated by a wash-out period of at least seven days (see Figure 1). Participants received a reminder via text message on the evening prior to each run-in period. For females, run-in periods and test days were not scheduled during the menses phase of their menstrual cycle, for which the wash-out period was prolonged if necessary.
On the evening of each test day and during the three run-in days, participants completed symptom diaries for GI and extra-intestinal symptoms, and the Bristol Stool Scale[58] to assess stool frequency and consistency.
All participants were asked to adhere to their symptom-free diet throughout the study period. Food records were completed during each run-in period and test day to assess compliance to the individual's symptom-free diet, and, combined with photos of the study breads sent on the test day, to assess compliance to the intervention.
Because of limited shelf life of the study breads, study A was completed before starting study B. Hence, participants who completed study A could thereafter also participate in study B.

Study bread
All study breads were manufactured by the Dutch Bakery Center, Wageningen, the Netherlands. Bread wheat (*Triticum aestivum* spp. *Aestivum*), spelt (*Triticum aestivum* ssp. *Spelta*), and emmer (*Tritordeum turgidum* var. *dicoccum*) were obtained from commercial growers. Breads made from bread wheat and spelt were chosen to represent modern bread products, whereas emmer represented ancient wheat species. All breads were prepared using 100% food-grade ingredients suitable for human consumption. Additions such as salt and minor processing additives were constant throughout and in accordance with standard commercial bread baking process, with minor adjustments to the addition of water and yeast to obtain uniform-looking breads. For the SF breads, the commercial sourdough starter culture 'Mailander Le Chef' (Böcker, Germany) was used.
The breads used in the present study were baked from the same materials according to the processing methods as described by Shewry *et al.* 2022.[59] More details about baking procedures, and analysis of the bread composition are included in the

Supplementary Materials (Tables S1-4 and Figure S1), with a description of the comparison included in the Supplementary Results ("Comparing nutrient composition of the different bread types").

Primary and secondary outcomes

The primary outcome was the effect of YF bread (study A) and SF bread (study B) made from either bread wheat, spelt, or emmer on overall GI symptoms. Secondary, the effects of these breads on individual GI symptoms (*i.e.* abdominal discomfort, abdominal pain, belching, bloating, constipation, diarrhoea, flatulence, fullness, nausea, urge to empty bowel) and extra-intestinal symptoms (*i.e.* confusion, headache, joint pains, loss of coordination, skin rash, tiredness) were investigated. All symptom scores were measured on a 100mm VAS as part of the symptom diary.

Statistical analysis

Sample size was calculated using G*power version 3.1 (Heinrich Heine Universität, Düsseldorf, Germany). Based on a study by Biesiekierski,[21] a mean difference in VAS of 10.3mm with standard deviation (SD) of 12.8mm was expected. With a power of 80% and a Bonferroni-corrected alpha of 0.0167, this resulted in a sample size of 20 participants per study. Expecting a drop-out rate of maximum 10%, permission was granted by the Medical Ethics Committee to include two extra participants per study if necessary.

Statistical analyses were conducted using IBM SPSS statistics version 26.0 (IBM Corp., Armonk (NY), United States) and figures were drawn using GraphPad Prism version 10.1.1 (GraphPad Software, Boston (MA), United States). Study A and B were analysed separately. Normality of data was evaluated using histograms and the Kolmogorov-Smirnov test. Baseline characteristics were presented as mean with SD for normally distributed continuous variables, as median with interquartile range (IQR) for non-normal distributed continuous variables, and as frequencies with percentages for categorical variables.

To assess primary and secondary outcomes, delta VAS symptom scores (∆VAS) were calculated per symptom for each bread as [score test day] – [average of 3-day run-in period], where the average of the 3-day run-in period was used as baseline. The ∆VAS per symptom was compared between breads using the non-parametric Friedman test, with post-hoc Wilcoxon test. Missing values for run-in days were imputed per symptom, using the mean of the other days of that run-in period. No values were missing for the test days.

The averages of each 3-day run-in period were compared to check for carry-over effects, and the ∆VAS of each test day to check for an order effect, both using the Friedman test with post-hoc Wilcoxon test.

Because of the large variation observed for each test day, in a post-hoc analysis responders and non-responders were further explored. Responders were defined as participants with an increase of at least 15mm on ∆VAS for overall GI symptoms and/or

for predominant symptoms abdominal discomfort, abdominal pain, bloating, or flatulence.[21,51,60] The number of responders for each bread was compared by Cochran's Q test with post-hoc McNemar test.
Exploratively, the effects of dough processing using either yeast- or sourdough fermentation was assessed in the subgroup of participants that completed both study A and B. Again, the Friedman test was used to compare symptom scores, and Cochran's Q test to compare the number of responders.

Results

Study A: yeast fermented (YF) breads

Study A was completed between 11 September 2020 and 20 April 2022. Fifty-seven potential participants received the study information. Of these, 39 completed the pre-screening and 26 the full screening. Main reasons for ineligibility were that their symptoms were self-reported not to result from bread (n=7), that CD was not ruled out (n=4), or that symptoms were too high despite following the symptom-free diet (n=2). Twenty participants started and completed study A (see Figure 2).
In study A, mean age was 42.8±12.8 years, mean BMI was 25.6±3.7 kg/m^2, and 15 participants were female (75%). Most participants never smoked (85%) and had an alcohol intake of less than 1 unit (*i.e.* 1 standard serving quantity) (35%) or 1-5 units per week (40%). Participants had been experiencing symptoms related to bread for 9.0 [IQR 3.5-28.0] years. Fifteen percent (3/20 participants) met de Rome IV criteria for IBS, and 5% (1/20) for FD. Full details are given in Table 1 and Supplementary Table S5.

No carry-over effect or order-effect was found for any of the symptoms (for all symptoms p>0.05) (Supplementary Figures S2-S3).

Overall GI symptoms (Figure 3A) were comparable between YF breads made of bread wheat (median ΔVAS 5.7mm [IQR 0-17.8mm]), spelt (median ΔVAS 0mm [IQR -7.6-9.4mm]), and emmer (median ΔVAS 1.3mm [IQR 0-21.3mm], p=0.267). Predominant GI symptoms were abdominal discomfort, abdominal pain, bloating and flatulence. None of the assessed GI symptoms showed significant differences between YF bread types (Figure 3B-K). Also, none of the assessed extra-intestinal symptoms showed significant differences between YF breads (Figure 4).

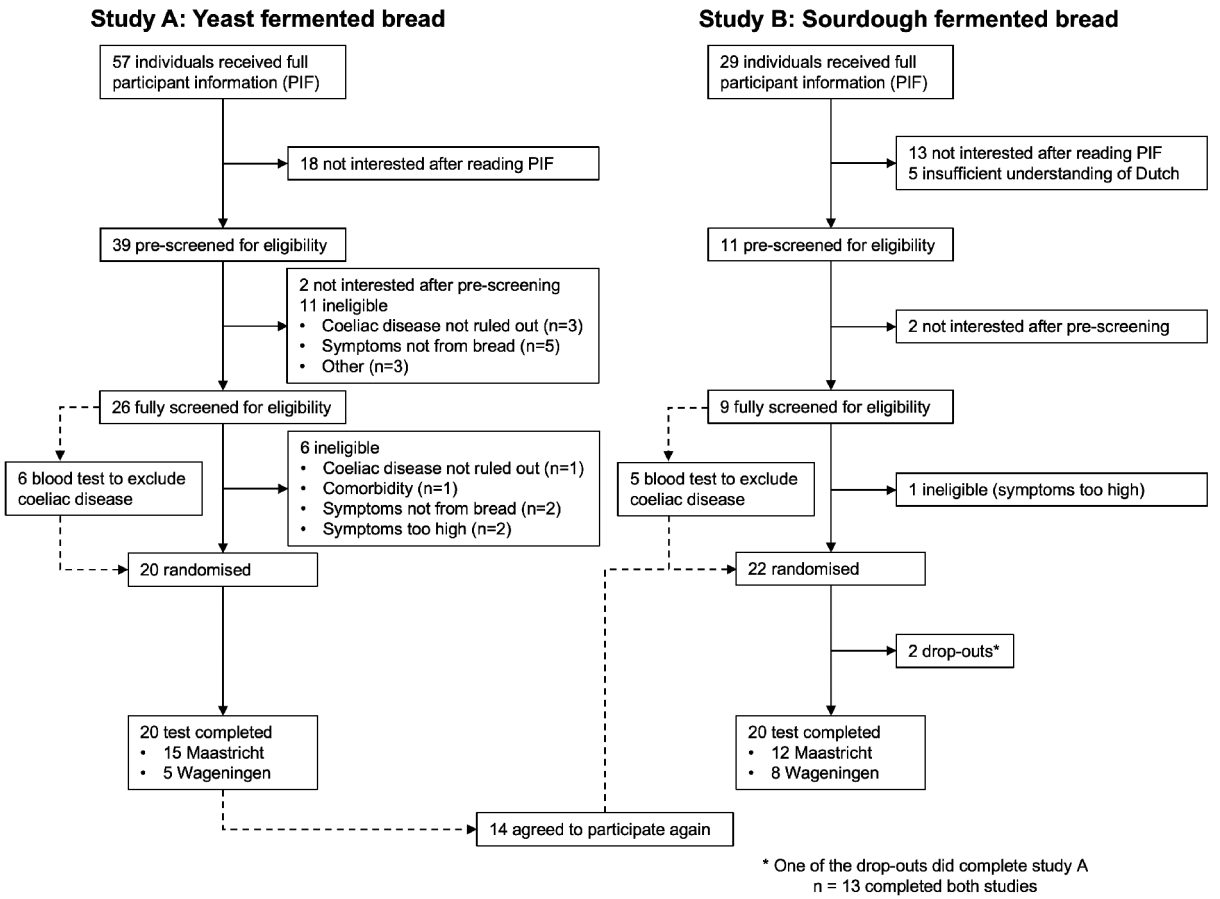

Figure 2. Flowchart of recruitment and inclusion.

Table 1. Baseline characteristics.

	Study A (n = 20) *	**Study B** (n = 20)
Female	15 (75%)	18 (85%)
Age (years)	42.8 ± 12.8	41.9 ± 12.9
BMI (kg/m^2) **	25.6 ± 3.7	25.1 ± 4.8
Smoking		
Never	17 (85%)	16 (80%)
Current smoker	0 (0%)	0 (0%)
Quit smoking	3 (15%)	4 (20%)
Alcohol intake ***		
None	4 (20%)	3 (15%)
< 1 unit per week	7 (35%)	7 (35%)
1-5 unit per week	8 (40%)	8 (40%)
6-7 unit per week	1 (5%)	1 (5%)
8-15 unit per week	0 (0%)	1 (5%)
Education level ****		
Low	1 (5%)	1 (5%)
Middle	4 (20%)	4 (20%)
High	15 (75%)	15 (75%)
Start of bread-related symptoms (number of years ago)		
Gastrointestinal *****	9.0 [3.5-28.0]	9.5 [5.0-23.5]
Extra-intestinal ******	18.0 [8.3-40.0]	11.0 [8.5-47.5]
Irritable bowel syndrome (Rome IV)	3 (15%)	3 (15%)
IBS-C	1 (5%)	1 (5%)
IBS-D	1 (5%)	0 (0%)
IBS-M	0 (0%)	0 (0%)
IBS-U	1 (5%)	2 (10%)
Functional dyspepsia (Rome IV)	1 (5%)	2 (10%)
Postprandial distress syndrome	0 (0%)	1 (5%)
Epigastric pain syndrome	0 (0%)	1 (5%)
Overlap syndrome	1 (5%)	0 (0%)
Anxiety (GAD-7)	0.0 [0.0-1.8]	1.0 [0.0-1.8]
Yes, anxiety (≥ 10)	0 (0%)	0 (0%)
Depression (PHQ-9)	1.0 [0.0-3.8]	1.0 [0.0-2.0]
Yes, depression (≥ 10)	1 (5%)	1 (5%)
Somatisation (PHQ-15)	4.8 ± 3.4	4.9 ± 2.5
Minimal (<5)	9 (45%)	9 (45%)
Low (5-9)	9 (45%)	11 (55%)
Medium (10-14)	2 (10%)	0 (0%)
High (15+)	0 (0%)	0 (0%)

Continuous variables are displayed as mean ± SD for normally distributed data and as median [interquartile range] for non-normal distributed data. Categorical variables are displayed as number (percentage).

BMI = body mass index; IBS-C = constipation predominant IBS; IBS-D = diarrhoea predominant IBS; IBS-M = mixed stool pattern IBS; IBS-U = unspecified subtype IBS; FD = functional dyspepsia; GAD-7 = Generalized Anxiety Disorder; PHQ-9 = Patient Health Questionnaire 9; PHQ-15 = Patient Health Questionnaire 15.

* 13 participants from study A also completed study B.

** BMI was calculated based on self-reported weight and height. If unknown, weight and height were measured during the screening visit.

*** Alcohol use was classified in these pre-defined categories according to the average number of units (1 unit = 1 standard serving quantity) per week.

**** Education level was categorised according to the Dutch education system.[61]

***** n=17 for study A, because the other 3 participants could not recollect how long they had already experienced symptoms.

****** n=8 for study A and n=5 for study B, because the other participants did not report extra-intestinal symptoms after bread consumption.

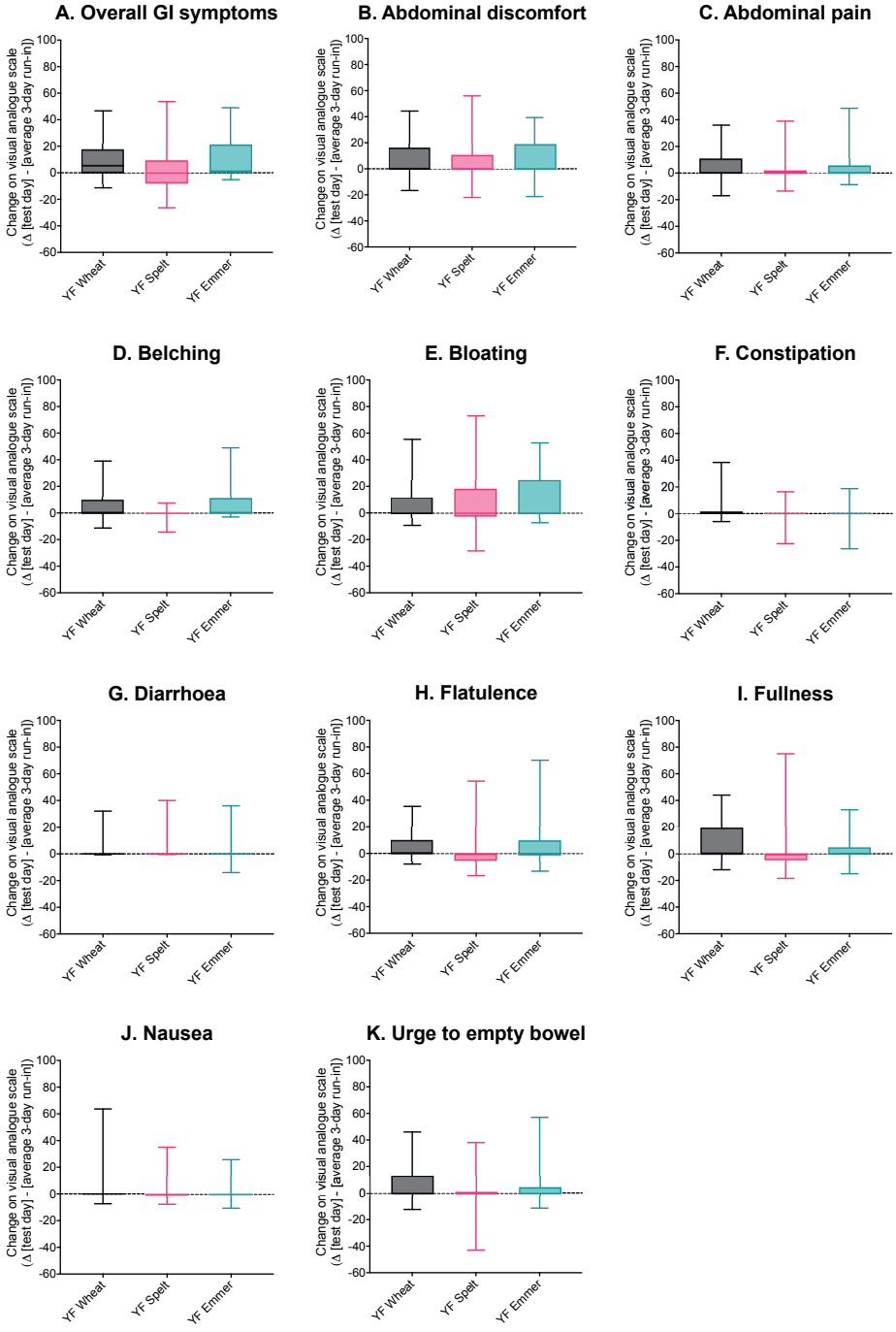

Figure 3. Gastrointestinal (GI) symptom scores, displayed as change on visual analogue scale (ΔVAS = [score test day] – [average of 3-day run-in period]) for yeast fermented (YF) breads made with bread wheat, spelt, or emmer (study A, n=20). ΔVAS per symptom was compared between breads using the non-parametric Friedman test, with post-hoc Wilcoxon test.

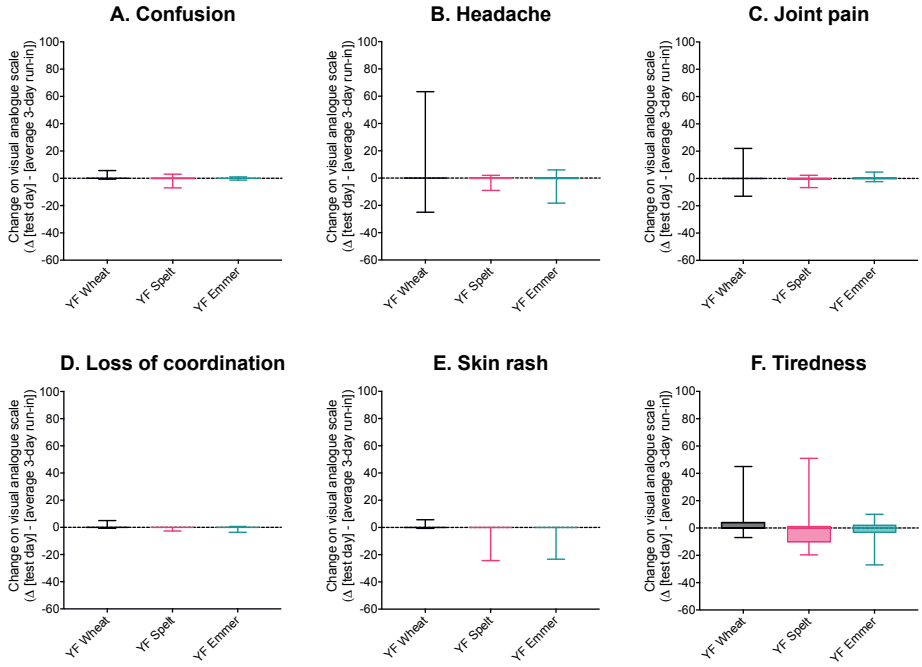

Figure 4. Extra-intestinal symptom scores, displayed as change on visual analogue scale (ΔVAS = [score test day] – [average of 3-day run-in period]) for yeast fermented (YF) breads made with bread wheat, spelt, or emmer (study A, n=20). ΔVAS per symptom was compared between breads using the non-parametric Friedman test, with post-hoc Wilcoxon test.

Study B: sourdough fermented (SF) breads

Study B was completed between 3 May 2022 and 29 November 2022. Fourteen participants from study A gave consent to also participate in study B. Additionally, 29 new potential participants received the study information. Eleven completed the pre-screening and nine the full screening. The main reason for ineligibility was insufficient understanding of Dutch (n=5), the other participants were no longer interested in participation. Twenty-two participants started the study, but two participants dropped out after test day 1 (because of severe symptoms (n=1), or found the study too time consuming (n=1)).

Twenty participants completed study B (see also Figure 2). Of these, 18 were female (85%), mean age was 41.9±12.9 years, and mean BMI was 25.1±4.8 kg/m^2. Most participants never smoked (80%) and had an alcohol intake of less than 1 unit (35%) or 1-5 units per week (40%). Participants had been experiencing symptoms related to bread for 9.5 [IQR 5.0-23.5] years. Fifteen percent (3/20 participants) met de Rome IV criteria for IBS and 10% (2/20) for FD. For full details, see Table 1 and Supplementary Table S5.

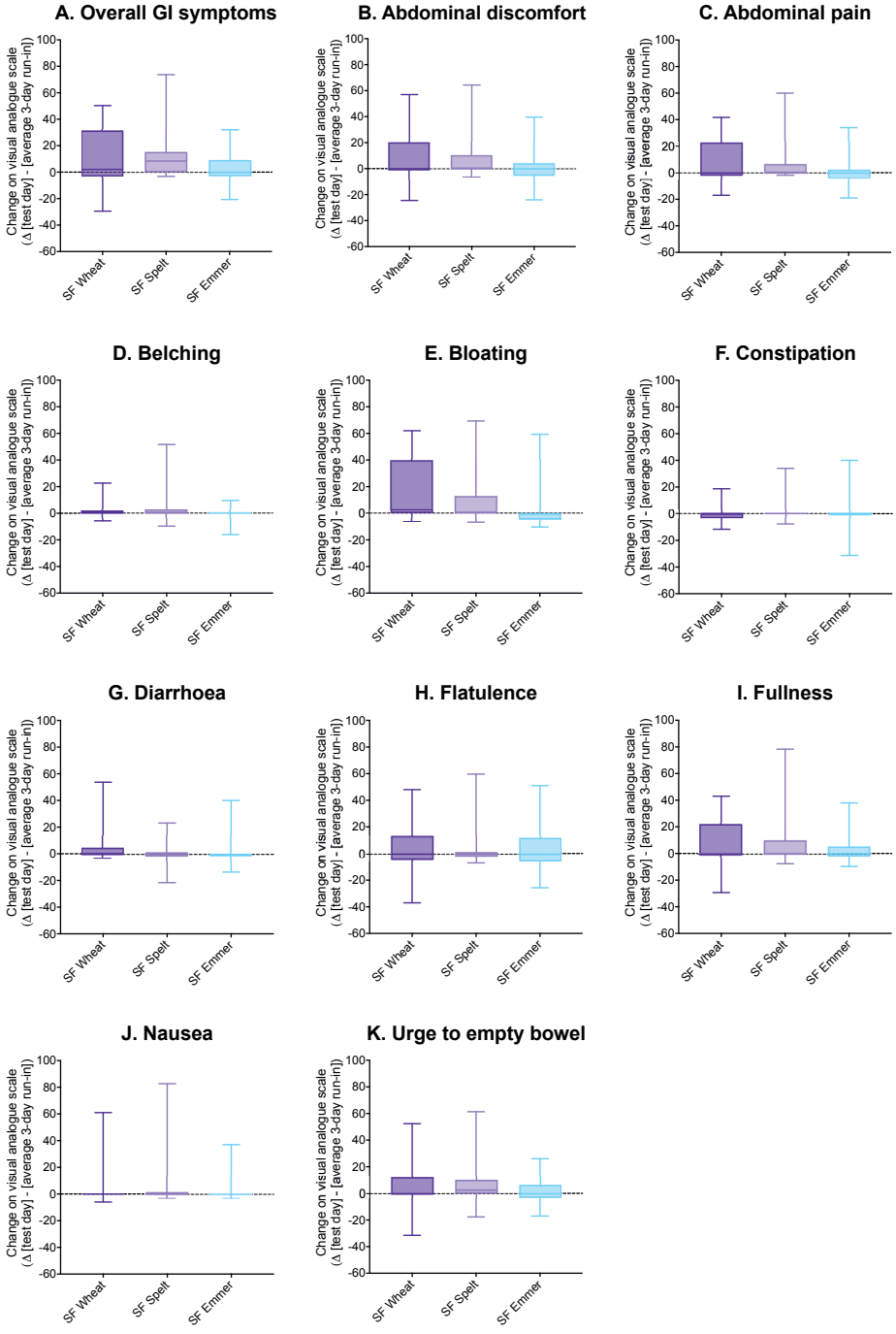

Figure 5. Gastrointestinal symptom scores, displayed as change on visual analogue scale (ΔVAS = [score test day] – [average of 3-day run-in period]) for sourdough fermented (SF) breads made with bread wheat, spelt, or emmer (study B, n=20). ΔVAS per symptom was compared between breads using the non-parametric Friedman test, with post-hoc Wilcoxon test.

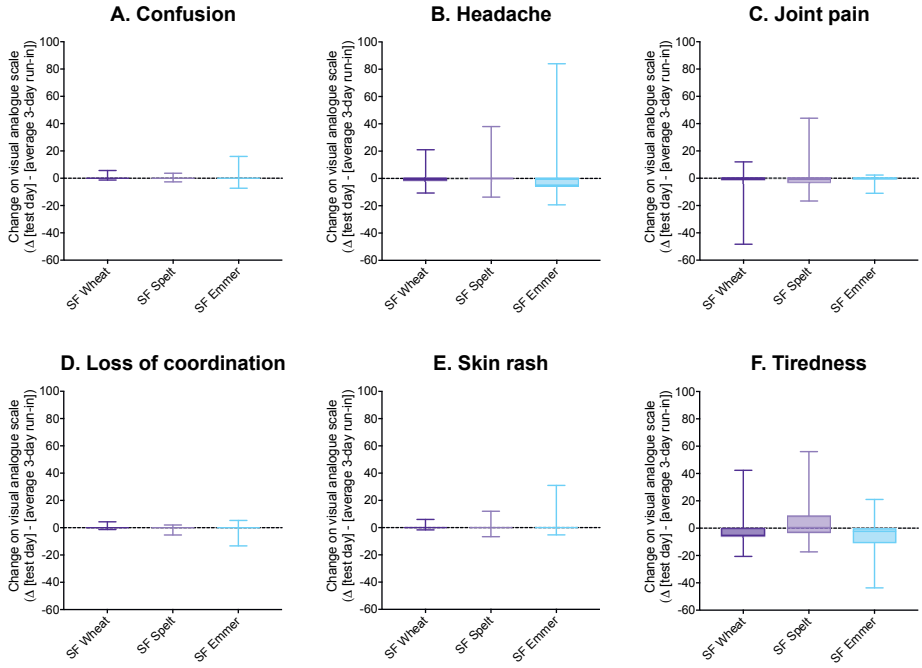

Figure 6. Extra-intestinal symptom scores, displayed as change on visual analogue scale (ΔVAS = [score test day] – [average of 3-day run-in period]) for sourdough fermented (SF) breads made with bread wheat, spelt, or emmer (study B, n=20). ΔVAS per symptom was compared between breads using the non-parametric Friedman test, with post-hoc Wilcoxon test.

No carry-over effect or order-effect was found for any of the symptoms (for all symptoms p>0.05) (Supplementary Figures S4-S5).

Overall GI symptoms (Figure 5A) were comparable between SF breads made of bread wheat (median ΔVAS 2.1mm [IQR -3.1-31.5mm]), spelt (median ΔVAS 8.5mm [IQR 0-15.3mm]), and emmer (median ΔVAS 0mm [IQR -2.9-9.3mm], p=0.144). Predominant GI symptoms were abdominal discomfort, abdominal pain, bloating, flatulence, and fullness. None of the assessed GI symptoms showed significant differences between SF bread types (Figure 5B-K). Also, none of the assessed extra-intestinal symptoms showed significant differences between SF breads (Figure 6).

Post-hoc analyses

Responders vs. non-responders

On group level, no differences in symptom scores were found between YF breads nor between SF breads. Nevertheless, we noted a wide range in symptom scores, suggesting inter-individual variation in response. To further explore this, responders were defined as participants with an increase of at least 15mm ΔVAS for overall GI symptoms, or for any of the predominant symptoms abdominal discomfort, abdominal pain, bloating, and flatulence.

For study A, the number of responders (Supplementary Table S6) was comparable between YF breads made of bread wheat (n=6), spelt (n=5), and emmer (n=7, p=0.761). Seven participants (35%) responded to one type of bread, four participants (20%) to two types of bread, and one (5%) to all three breads (Supplementary Table S7). In total, 40% of participants were considered non-responders.

For study B, the number of responders (Supplementary Table S8) was comparable between SF breads made of bread wheat (n=9), spelt (n=7), and emmer (n=8, p=0.761). Seven participants (35%) responded to one type of bread, four participants (20%) to two types of bread, and three (15%) to all three breads (Supplementary Table S9). In total, 30% of participants were considered non-responders.

Yeast vs. sourdough (n = 13)

Fourteen participants from study A volunteered to also participate in study B. One of these participants dropped out of study B after test day 1, resulting in 13 participants that completed both studies (see Figure 2).

Overall GI symptoms scores (Supplementary Figure S6A) were comparable between all YF and SF bread types (p=0.396). None of the assessed individual GI symptoms (Supplementary Figure S6B-K) or extra-intestinal symptoms (Supplementary Figure S7) showed significant differences between the six bread types. The number of responders (Supplementary Table S10) was comparable between all YF and SF breads (p=0.835). None of the participants responded to the same combination of bread types across fermentation types (Supplementary Table S11).

Discussion

The present study investigated the effects of YF and SF breads made of bread wheat, spelt, or emmer on symptoms in individuals with self-reported NCWS. NCWS was defined as symptom development within 12 hours after bread consumption, while CD and WA were ruled out. When comparing the three wheat types, we found no differences in GI and extra-intestinal symptoms between the YF nor between the SF breads. On an individual level, however, we noted that more than half of the participants responded with GI symptoms to at least one of the breads. Since all bread types contained FODMAPs, gluten, and ATIs, it was not possible to assign any of the reported symptoms to one of these components. Nevertheless, the number of responders did not different between bread types.

Breads made from bread wheat, spelt, or emmer did not result in differences in GI symptoms in our study population. Although previous studies investigated the effects of gluten[20,22-26,62-66] and/or FODMAPs[21,67-74] on symptoms in NCGS/NCWS, only a few studies compared breads made of different wheat species or using yeast or sourdough fermentation. In line with our results, the only study using yeast fermented bread wheat and spelt also found no differences between bread types in NCWS individuals.[40] In contrast, a reduction of IBS symptoms was found from intake of ancient compared to

modern durum wheat products,[47] from tritordeum-based products compared to habitual wheat-containing diet,[75] and a tritordeum-based diet was just as effective as a low-FODMAP diet.[72] We included emmer as ancient grain in the current study. Although some differences were found in total fibre and fructans content,[59] the absolute differences were rather small, and no clear benefit was found for emmer bread. However, a comparison to our study population should be done with care, as the aforementioned studies included IBS patients in whom CD was excluded, but not specifically characterised as NCWS.[47,72,75]

Our study also showed no differences in extra-intestinal symptoms between study breads. To our knowledge, this has been investigated in only one other study, showing significant improvement of fatigue when eating ancient wheat products.[47] Possibly, the longer intervention (6 weeks) was better suited to investigate extra-intestinal symptoms, which usually have a longer time until onset.[76]

The majority of previous studies on the effects of bread used different grains[77,78] or processing methods[70,79-82] to compare differences in specific compounds, usually FODMAPs or gluten, as potential trigger in NCWS. However, their joint presence in bread in varying amounts[38,44,83] hinders attributing effects of different breads to one specific compound. Additionally, growing conditions such as the location and soil type, environment, and agronomic practices also affect the composition of grain.[84] We therefore performed detailed analyses of our study breads,[59] showing effects of wheat type and processing method. The clinical relevance of observed differences is unclear, but may contribute to the large variation between symptom responses of participants to individual breads, with no single bread causing the lowest symptoms.

Exploratively, we also compared YF and SF in a subset of participants, finding no significant differences in GI symptom response. Also, these results should be interpreted with caution as the study was not designed nor powered for this direct comparison. Our findings are in line with a pilot study by Laatikainen *et al.*,[51] but they did show SF resulted in higher extra-intestinal symptom scores, which they suggest may be explained by a nocebo response. The role of the nocebo effect in NCGS was recently confirmed by a randomised, double-blind, placebo-controlled, international multicentre study designed to assess the role of expectancy on adverse reactions after gluten intake.[60] As a nocebo response may induce an order effect in crossover studies, this was checked for the current study, but not found. Nevertheless, we cannot exclude any potential influence of a nocebo effect throughout the study.

There is no consensus on the definition and diagnostic criteria of NCWS as the trigger(s) remain unclear. The only diagnostic criteria so far are the Salerno Experts' Criteria,[10] which focus on gluten and therefore may not always apply. We consider our definition of NCWS, *i.e.* symptoms after the consumption of bread, clinically relevant in the Netherlands where bread is an important staple gluten-containing food product,[85] but this may limit generalisability in other countries.

We feel that studies investigating wheat-based foods consumed "as part of a daily diet" are required to provide data that are useful for optimizing food processing, product development, and dietary recommendations. Participants consumed five slices of study bread per day, based on the Dutch healthy diet guidelines and average daily

consumption, therefore considered sufficient to induce GI symptoms and have clinical relevance.[85,86] Since we wanted to compare breads that were as similar as possible to commercially available bread and mimic the real-life situation, levels of gluten, ATIs or other components did not differ from commercially available bread. As only a few individuals responded to all different breads, this highlights the need for individualised dietary treatment. NCWS individuals in whom CD and WA have been excluded may benefit from trying different bread types.

We observed large heterogeneity in our study population in symptom response and bread type(s) triggering symptoms, which may have contributed to no significant differences on group level. However, a strength of the study was the crossover design comparing the effects within individuals, who themselves indicated to develop symptoms after consuming bread. The variation observed may also indicate a variety of biological and/or psychological factors that may contribute to symptoms in individuals. Given the fact that GI symptoms generally arise rather fast and as predominant symptoms are abdominal pain, bloating and flatulence,[19] the intestinal microbiota may be a relevant factor in symptom generation.[87]

Contrary to previous studies, our intervention only consisted of one test day. Although we may have missed symptom responses after prolonged intake, previous studies show that most NCWS individuals report symptoms within 12 hours.[19] This was also the group included in the current study. Another possible limitation of our study is the small sample size. Although this was considered sufficient based on the sample size calculation, the heterogeneity found in symptom response may require a larger number to show differences between interventions. Furthermore, this limited the interpretation of the comparison between YF and SF breads.

With a crossover design, there is always the risk of a carry-over effect, especially with longer lasting symptoms.[19] However, symptom scores did not differ between run-in periods. Furthermore, although participants adhered to a symptom-free diet throughout the study, we found some participants had higher symptom scores during run-in than on the test day. This may be due to the overlap with IBS and/or other factors, such as stress, that were not assessed in our study.

Conclusion

The majority of NCWS individuals experienced GI symptoms for at least one of the breads, but on the group level, no differences were found between different YF or SF breads. Nevertheless, these individual differences confirm the need for personalised dietary treatment of NCWS.

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Supplementary Materials & Results

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Supplementary Materials

Supplementary Table S1. Recipes and conditions used for making yeast- or sourdough fermented breads from bread wheat, spelt, or emmer, as previously described by Shewry *et al.*[59]

Supplementary Table S1A. Bread wheat, yeast fermentation.

Ingredient	**%**	**gram**
Wheat flour	100	3500
Instant Dry Yeast	0.6	21
Salt (with iodine)	1.5	52.5
Sugar	1	35
Sunflower oil	2	70
Water	80	2870

Controlled temperature/relative humidity environment, logging total proof time		
Flour temperature	23	°C
Water temperature / ice water	2	°C
Mixing	3	minutes
Rest 30 minutes, autolyse		
Mixing	3	minutes
Kneading, 2nd speed	circa 10	minutes
Energy	393	kilojoules
Finished dough time		
Dough temperature	25	°C
Dough assessment	elastic/ a bit sticky	
Bulk fermentation	45	minutes
Temperature bulk fermentation	27	°C
Scale	870	gram
Modelling	equal/ 28 cm	
Dough assessment	flexible/ liquid	
Final proof	30	°C
Time	70	minutes
Baking program WOW 10	235 / 255	°C
Total baking time	38	minutes
Cooling down	1	hours
Packaging		

Supplementary Table S1B. Spelt, yeast fermentation.

Ingredient	**%**	**gram**
Spelt flour	100	3500
Instant Dry Yeast	0.6	21
Salt (with iodine)	1.5	52.5
Sugar	1	35
Sunflower oil	2	70
Water	73	2555

Controlled temperature/relative humidity environment, logging total proof time		
Flour temperature	23	°C
Water temperature / ice water	2	°C
Mixing	3	minutes
Rest 30 minutes, autolyse		
Mixing	3	minutes
Kneading, 2nd speed	circa 10	minutes
Energy	393	kilojoules
Finished dough time		
Dough temperature	25	°C
Dough assessment	elastic/ sticky	
Bulk fermentation	45	minutes
Temperature bulk fermentation	27	°C
Scale	870	gram
Modelling	equal/ 28 cm	
Dough assessment	flexible/ liquid	
Final proof	30	°C
Time	70	minutes
Baking program WOW 10	235 / 255	°C
Total baking time	38	minutes
Cooling down	1	hours
Packaging		

Supplementary Table S1C. Emmer, yeast fermentation.

Ingredient	**%**	**gram**
Emmer flour	100	3500
Instant Dry Yeast	0.8	28
Salt (with iodine)	1.5	52.5
Sugar	1	35
Sunflower oil	2	70
Water	70	2450

Controlled temperature/relative humidity environment, logging total proof time		
Flour temperature	23	°C
Water temperature / ice water	2	°C
Mixing	3	minutes
Rest 30 minutes, autolyse		
Mixing	3	minutes
Kneading, 2nd speed	circa 10	minutes
Energy	377	kilojoules
Finished dough time		
Dough temperature	25	°C
Dough assessment	elastic/ liquid	
Bulk fermentation	45	minutes
Temperature bulk fermentation	27	°C
Scale	900	gram
Modelling	equal/ 28 cm	
Dough assessment	flexible/ very liquid	
Final proof	30	°C
Time	70	minutes
Baking program WOW 10	235 / 255	°C
Total baking time	37	minutes
Cooling down	1	hours
Packaging		

Supplementary Table S1D. Bread wheat, sourdough fermentation.

Ingredient	**%**	**gram**	
Wheat flour	100	3500	(calculating 3850)
Wheat sourdough	20	700	(50%/50% Flour/Water)
Salt (with iodine)	1.5	57.8	
Sugar	1	38.5	
Sunflower oil	2	77	
Water	72.7	2450	Note 73% total: Flour 3500+350=3850 / Water 2450+350=2800

Controlled temperature/relative humidity environment, logging total proof time		
Flour temperature	23	°C
Sourdough temperature	25	°C
Water temperature	6-8	°C
Mixing	1	minute
Kneading, only slow speed	15	minute
Energy	421.09	kilojoules
Dough temperature	26	°C
Dough assessment	elastic/ flexible	
Bulk fermentation	15	hours
Temperature bulk fermentation	5	°C
Controlled Dough Climate	4	hours
Dough acclimatisation until	16	°C
Scale	900	gram
Modelling	equal/ 28 cm	
Dough assessment	flexible/ tension	
Final proof	27	°C
Time	4	hours
Baking program WOW 11 SD	235 / 255	°C
after 10 minutes	225 / 235	°C
Total baking time	37	minutes
Cooling down	1	hours
Packaging		

Supplementary Table S1E. Spelt, sourdough fermentation.

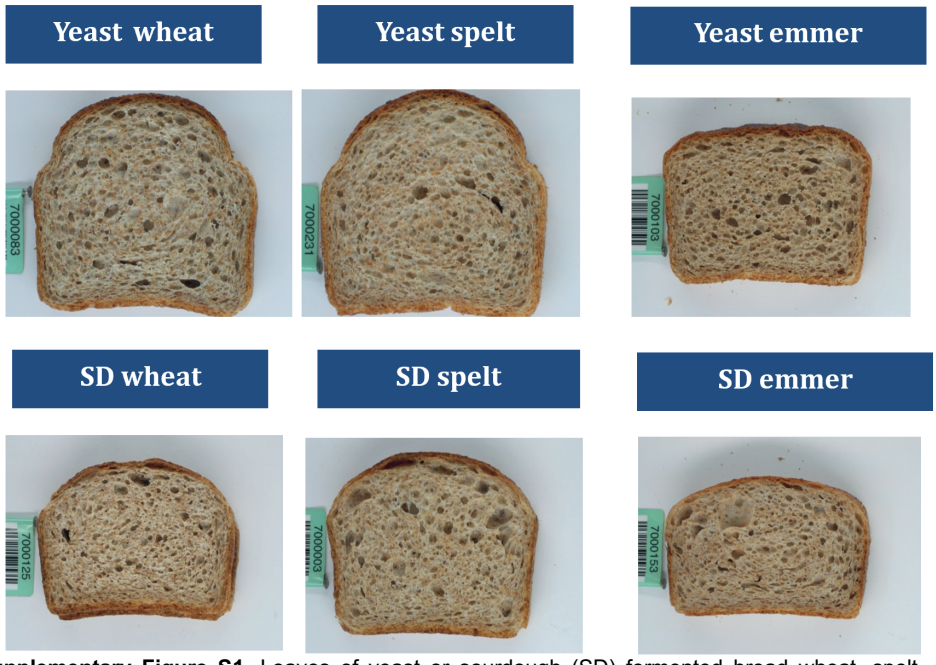
Ingredient	**%**	**gram**	
Spelt flour	100	3500	(calculating 3850)
Spelt sourdough	20	700	(50%/50% Flour/Water)
Salt (with iodine)	1.5	57.8	
Sugar	1	38.5	
Sunflower oil	2	77	
Water	72,7	2450	Note 73% total: Flour 3500+350=3850 / Water 2450+350=2800

Controlled temperature/relative humidity environment, logging total proof time		
Flour temperature	23	°C
Sourdough temperature	25	°C
Water temperature	6-8	°C
Mixing	1	minute
Kneading, only slow speed	15	minute
Energy	421.09	kilojoules
Dough temperature	26	°C
Dough assessment	elastic/ flexible	
Bulk fermentation	15	hours
Temperature bulk fermentation	5	°C
Controlled Dough Climate	4	hours
Dough acclimatisation until	16	°C
Scale	900	gram
Modelling	equal/ 28 cm	
Dough assessment	flexible/ tension	
Final proof	27	°C
Time	4	hours
Baking program WOW 11 SD	235 / 255	°C
after 10 minutes	225 / 235	°C
Total baking time	37	minutes
Cooling down	1	hours
Packaging		

Supplementary Table S1F. Emmer, sourdough fermentation.

Ingredient	**%**	**gram**	
Emmer flour	100	3500	(calculating 3850)
Emmer sourdough	20	700	(50%/50% Flour/Water)
Salt (with iodine)	1.5	57.8	
Sugar	1	38.5	
Sunflower oil	2	77	
Water	72.7	2450	Note 73% total: Flour 3500+350=3850 / Water 2450+350=2800

Controlled temperature/relative humidity environment, logging total proof time		
Flour temperature	23	°C
Sourdough temperature	25	°C
Water temperature	6-8	°C
Mixing	1	minute
Kneading, only slow speed	15	minute
Energy	419	kilojoules
Dough temperature	26	°C
Dough assessment	elastic/ flexible	
Bulk fermentation	15	hours
Temperature bulk fermentation	5	°C
Controlled Dough Climate	4	hours
Dough acclimatisation until	16	°C
Scale	900	gram
Modelling	equal/ 28 cm	
Dough assessment	flexible/ tension	
Final proof	27	°C
Time	4	hours
Baking program WOW 11 SD	235 / 255	°C
after 10 minutes	225 / 235	°C
Total baking time	37	minutes
Cooling down	1	hours
Packaging		



Supplementary Figure S1. Loaves of yeast or sourdough (SD) fermented bread wheat, spelt, or emmer.

Supplementary Table S2. Energy, macro- and micronutrient composition of study breads per 100g fresh weight.

	Yeast fermented bread			Sourdough fermented bread		
Component	**Wheat**	**Spelt**	**Emmer**	**Wheat**	**Spelt**	**Emmer**
Energy (KJ)	878	822	929	960	966	1051
Energy (kcal)	209	196	221	229	227	250
Carbohydrates (g)	38	35	37	42	41	42
Sugar (g)	2.9	1.8	3.7	3.2	2.0	4.1
Protein (g)	8.6	10	9.1	9.3	11.4	10.1
Lipids (g)	2.7	2.5	2.3	2.9	2.9	2.6
Dietary fibre (g)	5.0	4.4	3.4	5.4	4.9	3.8
Sodium (mg)	363	372	359	400	429	404
Salt (g)	0.9	0.9	0.9	1.0	1.1	1.0
Potassium (mg)	254	219	223	280	253	251
Calcium (mg)	22	20	18	24	24	21
Iron (mg)	1.9	2.6	2.5	2.1	3.0	2.8
Magnesium (mg)	73	79	85	81	91	96
Copper (mg)	0.2	0.3	0.2	0.2	0.3	0.2
Zinc (mg)	1.8	2.5	2.7	2.0	2.8	3.1

The composition was determined by Nutrilab B.V. (Giessen, the Netherlands) using Association of Official Agricultural Chemists (AOAC) method 991.43. KJ = kilojoules; kcal = kilocalories; g = gram; mg = milligram.

Supplementary Table S3. Amount of fibre components and fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) (means and standard deviation) in the breads calculated from the analyses in Shewry *et al.*[59]

		Yeast fermented bread			Sourdough fermented bread		
	Component	**Wheat**	**Spelt**	**Emmer**	**Wheat**	**Spelt**	**Emmer**
Fibre	Arabinoxylan	3.96	3.43	2.46	3.87	3.30	2.48
	(% dry wt.)	0.479	0.095	0.028	0.325	0.065	0.012
Fibre	B-glucan	1.94	2.44	1.94	1.74	2.05	1.55
	(arbitrary units)	0.055	0.153	0.064	0.105	0.178	0.120
Fibre/	Fructans (F)	0.41	0.27	0.14	0.57	0.34	0.25
FODMAP	(% dry wt.)	0.013	0.025	0.023	0.034	0.020	0.022
	(mg/g 40% W)	2.46	1.62	0.84	3.42	2.04	1.5
FODMAP	Mannitol (M)	21.149	24.468	22.742	42.260	45.371	45.127
	(mg/g dry wt.)	0.247	0.429	1.257	0.840	1.000	1.278
FODMAP	Raffinose (R)	2.986	3.013	3.483	3.202	4.014	3.830
	(mg/g dry wt.)	0.066	0.020	0.234	0.289	0.256	0.463
FODMAP	Glycerol (G)	12.959	13.946	17.667	10.856	10.807	16.724
	(mg/g dry wt.)	0.234	0.322	0.421	1.202	0.308	1.562
Fibre/	F+M+R+G	24.82	26.47	27.19	37.33	38.15	40.91
FODMAP	(mg/g 40% W)						

wt = weight; g = gram; mg = milligram; mg/g 40% W = mg/g in bread with 40% water.

Methods proteomics analysis

Bread samples were freeze-dried, ground to powder and aliquoted.

50 milligram of powder was extracted with 1ml of 8M urea, 50mM Tris-HCl pH 7.2, 10mM DTT and 5mM TCEP (tri-chloro-ethyl-phosphine) using vortexing and waterbath sonicator.
Extracts were performed in five-fold replicates.

After centrifugation, the supernatant was collected. Protein concentration was assayed using Bradford assay. An aliquout of 50 μg of protein per replicate was incubated with 20mM iodo-acetamide in water (during 30 minutes at RT), and subsequently diluted with 5 volumes of mQ water to 1.5M urea. Digestion was performed by adding 1 μg of chymotrypsin (ThermoFisher/ Pierce) and incubated at 37°C overnight. Resulting peptides were isolated by SPE (solid-phase extraction) using OASIS HLB microplates (Waters inc., Milford, USA) according to manufacturer instructions. Peptides were dried and redissolved in 0.1% formic acid.

Peptide samples were injected onto a C18 HSS column (Waters inc., Milford, USA) (Dionex UPLC, ThermoFisher, Palo Alto, USA) and directly eluted into a Qexactive Plus high-resolution mass spectrometer. Peptide ions were detected using a standard DDA Top10 detection method. Raw data were processed using FragPipe workflow (FragPipe version 16.0, MSFragger version 3.3, Philosopher version 4.0.0 (Peptide- and ProteinProphet)). The search space for identification was the concatenated list of proteins entries in Uniprot from the taxons 4565 (*Triticum aestivum*, bread wheat), 85692 (*Triticum dicoccoides*, wild emmer) and 58933 (*Triticum spelta*, spelt) downloaded at 3rd September 2021.

Combined peptide table was processed with custom made script (in R) to aggregate peptide intensity values at protein class level. Protein classes were manually appended to individual protein entries, based on the description and gene ontology (GO) information from UniProt of the respective protein entries. Protein classes “Gliadin", "Glutenin", "Globulin", "ATI", "Protease inhibitor" and "amylase" were specifically selected for this study. Peptide intensities were summed per protein class, standard deviation and variance coefficient were calculated over the 5 replicate values per sample type, and subsequently averaged at protein class aggregation level.

Supplementary Table S4. The summed signal intensities of all peptide peaks that were grouped at protein class level with the averaged standard deviation (SD) and coefficient (Coeff) of Variance calculated per peptide averaged over 5 replicates per sample condition.

Protein	Bread type		Summed Intensity	Intensity SD	Coeff Variance	Sum Count of Peptides
Amylase	BW	YF	1.30B	26.59M	0.02	48.00
Amylase	BW	SF	1.19B	44.23M	0.04	47.80
Amylase	S	YF	1.73B	42.38M	0.02	46.00
Amylase	S	SF	1.53B	71.25M	0.05	47.00
Amylase	E	YF	1.75B	52.81M	0.03	47.00
Amylase	E	SF	1.45B	54.35M	0.04	47.80
ATI	BW	YF	5.96B	17.82M	0.02	118.80
ATI	BW	SF	6.15B	18.43M	0.03	120.20
ATI	S	YF	6.08B	23.71M	0.05	118.00
ATI	S	SF	6.12B	33.95M	0.06	114.20
ATI	E	YF	6.34B	18.66M	0.04	119.40
ATI	E	SF	6.85B	21.00M	0.04	112.80
Gliadin	BW	YF	5.67B	67.70M	0.04	125.80
Gliadin	BW	SF	5.95B	86.65M	0.06	123.40
Gliadin	S	YF	6.74B	69.08M	0.12	126.00
Gliadin	S	SF	7.05B	122.50M	0.07	123.80
Gliadin	E	YF	6.44B	78.49M	0.05	124.80
Gliadin	E	SF	6.79B	68.21M	0.07	123.20
Globulin	BW	YF	1.94B	27.29M	0.04	117.60
Globulin	BW	SF	1.74B	41.41M	0.06	113.20
Globulin	S	YF	1.88B	23.00M	0.04	112.20
Globulin	S	SF	1.61B	21.21M	0.04	104.80
Globulin	E	YF	3.40B	22.68M	0.02	128.20
Globulin	E	SF	2.71B	36.29M	0.04	119.60
Glutenin	BW	YF	12.89B	158.56M	0.03	132.00
Glutenin	BW	SF	13.40B	141.75M	0.02	133.00
Glutenin	S	YF	10.45B	233.52M	0.04	158.20
Glutenin	S	SF	10.93B	511.36M	0.07	156.20
Glutenin	E	YF	9.36B	283.53M	0.05	150.00
Glutenin	E	SF	10.23B	269.91M	0.04	147.80
Protease inhibitor	BW	YF	1.47B	15.80M	0.08	140.80
Protease inhibitor	BW	SF	1.67B	6.33M	0.05	144.60
Protease inhibitor	S	YF	1.18B	15.05M	0.09	119.80
Protease inhibitor	S	SF	1.38B	13.89M	0.12	120.40
Protease inhibitor	E	YF	1.01B	9.86M	0.10	129.60
Protease inhibitor	E	SF	1.15B	7.94M	0.10	123.80

The total number of peptides per protein class was summed and averaged over the 5 replicates per sample. BW = bread wheat, S = spelt, E = emmer; YF = yeast fermented; SF = sourdough fermented; B = billion *i.e.* 10E9, M = million *i.e.* 10E6.

Supplementary Results

Comparing nutrient composition of the different bread types

Energy, macro- and micronutrient composition of study breads are given in Supplementary Table S2. Energy and carbohydrate content are generally (about 10%) higher in all sourdough fermented (SF) breads as compared to yeast fermented (YF) breads. These values are considered to be related. Further, Sodium, Potassium and Magnesium are also (overall about 10%) higher in SF breads. The data for the other micronutrients are similar in all bread types.

The amounts of fibre components and FODMAPs in the breads have been calculated from the analyses in Shewry *et al.*[59] and summarized in Supplementary Table S3. With regard to the fibre compounds, arabinoxylan is highest in YF and SF wheat bread, about 15% lower in spelt bread and remarkably almost 40% lower in emmer bread. Beta-glucans are ~25% higher in YF and SF spelt bread than in bread wheat bread and emmer bread. Fructans are highest in YF and SF bread wheat bread and one-third to half in YF and SD emmer bread, respectively. Regarding FODMAPs, mannitol is generally about two times higher in all SF breads, also raffinose tends to be higher in all SF breads. Glycerol is highest in YF and SF emmer bread.

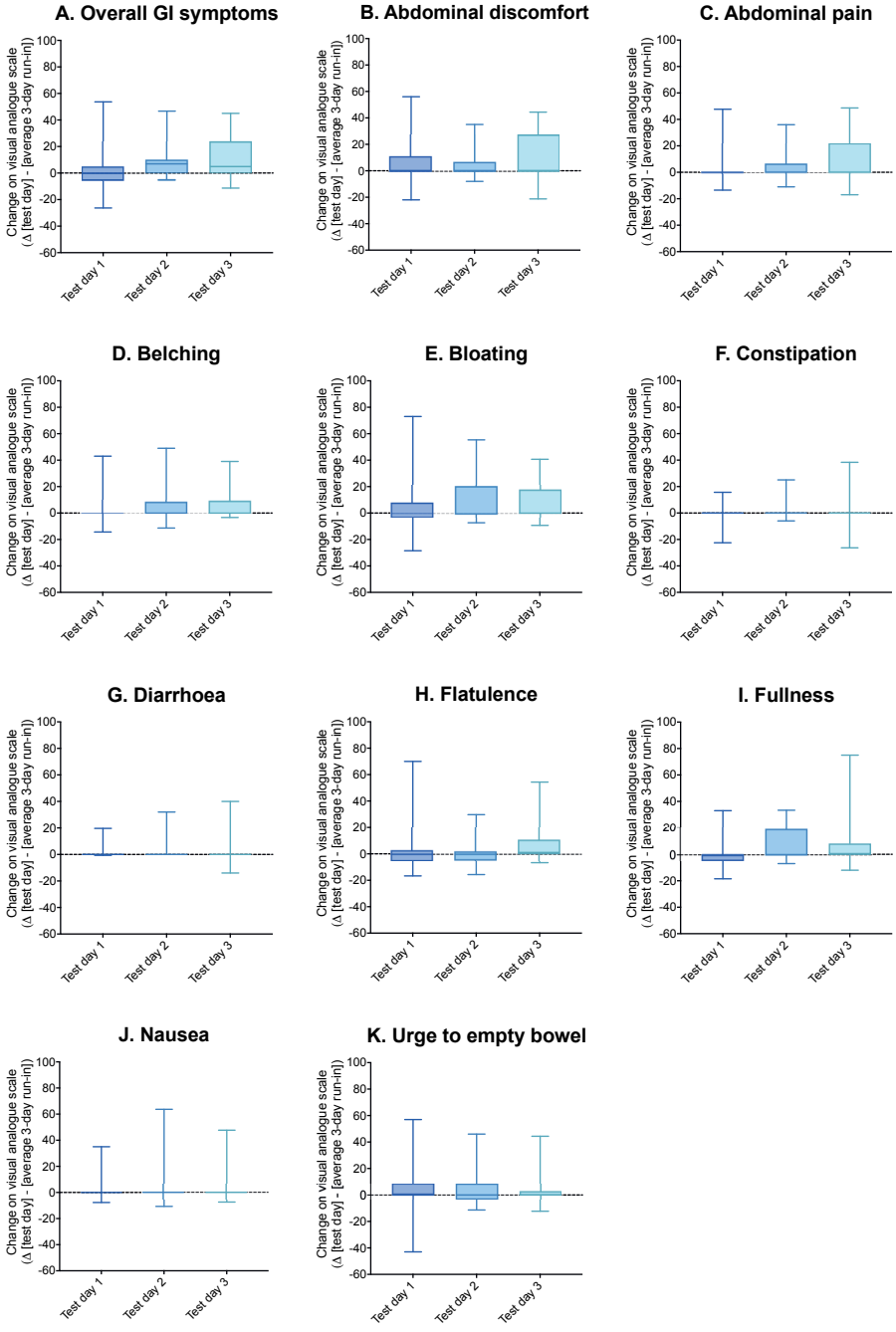
Of the proteins (Supplementary Table S4), the amounts of amylase, ATI and gliadin were similar in all bread types. Glutenin was about 30% higher in bread wheat bread, globulins were higher in emmer bread (almost double in its YF bread), and protease inhibitor was equally high in YF and SF bread wheat bread and lowest in both types of emmer bread.

Baseline characteristics

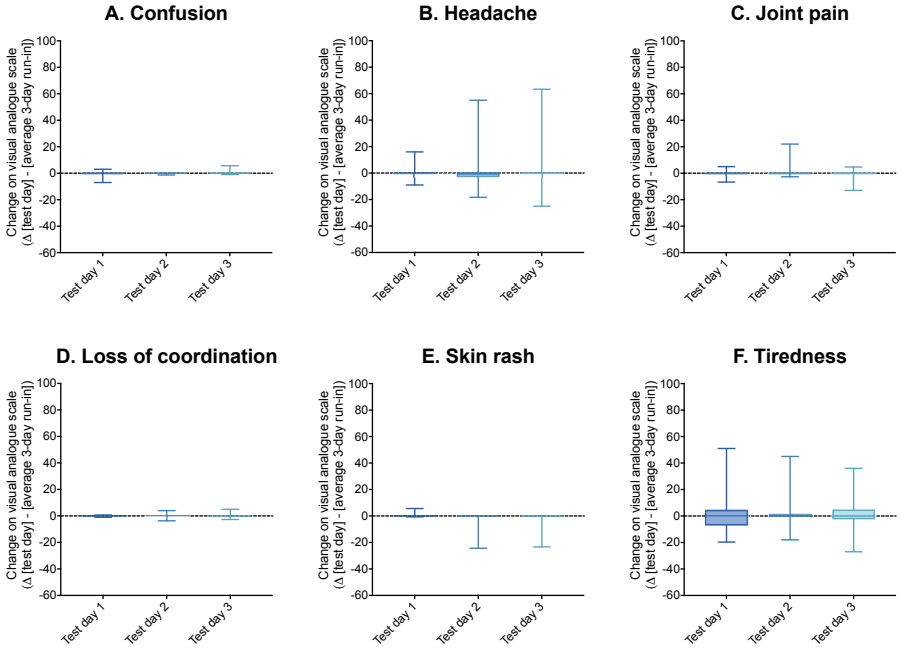
Supplementary Table S5. Use of medication and nutritional supplements, as reported during the screening visit, for study A (yeast fermented bread) and study B (sourdough fermented bread).

	Study A (n = 20)	**Study B** (n = 20)
Medication		
None	13 (65%)	12 (60%)
Antihistamine	0 (0%)	1 (5%)
Antihypertensive	2 (10%)	1 (5%)
Inhaled steroids	0 (0%)	1 (5%)
Insulin	1 (5%)	0 (0%)
Laxatives	1 (5%)	0 (0%)
Mucosal protective agent	0 (0%)	1 (5%)
Oestrogen hormones	0 (0%)	1 (5%)
Oral contraceptives	1 (5%)	2 (10%)
Proton pump inhibitors	3 (15%)	2 (10%)
Spasmolytics	0 (0%)	1 (5%)
SSRI	0 (0%)	1 (5%)
Thyroid hormones	2 (10%)	0 (0%)
Other	1 (5%)	1 (5%)
Nutritional supplements		
None	12 (60%)	13 (65%)
Fibres	0 (0%)	2 (10%)
Iron	1 (5%)	0 (0%)
Minerals	4 (20%)	2 (10%)
Multivitamin	1 (5%)	1 (5%)
Omega 3	3 (15%)	1 (5%)
Vitamin B12	3 (15%)	3 (15%)
Vitamin C	3 (15%)	2 (10%)
Vitamin D	5 (25%)	4 (20%)
Vitamin - other	1 (5%)	1 (5%)
Other	5 (20%)	2 (10%)

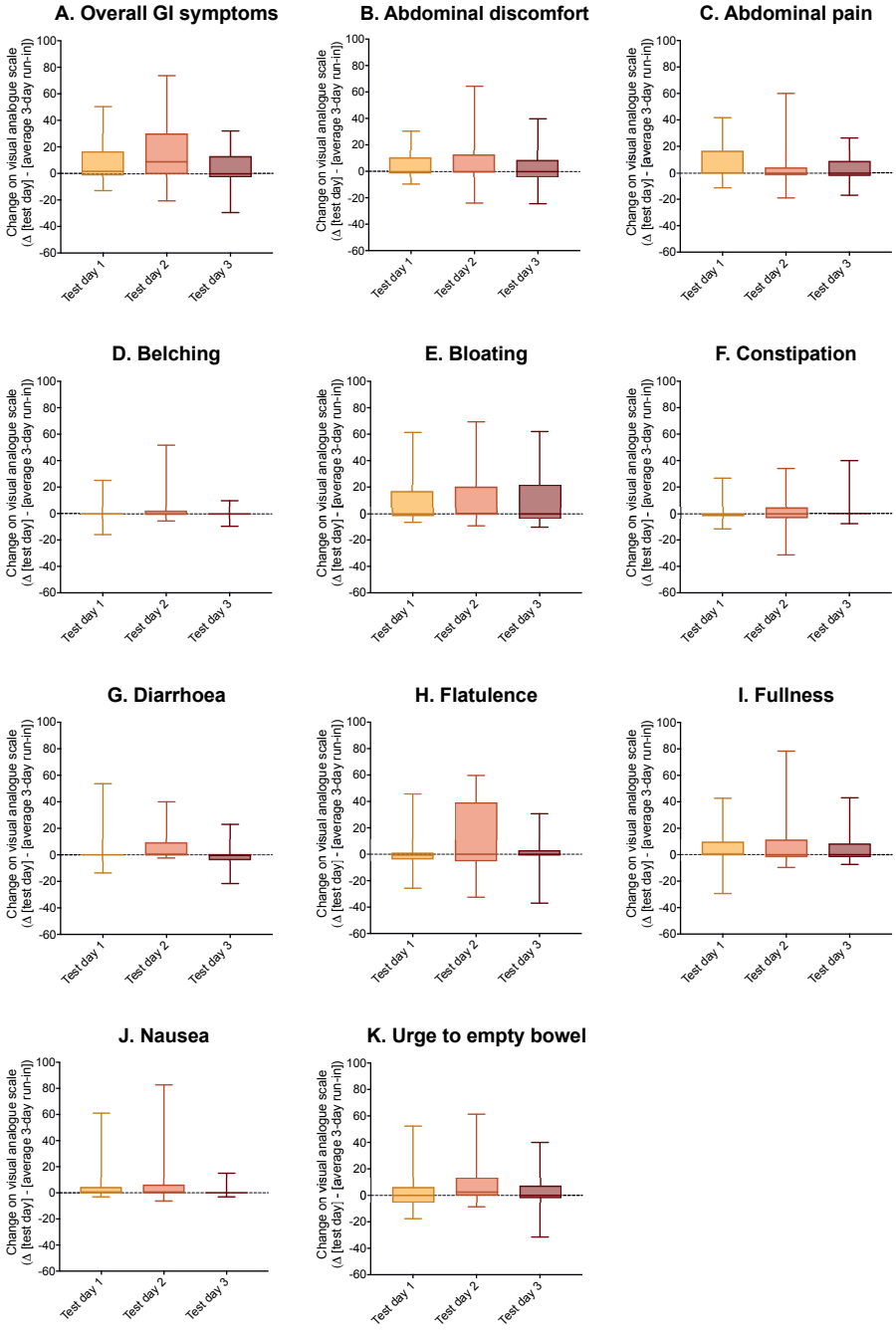
Order effect analyses



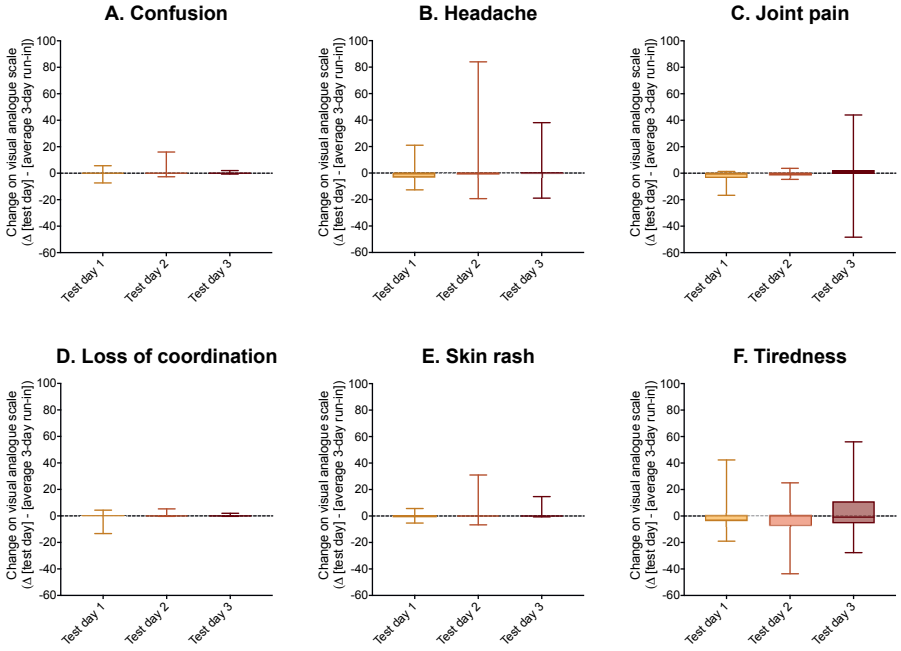
Supplementary Figure S1. Gastrointestinal (GI) symptom scores, displayed as change on visual analogue scale (ΔVAS = [score test day] – [average of 3-day run-in period]) for yeast fermented breads per test day, irrespective of grain type (study A, n=20). ΔVAS per symptom was compared between breads using the non-parametric Friedman test, with post-hoc Wilcoxon test.



Supplementary Figure S2. Extra-intestinal symptom scores, displayed as change on visual analogue scale (ΔVAS = [score test day] – [average of 3-day run-in period]) for yeast fermented breads per test day, irrespective of grain type (study A, n=20). ΔVAS per symptom was compared between breads using the non-parametric Friedman test, with post-hoc Wilcoxon test.



Supplementary Figure S3. Gastrointestinal (GI) symptom scores, displayed as change on visual analogue scale (ΔVAS = [score test day] – [average of 3-day run-in period]) for sourdough fermented breads per test day, irrespective of grain type (study B, n=20). ΔVAS per symptom was compared between breads using the non-parametric Friedman test, with post-hoc Wilcoxon test.



Supplementary Figure S4. Extra-intestinal symptom scores, displayed as change on visual analogue scale (ΔVAS = [score test day] – [average of 3-day run-in period]) for sourdough fermented breads per test day, irrespective of grain type (study B, n=20). ΔVAS per symptom was compared between breads using the non-parametric Friedman test, with post-hoc Wilcoxon test.

Post-hoc analyses

Supplementary Table S6. Responders vs. non-responders for study A (yeast fermented breads, n=20).

+ 15mm for…	Wheat	Spelt	Emmer	p-value
Overall GI symptoms	6	4	6	0.695
Abdominal discomfort	5	4	5	0.895
Abdominal pain	4	2	4	0.565
Bloating	3	5	5	0.641
Flatulence	2	2	3	0.819
Total number of responders	6	5	7	0.761

Definition of responder: +15 mm on visual analogue scale compared to 3-day run-in period for overall GI symptoms, and/or predominant symptoms abdominal discomfort, abdominal pain, bloating or flatulence. The number of responders for each bread was compared by Cochran's Q test with post-hoc McNemar test.

Supplementary Table S7. Number of responders per (combination of) bread type(s) for study A (yeast fermented breads, n=20).

	Study A
None	8
Wheat only	3
Spelt only	1
Emmer only	3
Wheat + spelt	1
Wheat + emmer	1
Spelt + emmer	2
Wheat + spelt + emmer	1

Definition of responder: +15 mm on visual analogue scale compared to 3-day run-in period for overall GI symptoms, and/or predominant symptoms abdominal discomfort, abdominal pain, bloating or flatulence.

Supplementary Table S8. Responders vs. non-responders for study B (sourdough fermented breads, n=20).

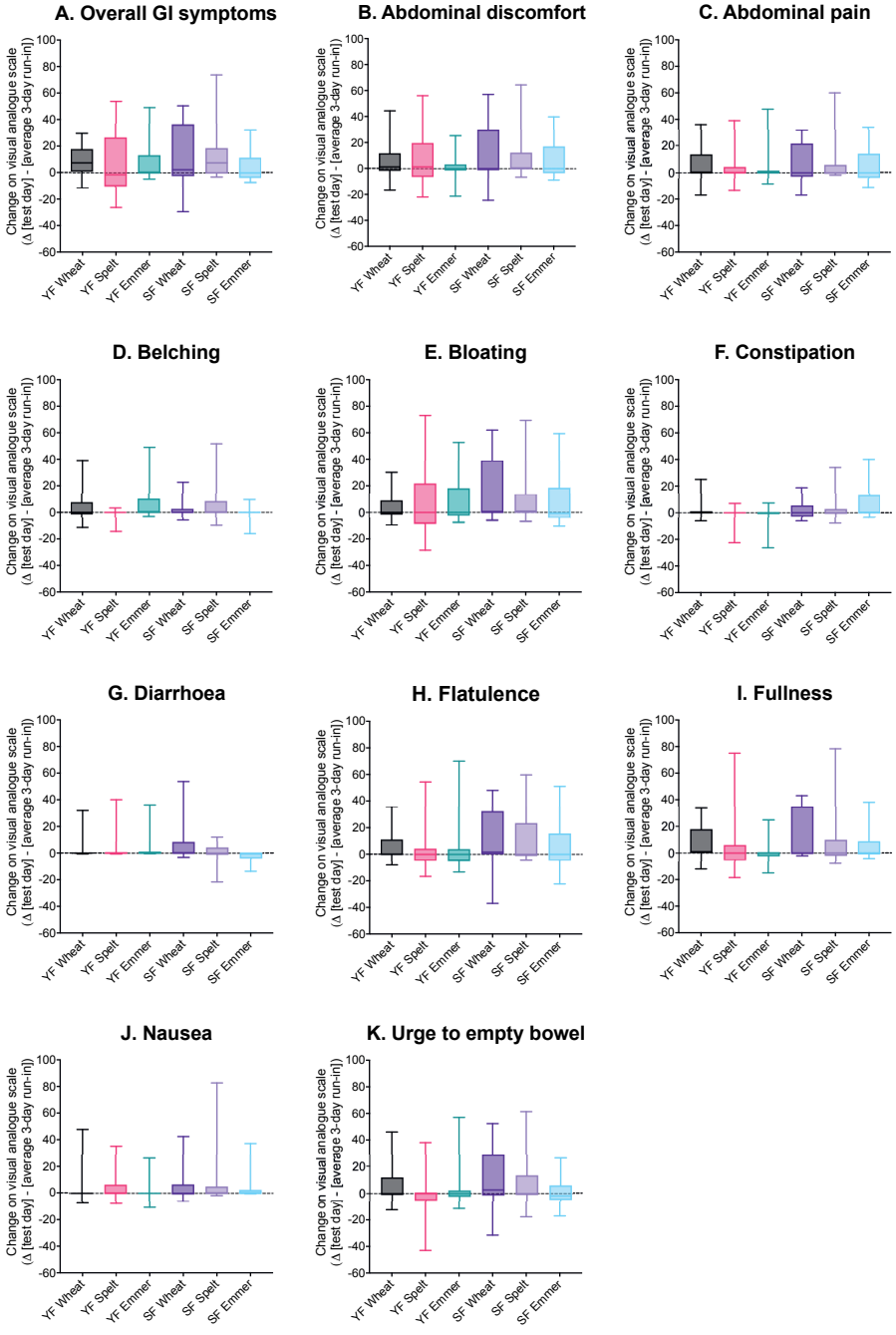
+ 15mm for…	Wheat	Spelt	Emmer	p-value
Overall GI symptoms	7	5	4	0.417
Abdominal discomfort	5	2	3	0.174
Abdominal pain	7	2	4	0.042
Bloating	7	4	4	0.276
Flatulence	5	4	4	0.867
Total number of responders	9	7	8	0.761

Definition of responder: +15 mm on visual analogue scale compared to 3-day run-in period for overall GI symptoms, and/or predominant symptoms abdominal discomfort, abdominal pain, bloating or flatulence. The number of responders for each bread was compared by Cochran's Q test with post-hoc McNemar test.

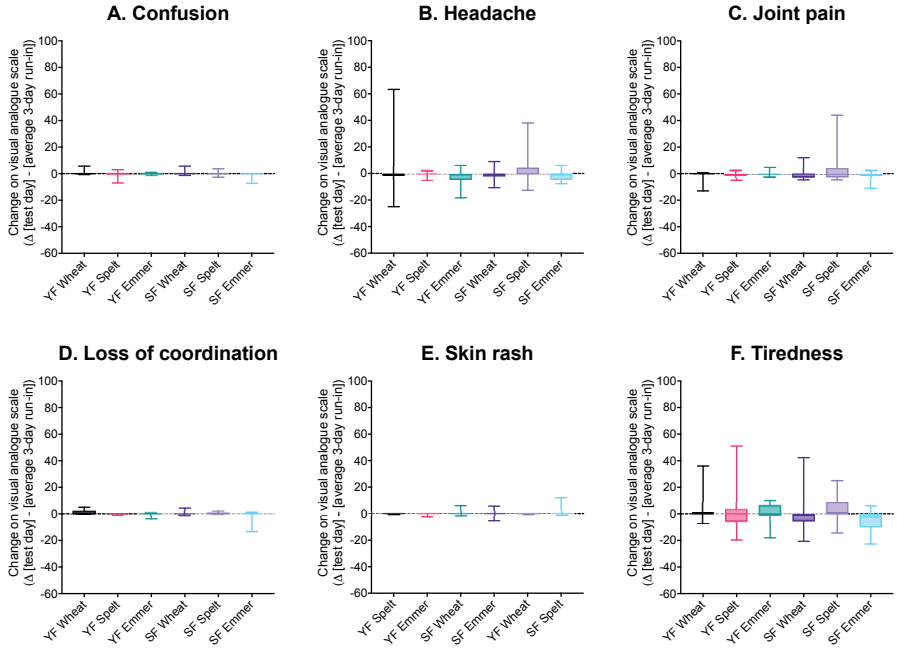
Supplementary Table S9. Number of responders (+15mm) per (combination of) bread type(s) for study B (sourdough fermented breads, n=20).

	Study B
None	6
Wheat only	3
Spelt only	1
Emmer only	3
Wheat + spelt	2
Wheat + emmer	1
Spelt + emmer	1
Wheat + spelt + emmer	3

Definition of responder: +15 mm on visual analogue scale compared to 3-day run-in period for overall GI symptoms, and/or predominant symptoms abdominal discomfort, abdominal pain, bloating or flatulence.



Supplementary Figure S6. Gastrointestinal (GI) symptom scores, displayed as change on visual analogue scale (ΔVAS = [score test day] – [average of 3-day run-in period]) for yeast fermented (YF) and sourdough fermented (SF) breads made with bread wheat, spelt, or emmer (Participants that completed both study A & B, n=13). ΔVAS per symptom was compared between breads using the non-parametric Friedman test, with post-hoc Wilcoxon test.



Supplementary Figure S7. Extra-intestinal symptom scores, displayed as change on visual analogue scale (ΔVAS = [score test day] – [average of 3-day run-in period]) for yeast fermented (YF) and sourdough fermented (SF) breads made with bread wheat, spelt, or emmer (Participants that completed both study A & B, n=13). ΔVAS per symptom was compared between breads using the non-parametric Friedman test, with post-hoc Wilcoxon test.

Supplementary Table S10. Responders vs. non-responders for participants that completed both study A (yeast fermented breads) and study B (sourdough fermented breads) (n=13).

	Yeast fermented			Sourdough fermented			
+ 15mm for…	**Wheat**	**Spelt**	**Emmer**	**Wheat**	**Spelt**	**Emmer**	**p-value**
Overall GI symptoms	4	3	3	5	4	3	0.900
Abdominal discomfort	3	3	2	4	2	3	0.807
Abdominal pain	3	1	1	4	2	3	0.296
Bloating	1	4	3	5	3	3	0.317
Flatulence	2	2	2	4	4	3	0.666
Total number of responders	4	4	4	6	6	5	0.835

Definition of responder: +15 mm on visual analogue scale compared to run-in for overall GI symptoms, and/or predominant symptoms abdominal discomfort, abdominal pain, bloating or flatulence. The number of responders for each bread was compared by Cochran's Q test with post-hoc McNemar test.

Supplementary Table S11. Number of responders (+15mm) per (combination of) bread type(s) (indicated with "x") for participants that completed both study A (yeast fermented (YF) breads) and study B (sourdough fermented (SF) breads) (n=13).

Number of breads	**Yeast fermented**			**Sourdough fermented**			**Responders (n)**
	Wheat	**Spelt**	**Emmer**	**Wheat**	**Spelt**	**Emmer**	
0							2
1	x						1
				x			1
					x		1
						x	1
2	x					x	1
		x		x			1
3			x	x	x		1
			x		x	x	1
5	x	x	x	x	x		1
	x	x		x	x	x	1
		x	x	x	x	x	1

Definition of responder: +15 mm on visual analogue scale compared to run-in for overall GI symptoms, and/or predominant symptoms abdominal discomfort, abdominal pain, bloating or flatulence.

CHAPTER 6

The effect of expectancy versus actual gluten intake on gastrointestinal and extra-intestinal symptoms in non-coeliac gluten sensitivity: a randomised, double-blind, placebo-controlled, international, multicentre study

Marlijne C.G. de Graaf, Clare L. Lawton, Fiona Croden, Agnieszka Smolinska, Bjorn Winkens, Martine A.M. Hesselink, Gonny van Rooy, Peter L. Weegels, Peter R. Shewry, Lesley A. Houghton, Ben J.M. Witteman, Daniel Keszthelyi, Fred J.P.H. Brouns, Louise Dye[$], Daisy M.A.E. Jonkers[$]

[$] Shared last author

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Abstract

Background: Many individuals without coeliac disease or wheat allergy reduce their gluten intake because they believe that gluten causes their gastrointestinal symptoms. Symptoms could be affected by negative expectancy. Therefore, we aimed to investigate the effects of expectancy versus actual gluten intake on symptoms in people with non-coeliac gluten sensitivity (NCGS).

Methods: This randomised, double-blind, placebo-controlled, international, multicentre study was done at the University of Leeds (Leeds, UK), Maastricht University (Maastricht, the Netherlands), and Wageningen University and Research (Wageningen, the Netherlands). People aged 18-70 years with self-reported NCGS (*i.e.*, gastrointestinal symptoms within 8 h of gluten consumption) without coeliac disease and wheat allergy were recruited. Participants had to follow a gluten-free or gluten-restricted diet for at least 1 week before (and throughout) study participation and had to be asymptomatic or mildly symptomatic (overall gastrointestinal symptom score ≤30 mm on the Visual Analogue Scale [VAS]) while on the diet. Participants were randomly assigned (1:1:1:1; blocks of eight; stratified by site and gender) to one of four groups based on the expectation to consume gluten-containing (E+) or gluten-free (E-) oat bread for breakfast and lunch (two slices each) and actual intake of gluten-containing (G+) or gluten-free (G-) oat bread. Participants, investigators, and those assessing outcomes were masked to the actual gluten assignment, and participants were also masked to the expectancy part of the study. The primary outcome was overall gastrointestinal symptom score on the VAS, which was measured at and corrected for baseline (before breakfast) and hourly for 8 h, with lunch served after 4 h, and analysed per-protocol. Safety analysis included all participants incorporated in the per-protocol analysis. The study is registered at ClinicalTrials.gov, NCT05779358, and has ended.

Findings: Between Oct 19, 2018, and Feb 14, 2022, 165 people were screened and 84 were randomly assigned to E+G+ (n=21), E+G- (n=21), E-G+ (n=20), or E-G- (n=22). One person in the E+G+ group was excluded due to not following test day instructions, leaving 83 participants in the per-protocol analysis. Median age was 27.0 years (IQR 21.0-45.0), 71 (86%) of 83 people were women, and 12 (14%) were men. Mean overall gastrointestinal symptom score was significantly higher for E+G+ (16.6 mm [95% CI 13.1 to 20.0]) than for E-G+ (6.9 mm [3.5 to 10.4]; difference 9.6 mm [95% CI 3.0 to 16.2], p=0.0010) and E-G- (7.4 mm [4.2 to 10.7]; difference 9.1 mm [2.7 to 15.6], p=0.0016), but not for E+G- (11.7 mm [8.3 to 15.1]; difference 4.9 mm [-1.7 to 11.5], p=0.28). There was no difference between E+G- and E-G+ (difference 4.7 mm [-1.8 to 11.3], p=0.33), E+G- and E-G- (difference 4.2 mm [-2.2 to 10.7], p=0.47), and E-G+ and E-G- (difference -0.5 mm [-7.0 to 5.9], p=1.0). Adverse events were reported by two participants in the E+G- group (itching jaw [n=1]; feeling lightheaded and stomach rumbling [n=1]) and one participant in the E-G+ group (vomiting).

Interpretation: The combination of expectancy and actual gluten intake had the largest effect on gastrointestinal symptoms, reflecting a nocebo effect, although an additional effect of gluten cannot be ruled out. Our results necessitate further research into the possible involvement of the gut-brain interaction in NCGS.

Research in context

Evidence before this study

We searched PubMed for randomised controlled trials, systematic reviews, and meta-analyses published in English from database inception to May 31, 2023, using the search terms (“non-celiac gluten sensitivity” OR “non-coeliac gluten sensitivity” OR “nonceliac gluten sensitivity” OR “noncoeliac gluten sensitivity” OR “NCGS”) AND (“nocebo” OR “expectancy” OR “expectation” OR “perception”) AND (“randomized controlled trials” OR “systematic review” OR “meta-analysis”). This search yielded one narrative review from 2019, which concluded that a large nocebo effect had been found in some studies. Central to this conclusion was the pooled analysis of all double-blind, placebo-controlled, gluten-challenge studies done in people with non-coeliac gluten sensitivity (NCGS) up to March 31, 2016, which showed a nocebo response in 94 (41%) of 231 participants. Additionally, we searched PubMed for systematic reviews and meta-analyses on NCGS in general. The most recent systematic review available in English, including all articles published between Jan 1, 1976 and June 1, 2020, concluded that the vast majority of studies reported a predominant nocebo effect, which the authors considered intrinsically related to the double-blind, placebo-controlled design. Moreover, the authors asserted that the carryover and order effects found in previous studies were strictly connected to the psychological background of the study participants and that these characteristics should be considered in all double-blind, placebo-controlled studies. We found no studies specifically designed to investigate the role of the nocebo effect in NCGS.

Added value of this study

To our knowledge, this study is the first to investigate the role of the nocebo effect in people with NCGS. Our randomised, double-blind, placebo-controlled, international, multicentre study showed that the combination of expectancy to receive gluten and actual gluten intake resulted in the highest scores for overall gastrointestinal symptoms, abdominal discomfort, and bloating. Repeated gluten exposure further accentuated differences between the intervention groups. We found no significant effect of actual gluten intake within each expectancy group. Although an additional effect of gluten could not be ruled out, our findings indicate that the nocebo effect has an important role in symptom occurrence in people with NCGS.

Implications of all the available evidence

To our knowledge, this study is the first to explicitly manipulate gluten expectancy and confirm the nocebo effect in people with NCGS, consistent with previous research. Our results point towards possible involvement of the gut-brain interaction in symptom occurrence in NCGS, warranting further research. However, as we could not rule out an effect of gluten, these findings also highlight the need to elicit possible biological mechanisms underlying gluten-related symptoms.

Introduction

Wheat is one of the most important staple foods consumed in the Western world. Whole-grain wheat products are an important source of carbohydrates, dietary fibres, proteins, vitamins, minerals, and phytochemicals, and globally provide a major contribution to daily energy intake and a healthy diet.[1] Based on epidemiological evidence, the consumption of whole-grain cereal foods has been associated with several beneficial health effects, including a reduced risk of obesity, type 2 diabetes, cardiovascular disease, cancer, and overall and cause-specific mortality.[2-5]

However, wheat products can also elicit adverse (immune-mediated) effects, such as in coeliac disease and wheat allergy. In addition, a proportion of the general population now avoid or have reduced their consumption of wheat products due to self-reported symptoms following wheat intake, without having positive tests for coeliac disease or wheat allergy. Gluten proteins (gliadins and glutenins) are often attributed to be the wheat components responsible for inducing negative reactions in these people, who are then considered to have non-coeliac gluten sensitivity (NCGS).

Individuals with NCGS mostly report gastrointestinal symptoms, such as abdominal pain or discomfort, bloating, and altered stool patterns, and, to a lesser extent, extra-intestinal symptoms like tiredness and headache.[6] The estimated prevalence of NCGS in various global regions ranges from 0.6% to 13%.[7] Due to unavailability of biomarkers, diagnosis is defined by the Salerno Experts' Criteria,[6] including a double-blind, placebo-controlled gluten challenge, which is not always feasible in clinical practice.

Furthermore, previous studies have reported the presence of NCGS in 6.8-46.1% of people with irritable bowel syndrome (IBS), indicating substantial overlap between these conditions.[8] IBS is a disorder of gut-brain interaction characterised by recurrent abdominal pain and altered bowel habits and affects 5-10% of the population globally.[9] Wheat is among the top five foods reported by people with IBS to trigger their symptoms.[8]

Gluten-free diets are becoming more popular, perhaps due to perceived symptom alleviation and negative media attention about gluten.[10,11] However, gluten-free diets are associated with an increased risk of nutritional deficiencies.[12,13]

To date, little evidence is available on the role of gluten in symptom occurrence in NCGS or on the underlying mechanisms. Previous studies suggest involvement of the immune system, intestinal inflammation, gut dysbiosis, or altered intestinal barrier function, but the exact mechanism remains unclear.[14] Furthermore, the role of psychological factors cannot be ruled out. Anxiety and depression are more prevalent in people with NCGS than in the general population.[15] This higher prevalence is in line with observations in IBS.[16]

Additionally, the double-blind, placebo-controlled, crossover study by Biesiekierski and colleagues showed statistically significant worsening of overall gastrointestinal symptoms and abdominal pain in people with NCGS irrespective of dietary intervention (placebo, a low-gluten diet, or a high-gluten diet).[17] Symptomatic responses were highest with the first intervention participants received, irrespective of the actual

content, suggesting a nocebo effect. The importance of the nocebo effect was further highlighted in a pooled analysis of ten double-blind, placebo-controlled, gluten-challenge trials, which found that 41% of participants with suspected NCGS showed similar or increased symptoms in response to placebo versus a gluten challenge.[18] These findings indicate that expectation could mediate a nocebo effect, for example by influencing gastrointestinal sensory and motor functions.[19] The relevance of the nocebo effect has previously been shown in patients with IBS, with a pooled nocebo response rate of 32% (95% CI 26-38) in clinical drug trials.[20] However, to our knowledge, the contribution of negative expectation about gluten consumption to NCGS symptom occurrence has never been investigated. Exploring the effect of expectation might further our understanding of the pathophysiology of NCGS and help to improve diagnostic procedures and dietary or psychological treatments.

Therefore, we aimed to investigate the effects of expectancy about gluten intake versus actual gluten intake on gastrointestinal and extra-intestinal symptoms in individuals with self-reported NCGS. In addition, we aimed to investigate the role of psychological factors in these symptoms and the effect of expectancy and gluten on mood. We hypothesised that expected gluten intake, but not actual gluten intake, would increase symptom severity. As an expectancy effect would reflect a psychological process, we hypothesised that measures of anxiety, depression, and somatisation would affect response to the intervention.

Methods

Study design and participants

This randomised, double-blind, placebo-controlled, international, multicentre study was done at the University of Leeds (Leeds, UK), Maastricht University (Maastricht, the Netherlands), and Wageningen University and Research (Wageningen, the Netherlands; see Supplementary Methods – Study sites). A crossover design was not deemed feasible due to the possibility of undermining or revealing the expectancy part of the study. Participants were recruited via advertisements on social media, on patient association websites, on noticeboards on the university campuses and in local public areas, and in local newspapers. After receiving written and verbal information, interested participants were pre-screened by telephone and then invited for a full screening visit to assess eligibility. People aged 18-70 years with self-reported gastrointestinal symptoms within 8 h of a single intake of gluten-containing products were eligible. Participants had to be willing to follow a gluten-free or gluten-restricted diet (as defined by a Biagi and colleagues[21] score of 2-4) for at least 1 week before (and throughout) study participation and had to be asymptomatic or only mildly symptomatic (overall gastrointestinal symptom score ≤30 mm on the Visual Analogue Scale [VAS]) while on the diet [rated at one timepoint to represent the mean over the previous week]). All concurrent medication had to be stable for at least 6 weeks before and during the study. Participants were excluded if they had been diagnosed with coeliac disease, wheat allergy, other organic gastrointestinal diseases, other diseases that could interfere with NCGS symptoms, or any malignancies, or if they had

previously had major abdominal surgery or radiotherapy that could interfere with gastrointestinal function (participants with uncomplicated appendicectomy, cholecystectomy, or hysterectomy—*i.e.*, performed without perioperative or postoperative complications—were considered eligible if the procedure was >6 months ago). If coeliac disease had not been excluded by previous serology or upper gastrointestinal endoscopy, and participants still consumed some gluten or were willing to reintroduce gluten into their diet for at least 6 weeks, an additional visit was scheduled for serological testing (total IgA and anti-tissue transglutaminase IgA) to exclude coeliac disease. Furthermore, the use of antibiotics, probiotics, or prebiotics, the use of investigational drugs or participation in other studies that might interfere with results in the 14 days before our study, excessive use of alcohol (>15 alcoholic units per week) or any use of illicit drugs, and intentional weight loss or a planned diet during the study period were not allowed. Female participants could not be pregnant or lactating. Current smokers were included but asked not to smoke during the test day. Participants had to have sufficient knowledge of Dutch or English to understand the nature of the study, give consent, and complete the measures.

The study protocol was written in close collaboration between the University of Leeds and Maastricht University and was approved by the Faculty Research Ethics Committee of the University of Leeds and by the Medical Research Ethics Committee of Academic Hospital Maastricht and Maastricht University, and was also accepted by the Board of Directors of Wageningen University and Research. The study protocol is available online. The study was done in compliance with Good Clinical Practice, the Declaration of Helsinki (2013), the US Food and Drug Administration, and the Netherlands Medical Research Involving Human Subjects Act. To maintain secrecy about the study design, special approval was granted by the Dutch Central Committee on Research Involving Human Subjects (reference number CCMO18.0344/lvV/ek) for the expectancy part of the study and for delayed registration on ClinicalTrials.gov. All participants gave their written informed consent before participation.

Randomisation and masking

By block randomisation (block size eight) and stratified by study site and gender, eligible participants were randomly assigned (1:1:1:1) to one of four groups based on expectancy and actual gluten intake (see Supplementary Figure S1): E+G+ (expectancy to consume gluten-containing bread, combined with actual intake of gluten-containing bread); E+G- (expectancy to consume gluten-containing bread, combined with actual intake of gluten-free bread), E-G+ (expectancy to consume gluten-free bread, combined with actual intake of gluten-containing bread), and E-G- (expectancy to consume gluten-free bread, combined with actual intake of gluten-free bread). Randomisation was done by a colleague independent from the trial and the randomisation list was generated by use of a publicly available internet procedure. For the internet procedure see http://randomizer.org. The independent colleague provided investigators with a participant's unique randomisation number that indicated the expectancy condition and corresponded to the participant identifier on the study bread label. The study breads were identical in appearance, and the actual intervention (G+

or G-) could not be identified from this code. Participants, investigators, and those assessing outcomes were masked to the actual gluten intervention, and participants were also not aware of the expectancy part of the study. Data analysis was done before unblinding.

Procedures

At the screening visit, we assessed eligibility and baseline characteristics via questionnaires (*e.g.*, demographics [including self-reported gender, with the options of male or female], medical history, comorbidities, gluten-free diet compliance [the Biagi questionnaire[21]], usual symptoms after gluten consumption, and overall gastrointestinal symptom score on the VAS during the preceding week [*i.e.*, while on a gluten-free or gluten-restricted diet]). Additionally, Rome IV criteria for IBS and functional dyspepsia were assessed. For participants for whom coeliac disease had not been excluded already, an additional visit was scheduled before the screening visit (*i.e.*, before starting the gluten-free or gluten-restricted diet) for serological testing (total IgA (0.7-4.0 g/L) and anti-tissue transglutaminase IgA (< 7.0 U/mL)) to exclude coeliac disease. Participants completed Generalized Anxiety Disorder-7 (GAD-7) to assess anxiety, Patient Health Questionnaire-9 (PHQ-9) to assess depression, and Patient Health Questionnaire-15 (PHQ-15) to assess somatic symptoms at home between the screening visit and the test day. Participants were instructed to adhere to a gluten-free or gluten-restricted diet from 1 week before test day 1 to days 2 and 3 of follow-up.

A 100% gluten-free oat-based bread mix (SonFit Gluten Free Original, Sonneveld Group, Papendrecht, the Netherlands) was used as the base material for the production of both the gluten-free and gluten-containing breads. The gluten-free oat bread was baked under gluten-free conditions and confirmed to be gluten-free by the R5 RIDASCREEN Gliadin test (R-Biopharm, Darmstadt, Germany). Vital wheat gluten (Kröner-Stärke, Ibbenbüren, Germany) was added to the gluten-free oat-based bread mix at 8.6% of the total dough weight to generate gluten-containing bread, amounting to around 3.35 g of gluten per slice. The amount of gluten to add was determined on the basis of mean daily gluten intake in the Netherlands, as described in previous studies.[22-24] The recipes were the same except for the addition of gluten, and both breads were similar in texture, taste, and appearance, as also confirmed by a blind test in healthy volunteers. Both breads were baked for this study by the European Bakery Innovation Centre (Papendrecht, Netherlands). Further details about the study breads can be found in the Supplementary Methods – Study breads.

On the test day (day 1; see Supplementary Figure S1), participants were asked to come to the study site in a fasted state at 08:00 h. The test day started with baseline questionnaires (0 h) before breakfast. The questionnaires consisted of a symptom diary with 100 mm VAS to assess overall and individual gastrointestinal symptoms and extra-intestinal symptoms, the Bristol Stool Scale (only after bowel movement), and the Positive and Negative Affect Schedule (PANAS) to assess mood. After completion of the baseline questionnaires, participants were informed by the researcher about the group that they had been assigned to (E+ or E-) and then received breakfast with two slices of bread (G+ or G-) with a gluten-free topping of their choice (margarine with one

standardised, gluten-free portion of cheese, cooked ham, or jam), which was noted. Throughout the test day, participants completed the same questionnaire each hour, starting directly after breakfast, for 8 h (Supplementary Figure S1). After 4 h, participants received lunch with the same expectancy information repeated and the same bread type (two slices, with any topping) as they had consumed for breakfast. Participants were allowed to drink coffee, tea, or water (ad libitum, but quantity was noted) during the test day, but no other foods or drinks were allowed. Between measurements, participants were requested to remain in the research unit and were free to watch television, read, or work. In the exception where participants were not able or willing to stay at the unit for the full day, they were instructed to return to the unit for the hourly questionnaires. After 8 h, participants could go home. The test day questionnaires were repeated on the evening of day 1 (the test day) and on the two following days (days 2 and 3) before going to bed (available between 20:00 h and 02:00 h), including a food record to assess diet adherence and reporting of medication use. Participants were allowed to consume gluten-free bread on days 2 and 3 as this bread would not interfere with the intervention. For female participants, test and follow-up days were not scheduled during menstruation. Participants could leave the study at any time if they wished to do so, and the investigator could decide to remove a participant for urgent medical reasons.

All adverse events—*i.e.*, any undesirable experience occurring to a participant, whether or not considered related to the food intervention, as reported spontaneously by the participant or observed by the investigator during the study—were recorded.

Outcomes

The primary outcome was the effect of expectancy related to gluten intake and actual gluten intake on the overall gastrointestinal symptom score, measured on a 100 mm VAS as part of the symptom diary, and was assessed centrally. Secondary outcomes were the effects of expectancy and actual gluten intake on individual gastrointestinal symptoms (*i.e.*, abdominal discomfort, abdominal pain, belching, bloating, constipation, diarrhoea, flatulence, fullness, nausea, and urge to empty bowel), extra-intestinal symptoms (*i.e.*, confusion or foggy mind, headache, and tiredness), and changes in mood (PANAS) throughout the test day, and stool frequency and consistency on the Bristol Stool Scale. A substantial proportion of the participants did not defecate at baseline or during the test day, and the remainder mostly had a single defecation at varying timepoints. Therefore, insufficient data were available for a reliable analysis of stool frequency and consistency, and these data were not analysed or reported. A tertiary endpoint was participant characteristics (*e.g.*, demographics and psychological variables) in relation to NCGS.

Statistical analysis

The sample size calculation was done with G*power (version 3.1) and based on the increase in overall gastrointestinal symptom scores reported by Biesiekierski and colleagues after gluten consumption in patients with IBS.[17] We assumed a difference between E+G- and E-G- of 15 mm (considered clinically relevant), a SD of 12.8 mm, a power of 80%, and a Bonferroni-corrected α of 0.0083, correcting for six pairwise comparisons. Per this calculation, 20 participants were required per group, resulting in 80 participants in total. We aimed to include 84 participants on the basis of an estimated dropout rate of 5%. Although this sample size provided sufficient power to examine the primary research question, initially we aimed to obtain this sample size in each country (the UK and the Netherlands) so that any differences between countries could be compared. Because of recruitment delays due to the COVID-19 pandemic, an interim analysis was done in July 2021. This analysis was not prespecified in the study protocol as the COVID-19 pandemic was unforeseen. The interim analysis compared E+ (n=37; 20 from the UK and 17 from the Netherlands) with E- (n=36; 19 from the UK and 17 from the Netherlands) without unblinding the gluten intervention. This analysis showed that overall and individual gastrointestinal symptom and extra-intestinal symptom profiles were similar between the countries (data not shown). On the basis of this interim analysis, we decided to recruit until a combined sample size of 84 was reached, as obtained from the power calculation. Thereafter, the data from the two countries were pooled for final analyses.

Statistical analyses were conducted by use of IBM SPSS Statistics version 26.0. Normality of data was evaluated by use of histograms and quantile-quantile plots. Baseline characteristics are presented as mean (SD) or median (IQR) for numerical variables, and as frequencies with percentages for categorical variables.

We planned for an intention-to-treat analysis comprising all participants who were randomly assigned. However, one participant, after completing the screening visit and being randomly allocated, did not follow the test day instructions, resulting in no data being available for this participant. Therefore, we excluded this participant and performed a per-protocol analysis for all outcomes.

The primary and secondary outcomes between the four groups were analysed by use of repeated-measures ANCOVA, with the intervention group as the between-participant factor, baseline (0 h) as a covariate, and time (1-8 h) as the repeated-measures factor. The mean VAS score over 1-8 h was compared between groups, correcting for baseline value. For the primary outcome, we first checked the expectancy effect on overall gastrointestinal symptoms by assessing the pairwise comparison of E+G- versus E-G-, and thereafter assessed the other pairwise comparisons independently. For the secondary outcomes, we first did an overall comparison of all four groups and only if that showed significant differences were post-hoc pairwise comparisons performed, with post-hoc Bonferroni correction applied as appropriate (per symptom the α was corrected for six pairwise comparisons). Only Bonferroni-corrected p-values are reported.

Three post-hoc sensitivity analyses of the primary and secondary outcomes were done separately for the morning (1-4 h), afternoon (5-8 h), and follow-up (1-3 days) by use

of repeated-measures ANCOVA with the intervention group as the between-participant factor, baseline (0 h) as a covariate, and time (1-4 h, 5-8 h, or 1-3 days) as repeated measures. Additionally, post-hoc sensitivity analyses were performed for the test day, morning, afternoon, and follow-up analyses, in which the following variables were added sequentially to each model as single covariates to assess their impact: study site, gender, age (continuous), body mass index (BMI) (continuous), education level (university-educated or not), smoking behaviour (current smoker, former smoker, or never smoked), alcohol consumption (none, <1, 1-5, 6-7, 8-15, or 16-30 units per week), IBS according to Rome IV criteria, functional dyspepsia according to Rome IV criteria, GAD-7 score, PHQ-9 score, and PHQ-15 score.

Missing values for the primary and secondary outcome measures were imputed by use of the median of the repeated measures from that participant for that symptom. This imputation method is straightforward, as the median is robust to non-normal data distributions and the overall central tendency of the variable is preserved, and was considered reliable as only three participants had single missing values out of nine timepoints (*i.e.*, 0-8 h). The follow-up measurements included three timepoints (1-3 days) and had more missing data (13 [16%] of 83 participants with missing measurements for at least one timepoint; two for day 1, six for day 2, and eight for day 3). Therefore, insufficient information was available to impute missing values using the median. Instead, for the follow-up measurements, multiple imputation (generating 20 imputed datasets, each subjected to 20 iterations, utilising fully conditional specification and predictive mean matching) was used. A two-sided p-value of less than 0.05 was considered statistically significant.

As we noted substantial variation in individual responses within the groups, we explored symptom patterns post-hoc using an explorative unsupervised random forest analysis. The unsupervised random forest analysis was performed with overall gastrointestinal symptoms and all individual gastrointestinal symptoms at timepoints 0-8 h. Results were visualised by use of a principal coordinate analysis plot to check for any ordination of data points, which axis explained the largest variation observed, and the influences of IBS status, age, gender, BMI, and country. In order to visualise the groupings in the data, various combinations of principal coordinates were used.

In the UK, all data collection and entry was monitored and checked by the principal investigator and coordinating investigator. Additionally, as part of local regulations in the Netherlands, the study (both in Maastricht and Wageningen) was monitored by a clinical study monitor, who checked, for example, informed consent forms, data collection and entry, compliance to protocols, and reporting of (serious) adverse events. The study is registered at ClinicalTrials.gov, NCT05779358.

Role of the funding source

Representatives from the funders were permitted to ask questions and provide suggestions to the academic research consortium team (ARCT) during biannual progress meetings, but were not involved in final decisions regarding the study design, data collection, data analysis, data interpretation, and writing of the report.

Results

Between Oct 19, 2018, and Feb 14, 2022, 683 individuals received the full study information (Figure 1). Of these, 301 (44%) individuals were pre-screened by telephone, and thereafter 165 (24%) completed full screening, with 49 (7%) participants also undergoing a blood test to exclude coeliac disease. The main reasons for ineligibility were that coeliac disease could not be ruled out (43 [6%]); that individuals linked their symptoms to bread, wheat, or other food products rather than to gluten (42 [6%]); comorbidities or medication use (24 [4%]); high gastrointestinal symptom scores despite following a gluten-free or gluten-restricted diet (20 [3%]); and symptoms reported to occur later than 8 h after gluten consumption (five [1%]). Furthermore, 25 (4%) eligible participants dropped out before randomisation, mainly due to delays to test day booking because of COVID-19 restrictions. 84 participants were randomly assigned to either E+G+ (n=21), E+G- (n=21), E-G+ (n=20), or E-G- (n=22). One participant in the E+G+ group was excluded due to not following the test day instructions, leaving 83 participants in the per-protocol analysis.

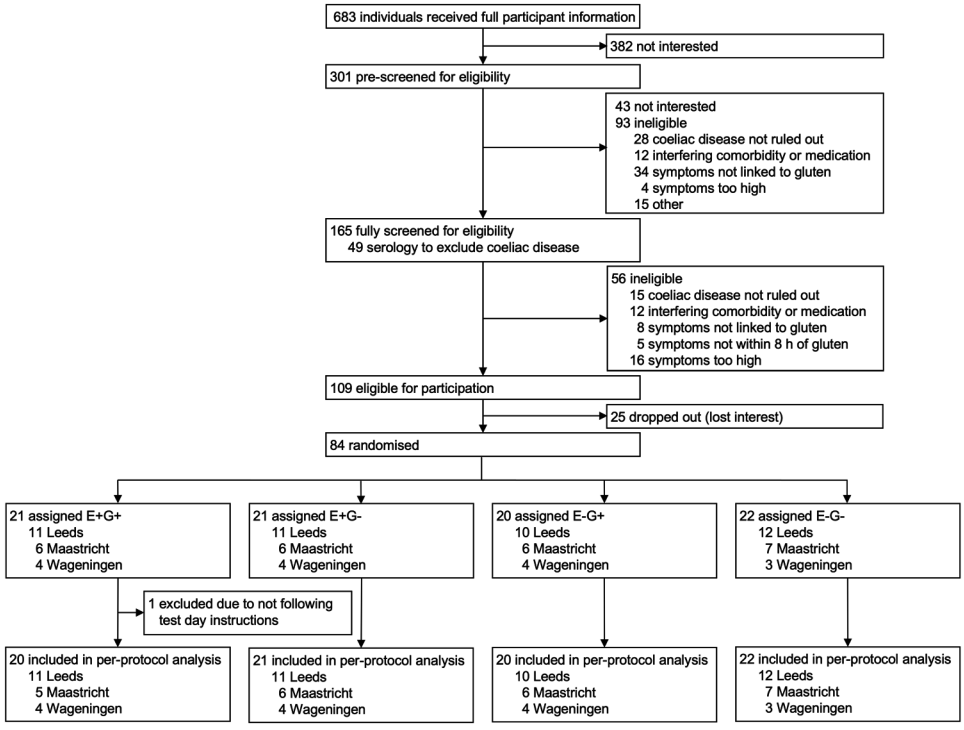

Figure 1. Trial profile. E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread.

Table 1. Baseline characteristics.

	All (n=83)	E+G+ (n=20)	E+G- (n=21)	E-G+ (n=20)	E-G- (n=22)
Gender					
Female	71 (86%)	16 (80%)	19 (90%)	17 (85%)	19 (86%)
Male	12 (14%)	4 (20%)	2 (10%)	3 (15%)	3 (14%)
Age (years)	27.0 [21.0-45.0]	23.5 [20.3-44.8]	25.0 [21.0-43.0]	28.5 [22.3-50.8]	29.0 [22.0-47.3]
BMI (kg/m^2)	23.8 ± 3.9	24.2 ± 4.5	23.0 ± 2.6	23.7 ± 5.0	24.2 ± 3.0
University-educated	50 (60%)	12 (60%)	14 (67%)	9 (45%)	15 (68%)
Smoking *					
Never smoked	66 (80%)	17 (85%)	18 (90%) of 20	14 (70%)	17 (77%)
Former smoker	11 (13%)	1 (5%)	2 (10%) of 20	5 (25%)	3 (14%)
Current smoker	5 (6%)	2 (10%)	0	1 (5%)	2 (9%)
Alcohol intake					
None	14 (17%)	6 (30%)	3 (14%)	3 (15%)	2 (9%)
< 1 unit per week	20 (24%)	5 (25%)	4 (19%)	6 (30%)	5 (23%)
1-5 units per week	34 (41%)	6 (30%)	11 (52%)	7 (35%)	10 (45%)
6-7 units per week	6 (7%)	2 (10%)	1 (5%)	2 (10%)	1 (5%)
8-15 units per week	8 (10%)	1 (5%)	2 (10%)	2 (10%)	3 (14%)
16-30 units per week	1 (1%)	0	0	0	1 (5%)
IBS (Rome IV)					
Yes	29 (35%)	5 (25%)	7 (33%)	8 (40%)	9 (41)
IBS-C	7 (8%)	2 (10%)	3 (14%)	0	2 (9%)
IBS-D	14 (17%)	1 (5%)	2 (10%)	6 (30%)	5 (23%)
IBS-M	5 (6%)	1 (5%)	0	2 (10%)	2 (9%)
IBS-U	3 (4%)	1 (5%)	2 (10%)	0	0
FD (Rome IV)					
Yes	19 (23%)	7 (35%)	2 (10%)	4 (20%)	6 (27%)
Postprandial distress syndrome	8 (10%)	4 (20%)	1 (5%)	0	3 (14%)
Epigastric pain syndrome	5 (6%)	0	1 (5%)	2 (10%)	2 (9%)
Overlap syndrome	6 (7%)	3 (15%)	0	2 (10%)	1 (5%)
Anxiety (GAD-7) *	2.0 [0.0-4.3]	1.5 [0.0-3.0]	2.0 [0.0-4.0]	2.5 [0.0-4.8]	2.0 [0.5-6.0]
Yes, anxiety (≥10)	2 (2%)	0	1 (5%)	0	1 (5%) of 21
Depression (PHQ-9) *	2.0 [0.0-4.0]	2.0 [1.0-4.0]	1.0 [0.5-2.5]	3.0 [0.0-5.0]	2.0 [1.0-5.5]
Yes, depression (≥10)	3 (4%)	0	1 (5%)	0	2 (10%) of 21

Table 1 (Continued). Baseline characteristics.

	All (n=83)	E+G+ (n=20)	E+G- (n=21)	E-G+ (n=20)	E-G- (n=22)
Somatisation (PHQ-15) *	6.2 ± 3.6	6.8 ± 3.4	5.7 ± 3.6	6.4 ± 3.0	6.0 ± 4.5
Minimal (<5)	27 (33%)	4 (20%)	8 (38%)	5 (25%)	10 (48%) of 21
Low (5-9)	40 (49%)	12 (60%)	10 (48%)	11 (55%)	7 (32%) of 21
Medium (10-14)	13 (16%)	4 (20%)	3 (14%)	4 (20%)	2 (10%) of 21
High (≥15)	2 (2%)	0	0	0	2 (10%) of 21
Baseline values primary and secondary outcomes (mm on VAS)					
Overall GI symptoms	4.5 ± 9.7	4.3 ± 11.3	7.0 ± 12.9	3.5 ± 6.4	3.3 ± 6.9
Abdominal discomfort	4.4 ± 7.7	5.1 ± 8.6	6.6 ± 9.2	2.5 ± 4.9	3.4 ± 7.0
Abdominal pain	3.0 ± 6.4	4.0 ± 7.7	4.5 ± 8.3	1.6 ± 4.7	1.8 ± 3.6
Belching	1.1 ± 3.2	0.7 ± 2.2	0.7 ± 2.4	1.0 ± 2.9	1.8 ± 4.7
Bloating	4.6 ± 12.9	4.9 ± 17.8	8.4 ± 16.8	3.7 ± 7.7	1.6 ± 3.5
Constipation	2.4 ± 9.5	1.0 ± 2.6	5.7 ± 17.6	0.6 ± 1.4	2.3 ± 5.9
Diarrhoea	0.7 ± 2.5	0.3 ± 1.0	0.6 ± 1.8	0.5 ± 1.2	1.3 ± 4.2
Flatulence	3.6 ± 8.4	1.6 ± 3.4	4.8 ± 7.5	7.0 ± 14.0	1.3 ± 3.7
Fullness	5.7 ± 12.3	5.5 ± 11.5	8.2 ± 16.8	3.8 ± 8.7	5.2 ± 10.9
Nausea	1.6 ± 4.3	1.7 ± 3.3	0.7 ± 2.6	1.1 ± 4.2	2.7 ± 6.0
Urge to empty bowel	4.5 ± 10.0	1.7 ± 4.3	7.7 ± 14.3	3.3 ± 5.6	5.2 ± 11.4
Confusion	4.3 ± 11.4	2.4 ± 3.9	3.0 ± 6.2	4.5 ± 13.0	7.1 ± 17.0
Headache	2.8 ± 7.1	3.8 ± 9.7	4.3 ± 8.7	1.3 ± 2.6	1.8 ± 4.8
Tiredness	17.7 ± 18.4	16.9 ± 18.4	16.5 ± 18.6	18.2 ± 17.7	19.1 ± 19.8
Positive affect	25.7 ± 8.4	23.7 ± 7.4	25.7 ± 9.6	29.7 ± 8.6	23.9 ± 6.8
Negative affect	11.9 ± 2.4	11.7 ± 1.4	11.7 ± 1.7	11.4 ± 1.6	12.8 ± 3.8

Data displayed as mean ± standard deviation (continuous variables with normal distribution), median [interquartile range Q1-Q3] (continuous variables with non-normal distribution) or n (%) (categorical).

E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread; BMI = body mass index; IBS = irritable bowel syndrome; IBS-C = constipation predominant IBS; IBS-D = diarrhoea predominant IBS; IBS-M = mixed stool pattern IBS; IBS-U = unspecified subtype IBS; FD = functional dyspepsia; GAD-7 = Generalized Anxiety Disorder; PHQ-9 = Patient Health Questionnaire 9; PHQ-15 = Patient Health Questionnaire 15; VAS = visual analogue scale; GI = gastrointestinal.

* Missing data from 1 subject.

Of these 83 participants, 71 (86%) were female and 12 (14%) were male. The median age was 27.0 years (IQR 21.0-45.0) and the mean BMI was 23.8 kg/m² (SD 3.9). 50 (60%) participants had a university education, 66 (80%) had never smoked, and overall alcohol intake was modest (Table 1; Supplementary Table S2). 29 (35%) participants met the Rome IV criteria for IBS, with diarrhoea-predominant IBS being the most common subtype, and 19 (23%) fulfilled the Rome IV criteria for functional dyspepsia. At the screening visit, participants reported bloating (72 [87%] of 83), abdominal discomfort (68 [82%] of 83), and abdominal pain (58 [70%] of 83) as predominant symptoms after gluten exposure (Supplementary Figure S3).

Mean overall gastrointestinal symptom score (Figure 2A) was not significantly different between E+G- (VAS 11.7 mm [95% CI 8.3 to 15.1]) and E-G- (7.4 mm [4.2 to 10.7]; difference 4.2 mm [95% CI -2.2 to 10.7], p=0.47; Supplementary Tables S3-S4). The mean overall gastrointestinal symptom score in the E+G+ group (16.6 mm [13.1 to 20.0]) was significantly higher than that in the E-G+ (6.9 mm [3.5 to 10.4]; difference 9.6 mm [3.0 to 16.2], p=0.0010) and E-G- (difference 9.1 mm [2.7 to 15.6], p=0.0016) groups, but not the E+G- group (difference 4.9 mm [-1.7 to 11.5], p=0.28). Additionally, no significant differences in mean overall gastrointestinal symptom score were found between E-G+ and E-G- (difference -0.5 mm [-7.0 to 5.9], p=1.0) or E+G- and E-G+ (difference 4.7 mm [-1.8 to 11.3], p=0.33).

When analysed separately in a post-hoc sensitivity analysis, differences in overall gastrointestinal symptoms between groups were more pronounced in the afternoon (E+G+ vs E-G+: difference 11.9 mm [95% CI 3.7 to 20.1], p=0.0011; E+G+ vs E-G-: difference 11.7 mm [3.7 to 19.8], p=0.0010) than in the morning (E+G+ vs E-G+: difference 7.4 mm [0.4 to 14.3], p=0.031; E+G+ vs E-G-: difference 6.5 mm [-0.3 to 13.3], p=0.068). There was no significant effect of gluten on overall gastrointestinal symptoms within each expectancy group in the morning or the afternoon (E+G+ vs E+G- and E-G+ vs E-G-; Supplementary Tables S5-S8). The other pairwise comparisons for overall symptoms during the test day showed no significant differences between groups (Supplementary Tables S5-S8). These observed differences in overall gastrointestinal symptom score for the test day, morning, and afternoon were still significant after correction for covariates (Supplementary Table S11). Observed differences between groups for overall gastrointestinal symptoms persisted throughout the follow-up measurements (Supplementary Tables S9-S10 and Supplementary Figure S5A), except for E+G+ versus E-G- after correction for covariates (Supplementary Table S11).

Evaluation of individual gastrointestinal symptoms showed that mean abdominal discomfort (Figure 2B) was significantly higher throughout the test day in the E+G+ group (19.1 mm [95% CI 14.5-23.7]) than in the E-G+ group (6.7 mm [2.1-11.4]; difference 12.4 mm [3.4-21.3], p=0.0020) and the E-G- group (8.6 mm [4.2-13.0]; difference 10.5 mm [1.8-19.2], p=0.010; Supplementary Tables S3-S4), again with differences more pronounced in the afternoon (Supplementary Tables S7-S8) than in the morning (Supplementary Tables S5-S6). Mean bloating (Figure 2C) was significantly higher throughout the test day for E+G+ (14.4 mm [10.3-18.5]) compared with E-G+ (4.7 mm [0.6-8.8]; difference 9.7 mm [1.8-17.6], p=0.0083; Supplementary

Tables S3-S4), but when morning and afternoon were analysed separately, this difference was only significant in the afternoon (Supplementary Tables S5-S8). Within each expectancy group, gluten had no significant effect on abdominal discomfort and bloating. Moreover, no differences were found between E+G- and E-G- (Supplementary Tables S3-S8). Observed test day differences for these symptoms were still significant during follow-up, except for abdominal discomfort in E+G+ vs E-G- (Supplementary Tables S9-S10 and Supplementary Figures S5B and S5E) and after inclusion of covariates (Supplementary Table S11). Mean fullness (Figure 2D) was significantly higher for E+G+ than for E-G+ and E-G- in the afternoon only (Supplementary Tables S3-S8). However, the differences between E+G+ and E-G+ or E-G- were no longer significant after adding certain covariates (Supplementary Table S11), nor during follow-up (Supplementary Tables S9-S10 and Supplementary Figure S5I).

The other gastrointestinal symptoms—abdominal pain, belching, constipation, diarrhoea, flatulence, nausea, and urge to empty the bowel—did not differ significantly between the groups, apart for abdominal pain and constipation between E+G+ and E-G+ during follow-up (Supplementary Tables S3-S10 and Supplementary Figures S4A-S4G, S5C-S5D, S5F-S5H, and S5J-S5K). Sequentially adding covariates post-hoc changed the significance of some differences (Supplementary Table S11).

For the extra-intestinal symptoms, mean confusion or foggy mind (Figure 2E; Supplementary Tables S3-S4) was significantly higher in the E+G+ group than in the E-G+ group throughout the test day (difference 7.3 mm [0.3-14.2], p=0.037), and remained so after the inclusion of covariates except smoking and alcohol intake (Supplementary Table S11). Mean headache (Figure 2F; Supplementary Tables S3-S4) was significantly higher for E+G+ than for E-G+ (difference 6.0 mm [0.6-11.4], p=0.020). After correction for gender, BMI, or GAD-7 score, headache was also significantly higher in the E+G+ group compared with the E+G- group (Supplementary Table S11). When analysed by time of day, these differences between groups for both confusion or foggy mind and headache were only significant in the morning (Supplementary Tables S5-S8). The differences also did not persist at follow-up (Supplementary Tables S9-S10 and Supplementary Figures S5L-S5M). Mean tiredness was not significantly different between groups (Supplementary Tables S3-S10 and Supplementary Figures S4H and S5N).

Overall, participants scored low on the screening questionnaires for anxiety, depression, and somatisation, with few participants meeting the cut-off of 10 points or greater (Table 1).[25-27] When added as covariates to the repeated-measures ANCOVA model, these psychological factors affected differences between intervention groups for headache and tiredness during the test day, for fullness and tiredness in the afternoon, and for overall gastrointestinal symptoms, abdominal discomfort, and abdominal pain during follow-up (Supplementary Table S11). Furthermore, throughout the test day (and morning and afternoon separately) and follow-up, positive and negative affect did not differ significantly between the four groups (Figures 2G-2H; Supplementary Tables S3-S10, Supplementary Figures S5G-S5H).

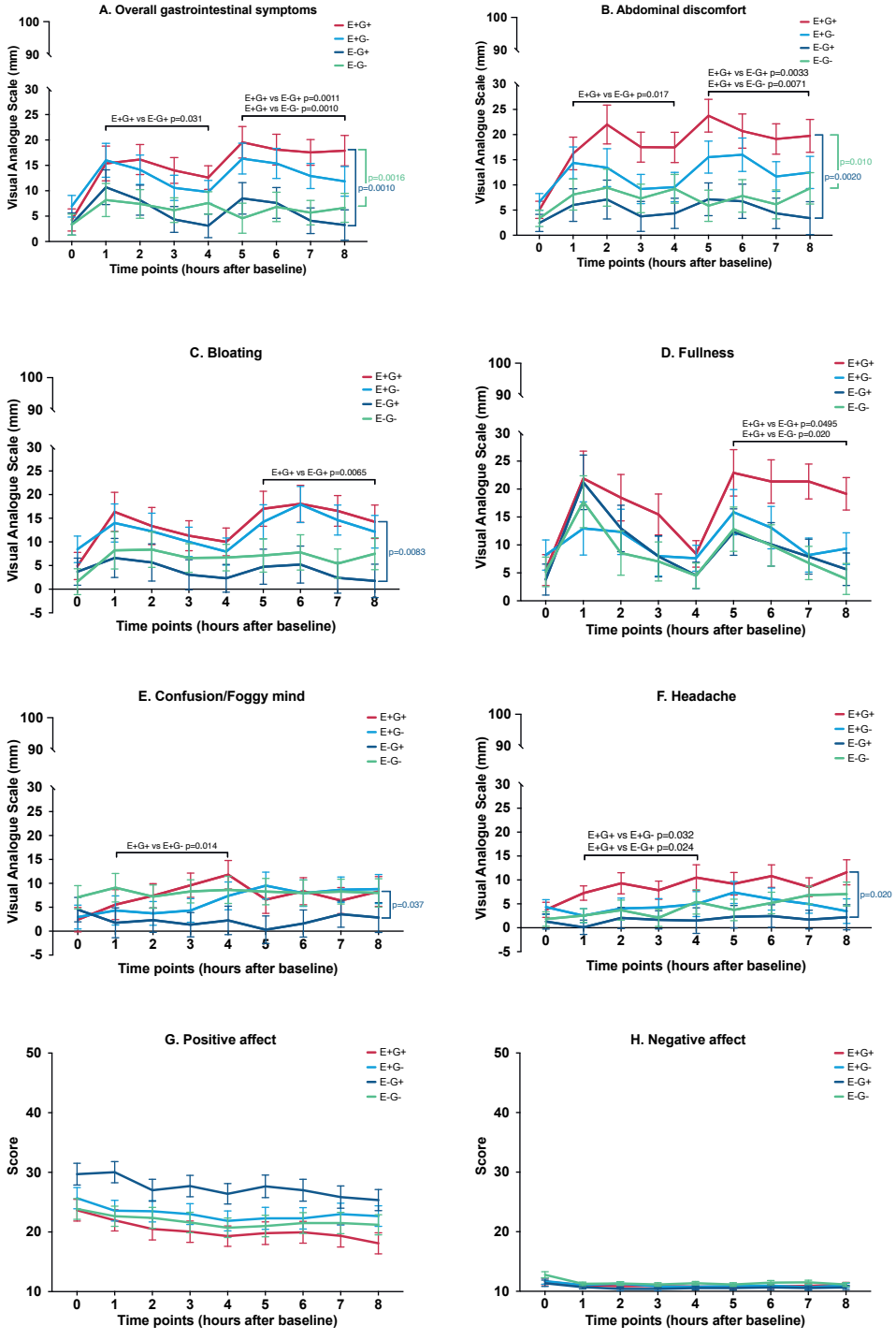

Figure 2. Test day scores. Mean score for gastrointestinal (A-D) and extra-intestinal (E-F) symptoms on the test day, assessed by visual analogue scale (0-100mm), with significant differences between groups

indicated (overall and for morning [1-4 h] and afternoon [5-8 h]). Positive (G) and negative (H) affect scores on PANAS (10-50). Error bars represent standard error. E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread; PANAS = Positive and Negative Affect Schedule; * p<005; ** p<001; *** p<0001; comparisons with p>0.05 are not indicated.

To further explore heterogeneity in symptom response, an unsupervised random forest post-hoc analysis was done. Principal coordinates 5 and 7 were selected because they exhibited better separation or clustering of data points in the score plot than other components. This can be attributed to their ability to highlight distinct groups or patterns in the data, and makes them best suited for visualising the underlying structure of the data. This analysis identified partial data separation by intervention group (Figure 3A). As can be seen within the specific intervention groups, a group of individuals (Supplementary Figure S6A) showed clear separation with respect to the measured symptoms, especially in groups E+G+ and E+G- in comparison with groups E-G- and E-G+. All symptoms had a role in this separation, with diarrhoea and constipation having the lowest importance (Figure 3B). For individuals with the highest responsiveness with respect to symptoms (*i.e.*, those in groups E+G+ and E+G-; Figure 3A), overall gastrointestinal symptoms, abdominal pain, abdominal discomfort, urge to empty the bowel, and fullness could be defined as the most important symptoms driving the separation. The observed data separation could not be explained by other demographic and clinical variables, such as IBS (Supplementary Figures S6B-S6F).

Three (4%) of 83 participants reported adverse events on the test day. In the E+G- group, one (5%) of 21 participants reported an itching sensation in their jaw between 0 h and 1 h and one (5%) participant reported a lightheaded feeling and rumbling stomach between 7 h and 8 h. In the E-G+ group, one (5%) of 20 participants vomited twice between 6 h and 8 h. No adverse events were reported during follow-up.

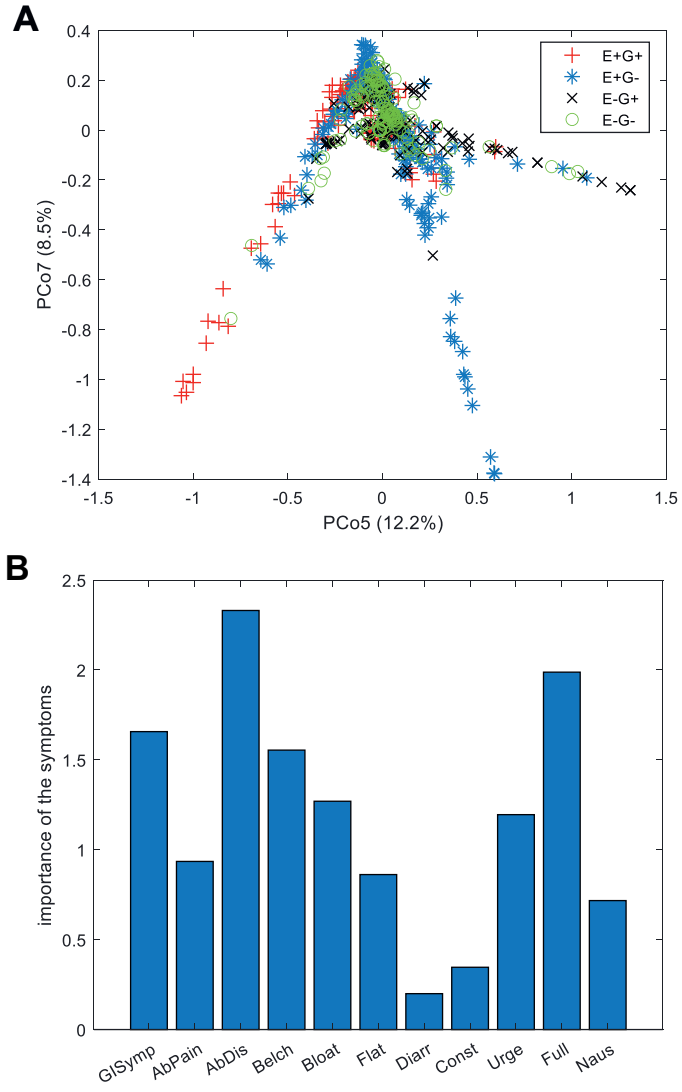

Figure 3. Unsupervised random forest analysis. (A) Principal coordinate analysis (PcoA) score plot based on post-hoc unsupervised random forest analysis with principle coordinate (PCo) 5 and 7 explaining the largest proportions of the variation observed—*i.e.*, 12.5% and 8.5%, respectively. This plot is colour-coded with respect to the intervention. E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread. (B) Relative contribution in the unsupervised random forest model of overall gastrointestinal (GI) symptoms (GISymp) and all individual GI symptoms (*i.e.* abdominal discomfort (AbDis), abdominal pain (AbPain), belching (Belch), bloating (Bloat), constipation (Const), diarrhoea (Diarr), flatulence (Flat), fullness (Full), nausea (Naus), and urge to empty bowel (Urge)) at t = 0-8 hours.

Discussion

This randomised, double-blind, placebo-controlled, international, multicentre study was, to our knowledge, the first study designed to investigate the role of the nocebo effect in NCGS. Our findings showed that the combination of expectancy and actual gluten intake had the largest effect on overall gastrointestinal symptoms. Repeated exposure compounded this effect, evidenced by the more pronounced differences between groups in the afternoon (after lunch) compared with the morning (after breakfast). Similar patterns were found for several individual gastrointestinal symptoms. Furthermore, expectancy within the two groups that received gluten significantly increased the extra-intestinal symptoms of confusion or foggy mind and headache. Some differences between intervention groups persisted throughout follow-up. These findings add weight to our hypothesis that a nocebo effect is involved in symptom occurrence in NCGS. We found no significant differences in overall or individual symptoms based on actual gluten intake within each expectancy group, but our data also indicate that a concurrent biological effect of gluten cannot be excluded. Additionally, contrary to our hypothesis, we found that emotional wellbeing—*i.e.*, anxiety, depression, or somatisation—did not affect differences between groups for overall and individual gastrointestinal symptom scores during the test day.

This study showed that the nocebo effect has an important role in symptom occurrence in NCGS. Hereby, we add to the findings from the study by Biesiekierski and colleagues,[17] which also indicated that the expectancy to receive gluten had a greater role than the actual consumption of gluten in people with NCGS, showing an order effect by which symptoms were highest with the first intervention. A study by Ponzo and colleagues[28] also found an order effect when comparing gluten with placebo in individuals with self-reported NCGS. Previous studies have considered the occurrence of the nocebo effect as a limitation of the double-blind, placebo-controlled study design rather than an important causal factor.[14,29,30] Expectancy, typically induced via verbal suggestions, and learning are the two best characterised mechanisms that mediate the nocebo effect. These processes are mediated centrally, involving multiple brain regions and influencing gastrointestinal sensory and motor functions along the bidirectional gut-brain axis between the gastrointestinal tract and the CNS.[19] The gut-brain axis involves multiple pathways, such as the autonomic and enteric nervous systems, the endocrine system, the hypothalamic-pituitary-adrenal axis, the immune system, and the gut microbiota and its metabolites.[31] The nocebo effect is also an important feature in patients with IBS, in whom the gut-brain interaction has a clear role.[20] We consider the role of the nocebo effect in NCGS symptom occurrence as a new lead for the possible involvement of the gut-brain interaction that warrants further study.

This consideration is further supported by the substantial overlap between NCGS and IBS, which is currently characterised as a disorder of gut-brain interaction.[9] 35% of our study population met the Rome IV criteria for IBS. This proportion is higher than that in the general population[9] and similar to the prevalence reported by previous studies, which ranged from 20% to 44%.[30] Diarrhoea-predominant IBS was the most prevalent

IBS subtype in our study, but numbers were too small for further analyses by subtype. Furthermore, the number of people with IBS in our study was similar between intervention groups, and symptom response was not different between those with and without IBS.

We found no significant effect of actual gluten intake within each of the expectancy groups. Nevertheless, the combination of expectancy and gluten had the largest effect on symptoms, pointing to an additive or synergistic effect of gluten exposure. Previous studies have shown conflicting evidence for the role of gluten in NCGS.[30] Although several studies have found that a gluten challenge induced higher symptom scores than placebo,[32-34] others have reported no effects,[35-37] no improvement of symptom scores on a gluten-free diet versus a gluten-containing diet in people with IBS,[38] or even a higher symptom response after placebo versus gluten.[39] Furthermore, several studies indicate that other wheat components, including fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs), such as fructans, and amylase trypsin inhibitors, might be more important triggers than gluten.[17,30,40-42] It is important to establish whether a strict gluten-free diet is needed to manage symptoms. Following a strict gluten-free diet without adequate guidance and food replacement could lead to unbalanced dietary intake and nutrient deficiency[12,13] and might not be necessary in the absence of coeliac disease.[14] Regardless, in clinical practice, it remains important that people with NCGS receive adequate dietary guidance to identify and replace potential trigger foods while maintaining a balanced diet.

The demand for an individualised dietary approach for people with NCGS was further supported by our exploratory, post-hoc unsupervised random forest analysis. We were able to identify separation in response within each intervention group, but could not fully explain the variation in symptom response by predominant symptoms or IBS status. Thus, these results suggest that symptom occurrence in NCGS is heterogeneous and cannot be explained by one clear mechanism. Therefore, further research should also focus on determining the biological mechanisms by which gluten and other wheat components can lead to gastrointestinal symptoms in NCGS, the cause for interindividual differences in symptom responses, and the need for a strict gluten-free diet in these individuals.

In line with some previous studies,[28,32,33,43] we found that expectancy had a significant effect on the extra-intestinal symptoms of confusion or foggy mind and headache during the test day. However, anxiety, depression, and somatic symptoms did not affect observed differences between groups for overall and individual gastrointestinal symptom scores during the test day. Furthermore, they had only a few effects on differences in extra-intestinal symptoms between groups during the test day and in gastrointestinal and extra-intestinal symptoms during follow-up. Mood was also not significantly affected by the intervention. Although previous studies have found a higher prevalence of psychological comorbidities in people with NCGS versus the general population[15] and that psychological wellbeing is affected by gluten intake,[15,35,41] our study did not confirm these findings. This result might be due to selection bias, as it is plausible that more anxious or symptomatic people were less willing to participate in our study. The effect of psychological factors should be considered in future studies.

The main strength of our study is that it was, to our knowledge, the first well designed study to investigate the role of the nocebo effect in people with NCGS by use of a physiologically relevant dose of gluten administered in a clinically controlled environment. The breads used in this study differed only in gluten content and had equal concentrations of fibres, including FODMAPs. Strict inclusion criteria were used and we did not include people with coeliac disease or wheat allergy, although wheat allergy was determined on the basis of medical history only. Another strength of our study was the hourly measurements during the 8 h test day, with a repeated exposure to expectancy and actual gluten intake. Subsequently, we noted that the differences between groups were generally higher in the afternoon than in the morning. Although the time course of gluten-evoked symptoms could be a plausible explanation in some individuals, we found that scores for several symptoms peaked first after 1-2 h, decreased before lunch, and again peaked after lunch. Therefore, we hypothesise that this result was mainly due to repeated exposure to the same condition.

Our study also has limitations. It should be noted that overall gastrointestinal symptom scores were rather low. We cannot exclude selection bias, as those with high symptoms or more anxious individuals might be less willing to participate. Additionally, we did not measure stress, despite it being known to affect gastrointestinal symptoms. Furthermore, because of delays in recruitment due to the COVID-19 pandemic, the study was terminated early, resulting in the pooling of data from the UK and the Netherlands. However, as the symptom profiles were similar in each country, we do not consider this pooling an issue. Although our analyses would have had more power with twice as many participants, lending more confidence to the generalisability of our results between the countries, we believe that the effects are clear and consistent. As our effect sizes are similar to those of previous studies,[17,34,37,40] we consider generalisability among European countries and Australia to be adequate. Although most of our study population was female, this result is in line with other studies[17,28,32-43] and indicates that being a woman can be considered a population characteristic or risk factor for NCGS.

On the basis of these findings, future research efforts should aim to identify biomarkers that distinguish heterogeneous symptom patterns of NCGS. Furthermore, the role of the gut-brain axis and psychological factors should be investigated, alongside the potential pathophysiological effects of gluten and other wheat components. For clinical management, both adequate dietary guidance, including proper identification of trigger foods and adequate replacement of these products guided by a dietitian, and potential psychological or behavioural factors should be considered.

To conclude, we found that the combination of expectancy and actual gluten intake had the largest effect on overall and several individual gastrointestinal symptoms, reflecting a considerable nocebo effect, although an additional effect of gluten could not be ruled out. Repeated exposure accentuated the effects of the intervention. The results of this study support the importance of further research into the possible involvement of the gut-brain interaction in NCGS.

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Supplementary Materials & Results

Contents

- Supplementary Figure S4. Test day scores for (A-G) gastrointestinal and (H) extra-intestinal symptoms, assessed by visual analogue scale (0-100mm), without significant differences between groups.
- Supplementary Table S9. Observed mean and estimated mean (corrected for baseline (t = 0 hours)) follow-up (t = 1-3 days) scores per intervention group, assessed by 0-100mm visual analogue scale (VAS), for gastrointestinal (GI) and extra-intestinal symptoms.
- Supplementary Table S10. Post-hoc sensitivity analysis for differences in estimated mean follow-up (t = 1-3 days) scores for gastrointestinal (GI) and extra-intestinal symptoms, assessed by visual analogue scale (0-100mm).
- Supplementary Figure S5. Follow-up scores for (A-K) gastrointestinal and (L-N) extra-intestinal symptoms, assessed by visual analogue scale (0-100mm), and (O-P) scores for positive and negative affect.
- Supplementary Table S11. Summary of significant differences between intervention groups after post-hoc sensitivity analysis of correction for covariates.
- Supplementary Figure S6. Principle coordinate analysis (PCoA) score plot based on unsupervised random forest analysis of overall gastrointestinal (GI) symptoms and all individual GI symptoms (*i.e.* abdominal discomfort, abdominal pain, belching, bloating, constipation, diarrhoea, flatulence, fullness, nausea, and urge to empty bowel) at timepoints 0-8 hours. Figures were colour coded for (A) individual participants, (B) irritable bowel syndrome (IBS) according to Rome IV criteria, (C) age, (D) gender, (E) body mass index, and (F) country.

Supplementary Methods – Study sites

University of Leeds (United Kingdom)

- Principal investigator: prof. dr. Louise Dye
- Number of patients: 44

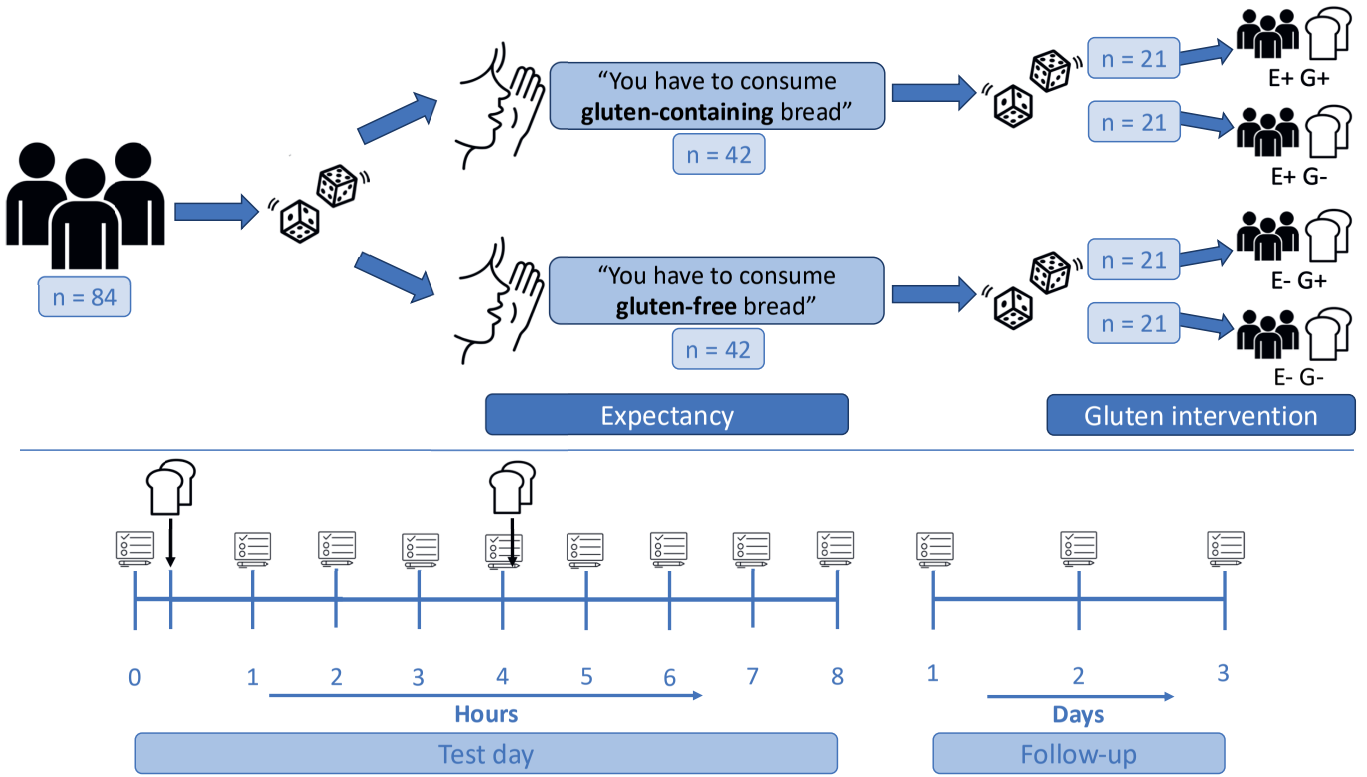
Maastricht University (the Netherlands)

- Principal investigator: prof. dr. Daisy M.A.E. Jonkers
- Number of patients: 25 (including 1 exclusion due to failure to understand the test day instructions)

Wageningen University and Research (the Netherlands)

- Principal investigator: prof. dr. Ben J.M. Witteman
- Number of patients: 15

Supplementary Methods – Study design



Supplementary Figure S1. Study design. Participants were randomised to one of four groups based on the expectation that they will consume "gluten-containing" (E+) or "gluten-free" (E-) oat bread for breakfast and lunch (two slices each), and actual intake of gluten-containing (G+) or gluten-free (G-) oat bread for breakfast (directly after t = 0 hours) and lunch (directly after t = 4 hours). Questionnaires were completed at baseline (t = 0 hours), hourly throughout the test day (t = 1-8 hours, starting as soon as the participant finished breakfast), on the evening of day 1 (the test day) and the two consecutive days (day 2 and 3).

Supplementary Methods – Study breads

Study breads were manufactured, packed, labelled, and frozen at the European Bakery Innovation Centre (Papendrecht, The Netherlands). Frozen packages were shipped to the study sites, where they were stored at -18°C until consumption.

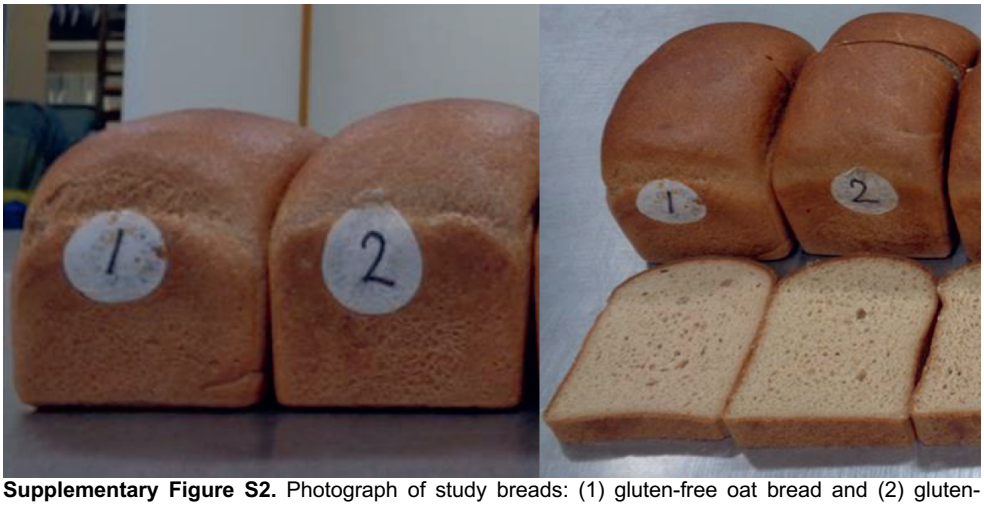
Both the gluten-containing and gluten-free bread were made using the same gluten-free oat based bread mix (SonFit Gluten Free Original/SGFO, Sonneveld Group B.V., Papendrecht, the Netherlands). The gluten-free oat bread was baked under gluten-free conditions and analysed to be gluten-free by the R5 Ridascreen Gliadin test. For gluten-free bread SGFO (47%), water (51%) and Fermipan Red (dry yeast, 19%) were mixed for 280 s slow and 1540 s fast in a Diosna spiral mixer. For the gluten containing bread* SFGO (43%), water (46%), Vital Wheat Gluten (Kröner Stärke, Ibbenbüren, Germany; 86%) and Fermipan Red (17%) were mixed for 280 s slow and 866 s fast in a Diosna mixer. Doughs were scaled at a weight of 700 g, moulded to a cylinder, proofed for 30 min at 32°C at 80% relative humidity and baked in a Deck oven for 45 min at 240°C upper and lower temperature. After cooling overnight, breads were sliced. The addition of gluten resulted in 335 g of gluten per slice of 46 g (8.6% Vital Wheat Gluten added to dough, after 10% baking loss resulting in 9.6%, with 75% gluten protein $\rightarrow$ 7.3% gluten in 100 g baked bread). A portion of 4 slices equals the amount of gluten in 155 g commercially available bread. See Supplementary Table S1 for the nutritional composition of the study breads.

Blinding was ensured by packaging the bread per 4 slices, *i.e.* the portion for one participant, and labelling each package with the randomisation number referring to the expectancy group. Preliminary testing with 15 healthy volunteers confirmed that the study breads were statistically not significantly different in texture, taste, and appearance. Additionally, at an annual “Well on Wheat?” project meeting, 30 partners participated in a blind test (based on texture, taste, and appearance) of the two study breads, as organised by Sonneveld Group B.V. About 63% did not correctly identify which of the two test breads contained gluten despite close proximal tasting. Accordingly, it was decided that the identicality of the study breads was good (See Supplementary Figure S2).

Supplementary Table S1. Nutritional composition of the study breads per slice of bread.

Nutrient composition per slice (46 g)	Gluten-containing bread	Gluten-free bread
Energy (kJ/kcal)	433.6 / 101.4	435.6 / 103.3
Total fat (g)	1.4	1.7
Saturated fat (g)	0.2	0.2
Mono-unsaturated fatty acids (g)	0.7	0.8
Poly-unsaturated fatty acids (g)	0.5	0.6
Linoleic acid (g)	0.1	0.1
Carbohydrates (g)	14.1	18.0
Mono/disaccharides (g)	0.9	1.1
Polysaccharides (g)	13.2	16.4
Dietary fibres (g)	2.5	3.0
Total protein (g)	6.8	3.1
Gluten (g(%))	3.35 (7.3)	0.0 (0.0)
Sodium (g)	0.4	0.6

* The amount of gluten to add was based on average daily gluten intake as described in previous studies. Several studies indicate that an average daily gluten intake in a Western population is within the range of 5-20 g/day.[1] More specifically, the gluten intake of the general Dutch population, which reflects a significant bread consuming population is 13.1 g/day.[2] Based on the latest nation-wide food consumption survey in the Netherlands[3] a total consumption of grain products is ca 191 g/day, including 115.5 g bread, but also 52 g rice and pasta with an unknown ratio. In addition, 37 g/day biscuits, pastry and gingerbread are consumed, containing between 15 and 20 g flour. Taking into account this unknown ratio of rice vs. pasta and the flour from pastry products, the total amount of gluten-containing grain consumption was assumed to be about 150 g. The amount of gluten added to our gluten-free oat bread was based on these consumption data, assuming a similar intake in the UK.
When assuming a protein content of wholemeal bread of 11.1%, and assuming that 80% of the protein is gluten, the consumption of gluten in the Netherlands is about 13.3 g/day similar to the data of van Overbeek *et al*.[2] mentioned above.



Supplementary Figure S2. Photograph of study breads: (1) gluten-free oat bread and (2) gluten-containing oat bread.

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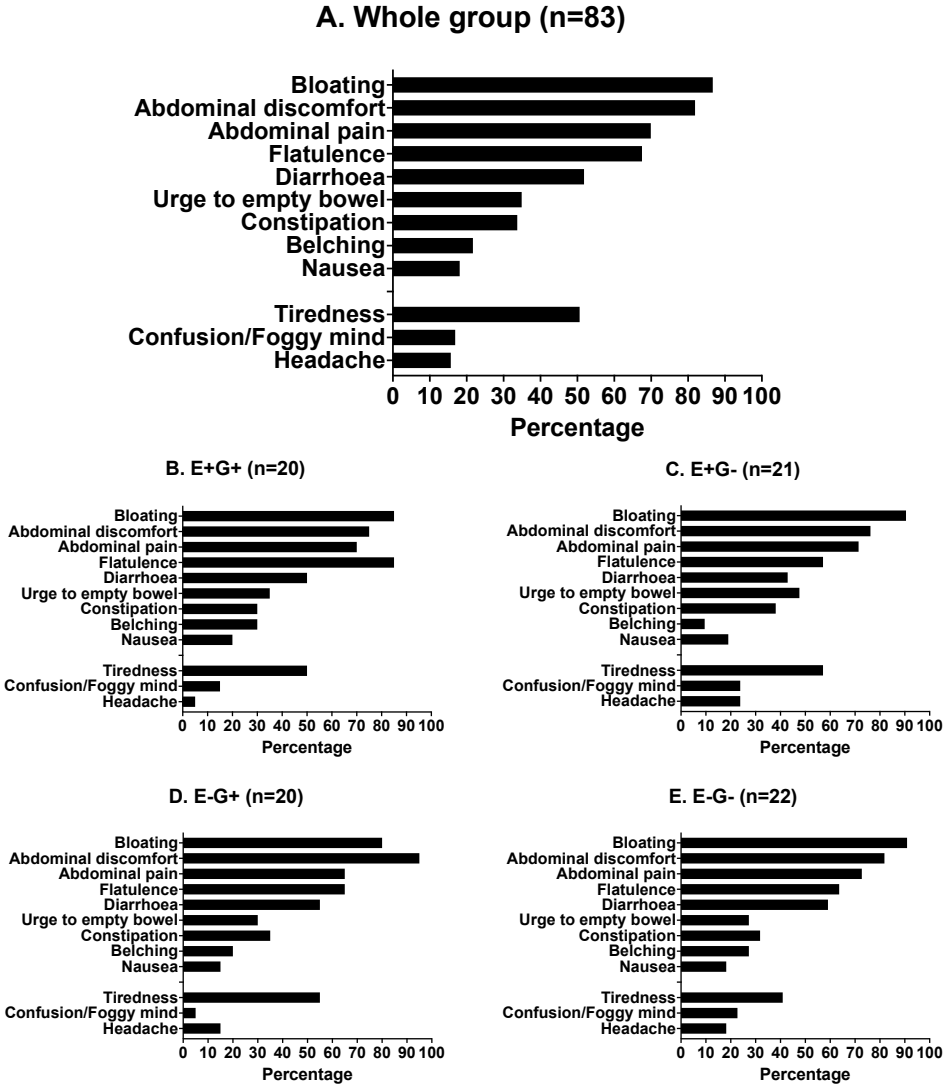
Supplementary Results – Baseline characteristics

Supplementary Table S2. Medication and nutritional supplements, as reported during the screening visit.

	All (n = 83)	E+G+ (n = 20)	E+G- (n = 21)	E-G+ (n = 20)	E-G- (n = 22)
Medication categories	35 (42.2%)	8 (40.0%)	5 (23.8%)	9 (45.0%)	13 (59.1%)
Acetanilide derivate	2 (2.4%)	0 (0.0%)	1 (4.8%)	1 (5.0%)	0 (0.0%)
Antacids	1 (1.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (4.5%)
Anticoagulant	1 (1.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (4.5%)
Antidepressants	1 (1.2%)	1 (5.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Antihistamine	7 (8.4%)	5 (25.0%)	0 (0.0%)	1 (5.0%)	1 (4.5%)
Antihypertensive	4 (4.8%)	0 (0.0%)	0 (0.0%)	3 (15.0%)	1 (4.5%)
Antipsychotics	1 (1.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (4.5%)
Inhaled steroids	3 (3.6%)	2 (10.0%)	1 (4.8%)	0 (0.0%)	0 (0.0%)
Laxatives	1 (1.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (4.5%)
NSAID	2 (2.4%)	1 (5.0%)	0 (0.0%)	0 (0.0%)	1 (4.5%)
Oral contraceptive	16 (19.3%)	3 (15.0%)	5 (23.8%)	4 (20.0%)	4 (18.2%)
PPI	4 (4.8%)	1 (5.0%)	0 (0.0%)	1 (5.0%)	2 (9.1%)
Spasmolytic	2 (2.4%)	1 (5.0%)	0 (0.0%)	0 (0.0%)	2 (4.5%)
SSRI	4 (4.8%)	0 (0.0%)	0 (0.0%)	1 (5.0%)	3 (13.6%)
Statins	1 (1.2%)	0 (0.0%)	1 (4.8%)	0 (0.0%)	0 (0.0%)
Thyroid hormone	4 (4.8%)	1 (5.0%)	2 (9.5%)	0 (0.0%)	1 (4.5%)
Other	7 (8.4%)	3 (15.0%)	2 (9.5%)	0 (0.0%)	2 (9.1%)
Nutritional supplements	28 (33.7%)	9 (45.0%)	8 (38.1%)	5 (25.0%)	6 (27.3%)
Fibres	2 (2.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (9.1%)
Iron	6 (7.2%)	0 (0.0%)	2 (9.5%)	3 (15.0%)	1 (4.5%)
Minerals	9 (10.8%)	3 (15.0%)	2 (9.5%)	0 (0.0%)	4 (18.2%)
Multivitamin	9 (10.8%)	5 (25.0%)	1 (4.8%)	2 (10.0%)	1 (4.5%)
Omega 3	5 (6.0%)	0 (0.0%)	3 (14.3%)	2 (10.0%)	0 (0.0%)
Vitamin C	6 (67.2%)	2 (10.0%)	1 (4.8%)	1 (5.0%)	2 (9.1%)
Vitamin D	15 (18.1%)	2 (10.0%)	6 (28.6%)	2 (10.0%)	5 (22.7%)
Vitamins - other	5 (6.0%)	2 (10.0%)	2 (9.5%)	0 (0.0%)	1 (4.5%)
Other	9 (10.8%)	0 (0.0%)	6 (28.6%)	2 (10.0%)	1 (4.5%)

E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread; NSAID = non-steroidal anti-inflammatory drugs; PPI = proton pump inhibitors, SSRI = selective serotonin reuptake inhibitors. Values displayed as n (%).



Supplementary Figure S3. Previously experienced symptoms after gluten consumption, as reported during the screening visit by (A) whole group, (B) E+G+, (C) E+G-, (D) E-G+, and (E) E-G-. E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread.

Supplementary Results – Test day

Supplementary Table S3. Observed mean and estimated mean (corrected for baseline (t = 0 hours)) test day scores (t = 1-8 hours) per intervention group, assessed by 0-100mm visual analogue scale (VAS), for gastrointestinal (GI) and extra-intestinal symptoms.

	E+G+ (n=20)	E+G- (n=21)	E-G+ (n=20)	E-G- (n=22)
Observed mean				
Overall GI symptoms	16.4 ± 14.6	13.4 ± 10.3	6.2 ± 5.6	6.6 ± 8.2
Abdominal discomfort	19.6 ± 17.5	12.8 ± 10.8	5.4 ± 6.1	7.9 ± 9.4
Abdominal pain	11.8 ± 16.8	7.5 ± 9.1	3.0 ± 4.8	4.0 ± 5.7
Belching	3.5 ± 5.7	2.9 ± 4.2	3.1 ± 6.1	2.8 ± 5.5
Bloating	14.6 ± 19.8	12.9 ± 15.8	4.0 ± 4.6	7.2 ± 10.2
Constipation	3.4 ± 11.0	2.6 ± 4.5	0.5 ± 1.2	0.5 ± 2.0
Diarrhoea	1.0 ± 2.2	1.5 ± 3.6	0.6 ± 2.1	1.2 ± 3.8
Flatulence	4.2 ± 6.0	3.6 ± 5.1	2.7 ± 4.5	3.2 ± 6.1
Fullness	18.6 ± 16.3	10.9 ± 13.6	10.3 ± 13.4	8.9 ± 8.4
Nausea	3.1 ± 5.8	1.9 ± 3.3	0.9 ± 2.2	2.9 ± 7.2
Urge to empty bowel	7.0 ± 7.4	6.4 ± 5.6	7.4 ± 5.6	5.3 ± 8.0
Confusion/Foggy mind	8.0 ± 7.7	6.8 ± 8.4	2.0 ± 2.4	8.2 ± 17.1
Headache	9.4 ± 10.6	4.7 ± 7.5	1.7 ± 5.5	4.6 ± 6.6
Tiredness	15.3 ± 10.2	17.6 ± 12.4	9.8 ± 11.1	17.0 ± 20.8
Positive affect	19.9 ± 6.8	22.8 ± 8.1	27.1 ± 8.8	21.6 ± 6.3
Negative affect	10.9 ± 0.9	10.9 ± 1.2	10.6 ± 0.7	11.3 ± 1.9
Estimated mean				
Overall GI symptoms	16.6 ± 1.7	11.7 ± 1.7	6.9 ± 1.7	7.4 ± 1.6
Abdominal discomfort	19.1 ± 2.3	11.2 ± 2.3	6.7 ± 2.3	8.6 ± 2.2
Abdominal pain	11.0 ± 2.0	6.4 ± 2.0	4.0 ± 2.0	4.9 ± 1.9
Belching	3.7 ± 1.2	3.0 ± 1.1	3.1 ± 1.2	2.4 ± 1.1
Bloating	14.4 ± 2.1	9.8 ± 2.0	4.7 ± 2.1	9.6 ± 2.0
Constipation	3.7 ± 1.3	2.0 ± 1.3	0.9 ± 1.3	0.6 ± 1.2
Diarrhoea	1.2 ± 0.6	1.6 ± 0.6	0.7 ± 0.6	0.9 ± 0.6
Flatulence	4.5 ± 1.2	3.4 ± 1.2	2.1 ± 1.2	3.6 ± 1.1
Fullness	18.7 ± 2.7	9.9 ± 2.7	11.1 ± 2.7	9.1 ± 2.6
Nausea	3.1 ± 1.0	2.5 ± 1.0	1.2 ± 1.0	2.2 ± 1.0
Urge to empty bowel	7.4 ± 1.5	6.0 ± 1.5	7.5 ± 1.5	5.2 ± 1.4
Confusion/Foggy mind	9.1 ± 1.8	7.6 ± 1.8	1.9 ± 1.8	6.6 ± 1.7
Headache	8.7 ± 1.4	3.7 ± 1.4	2.7 ± 1.4	5.2 ± 1.3
Tiredness	15.7 ± 2.6	18.1 ± 2.6	9.5 ± 2.6	16.3 ± 2.5
Positive affect	21.3 ± 1.1	22.8 ± 1.1	24.3 ± 1.1	22.9 ± 1.0
Negative affect	11.0 ± 0.3	11.0 ± 0.2	10.7 ± 0.3	11.1 ± 0.2

E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread. Values are displayed as mean ± standard deviation for the observed mean, and mean ± standard error for the estimated mean. Estimated means were obtained using repeated measures analysis of covariance (RM ANCOVA) with intervention group as the between-subject factor, baseline (t = 0 hours) as a covariate, and time (t = 1-8 hours) as the repeated measures factor.

See Supplementary Table S4 for differences between groups.

Supplementary Table S4. Differences in estimated mean test day scores, corrected for baseline (t = 0 hours), for gastrointestinal (GI) and extra-intestinal symptoms, assessed by visual analogue scale (0-100mm).

Outcome parameter	Overall p-value	E+ G+ vs E+ G-	E+ G+ vs E- G+	E+ G+ vs E-G-	E+ G- vs E- G+	E+ G- vs E- G-	E- G+ vs E- G-
Overall GI symptoms	p=0.0004	4.9mm [-1.7-11.5mm] p=0.28	9.6mm [3.0-16.2mm] p=0.0010	9.1mm [2.7-15.6mm] p=0.0016	4.7mm [-1.8-11.3mm] p=0.33	4.2mm [-2.2-10.7mm] p=0.47	-0.5mm [-7.0-5.9mm] p>0.99
Abdominal discomfort	p=0.0018	7.9mm [-0.9-16.7mm] p=0.10	12.4mm [3.4-21.3mm] p=0.0020	10.5mm [1.8-19.2mm] p=0.010	4.5mm [-4.5-13.4mm] p>0.99	2.6mm [-6.1-11.2mm] p>0.99	-1.9mm [-10.6-6.8mm] p>0.99
Abdominal pain	p=0.080	··	··	··	··	··	··
Belching	p=0.90	··	··	··	··	··	··
Bloating	p=0.016	4.6mm [-3.3-12.4mm] p=0.72	9.7mm [1.8-17.6mm] p=0.0083	4.7mm [-3.0-12.5mm] p=0.61	5.1mm [-2.7-13.0mm] p=0.49	0.2mm [-7.6-8.0mm] p>0.99	-4.9mm [-12.7-2.8mm] p=0.53
Constipation	p=0.30	··	··	··	··	··	··
Diarrhoea	p=0.75	··	··	··	··	··	··
Flatulence	p=0.58	··	··	··	··	··	··
Fullness	p=0.055	··	··	··	··	··	··
Nausea	p=0.61	··	··	··	··	··	··
Urge to empty bowel	p=0.64	··	··	··	··	··	··
Confusion/ Foggy mind	p=0.037	1.5mm [-5.4-8.4mm] p>0.99	7.3mm [0.3-14.2mm] p=0.037	2.6mm [-4.3-9.4mm] p>0.99	5.8mm [-1.1-12.7mm] p=0.16	1.1mm [-5.7-7.9mm] p>0.99	-4.7mm [-11.5-2.1mm] p=0.40
Headache	p=0.017	5.1mm [-0.2-10.3mm] p=0.066	6.0mm [0.6-11.4mm] p=0.020	3.5mm [-1.7-8.8mm] p=0.43	0.9mm [-4.4-6.3mm] p>0.99	-1.5mm [-6.7-3.6mm] p>0.99	-2.5mm [-7.7-2.7mm] p>0.99
Tiredness	p=0.11	··	··	··	··	··	··
Positive affect	p=0.33	··	··	··	··	··	··
Negative affect	p=0.74	··	··	··	··	··	··

E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread.

Values are displayed as difference in estimated mean [95% CI]. Differences between groups were analysed using repeated measures analysis of covariance (RM ANCOVA) with intervention group as the between-subject factor, baseline (t = 0 hours) as a covariate, and time (t = 1-8 hours) as the repeated measures factor. Post-hoc comparison of intervention groups (with per symptom a Bonferroni correction for 6 pairwise comparisons) was only done if the overall p-value was below 0.05; ·· = no pairwise comparison was done.

Supplementary Results – Post-hoc analyses

Supplementary Table S5. Observed mean and estimated mean (corrected for baseline (t = 0 hours)) test day scores in the morning (t = 1-4 hours) per intervention group, assessed by 0-100mm visual analogue scale (VAS), for gastrointestinal (GI) and extra-intestinal symptoms.

	E+G+ (n=20)	E+G- (n=21)	E-G+ (n=20)	E-G- (n=22)
Observed mean				
Overall GI symptoms	14.5 ± 14.1	12.6 ± 11.1	6.6 ± 6.9	7.3 ± 9.5
Abdominal discomfort	18.3 ± 19.5	11.6 ± 10.4	5.3 ± 6.9	8.5 ± 10.0
Abdominal pain	11.6 ± 18.4	8.5 ± 10.7	2.1 ± 4.4	4.0 ± 6.3
Belching	2.9 ± 5.4	2.3 ± 3.6	3.8 ± 7.7	3.0 ± 6.8
Bloating	12.8 ± 20.3	11.0 ± 15.6	4.4 ± 6.3	7.5 ± 12.2
Constipation	2.2 ± 7.6	2.1 ± 4.0	0.3 ± 0.8	0.7 ± 2.4
Diarrhoea	0.9 ± 2.1	2.0 ± 4.8	0.1 ± 0.3	1.8 ± 5.2
Flatulence	4.3 ± 6.2	3.9 ± 5.6	2.1 ± 3.1	4.4 ± 8.6
Fullness	16.1 ± 16.7	10.2 ± 15.4	11.7 ± 14.8	9.5 ± 12.1
Nausea	3.5 ± 8.3	1.9 ± 4.4	1.1 ± 4.1	2.9 ± 7.9
Urge to empty bowel	7.4 ± 7.3	7.5 ± 8.3	9.0 ± 9.3	6.8 ± 11.1
Confusion/Foggy mind	8.6 ± 9.5	4.9 ± 7.5	1.9 ± 2.4	8.3 ± 17.8
Headache	8.7 ± 12.3	3.9 ± 7.3	1.3 ± 3.9	3.4 ± 6.1
Tiredness	15.9 ± 13.2	16.1 ± 14.9	10.8 ± 12.1	15.9 ± 22.0
Positive affect	20.5 ± 6.9	23.0 ± 8.3	27.8 ± 8.5	21.8 ± 6.5
Negative affect	10.9 ± 0.8	11.0 ± 1.3	10.5 ± 0.6	11.3 ± 1.7
Estimated mean				
Overall GI symptoms	14.7 ± 1.8	10.8 ± 1.8	7.3 ± 1.8	8.2 ± 1.7
Abdominal discomfort	17.8 ± 2.5	10.0 ± 2.5	6.7 ± 2.5	9.3 ± 2.4
Abdominal pain	10.7 ± 2.2	7.1 ± 2.1	3.3 ± 2.2	5.0 ± 2.1
Belching	3.1 ± 1.3	2.5 ± 1.3	3.9 ± 1.3	2.6 ± 1.2
Bloating	12.5 ± 2.2	7.8 ± 2.1	5.2 ± 2.1	10.0 ± 2.1
Constipation	2.5 ± 0.9	1.3 ± 0.8	0.7 ± 0.9	0.7 ± 0.8
Diarrhoea	1.1 ± 0.8	2.1 ± 0.8	0.2 ± 0.8	1.5 ± 0.8
Flatulence	4.5 ± 1.4	3.7 ± 1.4	1.8 ± 1.4	4.6 ± 1.3
Fullness	16.1 ± 3.0	9.0 ± 3.0	12.6 ± 3.0	9.7 ± 2.9
Nausea	3.5 ± 1.3	2.5 ± 1.3	1.4 ± 1.3	2.1 ± 1.2
Urge to empty bowel	7.8 ± 2.1	7.0 ± 2.0	9.2 ± 2.0	6.7 ± 1.9
Confusion/Foggy mind	9.8 ± 1.8	5.8 ± 1.8	1.7 ± 1.8	6.5 ± 1.7
Headache	8.0 ± 1.3	2.8 ± 1.3	2.5 ± 1.3	4.2 ± 1.2
Tiredness	16.3 ± 2.8	16.7 ± 2.7	10.5 ± 2.8	15.1 ± 2.6
Positive affect	22.0 ± 1.0	23.0 ± 1.0	24.8 ± 1.1	23.2 ± 1.0
Negative affect	11.0 ± 0.2	11.0 ± 0.2	10.7 ± 0.2	11.0 ± 0.2

E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread. Values are displayed as mean ± standard deviation for the observed mean, and mean ± standard error for the estimated mean. Estimated means were obtained using repeated measures analysis of covariance (RM ANCOVA) with intervention group as the between-subject factor, baseline (t = 0 hours) as a covariate, and time (t = 1-4 hours) as the repeated measures factor.

See Supplementary Table S6 for differences between groups.

Supplementary Table S6. Post-hoc sensitivity analysis for differences in estimated mean test day scores in the morning (t = 1-4 hours) for gastrointestinal (GI) and extra-intestinal symptoms, assessed by visual analogue scale (0-100mm).

Outcome parameter	Overall p-value	E+ G+ vs E+ G-	E+ G+ vs E- G+	E+ G+ vs E-G-	E+ G- vs E- G+	E+ G- vs E- G-	E- G+ vs E- G-
Overall GI symptoms	p=0.023	3.9mm [-3.0-10.8mm] p=0.81	7.4mm [0.4-14.3mm] p=0.031	6.5mm [-0.3-13.3mm] p=0.068	3.5mm [-3.4-10.4mm] p>0.99	2.7mm [-4.1-9.4mm] p>0.99	-0.9mm [-7.7-5.9mm] p>0.99
Abdominal discomfort	p=0.017	7.8mm [-1.7-17.4mm] p=0.17	11.1mm [1.4-20.7mm] p=0.017	8.5mm [-0.9-17.9mm] p=0.10	3.2mm [-6.5-12.9mm] p>0.99	0.7mm [-8.7-10.1mm] p>0.99	-2.5mm [-11.9-6.9mm] p>0.99
Abdominal pain	p=0.11	..	..	..	..	..	..
Belching	p=0.87	..	..	..	..	..	..
Bloating	p=0.11	..	..	..	..	..	..
Constipation	p=0.40	..	..	..	..	..	..
Diarrhoea	p=0.40	..	..	..	..	..	..
Flatulence	p=0.49	..	..	..	..	..	..
Fullness	p=0.31	..	..	..	..	..	..
Nausea	p=0.72	..	..	..	..	..	..
Urge to empty bowel	p=0.81	..	..	..	..	..	..
Confusion/ Foggy mind	p=0.023	4.0mm [-2.8-10.9mm] p=0.70	8.1mm [1.1-15.0mm] p=0.014	3.4mm [-3.5-10.2mm] p>0.99	4.1mm [-2.8-10.9mm] p=0.69	-0.6mm [-7.4-6.1mm] p>0.99	-4.7mm [-11.5-2.1mm] p=0.39
Headache	p=0.014	5.2mm [0.3-10.2mm] p=0.032	5.5mm [0.5-10.5mm] p=0.024	3.8mm [-1.1-8.7mm] p=0.24	0.3mm [-4.7-5.3mm] p>0.99	-1.4mm [-6.3-3.4mm] p>0.99	-1.7mm [-6.6-3.2mm] p>0.99
Tiredness	p=0.36	..	..	..	..	..	..
Positive affect	p=0.31	..	..	..	..	..	..
Negative affect	p=0.62	..	..	..	..	..	..

E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread.

Values are displayed as difference in estimated mean [95% CI]. Differences between groups were analysed using repeated measures analysis of covariance (RM ANCOVA) with intervention group as the between-subject factor, baseline (t = 0 hours) as a covariate, and time (t =1-4 hours) as the repeated measures factor. Post-hoc comparison of intervention groups (with per symptom a Bonferroni correction for 6 pairwise comparisons) was only done if the overall p-value was below 0.05; ·· = no pairwise comparison was done.

Supplementary Table S7. Observed mean and estimated mean (corrected for baseline (t = 0 hours)) test day scores in the afternoon (t = 5-8 hours) per intervention group, assessed by 0-100mm visual analogue scale (VAS), for gastrointestinal (GI) and extra-intestinal symptoms.

	E+G+ (n=20)	E+G- (n=21)	E-G+ (n=20)	E-G- (n=22)
Observed mean				
Overall GI symptoms	18.3 ± 17.4	14.1 ± 10.6	5.9 ± 7.3	5.9 ± 8.2
Abdominal discomfort	20.8 ± 18.1	13.9 ± 12.3	5.4 ± 8.7	7.3 ± 10.7
Abdominal pain	11.9 ± 17.7	6.6 ± 8.3	3.8 ± 7.6	4.1 ± 6.9
Belching	4.1 ± 6.6	3.4 ± 5.4	2.4 ± 5.0	2.5 ± 6.3
Bloating	16.5 ± 21.2	14.7 ± 17.1	3.5 ± 6.0	7.0 ± 10.3
Constipation	4.7 ± 14.5	3.0 ± 6.5	0.8 ± 2.2	0.4 ± 1.7
Diarrhoea	1.0 ± 2.9	1.0 ± 3.0	1.1 ± 4.2	0.7 ± 3.1
Flatulence	4.1 ± 7.3	3.3 ± 5.3	3.3 ± 7.3	2.1 ± 4.0
Fullness	21.2 ± 18.2	11.6 ± 13.5	9.0 ± 14.6	8.4 ± 8.6
Nausea	2.7 ± 4.9	2.0 ± 4.0	0.7 ± 1.9	2.9 ± 7.3
Urge to empty bowel	6.6 ± 9.0	5.3 ± 5.9	5.7 ± 8.6	3.8 ± 6.5
Confusion/Foggy mind	7.4 ± 10.3	8.7 ± 10.1	2.1 ± 4.0	8.1 ± 17.1
Headache	10.0 ± 11.7	5.5 ± 8.8	2.2 ± 7.2	5.7 ± 8.0
Tiredness	14.8 ± 11.7	19.1 ± 11.9	8.8 ± 12.2	18.1 ± 20.7
Positive affect	19.3 ± 7.1	22.6 ± 8.2	26.5 ± 9.3	21.3 ± 6.8
Negative affect	10.9 ± 1.0	10.9 ± 1.2	10.6 ± 0.9	11.3 ± 2.1
Estimated mean				
Overall GI symptoms	18.4 ± 2.1	12.5 ± 2.1	6.5 ± 2.1	6.7 ± 2.0
Abdominal discomfort	20.4 ± 2.7	12.4 ± 2.6	6.7 ± 2.7	8.0 ± 2.5
Abdominal pain	11.3 ± 2.3	5.8 ± 2.3	4.6 ± 2.3	4.8 ± 2.2
Belching	4.2 ± 1.3	3.5 ± 1.3	2.4 ± 1.3	2.3 ± 1.3
Bloating	16.3 ± 2.5	11.8 ± 2.5	4.2 ± 2.5	9.3 ± 2.4
Constipation	4.9 ± 1.8	2.6 ± 1.8	1.0 ± 1.8	0.4 ± 1.7
Diarrhoea	1.2 ± 0.7	1.1 ± 0.6	1.2 ± 0.7	0.3 ± 0.6
Flatulence	4.6 ± 1.3	3.0 ± 1.3	2.4 ± 1.3	2.7 ± 1.2
Fullness	21.2 ± 3.1	10.9 ± 3.0	9.5 ± 3.1	8.5 ± 2.9
Nausea	2.7 ± 1.0	2.4 ± 1.0	1.0 ± 1.0	2.3 ± 1.0
Urge to empty bowel	6.9 ± 1.7	5.0 ± 1.7	5.8 ± 1.7	3.7 ± 1.6
Confusion/Foggy mind	8.4 ± 2.2	9.4 ± 2.2	2.0 ± 2.2	6.7 ± 2.1
Headache	9.5 ± 1.8	4.6 ± 1.8	3.0 ± 1.8	6.2 ± 1.8
Tiredness	15.0 ± 3.0	19.5 ± 2.9	8.6 ± 3.0	17.6 ± 2.9
Positive affect	20.7 ± 1.3	22.6 ± 1.2	23.8 ± 1.3	22.5 ± 1.2
Negative affect	11.0 ± 0.3	11.0 ± 0.3	10.7 ± 0.3	11.1 ± 0.3

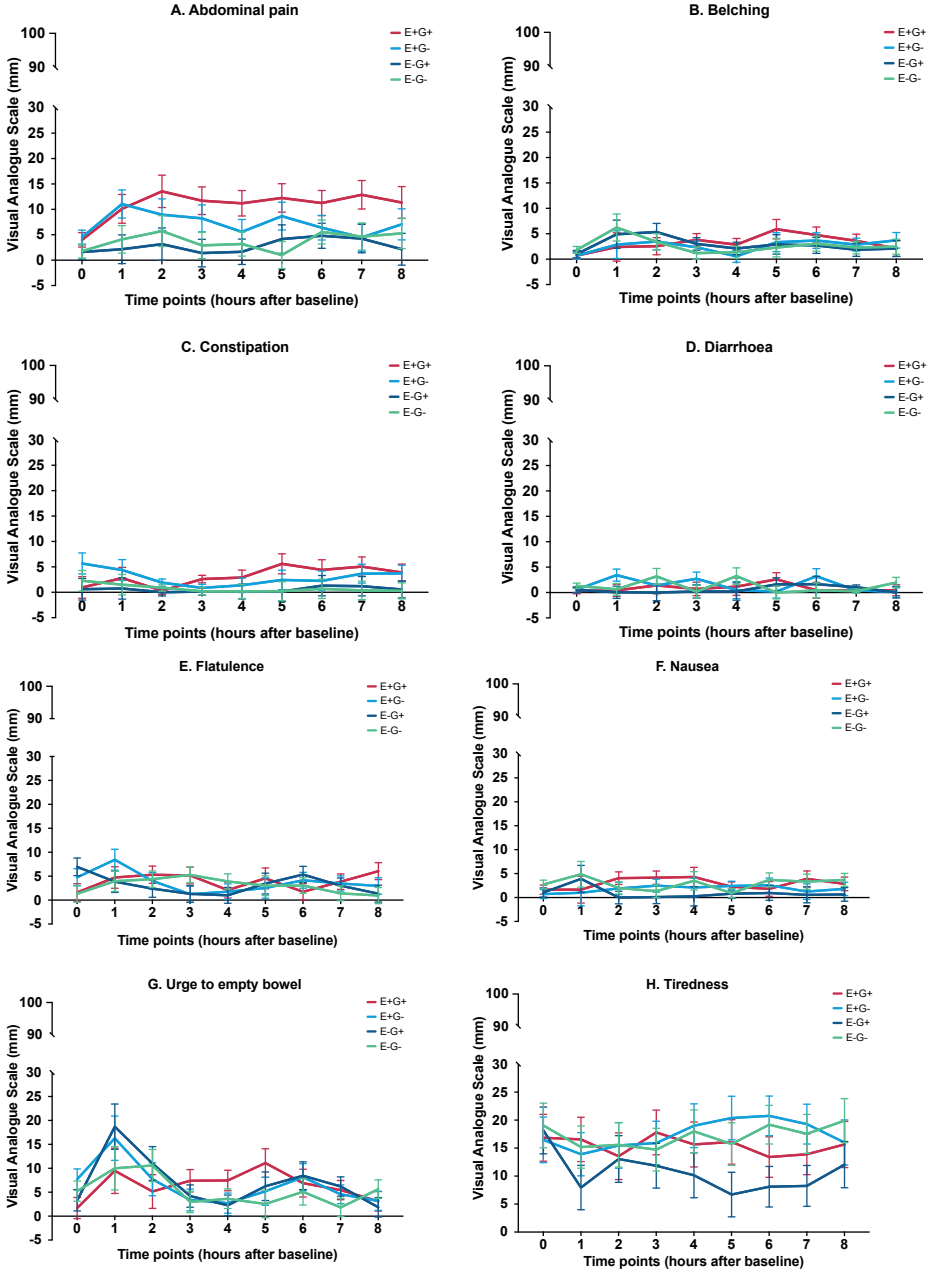
E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread. Values are displayed as mean ± standard deviation for the observed mean, and mean ± standard error for the estimated mean. Estimated means were obtained using repeated measures analysis of covariance (RM ANCOVA) with intervention group as the between-subject factor, baseline (t = 0 hours) as a covariate, and time (t = 5-8 hours) as the repeated measures factor.
See Supplementary Table S8 for differences between groups.

Supplementary Table S8. Post-hoc sensitivity analysis for differences in estimated mean test day scores in the afternoon (t = 5-8 hours) for gastrointestinal (GI) and extra-intestinal symptoms, assessed by visual analogue scale (0-100mm).

Outcome parameter	Overall p-value	E+ G+ vs E+ G-	E+ G+ vs E- G+	E+ G+ vs E-G-	E+ G- vs E- G+	E+ G- vs E- G-	E- G+ vs E- G-
Overall GI symptoms	p=0.0003	5.9mm [-2.2-14.1mm] p=0.315	11.9mm [3.7-20.1mm] p=0.0011	11.7mm [3.7-19.8mm] p=0.0010	6.0mm [-2.2-14.1mm] p=0.31	5.8mm [-2.2-13.8mm] p=0.32	-0.2mm [-8.2-7.9mm] p>0.99
Abdominal discomfort	p=0.0020	8.0mm [-2.1-18.1mm] p=0.215	13.7mm [3.4-23.9mm] p=0.0033	12.4mm [2.4-22.4mm] p=0.0071	5.7mm [-4.6-16.0mm] p=0.82	4.4mm [-5.5-14.4mm] p>0.99	-1.3mm [-11.2-8.7mm] p>0.99
Abdominal pain	p=0.14	..	..	..	..	..	..
Belching	p=0.69	..	..	..	..	..	..
Bloating	p=0.010	4.4mm [-5.1-13.9mm] p>0.99	12.0mm [2.4-21.6mm] p=0.0065	7.0mm [-2.4-16.4mm] p=0.29	7.6mm [-2.0-17.2mm] p=0.21	2.6mm [-6.9-12.0mm] p>0.99	-5.0mm [-14.4-4.3mm] p=0.90
Constipation	p=0.27	..	..	..	..	..	..
Diarrhoea	p=0.69	..	..	..	..	..	..
Flatulence	p=0.65	..	..	..	..	..	..
Fullness	p=0.014	10.3mm [-1.2-21.9mm] p=0.11	11.7mm [0.4-23.4mm] p=0.0495	12.7mm [1.3-24.2mm] p=0.020	1.4mm [-10.3-13.0mm] p>0.99	2.4mm [-8.9-13.7mm] p>0.99	1.0mm [-10.4-12.5mm] p>0.99
Nausea	p=0.65	..	..	..	..	..	..
Urge to empty bowel	p=0.59	..	..	..	..	..	..
Confusion/ Foggy mind	p=0.088	..	..	..	..	..	..
Headache	p=0.085	..	..	..	..	..	..
Tiredness	p=0.060	..	..	..	..	..	..
Positive affect	p=0.42	..	..	..	..	..	..
Negative affect	p=0.85	..	..	..	..	..	..

E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread.

Values are displayed as difference in estimated mean [95% CI]. Differences between groups were analysed using repeated measures analysis of covariance (RM ANCOVA) with intervention group as the between-subject factor, baseline (t = 0 hours) as a covariate, and time (t = 5-8 hours) as the repeated measures factor. Post-hoc comparison of intervention groups (with per symptom a Bonferroni correction for 6 pairwise comparisons) was only done if the overall p-value was below 0.05; .. = no pairwise comparison was done.



Supplementary Figure S4. Test day scores for (A-G) gastrointestinal and (H) extra-intestinal symptoms, assessed by visual analogue scale (0-100mm), without significant differences between groups. Participants consumed breakfast directly after t = 0 hours, and lunch directly after t = 4 hours. Differences between groups were analysed using repeated measures analysis of covariance (RM ANCOVA) with intervention group as the between-subject factor, baseline (t = 0 hours) as a covariate, and time (t = 1-8 hours) as the repeated measures factor. Sensitivity analyses were done for the morning (t = 1-4 hours) and the afternoon (t = 5-8 hours). E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread.

Supplementary Table S9. Observed mean and estimated mean (corrected for baseline (t = 0 hours)) follow-up (t = 1-3 days) scores per intervention group, assessed by 0-100mm visual analogue scale (VAS), for gastrointestinal (GI) and extra-intestinal symptoms.

	E+G+ (n=20)	E+G- (n=21)	E-G+ (n=20)	E-G- (n=22)
Observed mean				
Overall GI symptoms	23.2 ± 14.1	17.4 ± 12.1	11.9 ± 7.7	13.4 ± 12.0
Abdominal discomfort	23.1 ± 15.7	14.6 ± 11.1	6.6 ± 6.5	14.2 ± 14.4
Abdominal pain	18.0 ± 17.8	9.6 ± 8.2	5.5 ± 8.3	8.0 ± 8.8
Belching	4.8 ± 6.1	4.6 ± 8.1	2.2 ± 2.9	6.4 ± 8.9
Bloating	21.7 ± 22.5	14.7 ± 14.9	6.4 ± 6.2	13.3 ± 13.2
Constipation	9.7 ± 10.7	8.4 ± 11.0	2.0 ± 3.5	6.3 ± 8.2
Diarrhoea	4.4 ± 9.4	0.9 ± 2.3	1.9 ± 4.9	1.4 ± 2.9
Flatulence	15.2 ± 15.4	12.8 ± 12.6	9.3 ± 10.9	10.5 ± 14.6
Fullness	18.6 ± 14.2	12.5 ± 12.0	12.7 ± 13.5	10.3 ± 12.0
Nausea	4.2 ± 7.3	4.0 ± 5.8	1.0 ± 2.2	2.9 ± 4.0
Urge to empty bowel	13.3 ± 11.5	10.4 ± 9.9	10.2 ± 10.1	8.8 ± 12.0
Confusion/Foggy mind	8.2 ± 11.0	7.2 ± 7.4	2.5 ± 3.7	7.9 ± 11.3
Headache	9.8 ± 11.1	7.3 ± 10.3	2.4 ± 4.9	5.5 ± 8.0
Tiredness	21.4 ± 16.1	17.1 ± 13.1	11.1 ± 11.0	18.5 ± 17.6
Positive affect	21.0 ± 6.7	25.6 ± 8.4	27.7 ± 6.5	22.6 ± 6.7
Negative affect	12.6 ± 1.7	11.7 ± 1.4	11.5 ± 2.0	12.7 ± 2.7
Estimated mean				
Overall GI symptoms	23.4 ± 2.5	16.2 ± 2.4	12.5 ± 2.5	14.0 ± 2.4
Abdominal discomfort	22.5 ± 2.6	13.0 ± 2.5	8.1 ± 2.6	15.0 ± 2.5
Abdominal pain	17.5 ± 2.6	9.0 ± 2.5	6.1 ± 2.6	8.5 ± 2.5
Belching	5.1 ± 1.5	4.9 ± 1.5	2.3 ± 1.5	5.8 ± 1.4
Bloating	21.5 ± 3.0	12.2 ± 3.0	7.0 ± 3.0	15.3 ± 2.8
Constipation	10.1 ± 2.0	7.5 ± 1.9	2.6 ± 2.0	6.3 ± 1.9
Diarrhoea	4.7 ± 1.2	1.0 ± 1.2	2.1 ± 1.2	0.9 ± 1.2
Flatulence	16.9 ± 2.7	11.9 ± 2.7	6.6 ± 2.8	12.4 ± 2.6
Fullness	18.6 ± 2.9	12.0 ± 2.9	13.1 ± 2.9	10.5 ± 2.8
Nausea	4.1 ± 1.2	4.2 ± 1.1	1.1 ± 1.2	2.6 ± 1.2
Urge to empty bowel	14.5 ± 2.4	9.2 ± 2.3	10.7 ± 2.4	8.5 ± 2.3
Confusion/Foggy mind	9.0 ± 1.8	7.7 ± 1.7	2.5 ± 1.7	6.7 ± 1.7
Headache	9.5 ± 2.0	6.8 ± 2.0	2.9 ± 2.0	5.8 ± 1.9
Tiredness	21.7 ± 2.9	17.5 ± 2.9	11.0 ± 2.9	17.9 ± 2.8
Positive affect	22.3 ± 1.2	25.6 ± 1.2	25.1 ± 1.2	23.8 ± 1.1
Negative affect	12.7 ± 0.4	11.8 ± 0.4	11.7 ± 0.4	12.4 ± 0.4

E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread. Values are displayed as mean ± standard deviation for the observed mean, and mean ± standard error for the estimated mean. Estimated means were obtained using repeated measures analysis of covariance (RM ANCOVA) with intervention group as the between-subject factor, baseline (t = 0 hours) as a covariate, and time (t = 1-3 days) as the repeated measures factor.

See Supplementary Table S10 for differences between groups.

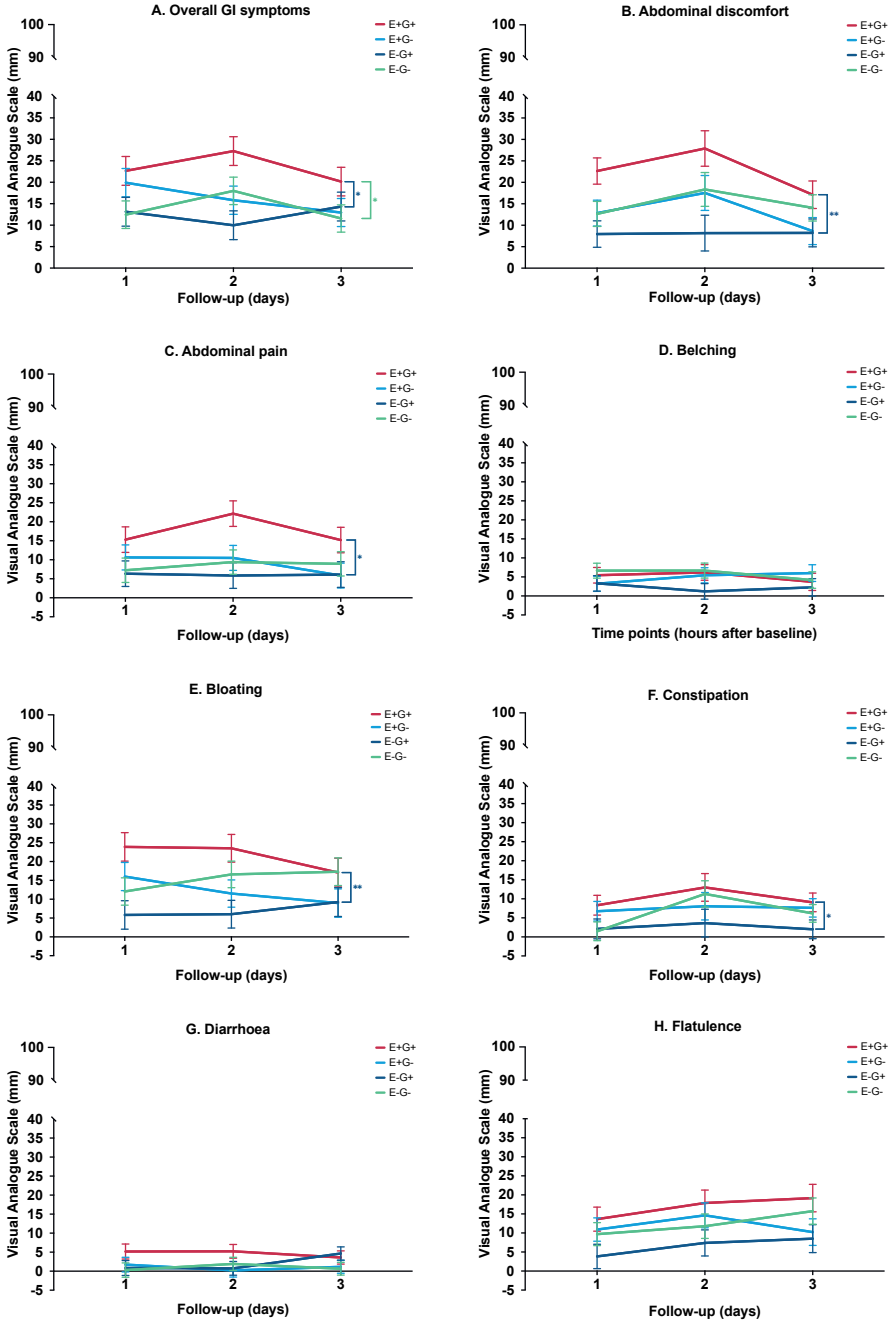
Supplementary Table S10. Post-hoc sensitivity analysis for differences in estimated mean follow-up (t = 1-3 days) scores for gastrointestinal (GI) and extra-intestinal symptoms, assessed by visual analogue scale (0-100mm).

Outcome parameter	Overall p-value *	E+ G+ vs E+ G-	E+ G+ vs E- G+	E+ G+ vs E-G-	E+ G- vs E- G+	E+ G- vs E- G-	E- G+ vs E- G-
Overall GI symptoms	0.010<p<0.020	7.1mm [-2.3-16.6mm] p=0.26	10.9mm [1.3-20.4mm] p=0.017	9.4mm [0.0-18.7mm] p=0.047	3.7mm [-5.8-13.2mm] p>0.99	2.2mm [-7.0-11.5mm] p>0.99	-1.5mm [-10.8-7.8mm] p>0.99
Abdominal discomfort	0.013<p<0.0031	9.5mm [-0.3-19.4mm] p=0.060	14.4mm [4.4-24.4mm] p=0.0011	7.5mm [-2.2-17.3mm] p=0.23	4.9mm [-5.1-14.9mm] p>0.99	-2.0mm [-11.7-7.7mm] p>0.99	-6.9mm [-16.6-2.8mm] p=0.34
Abdominal pain	0.010<p<0.019	8.6mm [-1.2-18.3mm] p=0.12	11.4mm [1.5-21.4mm] p=0.0150	9.0mm [-0.7-18.7mm] p=0.082	2.9mm [-7.0-12.8mm] p>0.99	0.5mm [-9.2-10.1mm] p>0.99	-2.4mm [-12.1-7.2mm] p>0.99
Belching	0.26<p<0.43	..	..	..	..	..	..
Bloating	0.0060<p<0.010	9.3mm [-2.0-20.6mm] p=0.17	14.5mm [3.1.25.9mm] p=0.0053	6.2mm [-5.0.17.4mm] p=0.82	5.1mm [-6.2-16.5mm] p>0.99	-3.2mm [-14.3-8.0mm] p>0.99	-8.3mm [-19.4-2.9mm] p=0.28
Constipation	0.043<p<0.078	2.7mm [-4.9-10.2mm] p>0.99	7.5mm [0.1-15.0mm] p=0.045	3.8mm [-3.5-11.1mm] P=0.96	4.9mm [-2.6-12.4mm] p=0.48	1.2mm [-6.1-8.4mm] p>0.99	-3.7mm [-11.1-3.6mm] p>0.99
Diarrhoea	0.095<p<0.14	..	..	..	..	..	..
Flatulence	0.064<p<0.099	..	..	..	..	..	..
Fullness	0.18<p<0.25	..	..	..	..	..	..
Nausea	0.16<p<0.22	..	..	..	..	..	..
Urge to empty bowel	0.25<p<0.35	..	..	..	..	..	..
Confusion/ Foggy mind	0.045<p<0.093	1.3mm [-5.5-8.0mm] p>0.99	6.5mm [-0.3-13.3mm] p=0.069	2.3mm [-4.5-9.0mm] p>0.99	5.2mm [-1.5-12.0mm] p=0.48	1.0mm [-5.6-7.6mm] p>0.99	-4.2mm [-10.9-2.4mm] p>0.99
Headache	0.11<p<0.19	..	..	..	..	..	..
Tiredness	0.066<p<0.10	..	..	..	..	..	..
Positive affect	0.13<p<0.24	..	..	..	..	..	..
Negative affect	0.18<p<0.39	..	..	..	..	..	..

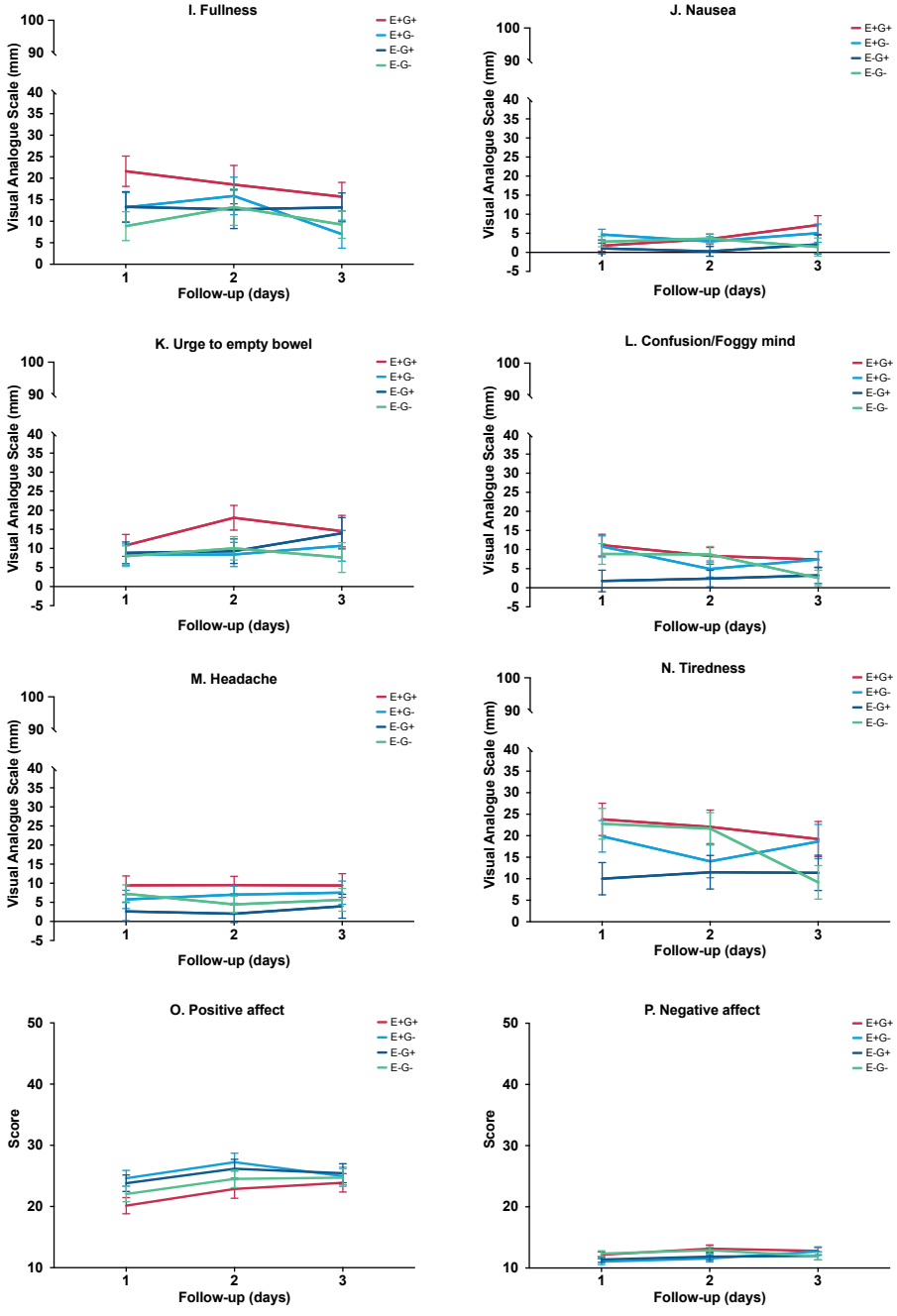
E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread.

* Missing values were imputed using multiple imputation. P-values for these analyses were checked for all imputations, and the range of p-values ([lowest value] < p < [highest value]) for these multiple imputations is listed.

Values are displayed as difference in estimated mean [95% CI]. Differences between groups were analysed using repeated measures analysis of covariance (RM ANCOVA) with intervention group as the between-subject factor, baseline (t = 0 hours) as a covariate, and time (t = 1-3 days) as the repeated measures factor. Post-hoc comparison of intervention groups (with per symptom a Bonferroni correction for 6 pairwise comparisons) was only done if the overall p-value was below 0.05; ·· = no pairwise comparison was done.



Supplementary Figure S5. Follow-up scores for (A-K) gastrointestinal and (L-N) extra-intestinal symptoms, assessed by visual analogue scale (0-100mm), and (O-P) scores for positive and negative affect. Differences between groups were analysed using repeated measures analysis of covariance (RM ANCOVA) with intervention group as the between-subject factor, baseline (t = 0 hours) as a covariate, and time (t = 1-3 days) as the repeated measures factor. E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread.



Supplementary Figure S5 (Continued). Follow-up scores for (A-K) gastrointestinal and (L-N) extra-intestinal symptoms, assessed by visual analogue scale (0-100mm), and (O-P) scores for positive and negative affect. Differences between groups were analysed using repeated measures analysis of covariance (RM ANCOVA) with intervention group as the between-subject factor, baseline (t = 0 hours) as a covariate, and time (t = 1-3 days) as the repeated measures factor. E+ = expectancy of getting gluten-containing bread; E- = expectancy of getting gluten-free bread; G+ = actual gluten-containing bread; G- = actual gluten-free bread.

Supplementary Table S11. Summary of significant differences between intervention groups after post-hoc sensitivity analysis of correction for covariates.

Outcome parameter	Time period *	Changes in significant differences between groups after addition of covariates **
Overall GI symptoms	Test day	None
	Morning	BMI (p=0.32) resulted in a significant difference between E+G+ and E-G- (p=0.044)
	Afternoon	None
	Follow-up	Study site (all p>0.05) resulted in the difference between E+G+ and E-G- no longer being significant (p=0.057) Education level (all p>0.05) resulted in the difference between E+G+ and E-G- no longer being significant (p=0.056) IBS (all p>0.05) resulted in the difference between E+G+ and E-G- no longer being significant (p=0.057) GAD-7 (all p>0.05) resulted in the difference between E+G+ and E-G- no longer being significant (p=0.057) PHQ-9 (all p>0.05) resulted in the difference between E+G+ and E-G- no longer being significant (p=0.059) PHQ-15 (all p>0.05) resulted in the difference between E+G+ and E-G- no longer being significant (p=0.052)
Abdominal discomfort	Test day	FD (p=0.11) resulted in a significant difference between E+G+ and E+G- (p=0.045)
	Morning	None
	Afternoon	None
	Follow-up	Alcohol consumption (all p>0.05) resulted in a significant difference between E+G+ and E+G- (p=0.048) FD (all p<0.05) resulted in a significant difference between E+G+ and E+G- (p=0.010) GAD-7 (all p>0.05) resulted in a significant difference between E+G+ and E+G- (p=0.044)
Abdominal pain	Test day	None
	Morning	None
	Afternoon	None
	Follow-up	FD (all p>0.05) resulted in a significant difference between E+G+ and E+G- (p=0.043) GAD-7 (all p>0.05) resulted in a significant difference between E+G+ and E-G- (p=0.0496)
Belching	Test day	None
	Morning	None
	Afternoon	None
	Follow-up	None
Bloating	Test day	None
	Morning	None
	Afternoon	None
	Follow-up	Alcohol consumption (all p>0.05) resulted in a significant difference between E+G+ and E+G- (p=0.049)
Constipation	Test day	None
	Morning	None
	Afternoon	None
	Follow-up	BMI (all p>0.05) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.085) Education level (all p>0.05) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.056) Alcohol consumption (all p>0.05) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.062) FD (all p>0.05) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.071)

Supplementary Table S11 (Continued).

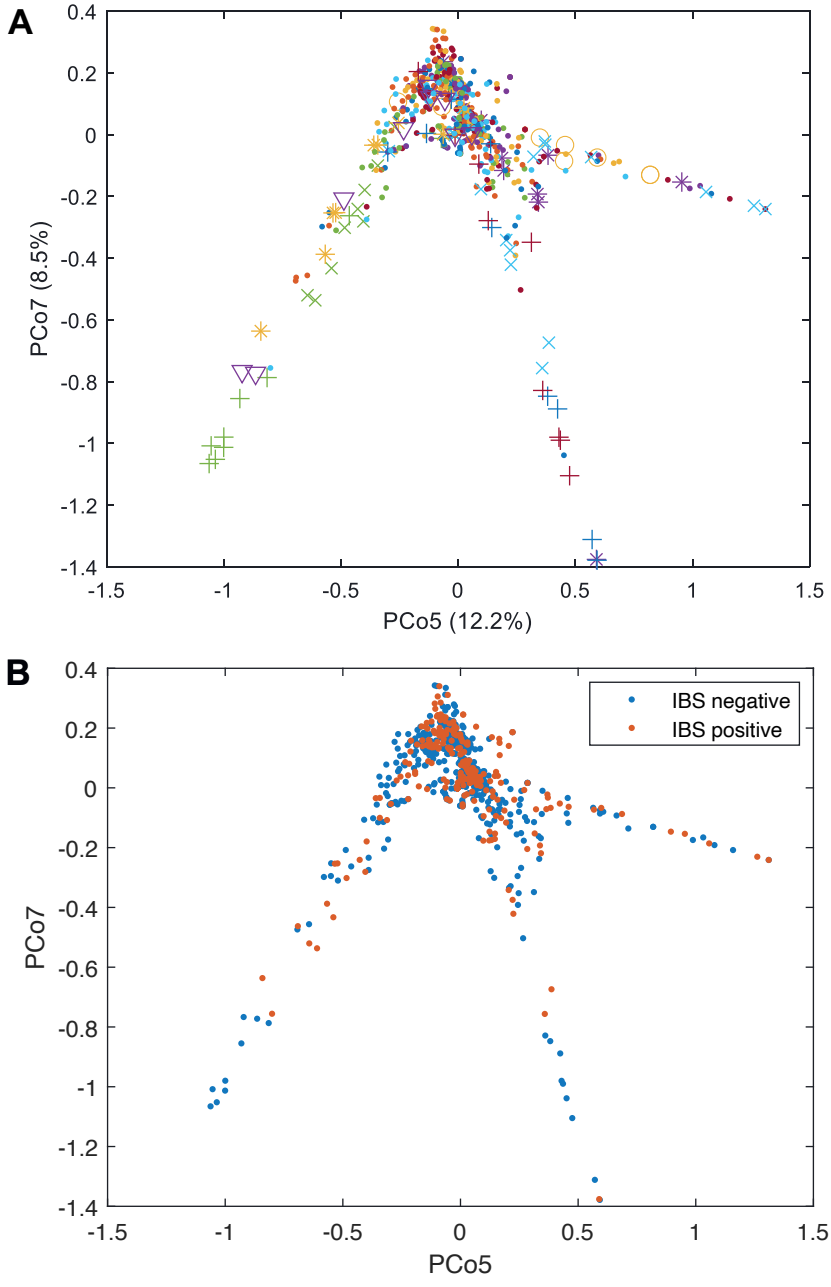
Outcome parameter	Time period *	Changes in significant differences between groups after addition of covariates **
Diarrhoea	Test day	None
	Morning	None
	Afternoon	None
	Follow-up	None
Flatulence	Test day	None
	Morning	None
	Afternoon	None
	Follow-up	Alcohol consumption (all p>0.05) resulted in a significant difference between E+G+ and E-G+ (p=0.021)
Fullness	Test day	Alcohol consumption (p=0.624) resulted in a significant difference between E+G+ and E-G- (p=0.040)
	Morning	None
	Afternoon	Study site (p=0.30) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.054) Sex (p=0.77) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.050) Age (p=0.65) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.058) BMI (p=0.98) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.15) Education level (p=0.38) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.068) Smoking behaviour (p=0.88) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.055) IBS (p=0.62) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.053) PHQ-15 (p=0.77) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.056) BMI (p=0.98) resulted in the difference between E+G+ and E-G- no longer being significant (p=0.066)
	Follow-up	None
Nausea	Test day	None
	Morning	None
	Afternoon	None
	Follow-up	None
Urge to empty bowel	Test day	None
	Morning	None
	Afternoon	None
	Follow-up	None

Supplementary Table S11 (Continued).

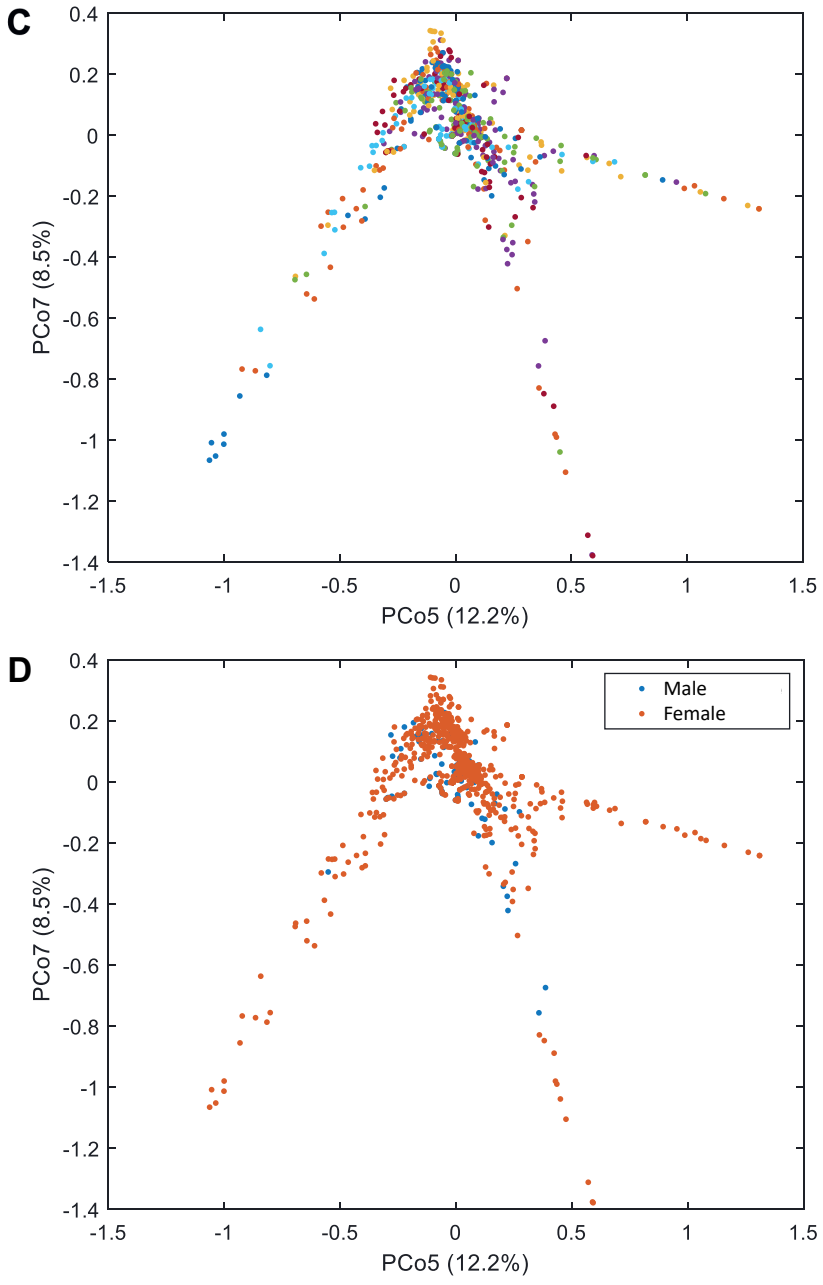
Outcome parameter	Time period *	Changes in significant differences between groups after addition of covariates **
Confusion/Foggy mind	Test day	Smoking behaviour (p=0.25) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.096) Alcohol consumption (p=0.97) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.057)
	Morning	None
	Afternoon	None
	Follow-up	None
Headache	Test day	Sex (p=0.14) resulted in a significant difference between E+G+ and E+G- (p=0.042) BMI (p=0.73) resulted in a significant difference between E+G+ and E+G- (p=0.045) GAD-7 (p=0.67) resulted in a significant difference between E+G+ and E+G- (p=0.0496)
	Morning	Smoking behaviour (p=0.58) resulted in the difference between E+G+ and E+G- no longer being significant (p=0.10) Alcohol consumption (p=0.50) resulted in the difference between E+G+ and E+G- no longer being significant (p=0.091) FD (p=0.194) resulted in the difference between E+G+ and E+G- no longer being significant (p=0.079) Smoking behaviour (p=0.58) resulted in the difference between E+G+ and E-G+ no longer being significant (p=0.054)
	Afternoon	Education level (p=0.052) resulted in a significant difference between E+G+ and E-G+ (p=0.048)
	Follow-up	None
Tiredness	Test day	Smoking behaviour (p=0.49) resulted in a significant difference between E+G- and E-G+ (p=0.021) PHQ-9 (p=0.0049) resulted in a significant difference between E+G- and E-G+ (p=0.034)
	Morning	None
	Afternoon	Smoking behaviour (p=0.192) resulted in a significant difference between E+G- and E-G+ (p=0.020) PHQ-9 (p=0.011) resulted in a significant difference between E+G- and E-G+ (p=0.020) PHQ-15 (p=0.0064) resulted in a significant difference between E+G- and E-G+ (p=0.032)
	Follow-up	None
Positive affect	Test day	None
	Morning	None
	Afternoon	None
	Follow-up	None
Negative affect	Test day	None
	Morning	None
	Afternoon	None
	Follow-up	None

* Time period lists repeated measures for the full test day (t = 1-8 hours), morning (t = 1-4 hours), afternoon (t = 5-8 hours), and follow-up (t = 1-3 days). Analyses for all time periods were analysed with baseline (t = 0 hours) as covariate.

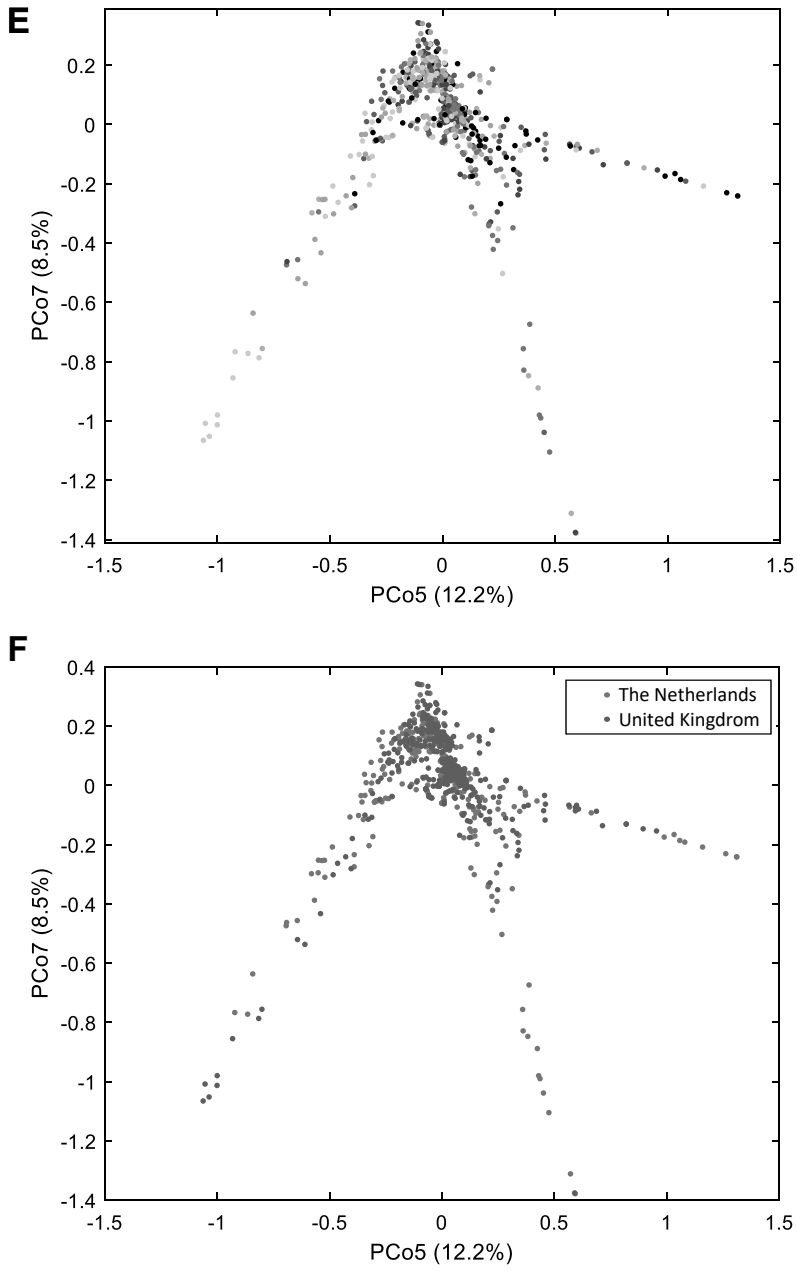
** The following variables were added sequentially to each model as single covariates to assess their impact: study site, gender, age, body mass index (BMI), education level (university educated or not), smoking behaviour (current smoker, former smoker, or never smoked), alcohol consumption, irritable bowel syndrome according to the Rome IV criteria (IBS), functional dyspepsia according to the Rome IV criteria (FD), Generalized Anxiety Disorder assessment score to assess anxiety (GAD-7), Patient Health Questionnaire-9 score to assess depression (PHQ-9), and Patient Health Questionnaire-15 score to assess somatic symptoms (PHQ-15).
For the follow-up measurements, missing values were imputed using multiple imputation. P-values for these covariates were checked for all imputations, but pooled p-values were not computed.



Supplementary Figure S6. Principle coordinate analysis (PCoA) score plot based on unsupervised random forest analysis of overall gastrointestinal (GI) symptoms and all individual GI symptoms (*i.e.* abdominal discomfort, abdominal pain, belching, bloating, constipation, diarrhoea, flatulence, fullness, nausea, and urge to empty bowel) at timepoints 0-8 hours. Figures were colour coded for (A) individual participants, (B) irritable bowel syndrome (IBS) according to Rome IV criteria, (C) age, (D) gender, (E) body mass index, and (F) country.



Supplementary Figure S6 (Continued)**.** Principle coordinate analysis (PCoA) score plot based on unsupervised random forest analysis of overall gastrointestinal (GI) symptoms and all individual GI symptoms (*i.e.* abdominal discomfort, abdominal pain, belching, bloating, constipation, diarrhoea, flatulence, fullness, nausea, and urge to empty bowel) at timepoints 0-8 hours. Figures were colour coded for (A) individual participants, (B) irritable bowel syndrome (IBS) according to Rome IV criteria, (C) age, (D) gender, (E) body mass index, and (F) country.



Supplementary Figure S6 (Continued)**.** Principle coordinate analysis (PCoA) score plot based on unsupervised random forest analysis of overall gastrointestinal (GI) symptoms and all individual GI symptoms (*i.e.* abdominal discomfort, abdominal pain, belching, bloating, constipation, diarrhoea, flatulence, fullness, nausea, and urge to empty bowel) at timepoints 0-8 hours. Figures were colour coded for (A) individual participants, (B) irritable bowel syndrome (IBS) according to Rome IV criteria, (C) age, (D) gender, (E) body mass index, and (F) country.

CHAPTER 7

General discussion

Key results

A variety of food products and components can trigger gastrointestinal (GI) symptoms in disorders like irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), and non-coeliac gluten/wheat sensitivity (NCGS/NCWS). These triggers can induce symptoms via different pathophysiological mechanisms. In this thesis, we further investigated overall diet, trigger foods and components, and their effect on GI symptoms and intestinal inflammation, as well as the role of psychological factors on these symptoms.

First, we studied the impact of the overall dietary pattern on intestinal inflammation and GI symptoms. In **Chapter 2**, we showed that diet quality was significantly lower in IBD and IBS patients as compared to healthy controls (HC). Lower diet quality was associated with more intestinal inflammation in IBD and more severe symptoms in IBS patients. Furthermore, although the dietary inflammatory potential was not significantly different between groups, in IBD patients a more pro-inflammatory diet was associated with higher abdominal pain scores.
Besides the overall dietary composition, also the processing of the food consumed, such as heat induced Maillard reactions, may impact intestinal health and thus disease. We found that the absolute intake of dietary dicarbonyls and advanced glycation endproducts (AGEs) was lower in IBS as compared to IBD and HC, but not after adjustment for energy intake (**Chapter 3**). The intake of these components was not significantly associated with faecal calprotectin, as marker for intestinal inflammation, in IBD and IBS patients, apart from a potential protective effect as indicated by a higher methylglyoxal (MGO) intake in individuals with low as compared to moderate faecal calprotectin levels.
Next, the role of specific food products in symptom generation was investigated, showing higher self-reported food intolerance and avoidance in IBS patients as compared to HC, with a wide variety of trigger foods (**Chapter 4**). Food avoidance was related to psychological factors, but not to type of symptoms.
Finally, we focussed on one of the common trigger foods in GI disorders, namely wheat. In NCWS individuals, we found that on a group level no differences were found between yeast fermented (YF) or sourdough fermented (SF) bread made of bread wheat, spelt, or emmer (**Chapter 5**). The majority experienced GI symptoms for at least one of the breads, but not to all of them. Additionally, we showed that although the role of gluten cannot be ruled out, the nocebo effect played a significant role in symptom induction in our study participants (**Chapter 6**), suggesting a role for the gut-brain interaction in NCGS.

In this general discussion, the findings of this thesis are put into perspective and future implications are discussed.

Food & gastrointestinal symptoms

There is substantial evidence that dietary patterns are associated with onset and disease course of both IBS and IBD. Several indices have been developed for the assessment of diet quality. We assessed overall diet quality using the Dutch Health Diet Index (DHD-2015),[1] measuring adherence to the Dutch dietary guidelines.[2] Diet quality was significantly lower in IBD and IBS patients as compared to HC (**Chapter 2**). Similar to previous studies,[3,4] a lower intake of dairy and high-fibre foods, such as wholegrain products, fruit, vegetables, and legumes, was found in IBD and IBS compared to HC. In IBS, a lower diet quality was associated with more abdominal pain, whereas in IBD no association was found between overall diet quality and symptom scores. This further supports the relevance to consider overall diet quality in these patients, as low diet quality could be both the result and the cause of food-related symptoms.

In **Chapter 4**, we confirmed that IBS patients report a significantly higher number of food products to be associated with perceived intolerance compared to HC. In line with previous studies,[4-7] we identified common triggers of food-related GI symptoms in IBS included gas-producing vegetables like cabbage, onion, and beans, dairy, fried snacks, alcohol, coffee, and carbonated soda. Additionally, because of the extensive nature of our questionnaire, we noted more details within these food groups. For example, within dairy, specifically the creamy, fatty and/or chocolate-based dairy products were most often reported to cause symptoms, as well as cream- or chocolate-based pastries. Several types of bread, including both whole-meal and white bread, were also frequently mentioned. Predominant food-related GI symptoms were abdominal pain and bloating, similar to previous studies.[4,5]

Wheat-containing products are among the top five trigger foods for IBS and IBD patients,[3,4] as also confirmed by our study (**Chapter 4**). They are considered the main culprit food for NCGS/NCWS, although the wheat component responsible for these symptoms is still under debate. There is conflicting evidence on the role of gluten.[8-16] We found a pronounced nocebo effect (see further discussion below) but cannot rule out that gluten also has an effect on symptom occurrence (**Chapter 6**). The exact underlying mechanism is not clear. A study in individuals with coeliac disease and healthy volunteers showed gluten, and especially gliadin, to increase intestinal paracellular permeability in both groups,[17] but this was not confirmed in NCGS individuals.[8] It should also be noted that most gluten preparations used in human studies contain significant amounts of amylase trypsin inhibitors (ATIs).[18] The same applies to our study, where vital wheat gluten was used. The potentially harmful effects of ATIs so far are mainly based on *in vitro* and animal studies,[19-24] and the impact in humans needs further study. Additionally, several studies indicate that fructans in wheat may be more important triggers than gluten.[9,25-28] This is further supported by the rather fast occurrence of symptoms after intake, *i.e.* within 1-4 hours (**Chapter 6**),

which may involve the intestinal microbiome, *e.g.* by fermentation of fructans. Interestingly, in a preliminary analysis as part of the TKI Well on Wheat? project, we noted in an *in vitro* setting exposing faecal samples of NCWS and HC donors to pre-digested bread, that there were no large differences in the microbiota composition. However, the metabolite profile, as studied by volatile organic compounds sampled from the headspace of the faecal dilutions in an anaerobic cabinet, did differ between NCWS as compared to HC, and showed larger variation over time of exposure. Furthermore, large inter-individual variation was observed (manuscript in preparation). Eliciting the contributions of different wheat components is further complicated by the fact that the biochemical composition differs between wheat species and varieties, and is further influenced by environmental and cultivation conditions, and bread processing methods.[29-31] In **Chapter 5**, the clinical relevance of these differences was studied by comparing the effects of well-characterised YF and SF breads, each made of bread wheat, spelt, or emmer, in individuals with self-reported NCWS. At group level, no differences were found between the YF breads, nor between the SF breads, but on an individual level more than half of the participants responded with GI symptoms, *i.e.* abdominal discomfort, abdominal pain, bloating, and/or flatulence, to at least one of the breads. However, most of the participants could tolerate one or two of the study breads, but which of the breads was tolerated varied between individuals. All bread types contained varying ratios of fructans, gluten, and ATIs, though with limited overall variation. Therefore, combined with the inter-individual differences in symptom response, it was not possible to assign any of the reported symptoms to one of these components.

It is important to establish whether a strict gluten-free diet (GFD) is needed to manage symptoms in NCGS/NCWS, as replacing the type of bread may be sufficient for part of the individuals. Wheat provides an important source of carbohydrates, dietary fibres, proteins, vitamins, minerals, and phytochemicals.[32] Following a strict GFD without adequate guidance and replacement can lead to an unbalanced dietary intake and nutrient deficiencies.[33]

Multiple previous studies reported both IBD and IBS patients adjust their diet because of food-related symptoms, resulting in a less healthy diet,[4-6,34-37] which was also confirmed by our findings (**Chapter 2**). Both IBS patients and HC avoided most food products associated with symptom occurrence. In line with self-reported food intolerance, food avoidance was more excessive in IBS patients than in HC. In general, onion, alcohol, and coffee were less often avoided despite these symptoms, suggesting symptoms induced by these food products may be ‘taken for granted’. We hypothesised that food avoidance behaviour may be related to the type of symptoms but could not confirm this (**Chapter 4**). The food products and groups identified to induce symptoms in IBS patients are in line with those incorporated in both the National Institute for Health and Care Excellence (NICE) guidelines[38] and the low-FODMAP (fermentable oligo-, di-, monosaccharides and polyols) diet.[39,40] Long-term effects of

these diets have not been well studied and negative impacts, for example on the gut microbiota, need to be considered.[41] Both diets should preferably be guided by a dietitian, which is often not the case.[42]

The identification of single food products and key components as triggers for food-related GI-symptoms can be useful for personalised dietary treatment. As illustrated by the number of and variation in culprit foods reported in **Chapter 4**, identification of the key trigger foods may already relieve symptoms, making a full elimination diet like the low-FODMAP diet or the GFD unnecessary. Nevertheless, it remains important to study these food products and components in the context of the whole dietary pattern, as individual components may enhance or counteract each other's effect. This additive effect is an important feature of the low-FODMAP diet.[39,40] Furthermore, the importance of this was also illustrated by the association between low diet quality and higher symptoms in IBS we found in **Chapter 2**.

Insight in type and co-occurrence of intolerances to specific food products may aid in eliciting the underlying mechanisms. The variety of food products and combinations reported in **Chapter 4** as well as the heterogeneity in symptom responses in **Chapter 5** confirm the complexity of food-related GI symptoms and substantiates that one size does not fit all. It seems unlikely that there is one underlying mechanism involved, which supports the need for an individualised approach.

Important to consider is that the associations between food intake and GI symptoms could be bidirectional. For example, elimination of high-fibre foods from the diet because of symptoms may result in gut microbiota dysbiosis, thereby further contributing to symptoms.[43] Longitudinal studies are needed to further elicit the mechanisms of food-related GI symptoms.

Food & intestinal inflammation

One of the potential mechanisms that may lead to GI symptoms is inflammation, either by direct immune modulating effects, or indirectly, mediated for example by intestinal barrier disruption or the intestinal microbiome.

Several dietary components have been associated with pro- or anti-inflammatory properties. We evaluated these properties combined by using the Adapted Dietary Inflammatory Index (ADII) as an indicator for the overall inflammatory potential of the diet.[44] We found a wide range from anti- to pro-inflammatory dietary intake in IBD, IBS and HC, with a slightly pro-inflammatory average of the overall diet that did not differ between groups (**Chapter 2**). Previous studies also found pro-inflammatory diets to be associated with these disorders.[45,46] A more pro-inflammatory diet, but not overall diet quality, was associated with higher abdominal pain scores in IBD patients. Based on our results, the inflammatory potential of the diet does not seem to be the driving factor in IBS.

Furthermore, we found no association between the ADII and faecal calprotectin as marker for intestinal inflammation in IBD nor in IBS. In addition, there was no difference in ADII score between IBD patients with active disease compared to those in remission. A more pro-inflammatory diet was also not significantly correlated with low diet quality. However, a lower diet quality was associated with more intestinal inflammation in IBD, and diet quality was lower in active versus remissive IBD. This was the first study using an objective marker of intestinal inflammation, as so far, previous studies only showed conflicting associations between a pro-inflammatory diet and clinical activity indices that do not necessarily correlate with active inflammation.[46,47]
Similar to the relation between diet quality and GI symptoms, also this relation between diet quality and intestinal inflammation could be bidirectional. Whereas a low intake of favourable nutrients like antioxidants and fibres can increase the risk of a flare,[43] patients with active disease also often attempt to mitigate their symptoms by changing their diet, which can result in poorer diet quality.[48] Furthermore, the effects of diet on inflammation may be confounded by medication use, *e.g.* anti-inflammatory or immunosuppressing drugs, especially in IBD patients with active inflammation.

A limitation of the food frequency questionnaire (FFQ) that we used to assess dietary intake is that it does not account for the effects of processing. Food processing, especially heating conditions like baking, grilling, and roasting, induces the Maillard reaction, resulting in the production of among others dicarbonyls and AGEs.[49] In **Chapter 3**, we found that the absolute intake of dietary dicarbonyls and AGEs was lower in IBS as compared to IBD and HC. However, this difference was no longer visible after correction for energy intake. Also important to note is that we only studied a selection of AGEs and dicarbonyls, whereas many more can be present in food.
Endogenously formed AGEs are considered to be involved in disease pathology by generating dysfunctional proteins and inducing pro-inflammatory signalling.[50] Currently, it is unclear to what extent dietary dicarbonyls and AGEs contribute to endogenous formation of AGEs. If present in the GI tract together with proteins from the food matrix or the intestinal environment, the Maillard reaction can occur, involving also a bidirectional interaction with the gut microbiome.[51-57] Additionally, rodent studies showed that increased intake of the FODMAPs lactose and fructo-oligosaccharides resulted in generation of toxic glycation metabolites like the dicarbonyl MGO in the gut lumen.[51,58] For dicarbonyls, both pro- and anti-inflammatory properties have been reported.[59-61] These highly reactive compounds can interact with proteins also present in the gut, resulting in formation of AGEs. In mice, this formation of AGEs from ingested FODMAPs has been correlated with visceral hypersensitivity, increased colonic epithelial expression of the receptor for advanced glycation endproducts (RAGE), increased mucosal mast cell count, and dysregulation of the colonic mucus barrier.[51,58] However, no such data is available from human studies.
We found no leads that the concentrations of dicarbonyls and AGEs present in the habitual diet of Dutch patients with IBD or IBS are associated with intestinal

inflammation (**Chapter 3**). On the contrary: a higher intake of the studied dicarbonyls and AGEs generally even correlated with a better diet quality and a more anti-inflammatory diet. This was supported by the fact that the main food products that contributed to the intake of these compounds were not only processed foods, but also foods generally considered as healthy, such as bread, vegetables, legumes, fruit, potatoes, rice and pasta, and coffee. Hereby, we cannot rule out that potential harmful effects might be counteracted by anti-inflammatory or otherwise bioactive components in the food matrix. Vitamin B6 for example is a known antiglycation agent.[51]
As described in a recent review,[62] limited evidence, mostly based on *in vitro* and animal studies, is available on what happens to dietary dicarbonyls and AGEs after ingestion. Whereas *in vitro* research shows a decreased digestibility of glycated protein, this has not been confirmed by *in vivo* studies. Additionally, studies so far are inconclusive about dietary dicarbonyls and AGEs interacting with or accumulating in intestinal epithelial cells. Furthermore, limited evidence from rodent models suggests that dietary AGEs may have different effects on healthy as compared to inflamed intestinal tissue. In healthy intestinal tissue, dietary AGEs affected tight junction expression and thereby intestinal barrier function, whereas in an IBD model they showed a protective effect against inflammation.[62] More advanced *in vitro* models, such as the TNO *in vitro* gastrointestinal models TIM-1 (stomach through small intestine) and TIM-2 (large intestine), intestinal organoids/microfluidics, as well as human intervention studies are needed to further elicit the effects of dietary dicarbonyls and AGEs in the gut.

Besides, by studying the intake and effects of dietary dicarbonyls and AGEs, we only investigated one aspect of food processing. Ultra-processed foods consist of a combination of substances derived from foods and food additives, and have been associated with many non-communicable diseases including IBD. Food additives, for example some emulsifiers, thickeners, colorants, or artificial sweeteners, have been reported to stimulate pro-inflammatory pathways, increase intestinal permeability, induce dysbiosis of the gut microbiota, or alter the mucus layer.[63] Further research is warranted to unravel the potential effects of these food additives in relation to their role in the development of GI disorders.

Complexity of diet and challenges of assessing diet

Despite the individual role of certain food products and components in food-related GI symptoms, it remains important to consider that these are always consumed as part of a whole dietary pattern. Several indices have been developed to study the overall effect of the diet, including the DHD-2015[1] and ADII[44] as described in **Chapter 2**. The DHD-2015 is very specific for the Netherlands.[1] Similar scores have been developed in other countries, for example the Healthy Eating Index (HEI) aligned with the Dietary Guidelines for Americans.[64] The fact that these questionnaires are country-specific hinders a direct comparison between studies. Additionally, it is important to note that the DHD-2015 was validated in healthy subjects, whereas IBS and IBD patients may

need other recommendations, for example because of active inflammation, increased loss via diarrhoea, and/or less absorption of nutrients.[65-67]
We used the ADII to assess the inflammatory potential of the diet, which is based on nutrients.[44] Again, it should be noted that this score was validated in healthy individuals, elderly, and those at risk of type 2 diabetes and cardiovascular disease,[44,68,69] but not IBS or IBD patients. Another score to assess the inflammatory potential of the diet is the Empirical Dietary Inflammatory Index, based on food groups.[70] We chose not to include this index in our analyses because the food groups were not representative for the Dutch dietary intake.
The main advantage of these dietary indices is that they account for the fact that foods are generally not consumed in isolation. The food matrix is important to consider when assessing the effects of specific compounds. As described above, pro-inflammatory effects may be counteracted by anti-inflammatory compounds like antioxidants, fibres, and micronutrients present in the food matrix.[71-73] The food matrix also plays an important role for the bioavailability of components. *In vitro* research showed that, whereas free-form AGEs may be more easily absorbed,[74] both dicarbonyls and protein-bound AGEs can survive gastric and small intestinal digestion, and reach the colon largely unaltered.[75,76]

It remains challenging to reliably measure food intake. For example, not all components of the DHD-2015 and the ADII could be calculated based on the FFQ used in **Chapters 2 and 3**. Several methods are available to address dietary intake, the choice depending on the research question and type of dietary data needed. In general, all methods are sensitive to bias, such as under-reporting, difficulties in portion size estimation, and recall-bias.[77] Furthermore, calculation of nutrient intake requires linkage to available and up-to-date databases on food composition, such as the NEVO table and other databases, like the ones we used for calculation of dicarbonyls[78] and AGEs[79] intake. At the moment, no such tables are available for components such as gluten, ATIs, fructans, and food additives.
The FFQ used in **Chapters 2 and 3**, as well as the dietary history method, were designed to assess overall dietary habits. An important limitation of these methods is that they do not include detailed information about food preparation methods and the intake of ready-to-eat products. They are also less useful to assess the effects of food on GI symptoms that occur within a few hours. For these purposes, dietary assessment methods such as 24-hour recalls, food records, or duplicate portions are more appropriate.[77] Combining these methods with biomarkers may also provide leads on the underlying mechanisms. Nevertheless, they are labour-intensive for participants, and therefore less suitable for longitudinal studies. Currently, digital food diary methods are being developed, *e.g.* use of artificial intelligence to estimate portion sizes and composition.[80]
When interpreting dietary assessment results and the health effects of food, it is always important to consider inter-individual differences, as also demonstrated in **Chapters 2,**

4, 5, and 6. Digestion and metabolism are affected amongst others by gut-transit time,[81] and gut microbiota composition and -activity.[82] Also, genetic susceptibility and underlying pathology can influence the effects of foods consumed.[83] Additionally, the individual's food choice is affected by environmental factors (including but not limited to socio-economic factors, lifestyle, living environment, behaviour, taste, and food preference) as well as psychological factors like anxiety, depression, and eating disorders.[84] These factors need to be accounted for in dietary intervention studies, but also in dietary treatment, further highlighting the need for an individualised approach.

Role of gut-brain interaction in food-related GI symptoms

The bidirectional gut-brain axis is important to consider in food-related GI symptoms, as psychological factors can influence GI symptoms and vice-versa.[85] In addition, psychological comorbidities like anxiety and depression are more prevalent in IBD,[86] IBS,[87] and NCGS[88] as compared to the general population.

Food intolerance is often accompanied by food avoidance to alleviate symptoms, as we also confirmed in **Chapter 4**. We found that excessive symptom-related food avoidance behaviour in IBS patients was associated with somatisation but not with anxiety and depression. Similar results were found in previous studies,[6,34,89] although more recent studies did find an association with depression and anxiety.[90,91] It is plausible we did not find an association because of the low prevalence rates of these psychological factors observed in our IBS population compared to previous studies,[87] which may be due to a selection bias.
We also found excessive food avoidance behaviour to be associated with higher screening scores for Avoidant/Restrictive Food Intake Disorder (ARFID). Although the Nine Item ARFID Screen (NIAS) questionnaire we used to assess ARFID is not validated to actually diagnose ARFID, it does imply an increased risk for disordered eating in those with high food avoidance, as was also indicated by previous studies.[92,93]

Food avoidance is also an issue in NCGS/NCWS, as these individuals often adopt a GFD, despite the fact that the role of gluten has not clearly been established yet.[8-16] Previous double-blind, placebo-controlled gluten challenges showed a high nocebo response in NCGS/NCWS.[94] In **Chapter 6**, we describe the first intervention study that actively manipulated the nocebo effect in NCGS individuals. We confirmed that expected gluten intake plays a bigger role in symptom generation than actual gluten intake. Thereby, we confirm the role of the nocebo effect in NCGS, suggesting involvement of the gut-brain axis in this disorder. We also assessed the effects of psychological factors *i.e.* anxiety, depression, and somatisation, on symptom responses in NCGS and found this was limited in our study, although this may again be a selection bias. Further research is needed to investigate if expectancy also plays a role related to other trigger foods.

Overlap between IBS and NCGS/NCWS

There is substantial overlap between NCGS/NCWS and IBS, with some studies even suggesting that NCGS/NCWS may be a subtype of IBS.[95-97] We found a prevalence of IBS, as defined by the Rome IV criteria, of 15% in NCWS (**Chapter 5**) and 34.9% in NCGS (**Chapter 6**). Previous studies have reported the presence of NCGS in 6.8-46.1% of IBS patients (Rome II-IV criteria),[12,98-102] or the other way around, the presence of IBS (Rome III criteria) in 20-44% of NCGS patients.[25] The difficulty with reliably establishing these (overlapping) prevalences is multifactorial. First of all, NCGS, thus with gluten clearly defined as the trigger, requires diagnosis by a double-blind placebo-controlled gluten challenge according to the Salerno Experts' Criteria,[103] but in clinical practice this is not always feasible, and no such criteria exist for NCWS. Also, no biomarkers are available for their diagnosis. Therefore, the prevalence of NCGS/NCWS is often self-reported. Secondly, studies do not always clearly distinguish between IBS and NCGS/NCWS. Several studies assessing the effect of gluten and/or the GFD[12,15,28,100,104-106] or different wheat products[107] in IBS patients, defined NCGS/NCWS as gluten or wheat sensitivity within an IBS population,[108,109] or IBS patients symptomatically controlled by a GFD.[8-10,110] At the same time, the effectiveness of the GFD in controlling IBS symptoms illustrates the overlap. Furthermore, wheat products are among the top five trigger foods in IBS.[4] Nevertheless, a clear distinction between IBS and NCGS/NCWS is that in IBS symptoms also occur in the absence of a dietary trigger, or due to food products other than wheat/gluten, but also non-food triggers such as stress.[111]

IBS patients typically present with abdominal pain and altered stool patterns, with other common symptoms including bloating, flatulence, and faecal urgency.[112,113] Similar symptoms are predominantly reported in NCGS/NCWS.[114,115] Additionally, a recent meta-analysis reported a pooled prevalence of 36.5% for overlap between disorders of gut-brain interaction (DGBI).[116] We also noted 5-22.9% of our NCGS/NCWS participants met the Rome IV criteria for functional dyspepsia (**Chapters 5 and 6**).

IBS and NCGS/NCWS also share similar patient characteristics. As described in the previous paragraph, both disorders show a higher prevalence of anxiety and depression than the general population.[87,88] Furthermore, IBS is significantly more prevalent in females and people below 50.[117] We noted female sex was significantly associated with more excessive food avoidance behaviour in IBS patients (**Chapter 4**) and observed a female predominance in NCGS/NCWS study participants (**Chapters 5 and 6**). Previous studies also noted a female predominance and an average age below 50 in NCGS/NCWS.[11,13,14,26,27,109,118,119] So far, a clear biological rationale for the higher prevalence of IBS and NCGS/NCWS in women, apart from hormonal influences on GI function,[120] is lacking. In our studies, test days were not scheduled during the menstrual phase, to limit the effect of menstrual symptoms on our outcomes.

The pathophysiology for both IBS and NCGS/NCWS is not clear. For IBS, altered intestinal motility, increased intestinal permeability, visceral hypersensitivity, altered gut-brain interaction, gut microbiota perturbations, and low-grade inflammation have been reported.[121] Although evidence is limited, also for NCGS/NCWS involvement of the immune system, intestinal inflammation, dysbiosis, and/or increased intestinal permeability have been indicated.[122] Additionally, we have shown the first leads that the gut-brain axis may also be involved in NCGS/NCWS (**Chapter 6**). Better understanding of these disorders as well as identification of validated biomarkers for diagnosis are required to better identify both IBS and NCGS/NCWS and to optimise (dietary) treatment strategies for both.

Conclusion and future perspectives

This thesis provides further insight into the heterogeneity of triggers and mechanisms for food-related GI symptoms in the context of IBS, IBD, and NCGS/NCWS. The importance of the overall dietary pattern as well as individual foods and components has been established. Possible mechanisms include intestinal inflammation and altered gut-brain interactions as exemplified by the nocebo effect and associations with psychological factors.

The associations between food intake, psychological factors, and GI symptoms may be bidirectional or even three-dimensional. Well-controlled (longitudinal) intervention studies with biological sampling are needed to further elicit mechanisms underlying food-related GI symptoms, taking into account inter-individual variation in disease phenotype and host-related factors, such as the intestinal microbiome, host genetics, and psychological factors. This may contribute to the identification of biomarkers for an individualised approach and enters the field of precision nutrition.

Treatment of food-related symptoms in GI disorders requires identification of potential triggers, both food and non-food related factors. Close attention should be paid to adequate replacement of the eliminated food items/components, including monitoring of nutritional status, as well as consideration of psychological factors. This requires an individualised and multidisciplinary approach with close collaboration between gastroenterologists, dietitians, and psychologists.

Finally, further development of (digital) dietary assessment tools is needed to improve the accuracy of nutrition research. These tools should be able to accurately measure the intake of various individual components, including the impact of food processing, such as gluten, food additives, and Maillard reaction products.

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Addendum

Summary

Various food products and components can trigger gastrointestinal (GI) symptoms in disorders like irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), and non-coeliac gluten/wheat sensitivity (NCGS/NCWS). These triggers can induce symptoms via different pathophysiological mechanisms, including immune responses and inflammation, intestinal barrier dysfunction, the gut microbiota, and/or the gut-brain axis. In this thesis, we further investigated overall diet, trigger foods and components, and their effect on GI symptoms and intestinal inflammation, as well as the role of psychological factors.

First, we studied the impact of the overall dietary pattern on intestinal inflammation and GI symptoms. In **Chapter 2**, we investigated the association of dietary indices with intestinal inflammation and GI symptoms in both IBD and IBS patients. Food frequency questionnaires (FFQ) from 238 IBD patients, 261 IBS patients, and 195 healthy controls (HC) were used to calculate the overall diet quality by the Dutch Healthy Diet index 2015 (DHD-2015) and its inflammatory potential by the Adapted Dietary Inflammatory Index (ADII). Intestinal inflammation was evaluated by faecal calprotectin and the Gastrointestinal Symptom Rating Scale was used to assess symptoms. We found that diet quality was significantly lower in IBD and IBS patients as compared to HC ($b=-4.009$; $p<0.001$). Lower diet quality was associated with more intestinal inflammation in IBD ($b=-0.016$; $p=0.006$) and more severe abdominal pain ($b=-0.012$, $p=0.023$) and reflux syndrome ($b=-0.016$, $p=0.004$) in IBS patients. Furthermore, although the dietary inflammatory potential was not significantly different between groups, in IBD patients a more pro-inflammatory diet was associated with higher abdominal pain scores ($b=0.194$, $p=0.004$). Longitudinal studies are needed to further investigate the role of dietary factors in the development of flares and predominant symptoms.

Besides the overall dietary composition, also the processing of the food consumed, such as the heat induced Maillard reaction, may impact intestinal health and thus disease. In **Chapter 3**, we investigated the intake of dietary dicarbonyls and advanced glycation endproducts (AGEs) as part of the habitual diet in IBD and IBS patients, and the association of these components with intestinal inflammation. The FFQ data from Chapter 2 were used to calculate the dietary intake of dicarbonyls methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG), and of the AGEs Nε-(carboxymethyl)lysine (CML), Nε-(1-carboxyethyl)lysine (CEL), and methylglyoxal-derived hydroimidazolone-1 (MG-H1). We found that the absolute intake of dietary dicarbonyls and AGEs was lower in IBS as compared to IBD and HC (all $p<0.05$), but not after adjustment for energy intake. The intake of these components was not significantly associated with faecal calprotectin, as marker for intestinal inflammation, in IBD and IBS patients, apart from a potential protective effect as indicated by a higher

MGO intake in individuals with low as compared to moderate faecal calprotectin levels (p=0.031). Thus, the concentrations of dietary dicarbonyls and AGEs generally present in the diet of Dutch patients with IBD or IBS are not associated with intestinal inflammation, although potential harmful effects might be counteracted by anti-inflammatory components in the food matrix.

As IBS patients often report symptoms to be triggered by food intake, the role of specific food products in symptom generation was investigated. In **Chapter 4**, we evaluated the extent and nature of food intolerance and avoidance in IBS patients and their relation to GI symptoms and psychological comorbidities. Food intolerance and avoidance behaviour were evaluated in 124 IBS patients and 113 HC using a questionnaire with 257 food products of 13 food groups. IBS patients reported a higher number of food products with perceived intolerance than HC (median of 18.0 [25th-75th percentile 10.0-33.5] vs 1.0 [0.0-8.0], respectively, p<0.001). A wide variety of trigger foods was reported, with gas-producing foods and fatty/cream-based dairy products most frequently reported by both groups. The number of avoided food products was higher in IBS (15.0 [8.0-27.0] vs 1.0 [0.0-7.0], p<0.001). Food avoidance was not significantly related to symptom type, but was significantly associated with an IBS diagnosis, female sex, and higher screening scores for somatisation and Avoidant/Restrictive Food Intake Disorder (ARFID).

Subsequently, we focussed on one of the common trigger foods in GI disorders, namely wheat. In **Chapter 5**, we investigated the effects of six different types of bread on GI symptoms in individuals with self-reported NCWS in whom coeliac disease and wheat allergy were ruled out. Two parallel, randomised, double-blind, crossover, multicentre studies were performed to evaluate yeast fermented (study A, n=20) or sourdough fermented (study B, n=20) bread made of bread wheat, spelt, or emmer in a randomised order on three separate test days. Each test day was preceded by a run-in period of 3 days and separated by a wash-out period of at least 7 days. Participants followed a symptom-free diet throughout the study. GI symptoms were evaluated by change in symptom score (test day minus average of the 3-day run-in period) on a 0-100mm visual analogue scale (ΔVAS). Responders were defined as an increase in ΔVAS of at least 15mm for overall GI symptoms, abdominal discomfort, abdominal pain, bloating and/or flatulence. The overall change in GI symptoms did not differ significantly between breads of different grains (YF p=0.267; SF p=0.144). The number of responders was also comparable for both YF (6 to wheat, 5 to spelt, and 7 to emmer, p=0.761) and SF breads (9 to wheat, 7 to spelt, and 8 to emmer, p=0.761). The majority of NCWS individuals experienced some GI symptoms for at least one of the breads and could also tolerate at least one of the breads. On a group level, no differences were found between different grain types for either YF or SF breads. Therefore, personalised dietary guidance is warranted in NCWS.

In addition to potential biological mechanisms, food-related symptoms may be affected by negative expectancy. Therefore, in **Chapter 6**, we investigated the effects of expectancy versus actual gluten intake on symptoms in 83 individuals with self-reported NCGS in whom coeliac disease and wheat allergy were ruled out. In this randomised, double-blind, placebo-controlled, international multicentre study participants were randomised to one of four groups based on the expectation to consume “gluten-containing” (E+) or “gluten-free” (E-) oat bread for breakfast and lunch (two slices each), and actual intake of gluten-containing (G+) or gluten-free (G-) oat bread. Mean overall GI symptoms were significantly higher in E+G+ compared with E-G+ ($p=0.0010$) and E-G- ($p=0.0016$), but not E+G- ($p=0.28$), nor between E+G- versus E-G+ ($p=0.33$), E+G- versus E-G- ($p=0.47$), and E-G+ versus E-G- ($p>0.99$). We concluded that the combined effect of expectancy and actual gluten intake had the largest effect on GI symptoms, reflecting a nocebo effect, although an additional effect of gluten could not be ruled out. The results of this study necessitate further research into possible involvement of gut-brain interaction in NCGS.

Finally, in **Chapter 7**, we summarised and discussed the main findings of this thesis. We concluded that the association between food intake, psychological factors, and GI symptoms may be bidirectional or even three-dimensional, and future studies should aim to further elicit mechanisms underlying food-related GI symptoms, taking inter-individual variation into account. We highlighted that treatment of these food-related symptoms in GI disorders requires an individualised and multidisciplinary approach with close collaboration between gastroenterologists, dietitians, and psychologists.

Samenvatting

Verschillende voedingsproducten en -componenten kunnen maagdarmklachten veroorzaken bij mensen met aandoeningen zoals het prikkelbare darmsyndroom (PDS), inflammatoire darmziekten (IBD) en niet-coeliakie gerelateerde gluten/tarwe sensitiviteit (NCGS/NCWS). Deze voedingsprikkels kunnen klachten induceren via diverse pathofysiologische mechanismen, waaronder inflammatoire en immuunreacties, intestinale barrièredysfunctie, darmmicrobiota, en/of de hersen-darm as. In dit proefschrift hebben we onderzocht hoe maagdarmklachten en intestinale inflammatie worden beïnvloed door het voedingspatroon, verschillende voedselproducten en -componenten, alsook door psychologische factoren.

Allereerst hebben we gekeken naar de impact van het totale voedingspatroon op intestinale inflammatie en maagdarmklachten. In **Hoofdstuk 2** hebben we onderzocht wat de associatie is tussen verschillende dieetindexen, intestinale inflammatie en maagdarmklachten bij IBD- en PDS-patiënten. De Dutch Healthy Diet index 2015 (DHD-2015), een score voor dieetkwaliteit op basis van de Nederlandse voedingsrichtlijnen, en de Adapted Dietary Inflammatory Index (ADII), een score voor het ontstekingspotentieel van het dieet, werden berekend op basis van data uit voedselfrequentievragenlijsten (FFQ) van 238 IBD-patiënten, 261 PDS-patiënten en 195 gezonde vrijwilligers. Intestinale inflammatie werd geëvalueerd met behulp van fecaal calprotectine. Daarnaast werd de 'Gastrointestinal Symptom Rating Scale' gebruikt om maagdarmklachten te scoren. We observeerden dat de dieetkwaliteit significant lager was in IBD- en PDS-patiënten vergeleken met gezonde vrijwilligers ($b=-4.009$; $p<0.001$). Een lagere dieetkwaliteit was geassocieerd met meer intestinale inflammatie in IBD-patiënten ($b=-0.016$; $p=0.006$) en met hogere scores voor buikpijn ($b=-0.012$, $p=0.023$) en reflux ($b=-0.016$, $p=0.004$) bij PDS-patiënten. Hoewel de inflammatoire potentie van het dieet niet significant verschilde tussen de groepen, zagen we in IBD-patiënten dat een meer pro-inflammatoir dieet geassocieerd was met hogere buikpijnscores ($b=0.194$, $p=0.004$). Longitudinale studies zijn nodig voor dieper inzicht in de rol van dieetfactoren bij het ontstaan van opvlammingen en predominante klachten.

Naast de algehele samenstelling van het voedingspatroon heeft ook de bewerking van voedsel mogelijk invloed op darmgezondheid en -ziekte. Een voorbeeld hiervan is het verhitten van voedsel, waardoor de Maillard-reactie plaatsvindt. In **Hoofdstuk 3** hebben we onderzoek gedaan naar de inname van dicarbonylen en versuikerde eiwitten (ofwel Advanced Glycation Endproducts (AGEs)) via de gebruikelijke voedingsinname van IBD- en PDS-patiënten om te onderzoeken of deze Maillard-reactieproducten geassocieerd waren met intestinale inflammatie. De FFQ-gegevens van Hoofdstuk 2 werden gebruikt om de inname te berekenen van de dicarbonylen methylglyoxaal (MGO), glyoxaal (GO) en 3-deoxyglucosoon (3-DG), en van de

versuikerde eiwitten Nε-(carboxy-methyl)lysine (CML), Nε-(1-carboxyethyl)lysine (CEL) en van methylglyoxaal afgeleide hydroimidazolone-1 (MG-H1). We vonden dat de absolute voedingsinname van dicarbonylen en versuikerde eiwitten lager was in PDS-patiënten vergeleken met IBD-patiënten en gezonde vrijwilligers (alle $p<0.05$). Echter, na correctie voor de energie-inname bleek dit niet meer het geval te zijn. De inname van deze componenten was niet significant geassocieerd met fecaal calprotectine, een marker voor intestinale inflammatie, in IBD- noch in PDS-patiënten. We vonden echter wel een mogelijk beschermend effect van een hogere MGO-inname bij personen met lage fecaal calprotectine-waardes vergeleken met gematigde fecaal calprotectine-waardes ($p=0.031$). We concludeerden dat de concentraties van dicarbonylen en versuikerde eiwitten in de voedingsinname van Nederlandse IBD- en PDS-patiënten dus niet geassocieerd waren met intestinale inflammatie. We kunnen echter niet uitsluiten dat potentieel schadelijke effecten mogelijk worden opgeheven door anti-inflammatoire componenten in de voedingsmatrix.

Tevens hebben we onderzoek gedaan naar de rol van specifieke voedingsproducten bij het ontstaan van maagdarmklachten. In **Hoofdstuk 4** hebben we de omvang en aard van voedselintolerantie en -vermijding bij PDS-patiënten geëvalueerd en de mogelijke relaties met maagdarmklachten en psychologische comorbiditeit onderzocht. Voedselintolerantie en -vermijding werden geëvalueerd in 124 PDS-patiënten en 113 gezonde vrijwilligers met behulp van een vragenlijst met 257 voedselproducten uit 13 productgroepen. PDS-patiënten rapporteerden een hoger aantal voedselproducten met zelfgerapporteerde intolerantie dan gezonde vrijwilligers (mediaan 18.0 [25^e-75^e percentiel 10.0-33.5] versus 1.0 [0.0-8.0], respectievelijk, $p<0.001$). Dit betrof een grote variatie aan producten, waarbij gasvormende producten en vette/romige zuivelproducten in beide groepen het meest werden genoemd. Het aantal voedselproducten dat vermeden werd, was ook het hoogst in de PDS-groep (15.0 [8.0-27.0] versus 1.0 [0.0-7.0], $p<0.001$). Voedselvermijding was niet geassocieerd met het type klachten, maar wel met de diagnose PDS, het vrouwelijke geslacht, en hogere screeningscores voor somatisatie en vermijdende/restrictieve voedselinname-stoornis (ARFID).

Vervolgens hebben we ons gericht op één van de voedingsprikkels die veel genoemd wordt door patiënten met gastro-intestinale aandoeningen, namelijk tarwe. In **Hoofdstuk 5** hebben we de effecten onderzocht van zes verschillende soorten brood op maagdarmklachten bij personen met zelfgerapporteerde NCWS. Coeliakie en tarwe-allergie werden bij deze mensen uitgesloten. We hebben twee parallelle, gerandomiseerde, dubbelblinde, cross-over, multicenterstudies uitgevoerd. Hiermee werden de effecten van gist-gefermenteerd (YF, studie A, n=20) of zuurdesem-gefermenteerd (SF, studie B, n=20) brood gemaakt van broodtarwe, spelt of emmer geëvalueerd op drie afzonderlijke testdagen, in een willekeurige volgorde. Iedere testdag werd voorafgegaan door een voorbereidingsperiode van 3 dagen en

gescheiden door een uitwasperiode van minimaal 7 dagen. Deelnemers volgden gedurende de hele studie een 'klachtenvrij dieet'. Klachten werden geëvalueerd door het verschil in symptoomscore (testdagscore minus het gemiddelde van de 3 dagen voorbereidingsperiode) op een 0-100mm visueel analoge schaal (ΔVAS). Een respons werd gedefinieerd als een toename van minimaal 15mm ΔVAS voor algehele maagdarmklachten, ongemak in de buik, buikpijn, opgeblazen gevoel en/of winderigheid. De ΔVAS voor algehele maagdarmklachten was niet significant verschillend tussen broodsoorten gemaakt van verschillende granen (YF p=0.267; SF p=0.144). Het aantal deelnemers met een respons was ook vergelijkbaar voor zowel de YF (6 voor broodtarwe, 5 voor spelt, en 7 voor emmer, p=0.761) als de SF-broden (9 voor broodtarwe, 7 voor spelt, en 8 voor emmer, p=0.761). De meerderheid van de mensen met NCWS ervaarde klachten voor ten minste één van de broden, maar kon ook ten minste één van de broden verdragen. Op groepsniveau zagen we geen verschil tussen de YF en SF-broden gemaakt van verschillende granen. We concludeerden dat een gepersonaliseerd dieetadvies wenselijk is voor NCWS.

Symptomen worden mogelijk ook beïnvloed door negatieve verwachtingen. Daarom hebben we in **Hoofdstuk 6** onderzoek gedaan naar het effect van verwachting versus daadwerkelijke gluteninname op klachten bij 83 personen met zelfgerapporteerde NCGS, waarbij coeliakie en tarwe-allergie waren uitgesloten. In deze gerandomiseerde, dubbelblinde, placebo-gecontroleerde, internationale multicenterstudie werden deelnemers willekeurig verdeeld in één van de vier groepen. Deze groepen waren gebaseerd op de verwachting om 'glutenbevattend' (E+) of 'glutenvrij' (E-) haverbrood te eten voor ontbijt en lunch (twee sneetjes per maaltijd), gecombineerd met daadwerkelijke inname van glutenbevattend (G+) of glutenvrij (G-) haverbrood. De gemiddelde score voor algehele maagdarmklachten was significant hoger in E+G+ vergeleken met E-G+ (p=0.0010) en E-G- (p=0.0016), maar niet vergeleken met E+G- (p=0.28), en ook niet voor E+G- versus E-G+ (p=0.33), E+G- versus E-G- (p=0.47), en E-G+ versus E-G- (p>0.99). We concludeerden dat het gecombineerde effect van verwachting en daadwerkelijke gluteninname het grootste effect had op maagdarmklachten. Dit wijst op een nocebo effect. We kunnen een additioneel effect van gluten echter niet uitsluiten. De resultaten van deze studie laten zien dat er verder onderzoek nodig is naar de mogelijke betrokkenheid van de hersen-darm as in NCGS.

Tot slot geven we in **Hoofdstuk 7** een samenvatting en discussie van de belangrijkste bevindingen van dit proefschrift. We concluderen dat de associatie tussen voedingsinname, psychologische factoren en maagdarmklachten mogelijk bi-directioneel of zelfs drie-dimensioneel is.
Toekomstige studies moeten zich richten op het verder ontrafelen van de onderliggende mechanismen van voedingsgerelateerde klachten, waarbij rekening moet worden gehouden met interindividuele variatie. We benadrukken dat de

behandeling van voedingsgerelateerde klachten in gastro-intestinale aandoeningen een gepersonaliseerde en multidisciplinaire aanpak vereist waarbij maag-, darm- en leverartsen, diëtisten en psychologen nauw moeten samenwerken.

Impact paragraph

The Western diet has been associated with an increased prevalence of gastrointestinal (GI) disorders.[1,2] Irritable bowel syndrome (IBS) is a disorder of gut-brain interaction (DGBI) that affects 5-10% of the Western population,[3] and is characterised by recurrent abdominal pain combined with altered stool patterns. Inflammatory bowel disease (IBD), a chronic inflammatory disease characterised by alternating sequences of active inflammation and remission, has a prevalence of 0.003% in Western countries.[4] About 35% of IBD patients in remission report IBS-like symptoms.[5] Food-related GI symptoms are common in these patients, with up to 90% of IBS patients, 56-68% of IBD patients with active disease, and 29-39% of IBD patients in remission indicating that GI symptoms like abdominal pain, bloating, and diarrhoea can be induced by meals and/or certain food products.[6,7] These symptoms severely impact patient's quality of life and are associated with substantial direct and indirect costs.[8,9] One of the common triggers, namely gluten-containing and/or wheat-based foods, has been indicated as the main culprit in non-coeliac gluten/wheat sensitivity (NCGS/NCWS). These individuals report symptoms despite the absence of coeliac disease and wheat allergy. NCGS/NCWS has an estimated prevalence of up to 15%.[10-12] The studies described in this thesis add to further insight into the role of potential trigger foods and food components, and their underlying mechanisms, thereby contributing to optimisation of (dietary) treatment of these patients.

Impact on research

Previous research focussed on the identification of trigger foods for GI symptom generation, but often lacked an extensive listing. The main challenge of understanding how these food products contribute to symptoms, is that they are generally not consumed in isolation, but as part of a whole diet. In this thesis, we investigated the effect of diet quality and dietary inflammatory potential as well as habitual consumption of individual potentially inflammatory components, *i.e.* dietary dicarbonyls and advanced glycation endproducts (AGEs), and food products on GI symptoms and intestinal inflammation in IBS and IBD patients (**Chapters 2-4**).

We highlighted the importance of investigating the effect of overall diet quality by showing its association with more intestinal inflammation in IBD and higher symptom levels in IBS. Furthermore, we noted a more pro-inflammatory diet was associated with higher abdominal pain scores in IBD (**Chapter 2**). Future studies on trigger foods and potential mechanisms should consider the matrix effects of the overall diet, because antagonistic as well as additive or synergistic effects will influence the *in vivo* effects of individual foods and compounds. Furthermore, inter-individual disease and host-related factors, such as the intestinal microbiome and host genetics, should be taken into account.

Not only overall diet composition, but also the processing of foods can impact health, such as 'browning' as part of the Maillard reaction during heating of food. We performed

the first study investigating the intake of dietary dicarbonyls and advanced glycation endproducts (AGEs) in IBS and IBD patients. Although these compounds are generally considered to be pro-inflammatory, we found no significant association with intestinal inflammation in these disorders (**Chapter 3**). As such, we concluded that the concentrations consumed seem insufficient to induce an inflammatory response. It should be noted that also other dicarbonyls and AGEs are present in food. Additionally, it is plausible that we found no inflammatory effects of dicarbonyls and AGEs because their effects may be counteracted by anti-inflammatory nutrients in the food matrix. Future studies should address the impact of the intestinal microbiota and the endogenous production of these compounds.
A lower diet quality may be the result of avoidance of culprit foods, without adequate replacement. Therefore, we added to the identification of known trigger foods and related food avoidance by evaluating 257 food items in patients with IBS. On one hand we found that reported sensitivity differed between foods within specific food groups (such as dairy and vegetables). On the other hand, we observed that many patients report a variety of food items that largely varied between patients. Based on these findings, it seems unlikely that just one underlying mechanism is involved. Finally, in our study population, food avoidance behaviour was associated with higher screening scores for somatisation and Avoidant/Restrictive Food Intake Disorder (ARFID), but not anxiety, depression, or type of symptoms (**Chapter 4**). Future studies should focus on an individualised approach and enter the field of precision nutrition, as well as including the impact of psychological factors.

A key culprit food, also among our top 25 of most frequently reported triggers in IBS patients, is wheat. The pathophysiological mechanism of individuals experiencing symptoms after consumption of wheat in general or gluten specifically (*i.e.* NCWS or NCGS), despite having ruled out wheat allergy and coeliac disease, is still under debate. This thesis includes the first study that actively investigated the nocebo effect in NCGS individuals and confirmed that it can play a substantial role in symptom generation. The nocebo effect was even more pronounced than the effect of actual gluten intake, and thereby suggests involvement of the gut-brain axis in this disorder (**Chapter 6**). Further research is needed to understand the role of the interaction between the gut and the brain in NCGS, and to understand whether it may be classified as a DGBI or possibly even a subtype of IBS.
Nevertheless, we also cannot rule out that specific wheat components, including gluten, trigger symptoms in NCGS/NCWS individuals. In **Chapter 6** we could not exclude an additive effect of gluten intake as highest symptom scores were found in the group that both expected and actually consumed gluten. Additionally, in **Chapter 5**, we showed that the majority of NCWS individuals responded with GI symptoms to at least one of the bread types (bread wheat, spelt, or emmer, made with either yeast- or sourdough fermentation) investigated. Based on our results, we were not able to identify which wheat component is the key culprit and whether symptoms are less

pronounced after consumption of bread from a specific grain type. Instead, we showed inter-individual differences in symptom response, suggesting that host factors, such as the gut microbiota, also play an important role. Future research should focus on better understanding of the mechanisms by which wheat (components) can induce GI symptoms, taking into account inter-individual variation, and aiming to identify biomarkers for the diagnosis of NCGS/NCWS.

The chapters of this thesis have been (or will soon be) published in international peer-reviewed scientific journals. Additionally, these results were presented to various audiences at multiple national and international conferences such as the Dutch Digestive Disease Days, the Digestive Disease Week, the United European Gastroenterology Week, the European Young Cereal Scientists and Technologists Workshop, and the International Gluten Workshop. Furthermore, we used the knowledge gained about NCGS/NCWS for education of dietitians from the Dutch gastroenterology network and the Dutch Coeliac Disease Association.

Impact on healthcare providers

The results from this thesis are relevant for healthcare providers involved in the care of patients with food-related symptoms, such as general practitioners, gastroenterologists, psychologists, and dietitians. One of the major challenges in treatment of these patients relates to the heterogeneity of food triggers and symptom responses.

This thesis has shown that treatment strategies for food-related symptoms in GI disorders require an individualised approach. The first step would be to identify which food products are mainly responsible for triggering symptoms. When eliminating these foods from the diet, also considering the lower diet quality found in IBS and IBD patients, referral to a dietitian is recommended. Dietitians can ensure adequate replacement of the eliminated food items/components and can monitor nutritional status. In NCGS/NCWS individuals, we found that a substantial group could tolerate at least one of the bread types tested. When coeliac disease and wheat allergy have been ruled out, it can therefore be advised to try different bread types to identify one(s) that can be tolerated.

Additionally, all healthcare providers should pay attention to psychological risk factors like excessive food avoidance behaviour, or coexistence of anxiety or depression. Elimination diets or excessive food avoidance are risk factors for eating disorders and worsening of psychological status. However, at the same time, concurrent psychological comorbidities may also impact symptoms occurrence.

Impact on patients and society

By increasing knowledge of the scientific community and healthcare providers, the research described in this thesis aims to improve treatment strategies for food-related GI symptoms in patients with IBS, IBD, and NCGS/NCWS. As media attention for negative effects of food is increasing, informing patients and society on the current evidence is of growing importance.

So far, a clear biological cause of NCGS/NCWS has not been identified. Also, potential causes for trigger foods may differ between various gastroenterology patients. Patients with a lot of food-related GI symptoms and therefore high food avoidance behaviour are at increased risk of nutritional deficiencies. Personal identification of the key trigger foods may already effectively relieve symptoms, making full elimination diets like the low-FODMAP (fermentable oligo-, di-, monosaccharides and polyols) or gluten-free diet unnecessary. Furthermore, a healthy relationship with food is important as it encompasses a complex interplay of biological, psychological, and social aspects. Food-induced symptoms and related anxiety can be an obstacle for a healthy diet as well as eating out and enjoying the social aspect of food. We showed the importance of paying attention to psychological factors, including an increased risk of eating disorders, which can go hand in hand with food-related GI symptoms. Adequate dietary treatment, if necessary combined with psychological intervention, can improve quality of life in these patients. The results from **Chapter 5** may contribute to dietary treatment by providing participants with further insight into which type(s) of bread they may tolerate best. Furthermore, findings of **Chapters 5 and 6** have been summarised and (will be) distributed among the participants.

Moreover, GI disorders are a major public health concern. Both IBS and IBD are associated with high direct and indirect costs.[8,9] For NCGS/NCWS this has not been studied yet, but gluten-free foods are expensive and because of the overlap with IBS we can hypothesise that costs may be similar. It has not been studied how much of these costs can be attributed to the role of food. Nevertheless, effectively managing food-related symptoms by adequate dietary and/or psychological therapy can reduce the socioeconomic impact.

Conclusion

The research described in this thesis has contributed to understanding the role of food in GI symptoms. We have evaluated the role of various food products and components, as well as the impact of psychological factors, in the common GI disorders IBS, IBD, and NCGS/NCWS. These results contribute to better understanding of food-related GI symptoms and add to optimisation of (dietary) treatment options for these patients.

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Beoordelingscommissie

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List of publications

MCG de Graaf, CEGM Spooren, EMB Hendrix, MAM Hesselink, EJM Feskens, A Smolinska, D Keszthelyi, MJ Pierik, Z Mujagic, DMAE Jonkers. Diet Quality and Dietary Inflammatory Index in Dutch Inflammatory Bowel Disease and Irritable Bowel Syndrome Patients. *Nutrients*. 2022;14(9). doi: 10.3390/nu14091945

S Wang, R Godschalk, C Spooren, **M de Graaf**, D Jonkers, FJ van Schooten. The role of diet in genotoxicity of fecal water derived from IBD patients and healthy controls. *Food and Chemical Toxicology*. 2022;168. doi: 10.1016/j.fct.2022.113393.

MCG de Graaf, JLJM Scheijen, CEGM Spooren, Z Mujagic, MJ Pierik, EJM Feskens, D Keszthelyi, CG Schalkwijk, DMAE Jonkers. The Intake of Dicarbonyls and Advanced Glycation Endproducts as Part of the Habitual Diet Is Not Associated with Intestinal Inflammation in Inflammatory Bowel Disease and Irritable Bowel Syndrome Patients. *Nutrients*. 2022;15(1). doi: 10.3390/nu15010083.

MCG de Graaf, CL Lawton, F Croden, A Smolinska, B Winkens, MAM Hesselink, G van Rooy, PL Weegels, PR Shewry, LA Houghton, BJM Witteman, D Keszthelyi, FJPH Brouns, L Dye[$], DMAE Jonkers[$]. The effect of expectancy versus actual gluten intake on gastrointestinal and extra-intestinal symptoms in non-coeliac gluten sensitivity: a randomised, double-blind, placebo-controlled, international, multicentre study. *The Lancet Gastroenterology & Hepatology.* 2024;9(2):110-123. doi: 10.1016/S2468-1253(23)00317-5

Submitted for publication

MCG de Graaf, E Timmers, B Bonekamp, G van Rooy, BJM Witteman, PR Shewry, A Lovegrove, AHP America, LJWJ Gilissen, D Keszthelyi, FJPH Brouns, DMAE Jonkers. Two randomised crossover multicentre studies investigating gastrointestinal symptoms after bread consumption in individuals with non-coeliac wheat sensitivity: do wheat species and fermentation type matter?

To be submitted

MCG de Graaf*, JTW Snijkers*, B Winkens, FA Zijlstra, D Keszthelyi[$], DMAE Jonkers[$]. Evaluation of Food Intolerance and Food Avoidance in Irritable Bowel Syndrome Patients.

* Shared first author [$] Shared last author

Scientific presentations

Congres Granen & Chronische Aandoeningen (September 2019) - Wageningen, the Netherlands

- Poster presentation: The effects of bread consumption on gastrointestinal and extra-intestinal symptoms, microbiota, and metabolism in individuals with non-coeliac gluten/wheat sensitivity

Annual NUTRIM Symposium (November 2019) - Maastricht, the Netherlands

- Poster presentation: The effects of bread consumption on gastrointestinal and extra-intestinal symptoms, microbiota, and metabolism in individuals with non-coeliac gluten/wheat sensitivity

Digestive Disease Days (March 2021) - Utrecht/online, the Netherlands

- Oral presentation - President Select (Abstract prize): Dietary inflammatory index and diet quality in IBD and IBS patients

Maastricht UMC+ Science Day (September 2021) - Maastricht, the Netherlands

- Poster presentation: The association of diet with intestinal inflammation and abdominal pain in inflammatory bowel disease and irritable bowel syndrome

External Review NUTRIM (November 2021) - Online

- Poster presentation: Dietary advanced glycation endproducts and intestinal inflammation in inflammatory bowel disease and irritable bowel syndrome patients

Digestive Disease Days (March 2022) - Online

- Oral presentation: Dietary advanced glycation endproducts and intestinal inflammation in inflammatory bowel disease and irritable bowel syndrome patients

Digestive Disease Week (May 2022) - San Diego, CA, United States

- Poster presentation: Diet Quality and Dietary Inflammatory Index in Inflammatory Bowel Disease and Irritable Bowel Syndrome Patients
- Poster presentation: Dietary Advanced Glycation Endproducts and Intestinal Inflammation in Inflammatory Bowel Disease and Irritable Bowel Syndrome Patients

Annual NUTRIM Symposium (June 2022) - Maastricht, the Netherlands

- Poster presentation: Dietary Advanced Glycation Endproducts and Intestinal Inflammation in Inflammatory Bowel Disease and Irritable Bowel Syndrome Patients

Digestive Disease Days (September 2022) - Veldhoven, the Netherlands
- Oral presentation: Dietary Dicarbonyls and Intestinal Inflammation in Inflammatory Bowel Disease and Irritable Bowel Syndrome Patients

United European Gastroenterology Week (October 2022) - Vienna, Austria
- Moderated poster presentation: Dietary Dicarbonyls and Intestinal Inflammation in Inflammatory Bowel Disease and Irritable Bowel Syndrome Patients

Annual NUTRIM Symposium (November 2022) - Maastricht, the Netherlands
- Oral presentation: The role of expectancy on gastrointestinal symptoms in non-coeliac gluten sensitive individuals (preliminary results)
- Poster presentation: The effects of different bread types on gastrointestinal symptoms in individuals with non-coeliac wheat sensitivity
- Poster presentation: The role of expectancy on gastrointestinal symptoms in non-coeliac gluten sensitive individuals (preliminary results)

Digestive Disease Days (March 2023) - Veldhoven, the Netherlands
- Oral presentation: The Effect of Expectancy versus Actual Gluten Intake on Gastrointestinal Symptoms in Non-Coeliac Gluten Sensitivity

20th European Young Cereal Scientists and Technologists Workshop (April 2023) - Leuven, Belgium
- Oral presentation: The effects of different bread types on gastrointestinal symptoms in individuals with non-coeliac wheat sensitivity

Digestive Disease Week (May 2023) - Chicago, IL, United States
- Poster presentation: The Effect of Expectancy versus Actual Gluten Intake on Gastrointestinal Symptoms in Non-Coeliac Gluten Sensitivity

XIV International Gluten Workshop (June 2023) - Madrid, Spain
- Oral presentation: The effect of expectancy versus actual gluten intake on gastrointestinal symptoms in non-coeliac gluten sensitivity
- Poster presentation: The effects of different bread types on gastrointestinal symptoms in individuals with non-coeliac wheat sensitivity

Curriculum vitae

Marlijne Cornelia Grietje de Graaf was born on November 21, 1994 in Eindhoven, the Netherlands. She attended primary school in Oirschot and completed her secondary education at Heerbeeck College in Best in 2012.

Subsequently, she obtained her bachelor's degree in Nutrition and Dietetics from HAN University of Applied Sciences in Nijmegen. During her studies, she participated in the Honours programme of the Faculty of Paramedical Studies, and completed a 4-month internship in London, United Kingdom, at the National Health Service and Islington Council. In 2016, she started her master's in Nutrition and Health at Wageningen University, graduating in 2018. Afterwards, she briefly worked as a data manager at the Amsterdam Medical Centre.

In February 2019, she started her PhD at the department of Gastroenterology-Hepatology at Maastricht University, under the supervision of prof. dr. Daisy Jonkers, prof. dr. Daniel Keszthelyi, and em. prof. dr. Fred Brouns. She worked on two clinical multicentre studies as part of the private-public partnership-funded Well on Wheat? project. Her research, conducted within the School of Nutrition and Translational Research in Metabolism (NUTRIM), is presented in this PhD thesis. Throughout her PhD trajectory, she presented her work at various (inter)national conferences, and she received the President Select Abstract Prize from the Dutch Society for Gastroenterology (NVGE). She was also a member and chair of the NUTRIM PhD council.

Currently, Marlijne lives in Oss and works as a Clinical Study Researcher at Danone Nutricia Research.