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Research Article

Two Randomized Cross-Over Trials Assessing the Impact of Dietary Gluten or Wholegrain on the Gut Microbiome and Host Metabolic Health

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Abstract

Background: Gut microbiota composition and activity may be changed by dietary factors and possibly affect metabolic health. Dietary gluten and wholegrain are suggested to influence metabolism in a negative and positive direction, respectively.

Objective: Describe the design and rational as well as baseline characteristics of two human intervention studies, within the Gut, Grain and Greens (3G) Center, investigating the effects of a gluten-poor and wholegrain-rich diet on microbiota composition and metabolic health.

Design: The gluten and wholegrain studies had a randomized, controlled, cross-over design each comprising two eight-week dietary intervention periods, separated by a six-week wash-out period. Each trial included 60 men and women exhibiting an increased metabolic risk. In the gluten study a gluten-poor diet was compared with a gluten-rich dietary fiber-controlled diet, and in the wholegrain study a wholegrain-rich diet was compared with a refined grain diet. The control diet was identical in both studies, being concomitantly high in gluten and refined. Participants substituted all cereal products with provided intervention products which they consumed *ad libitum*. Before and after each intervention period, fecal samples for quantitative metagenomic analyses were collected and an examination day was conducted. The primary outcome of the gluten intervention study was changes in the gut microbiota composition, while insulin sensitivity was an additional primary outcome of the wholegrain study. Further, a number of secondary outcomes were investigated.

Results: 52 and 50 participants completed the gluten and wholegrain intervention study, respectively. Participants had slightly elevated fasting glucose levels and increased waist circumference. Biological outcomes of the two studies will be published elsewhere.

Conclusion: The studies have the potential to provide new insights into the interplay of gut microbiota and metabolic health in individuals with increased risk of developing metabolic disorders.

Keywords: Dietary intervention; Gluten; 3G Center; Intestinal microbiota; Metabolic health; Wholegrain

Abbreviations: 3G: Gut, Grain And Greens; BMI: Body Mass Index; CVD: Cardiovascular Diseases; GI: Gastrointestinal; GLP-1: Glucagon-Like Peptide-1; GM: Gut Microbiota; HDL: High-Density Lipoprotein; HOMA-IR: Homeostasis Model Assessment Of Fasting Insulin Resistance; Iga: Immunoglobulin A; Igg: Immunoglobulin G; IL-1ß: Interleukin 1 Beta; IL-6: Interleukin 6; NEXS: Department Of Nutrition, Exercise And Sports; PYY: Pancreatic Peptide YY; RCPH: Research Center For Prevention And Health; SCFA: Short-Chain Fatty Acids; T2D: Type 2 Diabetes; TNF-A: Tumor Necrosis Factor Alpha; VAS: Visual Analogue Scale

Introduction

Recent research has provided indications for a link between altered Gut Microbiota (GM) composition and the risk of chronic metabolic disorders and low grade inflammation, contributing to the pathogenesis of Type 2 diabetes (T2D) and cardiovascular diseases (CVD) [1-3]. It has been known for decades that diet plays an important role in the development of lifestyle related diseases, but the mechanisms are still insufficiently elucidated. The mammalian GM comprises approximately 10^{14} bacteria, representing about 1,000 abundant bacterial species [4]. Its composition is influenced by several factors such as diet, health state and genetic predispositions [5,6]. The composition and metabolic activity of the GM is hypothesized to have an impact on gut permeability, systemic inflammation, and metabolic functions of the host [7,8]; it is, however, a major scientific challenge to establish the causalities driving the interactions between GM, host health, and environmental factors.

It is established from both animal and human studies that dietary interventions affect the composition of the GM communities in a rapid

and diet specific manner [9-14], but knowledge about the role of diet on GM functionality in humans is still limited. Evidence reveals that several chronic disorders are characterized by a reduced intestinal bacterial diversity and that different GM enterotypes may be associated with long-term intake of protein, fat and carbohydrates [14]. Thus, it may be hypothesized that also dietary components such as gluten and wholegrain affect bacterial diversity within various taxa and enterotypes.

Gluten is a structural protein component of wheat, rye and barley and present in high amounts in Western-type diets [15]. From *in vitro* models it is known that gluten is related to gut inflammation [16] and increased gut permeability [17]. Consumption of gluten has been linked to celiac disease in genetically pre-disposed individuals [16] and gut symptoms and fatigue in irritable bowel syndrome patients [18]. However, little is known about the effect of gluten on risk of chronic systemic low-grade inflammation in apparently healthy individuals. A recent study of ten healthy individuals consuming a gluten-free diet for one month observed alterations in GM composition and lowered immune response [19]. Wholegrain consumption has been associated with a decreased risk of T2D and CVD in prospective studies [20,21] and has been suggested to beneficially influence glucose metabolism [22], adiposity [23,24], and blood lipids [25,26]. Wholegrains have prebiotic effects, due to the high content of dietary fibers [27], which by microbial fermentation lead to production of short-chain fatty acids (SCFA) [28]. SCFA may exert metabolic effects on secretion of gut hormones [29], glucose and fat metabolism [30,31], low-grade inflammation [1], and gut permeability [32]. Most studies focused on specific dietary fibers and further investigation is needed to establish how a diet rich in various wholegrain foods affects GM composition and whether this is associated with changes in host metabolism.

Here, the rationale and design of two human intervention studies on the effects of a gluten-poor and a wholegrain-rich diet on host-GM interactions and host health are described.

Methods

Two human intervention studies were performed as a part of the Center for Gut Microbiota, Metabolic disorders, and Grain/Fiber based Diets (3G: Gut, Grain and Greens, www.3g-center.dk).The center aims to generate novel knowledge about the impact of specific dietary compounds on GM composition and function and host metabolic health by applying 'metagenomic sequencing' and quantitative metagenomics as well as analysis of host metabolic variables.

The wholegrain study was conceived and managed by investigators from the Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen (NEXS), while the gluten study was conceived and managed by investigators from the Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen. Both trials were run in parallel at NEXS.

The studies applied a randomized, controlled, cross-over design comprising two dietary intervention periods of eight weeks duration, separated by a wash-out period of at least six weeks. A total of 60 participants were included in each of the two trials. The outline of the studies is presented in Figure 1. The gluten and wholegrain study were registered at www.clinicaltrials.gov (NCT01719913 and NCT01731366, respectively) and were approved by the Municipal Ethical Committee of the Capital Region of Denmark in accordance with the Helsinki declaration (H-2-2012-064 and H-2-2012-065, respectively) and the Data Protection Agency (2012-54-0170 and 2007-54-0269, respectively).

Hypotheses

The 3G center investigates the hypothesis that interplays between human host and GM affect the risk of chronic metabolic disorders and that interventions targeting the GM modulate the risk of developing metabolic dysfunctions.
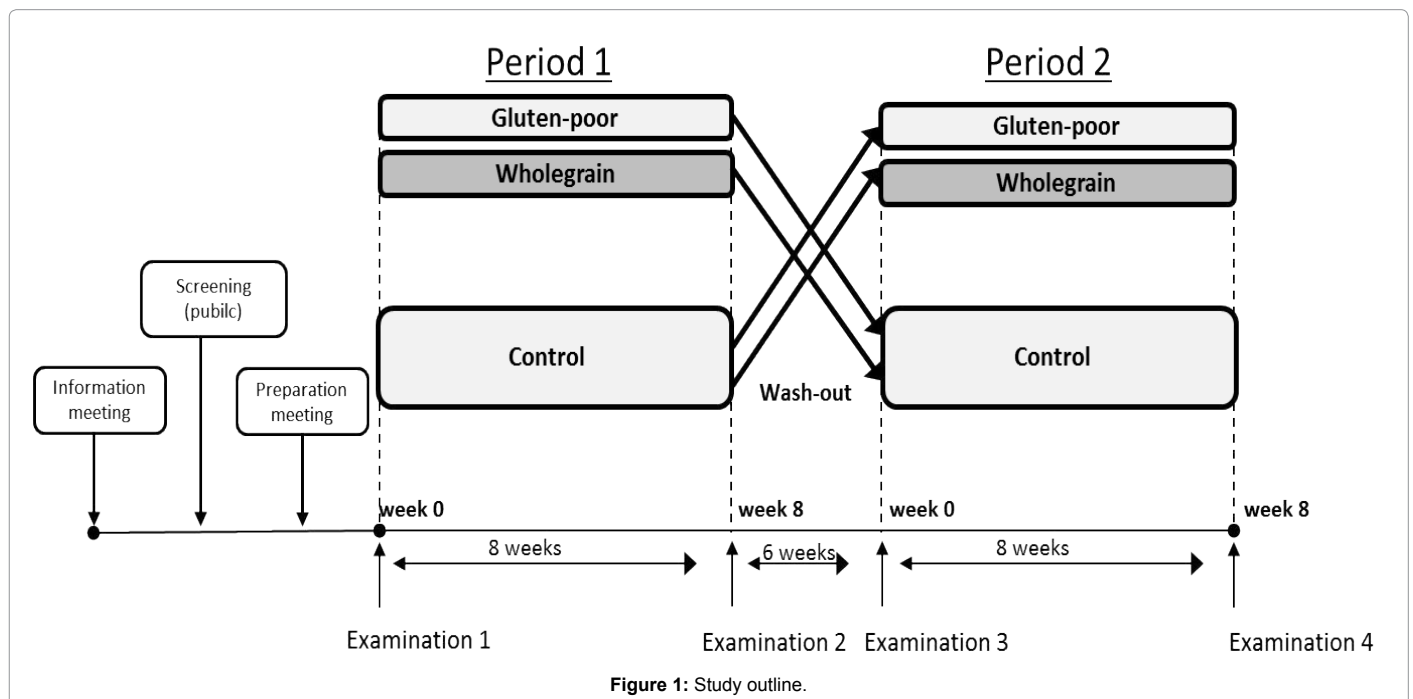

Figure 1: Study outline.

Hypotheses of gluten intervention

1) Compared to a gluten-rich diet a gluten-poor diet induces changes of GM composition and functional potential.

2) Compared to a gluten-rich diet a gluten-poor diet beneficially influences host metabolic and inflammatory markers. These changes in host physiology correlate with dietary gluten-related changes in GM.

3) Compared to a gluten-rich diet a gluten-poor diet lowers gastrointestinal permeability.

4) Compared to a gluten-rich diet a gluten-poor diet causes less subjective gastrointestinal discomfort.

Hypotheses of wholegrain intervention

1) A wholegrain-rich diet improves insulin sensitivity, which is associated with increased richness and diversity of the GM, and increased levels of saccharolytic bacteria and SCFA formation.

2) A wholegrain-rich diet improves postprandial glucose tolerance and appetite regulation and reduces food intake and adiposity *via* effects on colonic fermentation.

3) A wholegrain-rich diet reduces total and LDL-cholesterol.

Common hypotheses

1) Intake of a gluten-poor or wholegrain-rich diet has immune-modulatory effects linked to changes in the inflammasome and cytokine levels (e.g. IL-1β, TNF-α, and IL-6), mediated by changes in gut permeability and GM composition.

2) Wholegrains are rich in methyl donor components (e.g. B-vitamins), while a gluten-deprived diet is vastly depleted of methyl donors; thus, these two diets are hypothesized to have opposite effects on markers of methylation capacity, which may *via* different pathways affect metabolic syndrome markers.

Primary and Secondary Outcomes

In the gluten protocol an altered GM composition and functional potential during consumption of a gluten-poor compared to a gluten-rich diet is the primary endpoint. This endpoint is determined by quantitative metagenomic analyses of microbial DNA isolated from stool samples and sequenced applying a combination of deep and untargeted shotgun sequencing, 16S gene-targeted sequencing, and for selected bacterial taxa real-time qPCR.

In the wholegrain protocol, besides an altered GM composition, altered insulin sensitivity, as estimated by the Homeostasis Model Assessment of fasting Insulin Resistance (HOMA-IR), is an additional relevant primary outcome.

Both studies comprise a number of secondary outcomes including selected measures of glucose and lipid metabolism, gut functionality (intestinal transit time, gastrointestinal symptoms, defecation patterns, and gut permeability), inflammatory markers (plasma cytokine levels, immune cell composition and surface marker, and *ex vivo* cytokine responses), appetite regulation and food intake, anthropometry (body weight, waist circumference, sagittal abdominal diameter, and body composition), blood pressure, markers of methylation, immune cell transcriptomics, and urine and plasma metabolomics.

Participants

Inclusion and exclusion criteria

Individuals were invited to participate in one of the two studies at random and recruitment for each study was stopped once 60 participants were randomized in each of the two protocols. The participants had to be Danish-speaking men and women exhibiting a metabolic "risk profile". In order to participate they had to meet a total of four compulsory inclusion criteria as well as at least one of four additional inclusion criteria:

Compulsory inclusion criteria:

- Age 20 - 65 years

- Apparently healthy

- BMI 25-35 kg/m² **or** waist circumference ≥ 94 cm for men and ≥ 80 cm for women

- Weight stable

Additional inclusion criteria:

- Fasting plasma glucose 6.1- 6.9 mmol/L

- Fasting serum HDL-cholesterol ≤ 1.03 mmol/L for men and ≤ 1.29 mmol/L for women

- Fasting plasma triacylglycerol >1.3 mmol/L

- Systolic blood pressure >130 mmHg **or** medical treatment of hypertension

Exclusion criteria:

- Diagnosis of chronic GI disorders, diabetes or chronic pancreatitis

- Pharmacological treatment of dyslipidemia

- Medically prescribed diet

- Antibiotic treatment (<3 months prior to study start) or intake of pre- or probiotic supplements (< 1 month prior to study start)

- Blood hemoglobin <7.0 mmol/L or blood donation <1 month prior to study start or intention to do so during study

- Participation in other biomedical trials (<1 month prior to study start)

- Pregnancy (<3 months prior to study start) or lactation (<6 weeks prior to study start)

- Intense physical activity (>10 h/week)

- Alcohol consumption >21 units/week for men and >14 units/week for women

In order to detect latent celiac diseases, levels of serum IgA and IgG transglutaminase were measured as a marker of celiac diseases at the first examination day. In case values exceeded the acceptable maximum (>8 units/mL for IgA and >10 units/mL for IgG) participants were excluded from the study. Participants for the gluten and wholegrain intervention were initially recruited from the general population studies "Health 2008" and "Health 2010", established at the Research Center for Prevention and Health (RCPH) at Glostrup University Hospital in Copenhagen, Denmark [33,34]. RCPH provided information on age, sex, anthropometry, and fasting values on blood glucose, triglycerides, and HDL-cholesterol of all participants. Eligible candidates, i.e. fulfilling the inclusion criteria, were contacted through an information letter encouraging them to contact NEXS by e-mail or telephone. Half of the eligible candidates received letters for the gluten and half for the wholegrain study. After approximately two weeks, persons who had

not contacted NEXS were contacted by telephone by the study staff to learn about their potential interest in participation. As an insufficient number of participants from the "Health 2008" and "Health 2010" cohorts were recruited, additional participants were recruited through the webpage www.forsøgsperson.dk as well as through advertisements in local newspapers.

Screening

Interested individuals who had participated in the general population studies were screened *via* telephone for compulsory inclusion and exclusion criteria. Individuals recruited through advertisement were also pre-screened by telephone. Subjects fulfilling the compulsory inclusion criteria and taking blood pressure lowering medicine were directly included in the study, otherwise they were invited for an instant measurement of their blood pressure. Individuals were included if the systolic blood pressure was >130 mmHg (3 repeated measures), otherwise they were screened for increased fasting plasma glucose, decreased fasting serum HDL-cholesterol, and increased fasting plasma triacylglycerol concentrations.

Informed consent

All individuals received written information and were invited to an information meeting, which was held separately for the wholegrain and gluten intervention study. During these meetings potential participants received extensive information on background and study procedures and afterwards written informed consent was obtained.

Before study start, participants attended a preparation meeting, where they were instructed on all study procedures that needed to be undertaken prior to the first examination day, i.e. four-day dietary registration, feces collection, and fasting procedure before examination days as well as intake of plastic markers and completion of a defecation diary. Further, hemoglobin concentration was assessed by a finger-prick measurement, to ensure that individuals with low hemoglobin values (<7 mmol/L) were not subjected to blood sampling.

Randomization, Allocation, Concealment and Blinding

In each study, 60 participants were randomized at the first examination day. Randomization was performed separately for each of the studies in blocks of variable size to ensure equal randomization throughout the enrolment phase of the study. The randomization sequence was made by an investigator without contact to the participants (www.radomization.com). The dietitian allocated participants to the sequence of intervention using a list of participant IDs matched with allocated sequences. Both, the participants and the investigators involved in outcome assessment were blinded until the first examination day. Thereafter, blinding was not feasible due to the nature of the intervention. However, blinding of the allocation sequence will be re-established during sample analysis and initial data analysis.

Interventions

In the gluten study the aim was to keep the daily gluten consumption to an absolute minimum in the gluten-poor period (<2 g/d) and as high as possible in the control period (>20 g/d). The average consumption of gluten among Danish adults is uncertain, however assuming comparability with the Dutch population, an average intake of 11 g gluten per day is suggested (34). All cereals for the gluten-poor period were gluten-free or contained marginal traces of gluten. The wholegrain study aimed for a daily consumption of ≥ 75 g/d of wholegrain during the wholegrain period and of <10 g/d in control period, corresponding to the 90th and 10th percentile of the population, respectively. Wholegrain cereals consist of the intact, ground, cracked, or flaked caryopses, where the starchy endosperm, germ, and bran are present in the same relative proportions as in the intact caryopsis according to the definition proposed by the HEALTHGRAIN consortium in 2013 [35]. All wholegrain foods used contained a minimum of 50 % of wholegrain per dry matter.

Study products

Participants were provided with a selection of cereal products, corresponding to intervention type and period. Participants were advised to replace all cereal products from their diet with the provided study products, which were consumed *ad libitum*. The study products were aimed to be compatible with a typical Danish diet and comprised different kinds of cereal flakes and rolled oats for breakfast, breads for lunch, pasta, kernels, rice and bulgur for dinner, and crisp breads for snacks. The two studies had different active intervention diets (gluten-poor or wholegrain, respectively), whereas the control diets in both studies were comprised of the same products (Table 1). The gluten-poor and the control diet were matched for dietary fiber content, in order to exclude dietary fiber as a potential confounder.

To ensure complete substitution of cereal products and to limit gluten intake from the background diet, participants were not allowed to consume any cereal products besides the provided study products. This also included flour-containing confectioneries, such as cakes and biscuits, and savory snacks. Further, flour-based "fast food" meals as well as ready-to-eat meals, such as pasta and lasagna, were restricted. Moreover, participants were asked not to consume starchy products, such as potatoes, more than once a week, as they might consume these products instead of the provided study products. The same dietary restrictions were applied in both studies in order to ensure comparability between studies.

Consumption of study products

A trained dietitian provided participants with instructions on how to incorporate the provided study products in the diet. Initially it was ensured that study products were provided in sufficient amounts to ensure *ad libitum* consumption. Thereafter, participants were provided individualized amounts of all study products every second week to

Gluten-poor	Wholegrain	Control
Product (Brand)	*Product (Brand)*	*Product (Brand)*
• **Cornflakes (COOP)** • **Oat flakes (AXA)** • **Gluten-free rye bread (Specialbageren)** • **Gluten-free home baked buns (FINAX)** • **Gluten-free pasta (Doves Farm)** • **Quinoa (Urtekram)** • **Rice (NorgesGruppen)** • **Gluten-free crisp bread (Wasa)**	• Rolled Oats (Lantmännen) • Oat flakes (AXA) • Wholegrain rye kernel bread (Kohberg) • Wholegrain wheat buns (Kohberg) • Wholegrain wheat pasta (Kungsörnen) • Wheat kernel (Lantmännen) • Wholegrain bulgur (Zelected foods) • Wholegrain rye crisp bread (Wasa)	• Wheat-rice flakes (Kelloggs) • Oat flakes (AXA) • Maslin bread (Kohberg) • Wheat buns (Kohberg) • Wheat pasta (Lantmännen) • Pearled spelt (Lantmännen) • Bulgur (Unifood) • Crisp bread (Wasa)

Table 1: Intervention products consumed *ad libitum* for substituting all dietary cereal products during the studies.

meet their personal choices and preferences. All breads and buns were provided as frozen products, and all foods were either delivered to the participants at their home or picked up at NEXS, if more convenient.

Compliance

Participants were instructed to keep a study diary, in which they registered daily consumption (amount and type) of study products throughout both intervention periods. Furthermore, participants noted any deviations from the dietary instructions in the diary. Any cases of illness or use of medication, including antibiotics, during the study periods were also noted in the study diary. The study diary was used as a measure of compliance to the intervention and to calculate absolute consumption of study products. A trained dietician conducted a follow-up telephone-call every second week prior to home delivery of study products, focusing on consumption of study products and adherence to the diet.

Study Examinations

Examination days were conducted at the beginning and in the end of each intervention period. All examinations were done at NEXS. Before each examination day, participants had to collect a fecal sample, divide it into one fresh (stored at 5°C) and one immediately frozen sub-sample, and bring them to NEXS.

Before examination days 1, 2 and 4 participants ingested non-absorbable radio-opaque transit markers for measurement of intestinal transit time and filled in a defecation diary for six consecutive days. Furthermore, they completed a four-day pre-coded dietary registration, developed by the National Food Institute at the Technical University of Denmark [36,37].

Participants arrived at NEXS in the morning after having fasted for at least ten hours and abstained from physical activity and alcohol consumption for ≥ 24 hours. Additionally, participants were asked to avoid smoking and tooth brushing in the morning of the examination day. A fasting blood sample was drawn, blood pressure and all anthropometric measurements (sagittal abdominal diameter, waist circumference, body weight and composition) were assessed and a saliva and nasal fluid sample were collected. Hereafter, the participants were provided a drink containing lactulose and mannitol as urinary excretion of these sugars reflects gut permeability. A minimum 4 h urine sample was collected for this purpose. Subsequently, a standardized breakfast, consisting of white wheat bread, a pastry, butter, jam, cheese and 200 ml water (approximately 3000 kJ, 52.6 E% fat, 39.7 E% carbohydrates, 7.8 E% protein), was served and postprandial

blood samples were drawn at 30, 60, 120, and 180 min, followed by an *ad libitum* lunch meal. Subjective appetite sensation, using Visual Analogue Scales (VAS), and breath hydrogen excretion were measured twice at fasting, and every 30 min following the standardized breakfast. Furthermore, participants rated their GI symptoms during the past week and provided information on intake of medications and dietary supplements. At examination days 1, 2, and 4 an X-ray of the abdomen was taken during the afternoon at Frederiksberg Hospital, Copenhagen, Denmark. All procedures are summarized in Table 2.

Laboratory analyses

Throughout all the sample analysis procedures, samples will be randomized across time-points; however, all samples from a single individual will be analyzed in the same batch in order to minimize intra-individual variation. GM will be analyzed by a comprehensive combination of shotgun metagenome sequencing, 16S gene-targeted sequencing, and real-time PCR of selected bacterial taxa.

Status of trials

Recruitment was initiated in July 2012, and all data collection, i.e. completion of intervention and examination days, was completed in November 2013. Currently, biochemical analyses are ongoing; however, analyses of the GM composition are not expected to be finished before 2015.

Statistical Considerations

Sample size estimation

Prior to recruitment the sample size estimations were calculated. For the gluten protocol estimations were based on 85 % statistical power to detect a difference of 0.4 standard deviation in metabolic quantitative traits, based on previous observations from the MetaHit study [38]. It was estimated that 51 individuals were needed, but to allow for a 15 % drop-out after randomization, a total of 60 participants were invited for participation. Overall the number of subgroups within each arm of the study is still blinded, but the MetaHit estimation of three major enterotypes, should be addressable with the current setup. The sample size for the wholegrain protocol was estimated based on an expected difference in HOMA-IR between the wholegrain and the control periods of 0.25 with a weighted standard deviation of 0.6. The expected difference was based on an average difference of 0.26 among three difference studies [22,25,39]. The weighted standard deviation of 0.6 was based on unpublished data on within-group variation of 0.52 and 0.72 in refined grain and WG groups, respectively, from the study

	Examination 1	Examination 2	Examination 3	Examination 4
Blood pressure	+	+	+	+
Anthropometrics	+	+	+	+
Meal challenge	+	+	+	+
Blood (0, 30, 60, 120, 180 min after meal challenge)	+	+	+	+
Breath hydrogen (8 times)	+	+	+	+
Appetite (8 times)	+	+	+	+
Saliva and nasal fluid	+	+	+	+
4h urine	+	+	+	+
Feces	+	+	+	+
GI-symptoms	+	+	+	+
4-days dietary registration	+	+	-	+
Transit time (X-ray)	+	+	-	+
***Ad libitum* meal**	+	+	+	+

Abbreviations: VAS: visual analogue scales; La/Ma: lactulose/mannitol; GI: gastrointestinal

Table 2: Measurements performed before and during examination days 1, 2, 3 and 4.

by Kristensen *et al.* 2012. When applying a 5 % significance level and 85 % statistical power, a sample size of 51 participants was needed. A 15 % drop-out after randomization was assumed, thus a total of 60 participants were invited to participation.

Analysis of primary and secondary outcomes

All data will be checked for normal distribution and homogeneity of variance. In case of non-normal distributions, data transformation will be attempted or non-parametric analyses applied. All analyses will be performed as completers case analyses as imputations for drop-outs is not considered feasible for many of the outcomes, also considering the cross-over design. GM composition data will be analyzed as previously reported [38].

Analysis of HOMA-IR and fasting biochemical variables, such as insulin, glucose and plasma cholesterol as well as body weight will be analyzed as randomized, meaning only considering the baseline value and the sequence of treatments. Subsequent analyses will be performed adjusting for relevant covariates, such as sex, age, and markers of health and nutritional status (e.g. BMI). In the wholegrain intervention body weight change will furthermore be analyzed in term of responders and non-responders. Potential differences between the effects in men and women will be tested by inclusion of a group-gender interaction term as well as by sub-group analyses. Effect on insulin, glucose and other metabolic outcomes will also be analyzed in sub-groups for individuals with high and low baseline levels.

For the primary outcome GM composition the genetic material (DNA) of each individual stool sample will be extracted following protocols established during the MetaHIT project (www.metahit.eu) and WGS sequenced using current Next-Generation sequencing technology (Illumina HiSeq, 100bp pair end, 300-400bp insert size). It is aimed at 6Gbp raw sequence data for each sample, resulting in ca. 720 gigabases microbiome sequence data. As human faces usually contain only a very small amount of human DNA, removal of non-microbial DNA will be performed in-sillico. DNA from each sample will undergo *de novo* assembly, gene calling and taxonomical as well as functional annotation, using pipelines established in the MetaHIT project. This will allow establishment of a 3G specific microbial gene catalogue, which will be compared and integrated with the previously established gene catalogues for Danish, Spanish, and Chinese populations [4,40]. All genes in the gene catalogue will be binned by co-abundance to identify co-varying gene modules and Co-Abundance gene groups (CAG) [40]. This new 3G specific microbial gene and genomes catalogue will then be used to identify genes, genomes and genetic modules as markers which associate to phenotypic outcome. Additionally, the cohort will be stratified into cohort Enterotypes [41] and patterns of microbial diversity in relation to diet.

Exploratory analysis for the metabolic outcomes will be conducted and changes in metabolic outcomes will also be correlated with compliance. Other outcomes will be analyzed according to the same principles using models with repeated measurement in the analysis of postprandial data, such as responses in glucose, insulin, and appetite hormones as well as subjective appetite sensation, GI-symptoms, gut permeability, inflammatory markers, and breathe hydrogen excretion.

Results

Sixty participants were randomized in each of the studies. A total of 52 and 50 participants completed the gluten and wholegrain intervention study, respectively. The flow of participants is depicted in Figure 2.

There were more women than men in both studies. Baseline data of participants recruited for the gluten and wholegrain intervention studies are presented in Tables 3 and 4, respectively. All participants who dropped out did so before entering the second intervention period, and most withdrawals were related to either examination procedures or intervention products.

Overall, participants in both studies had slightly elevated fasting glucose levels according to the glucose regulation definitions of the American Diabetes Association [42] and increased waist circumference according to International Diabetes Federation [43]. For both studies, there were found no significant differences between randomization groups at baseline.

Discussion

To the authors' knowledge, the presented intervention protocols on gluten and wholegrain, respectively, are the largest studies to date examining the effects of both dietary gluten-withdrawal and wholegrain-addition on metabolic health markers and changes in the GM composition in a metabolic at-risk population. Participants in both studies were apparently and by self-report healthy individuals, yet with an elevated metabolic risk, due to increased adiposity. This renders the study population an optimal target group for investigating potential health improvements resulting from dietary gluten reduction or increased wholegrain consumption.

The main objective of the human intervention studies was to examine the effect of the dietary gluten deprivation or the increased intake of wholegrain on changes in the GM composition and associate this with changes in whole-body insulin sensitivity, measured as HOMA-IR, as well as changes in a large number of other host metabolic variables. Fecal samples were collected and bacterial DNA will be extracted, sequenced and analyzed applying the state of the art methods also used in the MetaHit project [4,41], which will provide extensive information on the GM composition. Additionally, untargeted shot-gun sequencing and targeted 16S-rRNA gene sequencing will be supplemented by quantitative PCR based determination of selected relevant bacterial taxa [44]. In an integrated approach, using complex bioinformatic methods, GM and metabolic health variables will be analyzed, giving new insights into the interactions between GM and host metabolic health. It needs to be noted that data from human interventions not involving GM transplantation will never *per se* allow an absolute establishment of causalities in the interplay between GM and host health. However, given the design of studies, comprising two interventions with very different expected effects, the possibilities to identify truly linked events will strongly increase. Furthermore, the integrated 3G research program also comprises animal trials involving transfer of human fecal samples from the two intervention studies to germ-free mice in order to uncover potential causal relations.

In both studies, all cereal products in the participants' diet were substituted with the provided study products, whereas it was aimed to keep the background diet the same during all periods. Thus, changes in metabolic variables as well as the GM composition are likely the direct consequence of the dietary interventions. As participants also daily registered the type and amount of study products consumed, correlations of particular products, e.g. wholegrain rye vs. wholegrain wheat, with certain health estimates and gut bacteria can be explored. Control diets in the two studies were identical and the same dietary restrictions were applied during all study periods allowing direct comparisons of the intervention periods within each study as well as between the two studies.
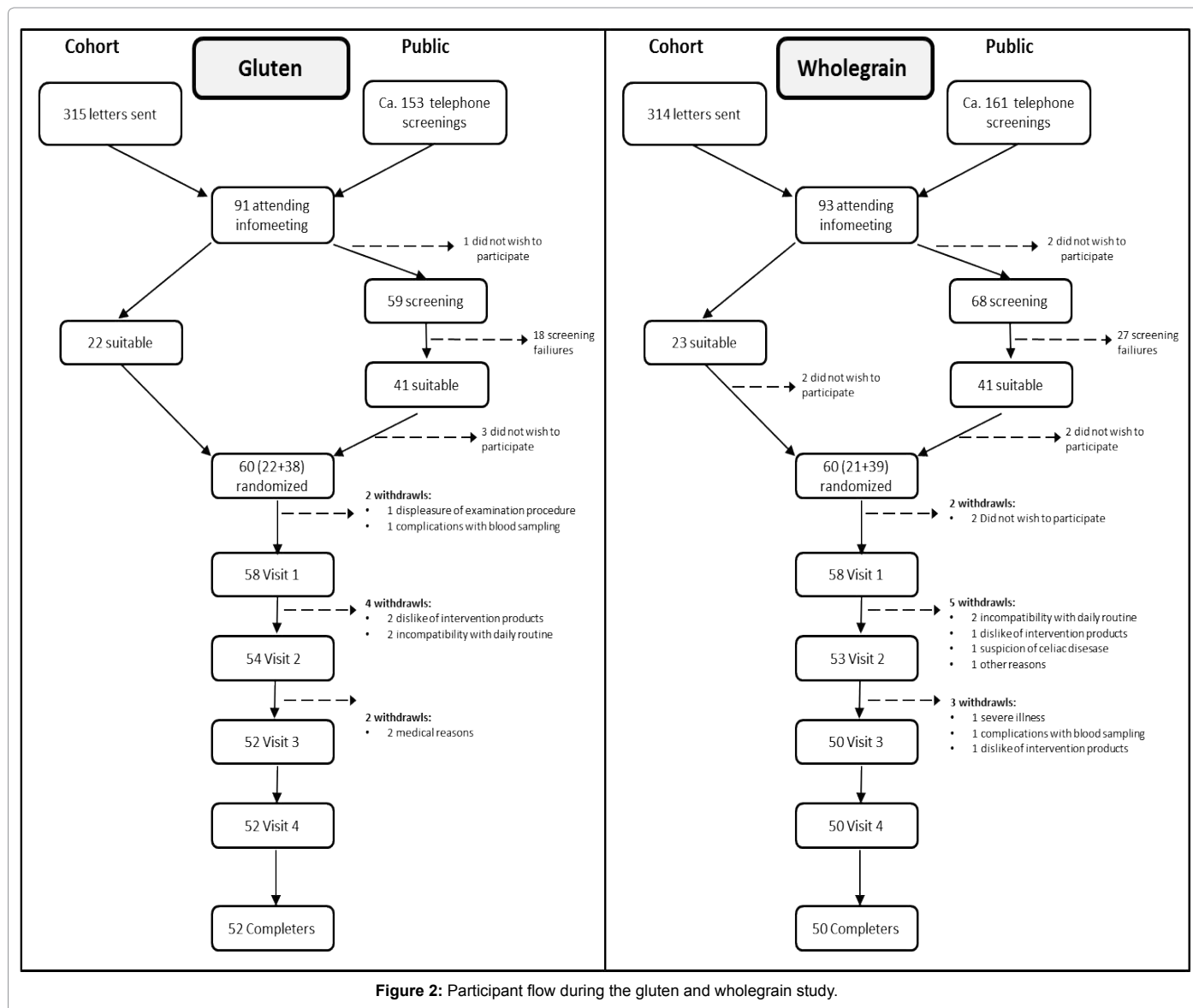
Figure 2: Participant flow during the gluten and wholegrain study.

So far, studies of a gluten-poor diet on health and GM have been limited in healthy individuals with varying degrees of adiposity [19], thus the gluten study will provide new evidence on the effects of gluten on a variety of inflammatory and metabolic variables in non-celiac individuals as well as on GM composition. In contrast, numerous studies have investigated health effects of wholegrain consumption, such as insulin sensitivity, adiposity, and inflammation [6,25,26]. However, results are inconsistent, which may be due to differences in study designs and lack of statistical power. Changes in GM composition as a consequence of wholegrain consumption have been investigated before; however, only focusing on selected bacterial groups [45].

One of the strengths of the studies described here is the high statistical power, which, due to the large sample size and the cross-over design, allows detecting even small differences in the outcome variables. It can be debated whether small differences are of clinical relevance; however, it may be relevant in a public health perspective for prevention of lifestyle diseases. Furthermore, an increased power of the studies enhances the chance of associating changes in host health with changes in GM composition and functionality. Deliberately, the two

studies did not focus on specific products or cereal types, but rather provided a variety of different cereals and products. Due to this and the *ad libitum* consumption of products, the studies reflect realistic patterns of consumption, which will render the results more applicable to the Danish population.

Conclusion

The gluten and wholegrain studies have the potential to provide new insights into the interplay of GM and metabolic health in individuals with increased risk of progressing to metabolic disorders.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

OP and LL are the principal investigators of the gluten and wholegrain research programs, respectively, while TRL is heading the 3G research center collaboration. LL, OP, MK, SI, RG, TRL, and

	All (n=59)	**Men** (n=26)	**Women** (n=33)	**Control in period 1** (n=29; n=17 men; n=12 women)	**Gluten-poor in period 1** (n= 30; n=10 men; n=20 women)
Age [y]	49 ± 12	50 ± 12	47 ± 12	50 ± 9	47 ± 13
Height [cm]	174 ± 10	182 ± 5	167 ± 7	176 ± 8	171 ± 11
Weight [kg]	87 ± 13	96 ± 9	79 ± 11	88 ± 12	85 ± 15
BMI [kg/m²]	29 ± 4	29 ± 2	28 ± 4	29 ± 3	28 ± 4
Waist circumference [cm]	99 ± 9	104 ± 6	96 ± 9	99 ± 3	99 ± 10
Sagittal height [cm]	23 ± 3	24 ± 3	22 ± 3	23 ± 3	23 ± 4
Body fat [%]	31 ± 8	25 ± 5	36 ± 6	29 ± 7	32 ± 8
Systolic blood pressure [mmHg]	128 ± 13	134 ± 13	122 ± 12	129 ± 11	124 ± 15
Diastolic blood pressure [mmHg]	81 ± 9	83 ± 8	80 ± 9	81 ± 9	81 ± 10
Fasting plasma glucose [mmol/L]	5.7 ± 0.6	5.9 ± 0.6	5.6 ± 0.7	5.8 ± 0.6	5.6 ± 0.7
Fasting plasma triacylglycerol [mmol/L]	1.4 ± 0.7	1.6 ± 0.8	1.2 ± 0.5	1.5 ± 0.8	1.3 ± 0.7
Fasting serum HDL-cholesterol [mmol/L]	1.3 ± 0.3	1.2 ± 0.3	1.4 ± 0.3	1.2 ± 0.3	1.4 ± 0.3
Intake blood pressure lowering medicine	5	3	2	3	2

Table 3: Baseline data of gluten intervention study. Data are presented as mean ± SD.

	All (n=58)	**Men** (n=18)	**Women** (n=40)	**Control in period 1** (n=27; n=9 men; n=18 women)	**Wholegrain in period 1** (n=31; n=9 men; n= 22 women)
Age [y]	48 ± 11	45 ± 11	50 ± 12	46 ± 12	51 ± 11
Height [cm]	172 ± 8	180 ± 8	169 ± 5	173 ± 8	172 ± 9
Weight [kg]	85 ± 13	94 ± 12	81 ± 12	87 ± 12	84 ±14
BMI [kg/m²]	29 ± 4	29 ± 3	29 ± 4	29 ± 4	28 ± 4
Waist circumference [cm]	101 ± 9	105 ± 7	99 ± 9	101 ± 8	11 ± 9
Sagittal height [cm]	23 ± 3	24 ± 2	22 ± 3	23 ± 3	23 ± 3
Body fat [%]	34 ± 9	24 ± 5	38 ± 6	34 ± 9	34 ± 9
Systolic blood pressure [mmHg]	127 ± 13	130 ± 12	125 ± 13	123 ± 11	129 ± 13
Diastolic blood pressure [mmHg]	81 ± 9	81 ± 9	80 ± 9	79 ± 7	82 ± 9
Fasting plasma glucose [mmol/L]	5.6 ± 0.6	5.8 ± 0.4	5.6 ± 0.6	5.6 ± 0.6	5.7 ± 0.6
Fasting plasma triacylglycerol [mmol/L]	1.3 ± 0.5	1.4 ± 0.5	1.2 ± 0.5	1.2 ± 0.5	1.3 ± 0.4
Fasting serum HDL-cholesterol [mmol/L]	1.3 ± 0.3	1.2 ± 0.2	1.4 ± 0.2	1.3 ± 0.2	1.4 ± 0.3
Intake blood pressure lowering medicine	5	0	5	1	4

Table 4: Baseline data of wholegrain intervention study. Data are presented as mean ± SD.

TH designed the studies. SI, RG, and MK carried out the studies. LL performed the randomization. SI and RG drafted the manuscript. All authors contributed to the writing of the manuscript and approved the final version.

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