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ORIGINAL CONTRIBUTION

Daily intake of wheat germ-enriched bread may promote a healthy gut bacterial microbiota: a randomised controlled trial

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Abstract

Purpose Wheat bran fibre has a beneficial effect on gastrointestinal function, but evidence for wheat germ is scarce. Accordingly, we evaluated the effects of daily intake of wheat germ on gastrointestinal discomfort and gut microbiota by adding wheat germ to refined (white) wheat bread, the most consumed bread type. We hypothesised that an improvement in the composition of refined bread could beneficially affect intestinal health without compromising consumers' acceptance.

Methods Fifty-five healthy adults were recruited for a randomised, double-blind, crossover, controlled trial comprising two 4-week intervention periods separated by a 5-week washout stage. During the first 4-week period, one group consumed wheat bread enriched with 6 g of wheat germ and the control group consumed non-enriched wheat bread.

Results Wheat germ-enriched bread was well-appreciated and the number of participants that demonstrated minimal gastrointestinal improvements after wheat-germ intake was higher than in the control arm. Importantly, intake of wheat germ-enriched bread decreased the perceived gastrointestinal discomfort-related quality of life (subscale worries and concerns) over refined white bread. The improvements in the gastrointestinal function were accompanied by favourable changes in gut microbiota, increasing the number of *Bacteroides* spp. and *Bifidobacterium* spp.

Conclusions Adding wheat germ to industrially made white bread without altering sensory properties may promote a healthy gut bacterial microbiota and the gastrointestinal health.

Keywords Gastrointestinal discomfort · Gut microbiota · Bread · Wheat germ · Randomised controlled trial

Introduction

The human gastrointestinal tract is colonised by microorganisms, mainly bacteria which have an important role in human metabolism and health. They are involved in the degradation of non-digestible food constituents/nutrients, production of secondary bile acids and the vitamins K and B, glucose and lipid metabolism as well as maturation and regulation of immune system [1–3]. In healthy adults, the

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gut microbiota is in a "steady-state" equilibrium dominated by two major bacterial phyla Bacteroidetes and Firmicutes, and in minor extent by Actinobacteria (e.g., *Bifidobacterium*), Verrucomicrobia (*Akkermansia*), and Proteobacteria (*Escherichia*) [4]. Changes in the composition, diversity, and richness of the gut microbiota can compromise its function, potentially leading to a disease-state. Indeed, several recent systematic reviews have demonstrated that patients diagnosed with functional gastrointestinal disorders, including irritable bowel syndrome (IBS), had alterations in gut microbiota characterised by a decrease in bacterial diversity and proliferation of potential pathogenic bacterial such as *Escherichia coli* [5, 6]. Diet is the major modifiable factor of human gut microbiota [7]. Numerous dietary intervention studies have been conducted to potentiate the enrichment of beneficial gut bacteria [5, 8]. A "substrate that is selectively utilized by host microorganisms conferring a health benefit" is called prebiotic [9]. However, outcomes are frequently controversial because of inter-individual variability in the response to a specific dietary intervention [10–12]. Because of that, some authors that defend personalised diets [11–13]. Nonetheless, the effect of food with beneficial ingredients for human health needs to be researched to be considered as general or specific dietary recommendations.

Whole grains contain bran and germ that are considered to be the healthy ingredients of whole grains. The germ is the definition for the embryonic axis and the scutellum of the seed; germ is rich in bioactive compounds such as α-linolenic acid, oligosaccharides, flavonoids, phytosterols, and vitamins (namely, thiamine, riboflavin, tocopherols, tocotrienols, and phylloquinone) [14]. The physiological effect of bran (designation for the outer layer of the grains) in gastrointestinal function has been demonstrated and health claims regarding these physiological effects were approved for wheat bran fibre in the European Union [15]. The dietary fibres mostly present in bran reduce intestinal transit time while increasing faecal bulk and water retention in the colon that, altogether, change stool characteristics and defaecation frequency [16–19]. The evidence suggests that dietary fibres confer most of their health benefits indirectly through the metabolic products derived from fibre's fermentation by the gut microbiota [20–23]. Regarding germ, an in vitro study using a gastrointestinal model that compared different commercial prebiotic products showed that a wheat-germ preparation efficiently enhanced the proportion of bifidobacteria from 15 to up to 24% of total bacteria in the faecal matter [24]. Likewise, a randomised clinical trial involving 32 healthy subjects, demonstrated that daily ingestion of wheat germ as a dietary supplement increased the number of gut bifidobacteria and lactobacilli, but only in individuals with low basal levels [25].

Considering the potential of wheat germ to improve the microbiota of healthy individuals and knowing that gut microbiota can affect gastrointestinal function, we hypothesised that wheat germ may improve the gastrointestinal function. Improving the gastrointestinal health has an important impact on the quality of life [2, 26, 27], and importantly, one-third of the worldwide population has one type of gastrointestinal discomfort, among which the most common are bloating and constipation [28]. Since a reduction in gastrointestinal discomfort is considered an indicator of improved gastrointestinal function [27], we designed a randomised clinical trial to evaluate the impact of daily intake of wheat germ on the gastrointestinal discomfort and the gut microbiota of healthy volunteers. Thus, to introduce the wheat-germ intervention in the diet, a refined wheat bread supplemented with 6 g of wheat germ was produced and its effects were compared with regular refined wheat germ, i.e., without supplementation (hereafter control bread). We chose bread as a vehicle for the intake of germ because of its important role in the human diet. Bread contributes to the 10% of daily caloric intake and it is also an important source of carbohydrate [12]. To understand the impact of wheat-germ intervention on daily life, we used a validated self-reported measurement of health-related quality of life (PAC-QOL) designed to evaluate patient's assessment of constipation. This outcome is crucial to infer the validity of wheat-germ intake intervention in terms of benefits for human health. Our study is the first clinical trial that investigates the effect of wheat germ on gastrointestinal health.

Materials and methods

Study participants and design

The clinical trial was conducted from June 2015 to October 2016 after obtaining approval from the Health Ethics Committee of the University Hospital Center of São João and the Ethics Committee of the Faculty of Medicine of the University of Porto (07/2015). The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and followed the Good Clinical Practice guidelines. Written informed consent was obtained from all participants before enrolment. The present study was registered in the ClinicalTrials.gov database (NCT02405507).

The detailed study protocol and baseline characteristics of the participants were previously published [29, 30]. Briefly, we report here a randomised, double-blind, crossover, controlled clinical trial. This trial comprised a 15-week follow-up with a 2-week run-in step, two crossover interventions of 4 weeks/each, separated by a 5-week washout period (Fig. 1). Fifty-five participants were recruited. The exclusion criteria included (1) bowel frequency lesser than twice per week, (2) use of medication or dietary supplements that influenced the intestinal microbiota, (3) use of prebiotics

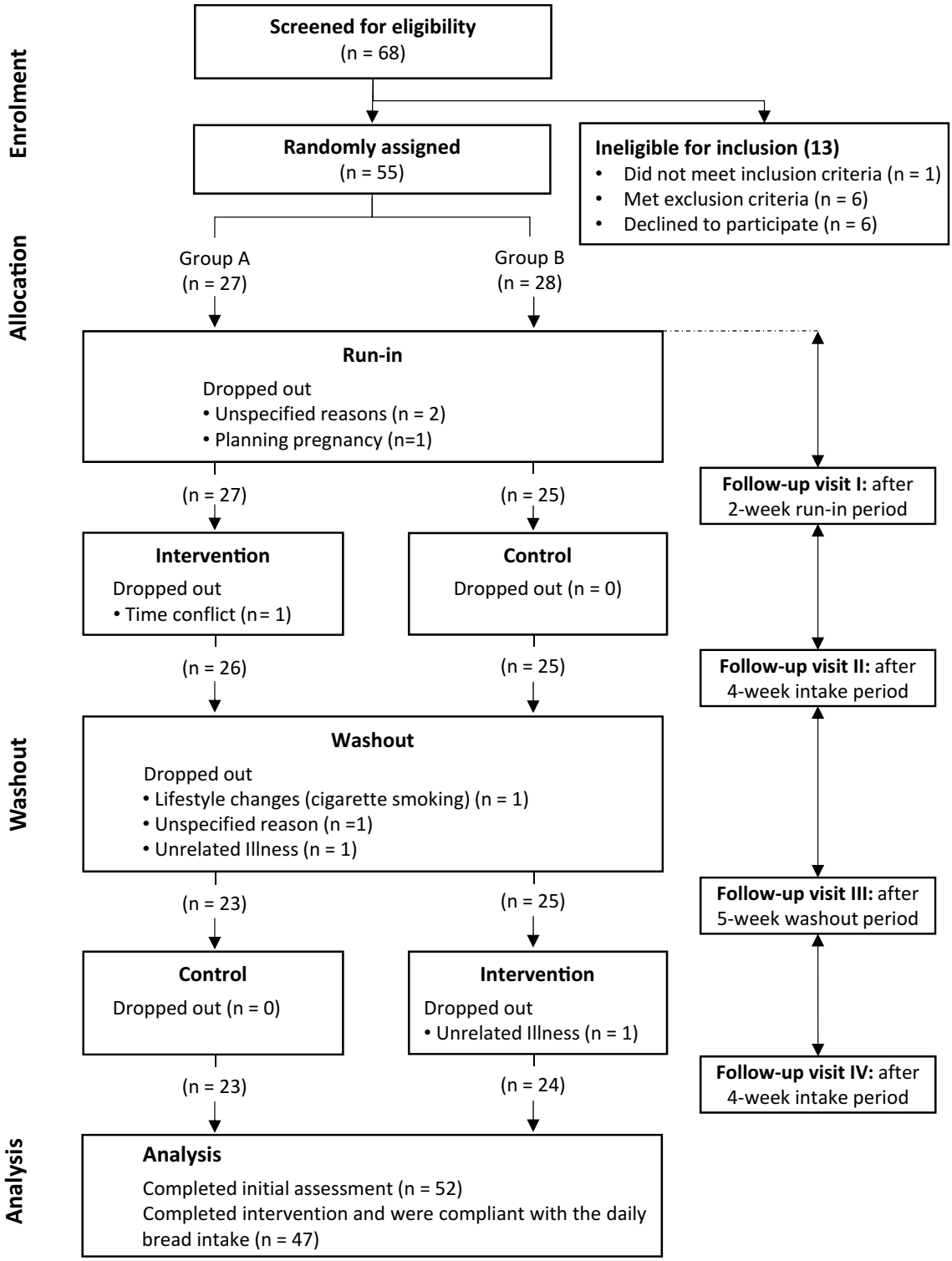

Fig. 1 Overview of study design and participants flow (adapted from Moreira-Rosário et al. [30])

and probiotics, and (4) change of dietary habits 4 weeks before recruitment. Moreover, participants were instructed not to change their physical activity and dietary habits and not to consume any food or dietary product supplemented with germ before and during the follow-up. The participants recruited for this study were non-smokers, had no gastric bypass surgery, and were free of any chronic or digestive diseases with relevant effect on the gastrointestinal system

or on visceral motility, including functional bowel disorders such as irritable bowel syndrome (IBS). None of the participants was medicated for digestive symptoms such as anti-spasmodic, laxatives, and anti-diarrheic drugs or other digestive auxiliaries.

Participants were randomly divided into two groups (ratio 1:1): intervention 1 consisted in consuming daily refined wheat bread (100 g) supplemented with 6 g of wheat germ, while intervention 2 comprised the daily intake of refined wheat bread (100 g) without any supplementation (the control arm). The formula for preparing the wheat germ-enriched bread was developed so as to mask the texture, volume, and flavour of the final product, whose formulation is described in our previous work [30]. Thus, control and wheat-germ-supplemented bread were indistinguishable, which allowed double blinding of the participants and researchers. The detailed description of the nutrient composition of both breads is presented in Supplemental Table 1. The unblinding occurred after statistical analysis. Compliance with the study protocol was monitored daily using self-reported questionnaires.

The outcome evaluations were performed at the end of each stage: (T1) run-in; (T2) intervention 1; (T3) washout; and (T4) intervention 2. The outcomes measured were grouped in two categories: (a) CVD risk factors and (b) gastrointestinal function. The results regarding the outcomes related to CVD risk markers are discussed separately [30]. Herein, we report the outcomes of the gastrointestinal function. We measured the gastrointestinal discomfort associated with constipation as a primary outcome. The secondary outcomes included (i) quality of life with regard to constipation, (ii) stool frequency and consistency, (iii) intestinal microbiota, and (iv) psychological stress.

Stool collection

One faeces sample was collected up to 72 h before the end of each period (T1, T2, T3, and T4) and stored in a tube containing 3 ml RNAlater Solution (Sigma-Aldrich) to preserve the DNA integrity. Samples were kept at − 80 °C until DNA extraction.

DNA extraction from stool samples

DNA was extracted from the stool samples using NZY Tissue gDNA Isolation Kit (NZYtech). Approximately 200 mg of faeces were homogenised in 1 ml TE buffer, centrifuged and the supernatant discarded. The remaining pellet was resuspended in 350 µl of buffer NT1 and incubated at 95 °C during 10 min for promoting bacterial cell lysis. Samples were spanned and the pellet was discarded. 25 µl of Proteinase K solution (NZYtech) was added to the supernatant and the mixture was incubated at 70 °C for 10 min. RNA contamination was removed by subsequent incubation with 400 µg RNase A (NZYtech) at room temperature during 15 min. After this, the DNA extraction was performed according to the manufacturer's instructions. The purified faecal DNA was quantified by measuring A_{260}. The A_{260}/A_{280} ratio was used to estimate the purity of the extracted RNA which should be between 1.8 and 2.0.

Gut microbiota quantification by real-time PCR

The quantification of genomic DNA was carried out using the LightCycler 96 Real-Time PCR system (Roche) with the FastStart Essential DNA Green Master quantification assay (Roche), following the manufacturer instructions. Each 20 µl reaction contained 20 ng of total faecal DNA and specific primers for 16S ribosomal RNA gene, which is a molecular marker to genus/specie identification. The quantitative PCR reactions were performed using the thermal cycling parameters default conditions; the annealing temperature was primer-specific (Supplemental Table 2). After amplification, reactions were checked for the presence of non-specific products through dissociation curve analysis.

For bacterial quantification, tenfold serial dilutions of genomic DNA extracted from ATCC bacterial reference strains were constructed for each 16S rRNA gene (Supplemental Table 2). The amount of DNA present per 20 µl reaction was converted into copy number based on bacterial genome size (base pairs) and the 16S rRNA gene number per genome. The number of copies was determined by extrapolation, using the specific standard curve from validated reference strains, and normalised to the amount of faecal DNA and amount of faeces in grams. The quantification values are expressed in $\log_{10}$ copies/g faeces and should be interpreted in terms of relative amounts.

Recording of gastrointestinal discomfort

Gastrointestinal discomfort was self-evaluated every 2 weeks using the validated Patient Assessment of Constipation Symptoms (PAC-SYM) and Patient Assessment of Constipation Quality-of-Life (PAC-QOL) questionnaires. The PAC-SYM includes 12 constipation-related symptoms grouped into three subscales related to abdominal, stool, and rectal symptoms. This questionnaire is rated on a 5-point scale, from 0 (no symptom) to 4 (very severe), aiming to measure the severity of gastrointestinal discomfort over the previous 2 weeks. In parallel, the participants evaluated the impact of gastrointestinal discomfort-related symptoms on health-related quality of life using a validated self-reported quality-of-life questionnaire (PAC-QOL). The PAC-QOL is composed of 28 items grouped into four subscales related to physical discomfort, psychosocial discomfort, worries and concerns, and satisfaction. This validated self-reported

questionnaire is rated on a 5-point scale, from 0 to 4 (where 0 = at no time/not at all, 4 = all the time/extremely), and is used over a 2-week recall period; scores of items 18 and 25–28 (satisfaction subscale) were reversed meaning as stated in the guidelines. A reduction in PAC-SYM and PAC-QOL scores (total or subscale) corresponds to an improvement in symptoms. Moreover, a −0.5 reduction corresponds to the smallest level of change in a self-reported outcome score that is perceived as an improvement [31]. Cultural adaptation and linguistic validation of the PAC-SYM and PAC-QOL for Portugal were performed by the Mapi Research Trust (France).

Recording of stool parameters

During the 15-week follow-up period, the participants reported all bowel movements according to the Bristol Stool Form Scale (7-point scale) in daily self-reported questionnaires. Based on this information, the average stool consistency (sum of Bristol Stool Form Scales divided by the number of stools) and the average stool frequency (number of stools divided by the number of days of diary recording) were assessed.

Confounding variables

Psychosocial factors such as psychological stress are a well-documented variable known to influence gut physiology and gastrointestinal symptoms [32]. For these reasons, the effect of wheat germ on gastrointestinal discomfort was controlled for psychological stress. A psychometric assessment was carried out every 4 weeks using the validated Perceived Stress Scale (PSS). This 13-item self-reported questionnaire measures the degree to which situations in a person's life are appraised as stressful. Cultural adaptation and linguistic validation of the PSS for Portugal were implemented by Pais Ribeiro and Marques [33].

Statistical analysis

Intervention effects were calculated as the difference between the change during each 4-week intervention period and the change during the 4-week control period. The change in gastrointestinal outcomes (PAC-SYM, PAC-QOL, stool frequency, and consistency) was the difference between the last 2 weeks of first intervention period and the 2 weeks of run-in period or the last 2 weeks of the second intervention period and the last 2 weeks of washout period. Total score and subscale scores of self-reported questionnaire (PAC-SYM, PAC-QOL and PSS) were computed based on non-missing item responses. Whenever more than 50% of items were missing the total scale or subscale score were not calculated and were designated as 'missing'. The same procedure was performed when more than 7 daily records of stool frequency or consistency were missing; the average of the 2-week interval was not calculated and, therefore, was designated as 'missing'. The proportion of participants with a minimal clinical improvement in PAC-SYM and PAC-QOL scores was calculated. A reduction of half a point in total and subscales scores was used, since it corresponds to minimal clinical improvement according with previous validation studies [31, 34]. We compared the proportion of participants with a minimal clinical improvement after a 4-week intervention period with the control period using the McNemar test.

The sample size was estimated taking into account the primary outcome that required the highest number of participants. For that, we calculated the sample size for each individually primary outcome, as described in the protocol manuscript [29]. To detect a 0.35 difference and an SD of 0.75 in the PAC-SYM total score, with 80% power and 95% confidence level, the estimated sample size was 40 participants.

A linear mixed model for repeated measures with compound symmetry as covariance structure was used to determine whether the intervention effects were statistically significant. Compound symmetry was used instead of the autoregressive or unstructured structure, because it resulted in the best fit according to a likelihood ratio test. Intervention, period, and sequence were included as fixed effects variables. To account for the between-subjects variability and to adjust any non-specific differences, subjects were included as random effects. We also included intervention–sequence interaction as a fixed effect in the model to assess potential carryover effects. When carryover was significant, we reported the first period in the analysis, following Pocock's recommendations [35].

Statistical analysis was performed using the SPSS version 23 software (SPSS Inc.). Numerical data are expressed as mean ± SD and treatment effects with 95% CI. Statistical significance was set at a two-sided P value of 0.05. However, because we had multiple primary outcomes, we decided to perform an adjustment for multiple comparison; thus, the type 1 error associated with any individual variable difference took into account all comparisons performed and were ruled significant after adjusting for the overall false discovery rate, using the Benjamini–Hochberg procedure (with $q^* = 0.05$) [36].

Results

Participants

Sixty-eight volunteers were screened and assessed for eligibility to participate in our study. After initial assessment,

55 participants were enrolled in the study; 39 women and 16 men, with a mean age of 33 years (range 18–59 years), and a BMI between 19 and 38 kg/m^2 (37 with normal weight, 14 overweight and 4 obese). This sample is representative of a healthy population. Eight participants dropped out in the course of the study: the first six declined to participate and the las last two withdrew because of unrelated illness (gastroparesis and pneumonia). Besides that, no other adverse events were reported throughout the study.

Compliance and confounders

Because of the absence of a biomarker for wheat-germ intake, the compliance was measured through self-reported daily questionnaires. The analysis of dietary intake data showed a good compliance with the study protocol (92.1% ± 9.3), and moreover, compliance was comparable between interventions (wheat germ: 92.2% ± 11.1; control: 92.0% ± 10.0; $P = 0.920$). Indeed, the control bread and the wheat germ-enriched bread were well-appreciated by the participants, and more importantly, both products have identical sensory properties (Supplemental Table 3). However, the two intervention breads differ in terms of nutritional composition. The addition of 6 g of wheat germ in 100 g of refined wheat bread increased the amounts of protein, dietary fibre, total phytosterols, and alpha-linolenic acid in the wheat germ-enriched bread. On the other hand, the content of carbohydrate and starch was higher in the control, while the content of fat and energy remained similar in both, intervention and control bread.

To control a potential bias due to psychological stress, a self-reported questionnaire evaluating individual perceived stress was used. This confounding variable was measured during run-in and intervention stages. Results from the Perceived Stress Scale (PSS) questionnaire showed no significant differences within wheat-germ intake group ($P = 0.572$), within the control ($P = 0.179$) or between groups ($P = 0.211$) during the study.

Gastrointestinal discomfort associated with constipation

Gastrointestinal discomfort was individually measured through the PAC-SYM. In this 5-point scale of symptoms, a score of 0 corresponds to the absence of symptoms, 1 to mild, 2 to moderate, 3 to severe, and 4 to very severe symptoms. The impact of constipation symptoms on the quality of life was evaluated using PAC-QOL. Descriptive statistics for the PAC-SYM and PAC-QOL scores are presented in Tables 1 and 2. The means of PAC-SYM and PAC-QOL overall and subscales scores varied between 0 and 1 at baseline and during the study, as expected for a healthy population. The exception is the PAC-QOL satisfaction subscale that was higher than 1.

The differences observed in the PAC-SYM questionnaire are not statistically significant ($P > 0.05$) when comparing baseline with post-intervention or when comparing wheat-germ intake with control (Table 1). The number of participants that improved ≥ 0.5 in the PAC-SYM score was analysed. Thus, despite not statistically significant, considerably more participants (14%, 7/50; $P = 0.063$) that consumed wheat germ-enriched bread during 4 weeks improved half a point or more in the total PAC-SYM score when compared with the same period of control bread intake (2%, 1/48) (Fig. 2). Evaluation of the defecation frequency and stool consistency using the Bristol scale does not suggest any effect associated with wheat-germ intake (Supplemental Table 4).

Table 1 Effect of 4-week intake of wheat germ on gastrointestinal discomfort associated with constipation

	Wheat germ-enriched bread			Control bread			Effect of wheat germ[b]	
	Baseline[a] ($n = 50$)	Post-intervention[a] ($n = 51$)	P value within group	Baseline[a] ($n = 49$)	Post-intervention[a] ($n = 48$)	P value within group	Effect (95% CI)	P value between group
PAC-SYM								
Overall score	0.50 ± 0.46	0.47 ± 0.45	0.714	0.44 ± 0.38	0.41 ± 0.42	0.547	0.00 (−0.12, 0.13)	0.962
Abdominal symptoms (4 items)	0.49 ± 0.43	0.55 ± 0.61	0.393	0.46 ± 0.42	0.46 ± 0.52	0.976	0.07 (−0.14, 0.27)	0.505
Stool symptoms (5 items)	0.61 ± 0.66	0.52 ± 0.58	0.187	0.57 ± 0.58	0.50 ± 0.59	0.175	−0.01 (−0.17, 0.14)	0.843
Rectal symptoms (3 items)	0.32 ± 0.68	0.28 ± 0.43	0.637	0.20 ± 0.40	0.22 ± 0.40	0.566	−0.06 (−0.24, 0.13)	0.531

The gastrointestinal discomfort was self-evaluated using the validated Patient Assessment of Constipation Symptoms (PAC-SYM) questionnaire

[a]Mean ± SD. *PAC-SYM* Patient Assessment of Constipation Symptoms

[b]Intervention effects were analysed using linear mixed model for repeated measures with compound symmetry as covariance structure

Table 2 Effect of 4-week intake of wheat germ on the quality of life regarding gastrointestinal discomfort associated with constipation

	Wheat germ-enriched bread			Control bread			Effect of wheat germ[b]	
	Baseline[a] ($n=50$)	Post-intervention[a] ($n=51$)	P value within group	Baseline[a] ($n=49$)	Post-intervention[a] ($n=48$)	P value within group	Effect (95% CI)	P value between group
PAC-QOL								
Overall score	0.57 ± 0.47	0.54 ± 0.49	0.488	0.51 ± 0.40	0.58 ± 0.54	0.178	−0.10 (−0.23, 0.03)	0.130
Satisfaction (5 items)	1.41 ± 0.98	1.52 ± 1.01	0.503	1.52 ± 1.00	1.57 ± 1.11	0.748	0.02 (−0.34, 0.37)	0.926
Physical discomfort (4 items)	0.54 ± 0.69	0.46 ± 0.68	0.378	0.41 ± 0.54	0.52 ± 0.69	0.203	−0.18 (−0.41, 0.06)	0.137
Psychosocial discomfort (8 items)	0.26 ± 0.43	0.20 ± 0.36	0.152	0.18 ± 0.28	0.22 ± 0.41	0.260	−0.10 (−0.21, 0.01)	0.082
Worries and concerns (11 items)	0.46 ± 0.48	0.38 ± 0.52	0.084	0.35 ± 0.41	0.44 ± 0.56	0.109	−0.16 (−0.27, −0.05)	0.007

The impact of gastrointestinal discomfort-related symptoms were self-evaluated using the validated Patient Assessment of Constipation Quality-of-Life (PAC-QOL) questionnaire

[a]Mean ± SD. *PAC-QOL* Patient Assessment of Constipation Quality of Life

[b]Intervention effects were analysed using linear mixed model for repeated measures with compound symmetry as covariance structure

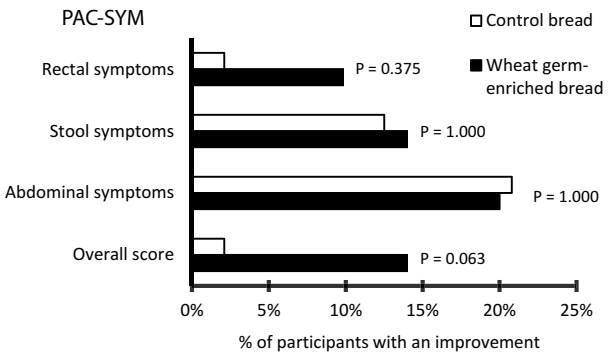

Fig. 2 Percentage of participants with minimal clinical improvement after 4-week daily intake of wheat germ-enriched bread and control bread. Score changes of half a point correspond to minimal clinical improvement. *PAC-SYM* Patient Assessment of Constipation Symptoms

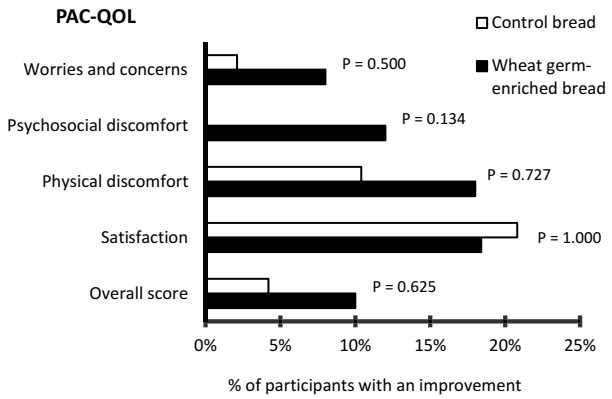

Fig. 3 Percentage of participants with minimal clinical improvement after 4-week daily intake of wheat germ-enriched bread and control bread. Score changes of half a point correspond to minimum important difference (improvement). *PAC-SYM* Patient Assessment of Constipation Symptoms

Concerning the PAC-QOL questionnaire, the 4-week consumption of wheat germ-enriched bread demonstrated a positive effect in the worries and concerns subscale (−0.16 [−0.27; −0.05], $P=0.007$) (Table 2). The effects measured in the overall, physical, and psychosocial discomfort subscales, indicate an improvement tendency although not statistically significant. This tendency is similar to the one observed in the analysis of the proportion of participants with a minimal clinical improvement (Fig. 3).

Gut microbiota

Specific genera or species that have been reported as beneficial for human health were selected for analysis by real-time PCR. Shifts in the number of copies of 16S rRNA genes for each target bacterial specie or genus were analysed in the faecal microbiota at baseline and after each intervention period. The statistical analyses of intervention–sequence interaction indicated the presence of carryover effects in a considerable part of the microbiota data, although our study

design included a 5-week washout period between the two intervention periods, aiming to minimise the risk of a carryover effect between interventions in period 1 and period 2. Thus, the crossover design was not considered for evaluating gut microbiota and only the first period was analysed. We emphasise that no significant differences were found between parallel groups in terms of subject characteristics (sex, age, weight, or BMI; Supplemental Table 5) or in baseline microbiota composition.

Our results show within-group differences in the intestinal bacterial microbiota of healthy volunteers that ingested wheat-germ bread (Fig. 4). Accordingly, we detected an increase in the number of *Bacteroides* spp. changing from 9.51 ± 1.11 to 9.96 ± 0.73 $\log_{10}$ copies/g faeces ($P = 0.004$). Likewise, the number of *Bifidobacterium* spp. rose from 10.03 ± 0.89 to 10.37 ± 0.88 $\log_{10}$ copies/g faeces ($P = 0.008$). Other bacteria known as beneficial such as *Clostridium leptum* and *Roseburia* spp. also increased, although the differences detected are not statistically significant. In general, the total number of bacteria increased after the 4-week intervention with wheat germ-enriched bread from 10.71 ± 0.46 at baseline to 10.92 ± 0.54 $\log_{10}$ copies/g faeces after intervention ($P = 0.070$). A relevant exception is the Gram-negative coliform *Escherichia coli* that remains unchanged (from 7.95 ± 0.90 to 7.93 ± 1.22 $\log_{10}$ copies/g faeces; $P = 0.925$); however, a tendency of reduction is observed (-0.75; $P = 0.084$) when the data are stratified by BMI and only participants with normal BMI are considered ($N = 31$). In contrast, the intake of control bread apparently increased the number of *E. coli* from 7.92 ± 1.05 to 8.22 ± 0.88 $\log_{10}$ copies/g faeces ($P = 0.248$) that, despite not being statistically significant, demonstrates an opposite tendency compared with the ingestion of wheat germ-enriched bread. Another effect of control bread was the likely decrease of *Akkermansia muciniphila* from 6.73 ± 2.16 to 6.51 ± 2.26 $\log_{10}$ copies/g faeces ($P = 0.249$) in opposition to wheat-germ intake that had an increase effect of 0.71 ($P = 0.137$).

Discussion

In this study, we found minimal differences in gastrointestinal function between the dietary interventions with wheat germ-enriched bread and refined wheat bread (control). The 4-week daily intake of wheat germ is very well tolerated by

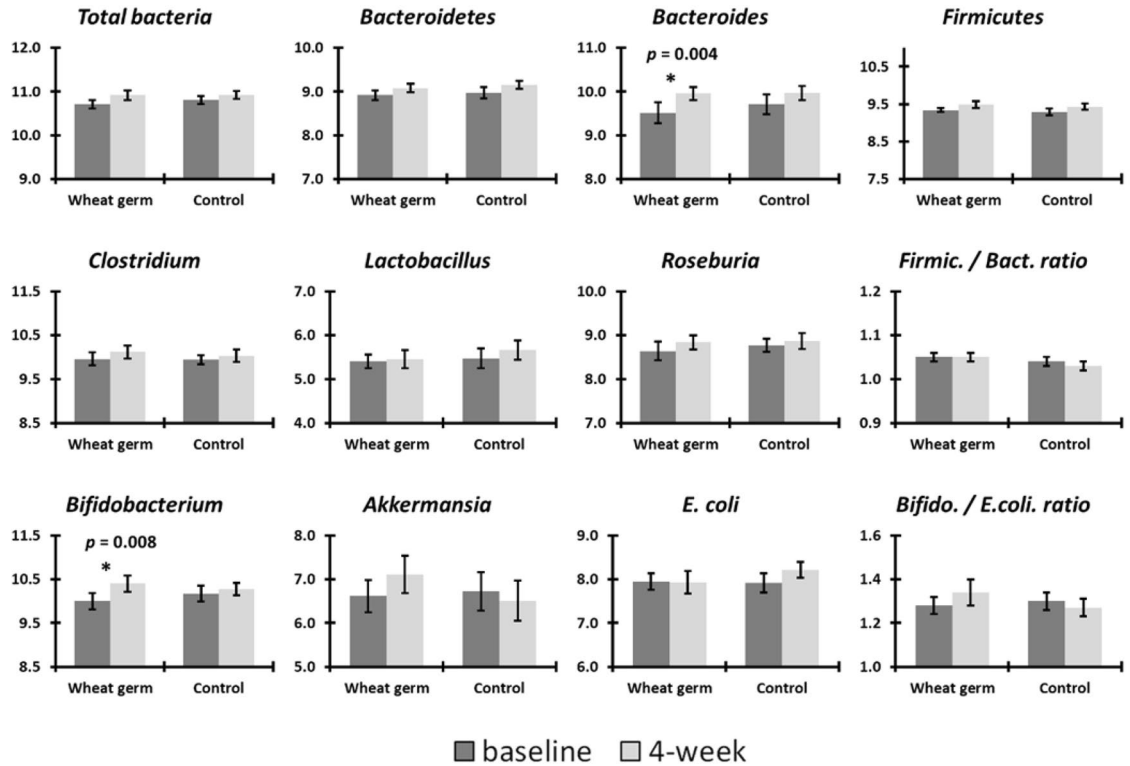

Fig. 4 Composition of the intestinal microbiota expressed as $\log_{10}$ copies/g faeces (mean ± SEM) of the study population at specie or genus level (*Clostridium leptum*, *Lactobacillus* spp., *Roseburia* spp., *Bacteroides* spp., *Bifidobacterium* spp., *Akkermansia muciniphila* and *Escherichia coli*) and phylum level (Firmicutes and Bacteroidetes). *Fimic./Bact. ratio* Firmicutes/Bacteroidetes ratio. *Bifido./E. coli ratio* Bifidobacteria/*Escherichia coli* ratio. Only the first period was used in the analysis due the existence of carryover effects. The statistically significant differences between baseline and 4 weeks were compared using the paired *t* test (*$P < 0.05$)

healthy volunteers, and in general, more participants tend to demonstrate minimal clinical improvement in PAC-SYM and PAC-QOL (total and subscales) after wheat-germ-enriched-bread ingestion than in the control arm. This difference is larger in the PAC-SYM total score (14% with wheat-germ intake versus 2% with control bread; $P=0.063$). Importantly, we demonstrated an improvement in the perceived gastrointestinal discomfort-related quality of life in the subscale "worries and concerns", after the 4-week intake of wheat germ regarding control ($P=0.007$). These results were unlikely to be influenced by psychological factors (confounding variable) (Supporting Information Table S4).

The gastrointestinal tolerability and functionality of wheat germ could be explained by favourable changes on the gut bacterial microbiota (Fig. 3 and Supplemental Table 6). Two different genera increased their abundance after wheat-germ intake: *Bifidobacterium* spp. and *Bacteroides* spp. Bifidobacteria are resident microbiota members which are known as probiotics [37] and their presence is associated with gut health. Recent data suggest that these bacteria can modulate T-cell responses reducing inflammation [38] and lower bifidobacteria abundance occurs in multiple inflammatory diseases including IBD. Lower levels of *Bacteroides* spp. are also associated with IBD by a mechanism similar to bifidobacteria [39]. *Bacteroides* spp. are part of the human gut microbiota well known for their ability to breakdown a wide variety of indigestible polysaccharides; they provide benefits to the human gut [40, 41]. Moreover, our data suggest an increase of *Clostridium leptum*, *Akkermansia muciniphila* and *Roseburia* spp. after wheat-germ intake, and these bacteria are known to exert positive effects on gut health [42]. Importantly, all alterations are not dependent on the initial microbiota composition of each subject, as previously observed by Matteuzzi et al. [25]. The changes herein reported were observed in the wheat-germ period, but not detected when the control and the wheat-germ groups were compared. However, the within-group comparison is scientifically limited. Thus, we conclude that wheat-germ intake may contribute to establish a healthy bacterial microbiota, unveiling wheat germ as a promising valuable prebiotic, which needs to be further investigated.

Likewise, we also detected relevant changes induced by the control bread that, despite not being statistically significant, should be highlighted, because they are clinically important. Thus, in opposition to wheat germ-enriched bread, the intake of refined bread without wheat germ seems to decrease the number of *A. muciniphila* while increasing *Escherichia coli* abundance. These changes are suggestive of a poor gut health. *E. coli* is belongs to the gut microbiota, whose high abundance is frequently associated with colorectal tumours [43], and *A. muciniphila* is associated with a healthier metabolic status and better clinical outcomes after calorie restriction in overweight/obese adults [44]. Some authors suggest that *A. muciniphila* can function as a diagnostic or prognostic tool to predict the potential success of dietary interventions [45]. The microbiota changes observed after control bread intake should be explored in the future, because for the first time, it is suggested that refined wheat bread could have a negative effect on gut microbiota. Clarifying this link is relevant in terms of public health, since refined bread is preferred by the majority of the populations, consumed more frequently, and in more occasions [46, 47].

In this study, the subjects were healthy volunteers with minimal gastrointestinal discomfort symptoms, as revealed by the PAC-SYM and the PAC-QOL overall and subscales score means at baseline. They had four times less gastrointestinal discomfort symptoms than the IBS patients or subjects with reduced bowel movements and gastrointestinal discomfort [48, 49]. Because of that, the number of participants enrolled should be higher than the number initially estimated to detect statistically significant differences. At the moment, this study was carried out, clinical trials evaluating the gastrointestinal discomfort in healthy subjects having PAC-SYM as an outcome were not available. For that reason, our sample size calculations were based on studies involving subjects with symptoms of gastrointestinal discomfort. This limitation prevents extensive conclusions regarding wheat germ-enriched-bread effect on gastrointestinal function and discomfort. Regarding the effect of wheat germ on the bacterial microbiota, the presence of carryover effects did not allow us to take the most advantage of the crossover design, and therefore, the washout period should be longer than 5 weeks. Other study limitations include diet-induced bias on the results reported. Despite the subjects were informed and asked not to change their dietary habits during the study, diet was not monitored to avoid what could be seen as an additional psychological pressure for the participants; and this is another potential confounding variable. Finally, the differences measured at the bacterial genus level can be interpreted in terms of changes in the diversity within each target genus or changes in the number of species already present at baseline. This is a limitation imposed by real-time PCR methodology.

In conclusion, adding wheat germ to refined wheat bread seems to promote a healthy gut bacterial microbiota which could be beneficial for gastrointestinal health. Participants that consumed wheat germ-enriched bread reported better health-related quality of life concerning worries and concerns. Our results indicate that wheat germ is able to overcome resilience of gut microbiota and may promote the growth of beneficial genera, while it has a protective role against proliferation of potential pathogenic detrimental bacterial species such as *E. coli*. Indeed, the participants that ingested wheat germ-enriched bread had a high bifidobacteria/*E. coli* ratio that is considered an important indicator of gut microbiota equilibrium and health. The

effect of wheat germ in the bifidobacteria/*E. coli* ratio was 0.10 [(−0.01; 0.21); $P=0.073$]. Accordingly, adding wheat germ to regular refined bread is an elegant strategy to overcome the consumers' preference for white bread and in this way, promoting better public health. In the future, it will be important to understand whether wheat-germ-induced changes on the gut bacterial microbiota are temporary or can be maintained by continuing the intake of this cereal grain ingredient.

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Compliance with ethical standards

Conflict of interest None of the authors has any conflict of interest regarding this study.

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