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Keywords:	wheat germ, lipid profile, glucose profile, randomised controlled trial, cardiovascular risk

RESEARCH

Does intake of bread supplemented with wheat germ have a preventive role on cardiovascular disease risk? A randomised, placebo-controlled, crossover trial.

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Keywords: wheat germ; lipid profile; glucose profile; randomised controlled trial; cardiovascular risk.

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ABSTRACT

Objective: Our aim was elucidating the role of germ in primary prevention of cardiovascular events and we selected a staple food for supplementation. Accordingly, the effects of daily consumption of refined bread having 6 g of wheat germ were evaluated in fasting cholesterol and triglycerides, fasting and postprandial glucose, insulin sensitivity and CRP. This germ supplementation corresponds to 6-fold increase in the global mean consumption of germ and, importantly, it preserves the sensory proprieties of refined bread which is crucial for consumer's acceptance.

Design: Fifty-five healthy adults were recruited in a randomised, double-blinded crossover trial with 15-week follow-up, comprising a 2-week run-in, two intervention periods of 4 weeks/each and a 5-week washout period. Fasting venous blood samples were collected at the end of each stage. Postprandial glucose was measured at different time points. The effect of daily intake of wheat germ-enriched bread was compared with control bread.

Results: Study subjects had mean age of 34 y and BMI between 19 and 38 kg/m^2. Among the 52 subjects, 15% and 4% of them were borderline-high and high fasting total plasma cholesterol, respectively. Two participants had high fasting total plasma triglycerides. We observed that daily intake of 6 g wheat germ had no significant effect on cholesterol and triglycerides levels, on postprandial glucose response, and on insulin sensitivity. No effect was also observed for the subgroups of participants who completed the outcome measures and complied with the daily bread intake (n = 47).

Conclusions: The absence of alterations on lipid and glucose profiles suggests that germ up to 6 g/day, may have no preventive effect on CVD risk. However, it is

important to investigate other food vehicles that can accommodate higher doses of wheat germ, in future studies.

Trial registration number: NCT02405507.

Strengths and limitations of this study:

- This study followed the best practices for designing, conducting and reporting clinical trials to support health claims on food products, namely random allocation, double blinding, reporting methods to measure and maximise compliance.
- We used validated outcomes which are considered beneficial physiological effects for human health.
- To the best of our knowledge, this is the largest study to assess the impact of germ intake in human subjects.
- Although compliance was high, it is uncertain whether this is due to over-reporting, since there is no biomarker for wheat germ intake.
- A longer intervention period would be desired for evaluating an effect on lipoprotein cholesterol, nonetheless could have a major impact on loss to follow-up in this crossover study.

INTRODUCTION

Cardiovascular diseases (CVD) remain the leading cause of mortality in the world, having accounted for 15 million of deaths in 2015.[1] Risk factors for CVD are well-identified and they include smoking, type 2 diabetes or high glucose levels, hypertension and elevated cholesterol levels.[2] Diet is therefore an important modifiable risk factor for CVD and, within the diet, some dietary components may have an important preventive role.[3-5] In this regard, cereal whole grains are a promising protective measure due to emerging evidence of an inverse association between their intake and CVD risk as shown by recent systematic reviews and meta-analyses.[5-8] The role of whole grains in reducing CVD risk is broader: 1) by improving glucose metabolism through better postprandial glucose and insulin responses;[9] and 2) by reductions in plasma cholesterol levels.[10] The health benefits associated with whole grains intake seems to be mediated by their high content in plant-derived redox-active compounds that may activate anti-oxidant pathways and thereby have anti-inflammatory proprieties.[11]

Cereal whole grains distinguish from refined grains by the presence of bran and germ fractions. These two fractions accumulate higher amounts of protective bioactive compounds, such as fibres, micronutrients, vitamins and phytochemicals. CVD prevention has been associated with bran intake,[7, 12-15] but findings about germ are conflicting.[7, 16] Recent systematic reviews addressing prospective studies reported no inverse association between germ intake and CVD risk, in contrast with prior clinical trials involving high-risk groups. But these two types of studies report different amounts of germ intake. The meta-analyses of the prospective studies reported a low germ intake

(1g/day average ranging from 0.2 to 2.9 g/day),[17-19] while the intervention studies used a daily supplementation of 20 or 30 g/day during 4-week period. Specifically, these clinical trials showed that intake of raw wheat germ can reduce cholesterol and triglycerides in rats[20-22] and also in hypercholesterolemic and hypertriglyceridemic humans.[23, 24]

Dietary guidelines around the world recommend 85 g daily intake of whole grains, which contains nearly 2.6 g of germ. However, their daily consumption is far below the recommendations and consumers prefer highly refined products.[25-27] In fact, supplementation with whole-grain ingredients is therefore an elegant way to overcome consumer's preferences while contributes for public health, as long as these ingredients are indeed beneficial. Clarification of the physiological effects of germ is needed. However, fortification of food products with germ is challenging because germ becomes rancid very rapidly due to high content in unsaturated lipids together with lipases and lipoxygenases,[28-30] and it also negatively affects the sensory properties of the final food product.[31] Thus, in order to address these specificities, germ stabilization[32] is necessary immediately after milling to inhibit enzymatic rancidity, while the percentage of germ in the final product should be tested whether long-term consumer acceptance is desired.

In this context, we designed a randomised, double-blinded, crossover, placebo-controlled clinical trial targeting the general population, in order to evaluate the physiological and metabolic effects of germ intake in a dose higher than the amount reported in the previous prospective studies, wherein no preventive CVD effect was demonstrated. The impact on CVD metabolic risk factors of daily consumption of 100 g

of wheat white-bread enriched with 6 g of wheat germ during 4-week, is here presented. This supplementation corresponds to 6-fold increase in the global mean consumption of germ. Bread was chosen as vehicle for germ intake because it is a staple food, major contributor to carbohydrates intake, whereas the wheat is globally a staple grain. Our aim was to elucidate the role of germ in the primary prevention of cardiovascular events and for this, we tested and developed a wheat germ-enriched bread without compromising the sensory characteristics of white refined-bread.[33] Besides allowing blinding and the analysis of the effect of added germ individually, this no-difference is crucial for consumer's acceptance. The strength of the current study also includes the use of outcomes which are considered to be beneficial physiological effects for human health.[33] Finally, to our knowledge, this is the largest study to assess the impact of wheat germ intake in human subjects and, also important, the statistical power of this study is higher than preceding studies. For that reason, smaller differences could be detected if they indeed existed.

METHODS

Participants

Fifty-five healthy volunteer subjects were recruited from the Porto metropolitan area in northern Portugal through public advertisements in the University and Faculty websites, and in online newspapers. The detailed study protocol has been previously described.[33] Briefly, volunteers were invited to visit our Research Unit (CINTESIS) for a physical exam and a brief questionnaire about their medical history and background diet in order to check their eligibility to participate in the study. Inclusion criteria included subjects age 18 to 60 years old, non-diabetic, and non-smoker. The exclusion criteria included the use of medication / dietary supplements, potentially interfering with this trial, not willing to avoid prebiotics and probiotics for the duration of the study, and change of dietary habits within the 4-week prior to screening (for instance, to start a diet high in fibre). In the protocol manuscript, we described sample size calculations; 40 participants were required to allow for an 80% power and 95% confidence level.[33]

The study protocol was approved by the Health Ethics Committee of São João Hospital Centre and the Ethics Committee of the Faculty of Medicine of the University of Porto, and all participants provided written informed consent. The clinical trial was conducted from June 2015 to October 2016, in accordance with ethical principles of the Declaration of Helsinki, international law and Good Clinical Practice guidelines. This study is registered in ClinicalTrials.gov database, reference NCT02405507, and followed the CONSORT reporting guidelines (online supplementary table S1).

Participant and public involvement

Participants and volunteers were involved in the development of the bread formula used in this study and then in its final sensory evaluation. They had no role in setting the research question, the outcome measures, the design, or implementation of the study. Nevertheless, we included inputs from participants, namely any burden associated with the procedures, throughout the study follow-up in order to optimize their involvement and compliance. Participants and volunteers were also involved in the recruitment process by encouraging others to participate. Upon publication, participants will be informed of the results of this study through direct email.

Study design and intervention

Our study was a 15-week, randomised, double-blinded, crossover, placebo-controlled clinical trial. The trial comprised four stages: a run-in period (2-week), two crossover intervention periods (4-week each), and a washout period between interventions (5-week). In the end of each stage, blood samples for measurement of plasma cholesterol (total, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL)), triglycerides, C-reactive protein (CRP), and postprandial glucose were collected from each study participant as primary outcomes. Blood samples were also collected for measurement of fasting glucose and insulin as secondary outcomes. Primary and secondary outcomes related with gastrointestinal discomfort were also evaluated in this trial, however they will be reported later.

Participants were instructed not to change their physical activity levels, maintain their dietary habits and do not consume any food or dietary product supplemented with germ during the study. Compliance to the study protocol (daily consumption of bread) was monitored through daily self-reported questionnaire, since there is no biomarker for

wheat germ intake. Participants were randomly assigned into two intervention groups (ratio 1:1) using a computer-generated allocation sequence by a statistician not involved in recruitment and intervention delivery. The intervention arm comprised the daily consumption of wheat bread (100 g) supplemented with wheat germ (6 g), whereas the control arm involved the daily intake of wheat bread (100 g) without any supplementation. The bread provided to participants replaced their usual bread intake during meals, namely during breakfast or afternoon snack.

The participants and the research team were blinded to the study breads (intervention/control). In this regard, we previously tested the best formula that masked wheat germ supplementation, in terms of bread texture, volume and flavour. Moreover, the bread was delivered to each participant in opaque bags with a label code (A/B); only the outsourced company responsible for bread production (Padaria Ribeiro Lda., Portugal) knew the correspondence code. The unblinding was performed after the statistical analysis had been completed.

Bread formulation

Control breads were prepared by mixing 6 kg refined wheat flour (Germen S.A., Portugal) with 3.3 L water, 97 g of salt, 60 g of bread improver mix (Germen, S.A., Portugal), and 180 g of baker's yeast. The mixture then fermented for 15 min at room temperature. Dough was divided and molded into pieces (123 g each), and then placed in a fermentation chamber with 80% relative humidity for 60 minutes at 30ºC. Finally, baking was done at 190 °C during 20 min. Preparation of intervention bread was similar to control bread; 480 g of refined wheat flour was replaced by raw wheat germ (Germen S.A., Portugal) only.

Nutrient composition of control and intervention breads was analysed by Silliker Portugal, S.A. (Mérieux Nutrisciences Corporation).

Blood sampling and analysis

The outcomes variables were measured on study participants under 12 hours overnight fasting conditions, at the end of 1) run-in, 2) first intervention, 3) washout and 4) second intervention. Accordingly, a venous blood sample was collected by venipuncture into serum separator tubes (BD Vacutainer SST II Advance, Becton, Dickinson and Company). For measuring glycated haemoglobin (HbA1c), blood was collected into tubes containing K_2EDTA (BD Vacutainer; Becton, Dickinson and Company). All venous blood samples were centrifuged at 1377 × g for 10 min at room temperature (CompactStar CS4; VWR), within 30 minutes after collection. A serum aliquot was immediately stored in a special cool transport container (at < -10°C) for insulin quantification. All biological samples were shipped to an outsourced certified medical laboratory (Clínica Laboratorial de Guimarães, S.A., Portugal) under refrigerated conditions. Analysis was performed within 24 hours after collection. Fasting glucose, total cholesterol and triglycerides were measured using specific enzymatic colorimetric methods, whereas LDL and HDL cholesterol were quantified by the elimination/catalase method. Serum CRP concentrations were measured by latex-enhanced immunoturbidimetric assay. These biochemical quantifications were done with the automatic analyser ADVIA 1800 (Siemens Healthcare Diagnostics). Insulin was determined by chemiluminescent microparticle immunoassay method using the automatic analyser Architect i2000 (Abbott Laboratories). The insulin resistance was

estimated using the homeostasis model assessment for insulin resistance (HOMA-IR):[34, 35]

(fasting insulin (μU/mL) × [fasting glucose (mg/dL) ÷ 18.01]) / 22.5

Regarding the postprandial glucose, four capillary blood samples were obtained by finger prick sampling using disposable lancet devices (Glucocard MX; Arkray) and a glucose meter. Postprandial glycaemia was measured in the fasting state (0) and at 30, 60 and 120 minutes after intervention or control bread intake. The postprandial glucose response was expressed as the incremental area under curve (IAUC), by using the trapezoidal rule ignoring the area below the fasting baseline, as previously described.[36]

Statistical Analysis

Statistical analysis was performed using SPSS version 23 software (SPSS Inc., Chicago, IL, USA). Data from all participants who were randomly assigned and completed an initial assessment were included in the intention-to-treat statistical analysis. Additionally, an analysis was also performed including the participants who adhered to the study protocol only, namely those that completed the outcome measures and complied with the daily bread intake (assessed by daily questionnaire). Numerical data are expressed as means ± SD, and treatment effects with 95% CI. Statistical significance was set at a 2-sided P value of 0.05. However, we also decided to perform an adjustment for multiple comparison because of the multiple primary outcomes analysed; thus the type 1 error and significance level 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).[37]

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. A linear mixed model for repeated measures, with compound symmetry as the 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 variables. In order to account for between subject variability and to adjust for any nonspecific 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 estimated intervention effect for each sequence.

RESULTS

Baseline characteristics

Of 68 subjects screened to assess eligibility to participate in the study protocol, 55 were randomly assigned, and 52 completed an initial assessment and were included in the statistical analysis (figure 1). Eight individuals dropped out: three did not wish to continue the study for unspecified reasons, three declined to participate due to personal reasons (see figure 1), and the last two because of unrelated illness (gastroparesis and pneumonia). Study participants were healthy men and women, with a mean age of 34 years (range: 18–59 years), and BMI (in kg/m^2) between 19 and 38 (34 normal weight, 14 overweight and 4 obese). All participants had normal fasting glucose and normal glucose tolerance. At baseline, forty-two had normal fasting total plasma cholesterol (< 200 mg/dL), 8 had borderline-high fasting total plasma cholesterol (200-239 mg/dL) and 2 had high fasting total plasma cholesterol (> 240 mg/dL). Fifty participants had normal fasting total plasma triglycerides (< 150 mg/dL) and two had high fasting total plasma triglycerides (200-499 mg/dL). Test results for haemoglobin and for liver function showed no evidence of ill health. Baseline characteristics of the 52 study participants that were included in the intention-to-treat statistical analysis are listed in table 1.

Table 1 Baseline characteristics of study participants included in the analysis[1]

Characteristics	*N*	Value
Sex, n		
Male		16
Female		36
Age, years	*52*	33.67 ± 11.69
Body weight, kg	*52*	66.69 ± 11.84
BMI, kg/m^2	*52*	23.98 ± 3.98
Total cholesterol, mg/dL	*52*	173.73 ± 32.17
HDL cholesterol, mg/dL	*52*	60.71 ± 15.04
LDL cholesterol, mg/dL	*52*	96.15 ± 26.33
Triglycerides, mg/dL	*52*	84.40 ± 56.42
Glucose, mg/dL	*52*	83.87 ± 6.44
Insulin, µU/mL	*50*	7.15 ± 3.56
HOMA-IR	*50*	1.49 ± 0.79
HbA1c, %	*51*	5.17 ± 0.25
IAUC glucose, mg.min/dL	*50*	3322.24 ± 2086.88
CRP, mg/dL	*52*	0.12 ± 0.18

[1]Mean ± SD. CRP, C-reactive protein; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; IAUC, incremental area under the curve: LDL, low-density lipoprotein.

Participant compliance

Daily questionnaires revealed good compliance to the study protocol. The daily consumption of bread was assessed and the average compliance was 92.1% ± 9.3 and did not vary depending of bread type. Intervention and control arm had the same percentage of compliance: 92.2% ± 11.1 and 92.0% ± 10.0, respectively (P = 0.920). None of the participants reported adverse effects.

Composition of intervention and control breads

The content of protein, and dietary fibre was higher in the wheat germ-enriched bread (9.6% and 7.5%, respectively), whereas control bread had a higher content of

carbohydrate and starch (5.5% and 6.0%, respectively). The content of fat and energy are comparable in both breads. As expected, the quantity of total phytosterols and alpha-linolenic acid was 73.3% and 41.7% higher in the intervention bread, respectively (table 2).

Table 2 Chemical composition of wheat germ-enriched and control breads per 100 g product weight

	Wheat germ-enriched bread	Control bread
Energy, kJ	1154.7	1182.8
Protein, g	9.7	8.8
Fat, g	5.4	5.5
Carbohydrate, g	44.4	47.0
Dietary fibre, g	4.3	4.0
Total sugar, g	3.4	3.4
Starch, g	40.8	43.4
Total phytosterols, mg	52.0	30.0
Moisture, g	34.5	32.9
Ash, g	1.7	1.9
Fatty acid, % of total fatty acids		
14:0	0.2	0.0
16:0	18.9	20.7
16:1	3.3	3.7
18:0	2.5	4.6
18:1n–9	14.9	16.0
18:1n–7	1.2	1.1
18:2n-6	52.9	49.8
20:0	0.2	0.0
18:3n–3	5.1	3.6
20:1n–9	0.8	0.5

Blood lipids

There was no statistically significant difference between wheat germ-enriched and control breads, after 4-week intake, for cholesterol (total, LDL and HDL) and serum triglycerides (table 3).

Table 3 The effect of 4-week wheat germ-enriched bread versus control bread on cardiovascular, inflammatory and metabolic outcomes

	Wheat germ-enriched bread					Control bread					Effect of wheat germ[2]		
	N	Baseline[1]	*N*	Post-Intervention[1]	*P* value within group	*N*	Baseline[1]	*N*	Post-Intervention[1]	*P* value within group	Effect	(95% CI)	*P* value between group
Total cholesterol, mg/dL	*52*	175.17 ± 31.82	*50*	172.98 ± 32.4	0.147	*48*	174.00 ± 32.17	*48*	170.00 ± 31.38	0.100	1.20	(-4.54, 6.95)	0.675
HDL cholesterol, mg/dL	*52*	60.92 ± 15.13	*50*	59.38 ± 16.07	0.010	*48*	60.65 ± 15.21	*48*	58.38 ± 13.77	0.012	0.14	(-1.83, 2.12)	0.886
LDL cholesterol, mg/dL	*52*	97.31 ± 26.07	*50*	96.92 ± 26.22	0.853	*48*	97.23 ± 25.79	*48*	95.21 ± 27.08	0.356	1.74	(-3.80, 7.28)	0.530
Triglycerides, mg/dL	*52*	85.13 ± 54.67	*50*	83.8 ± 42.62	0.565	*48*	81.10 ± 49.96	*48*	82.48 ± 59.66	0.763	-3.60	(-16.28, 9.08)	0.571
Glucose, mg/dL	*52*	83.5 ± 6.35	*50*	81.56 ± 8.02	0.025	*48*	83.50 ± 6.45	*48*	82.35 ± 8.38	0.219	-0.80	(-2.93, 1.34)	0.458[3]
Insulin, µU/mL	*51*	7.00 ± 3.08	*50*	7.04 ± 2.83	0.729	*47*	6.84 ± 3.45	*48*	7.32 ± 5.75	0.524	-0.39	(-2.03, 1.24)	0.629
HOMA-IR	*51*	1.46 ± 0.68	*50*	1.44 ± 0.67	0.973	*47*	1.43 ± 0.78	*48*	1.54 ± 1.38	0.541	0.12	(-0.49, 0.26)	0.529
HbA1c, %	*52*	5.19 ± 0.25	*50*	5.25 ± 0.26	0.004	*47*	5.23 ± 0.23	*48*	5.25 ± 0.22	0.393	0.04	(0.00, 0.08)	0.039[4]
IAUC glucose, mg.min/dL	*51*	3399.58 ± 2095.88	*48*	3017.74 ± 1959.09	0.166	*46*	3379.91 ± 2149.45	*44*	3334.59 ± 2101.47	0.812	-221.13	(-901.34, 459.08)	0.524
CRP, mg/dL	*52*	0.13 ± 0.21	*50*	0.18 ± 0.42	0.374	*48*	0.12 ± 0.17	*48*	0.25 ± 0.57	0.093	-0.07	(-0.26, 0.13)	0.481

[1]Mean ± SD. CRP, C-reactive protein; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; IAUC, incremental area under the curve: LDL, low-density lipoprotein.
[2]Intervention effects were analysed using linear mixed model for repeated measures with compound symmetry as covariance structure.
[3]The intervention-sequence interaction was significant (-5.73 [-10.00, -1.46], P = 0.010), the intervention effect was 2.07 in the first sequence and -3.66 in the second sequence.
[4]The intervention-sequence interaction was significant (0.15 [0.07, 0.23]; P < 0.001), the intervention effect was -0.04 in the first sequence and 0.12 in the second sequence.

No difference was also observed for participants who completed the outcome measures and complied with the daily bread intake (n = 47; total cholesterol: P = 0.797, HDL cholesterol: P = 0.996, LDL cholesterol: P = 0.665, and triglycerides: P = 0.762). The same result was obtained when participants with normal fasting total plasma cholesterol and triglycerides are analysed (n = 41; total cholesterol: P = 0.981, HDL cholesterol: P = 0.413, LDL cholesterol: P = 0.833, and triglycerides: P = 0.718). The results were not statistically different when participants with borderline or high fasting total plasma cholesterol were included (n = 10; total cholesterol: P = 0.432, HDL cholesterol: P = 0.170, LDL cholesterol: P = 0.781, and triglycerides: P = 0.508). Statistical analysis of blood lipid outcomes showed no interaction between intervention and sequence, potentially excluding a carryover effect.

Glucose metabolism and C-reactive protein

There were no significant changes in postprandial glucose peak values (at 30 minutes) after 4-week consumption of wheat germ-enriched bread (-5.13 ± 3.03 mg/dL; P = 0.097) or control bread (2.35 ± 3.73 mg/dL; P = 0.531), and neither between interventions (P = 0.182). Blood glucose response curves for 2 hours after the intake of intervention and control breads are shown in figure 2.

Comparing with control, wheat germ-enriched bread had no significant effect on IAUC glucose, fasting insulin or HOMA-IR (table 3), suggesting that 6 g of wheat germ did not improve postprandial glycaemia or glucose metabolism. No differences in CRP were observed between wheat germ-enriched and control breads. CRP and glucose results are not statistically different when only participants who adhered to the study

protocol were analysed (n = 47; IAUC: P = 0.597, Insulin: P = 0.709, HOMA-IR: P = 0.597, and CRP: P = 0.959).

The intervention-sequence interaction was significant for fasting glucose (-5.73 [-10.00; -1.46], P=0.010) and for HbA1c (0.15 [0.07; 0.23], P<0.001), revealing the existence of a carryover effect in these two outcomes. HbA1c reflects the average blood glucose level in previous 2 to 3 months.[38]

DISCUSSION

Several authors argue that diet should be seen as a key component of overall CVD prevention and health management care plan.[47-49] It is well known that corrective dietary interventions can positively alter lipid profile, blood pressure, BMI, endothelial function, insulin sensitivity, and several inflammatory markers, not only for individuals with genetic predisposition for CVD[50] but also for general population.[3, 4, 35, 48] In order to correctly inform consumers and food and nutrition policy makers about the benefits of supplementing food products with whole grain ingredients, there is an urgent need for clarifying the differences (whether any) between whole-grains and bran or germ individually. First, adding bran or germ individually may not have identical physiologic benefits as the whole grain; and secondly, these two fractions alone may have distinct physiological effects on cardiovascular-health promotion when compared with the whole-grain.[52]

In the present study, we evaluated the health effects of wheat germ intake in 52 healthy individuals. Blood lipids (cholesterol and triglycerides), fasting and postprandial glucose, CRP and insulin were evaluated during 15-week in a randomised crossover design. The results presented show that the intake of 6 g/day of wheat germ for 4-week, has no effect on cholesterol and triglycerides levels, on glucose metabolism, namely in IAUC glucose, and on insulin sensitivity (HOMA-IR). Thus, our findings strongly suggest that wheat germ by itself has no impact on the cardiovascular risk factors evaluated and thus, a beneficial physiological effect of wheat germ up to 6 g/day, by decreasing the risk of CVD in normal adults, is unlikely. Six grams of wheat germ intake per day corresponds to 2.4-fold increase in the germ present in the daily

recommendation for whole grains intake and to a 6-fold increase in the global mean consumption of germ.

Germ of wheat was chosen due to its potential to reduce blood cholesterol and triglycerides concentrations, as suggested in previous studies.[23, 24] The proposed mechanisms for lowering plasma cholesterol include a) the inhibition of pancreatic lipase activity by soluble proteins present on wheat germ;[39] b) the reduction in triglyceride lipolysis;[40] and c) reduction in cholesterol absorption by the endogenous wheat germ phytosterols.[41] However, the existing evidence regarding the lipid-lowering properties of wheat germ from previous studies is controversial. First, reduction of total cholesterol and LDL was only evaluated in hypercholesterolemic individuals after diet supplementation with 20 g/day for 4-week period. Second, daily ingestion of 30 g wheat germ supplement for 4-week markedly decreases (-39%) plasma triglycerides in hypertriglyceridemic individuals, whereas no reduction was observed in the normotriglyceridemic subgroup.[23]

In our study, we enrolled a moderately large and heterogeneous sample of participants which is representative of general population, with normal, borderline-high and high fasting total plasma cholesterol and triglycerides. The statistical power of this study is higher than preceding studies and for that reason, smaller differences could be detected if they indeed existed. Thus, it is not plausible that the absence of any statistical significance on the wheat germ-induced changes is due to the lack of statistical power. However, the inexistence of wheat germ effects reported here does not necessarily disprove the potential cholesterol- and triglyceride-lowering effect described in previous studies. In particular, because in those studies, wheat germ was consumed in higher

doses and by individuals at risk. In our opinion, a dose-response analysis for wheat germ intake and lipid profile should be investigated in the future, for general population and high-risk groups.

Effect of wheat germ in improving postprandial glycaemia or glucose metabolism was not demonstrated in our study, in accordance with previous studies. Earlier studies addressing the long-term impact of wheat germ on blood lipids of hypercholesterolemic individuals did not observe any significant variation on fasting glucose, insulin, fructosamine and HbA1c;[24] and one single study with six healthy participants showed that wheat germ had no effect on postprandial glucose and insulin concentrations.[43] Even so, we decided to investigate the impact of wheat germ on glucose metabolism because it was recently suggested that intake of alpha-linolenic acid has been associated with lower insulin resistance[44] and our wheat germ enriched-bread contains more 41.7% of alpha-linolenic than control bread (table 2).

Up until now, the beneficial effect of wheat germ in lowering blood cholesterol and triglycerides was only demonstrated when used as a diet supplementation with 20 g[24] or 30 g/day[23] during 4-week periods. Incorporation of such high proportions of wheat germ in food products, without impairing their nutritional profiles, is difficult since germ changes their sensory proprieties. Our study is the first interventional study evaluating the effect of wheat germ in higher doses than the global mean consumption of germ and higher than the germ present in the recommended doses of whole grains in the diet, in a randomised, double-blinded, placebo-controlled clinical trial using a functional food product, and not as a dietary supplement. More importantly, the nutritional status (table 2), texture and flavour in the wheat germ-enriched bread were

not compromised (online supplementary table S2). The aim was to develop a product that could reduce CVD risk, whilst maintaining the sensory characteristics of white refined-bread. Importantly, besides allowing blinding and the analysis of the effect of added germ individually, this no-difference is crucial for consumer's acceptance if this is envisioned as a long-term goal.

Our study followed the best practices for designing, conducting and reporting clinical trials to support health claims on food products, namely random allocation, double blinding, reporting methods to measure and maximise compliance. The strength of the current study also includes the use of validated outcomes which are considered beneficial physiological effects for human health.[33] Finally, to our knowledge, this is the largest study to assess the impact of germ intake in human subjects and, also important, targets the general population instead of a high-risk group.

The limitations of this study include the duration of the intervention period. Although 4-week is considered the minimal intervention for evaluating an effect on lipoprotein cholesterol, 8-week would be more desirable; however, such intervention period in a crossover study could have a major impact on loss to follow-up. Secondly, the absence of a biomarker specific for germ intake is also a limitation; adherence was monitored through daily self-reported questionnaire and, though compliance with the study protocol was optimal, it is uncertain whether there was over-reporting. Lastly, we decided not to collect information about diet and physical activity levels during the study in order to avoid changes in general participants' lifestyle and dietary patterns; and this could be seen as a limitation.

In summary, our goal was to demonstrate the effect of a staple food supplemented with wheat germ in its maximal concentration without compromise its sensory properties. However, the intake of 6 g/day of wheat germ does not contribute to reduce the cardiovascular risk factors: plasma triglycerides, total cholesterol, LDL cholesterol, or increase the HDL cholesterol levels, or even improve glycaemic control, in a generally healthy normal population. In order to justify an enrichment of food products with wheat germ as a public health approach to prevent CVD, the beneficial effects of wheat germ on human health should be investigated in other food vehicles that can accommodate higher doses of germ. Chemical and sensory proprieties of biscuits, noodles and cakes supplemented with increasing amounts of wheat germ (up to 30%) have been recently analysed and improved.[31, 55, 56] These technological advances let us to envisage that other food products functionalised with higher wheat germ content can be developed. Future studies following our clinical trial design are needed to elucidate if high amounts of daily intake of wheat germ are effective in reducing CVD risk.

Abbreviations CRP, C-reactive protein; CVD, cardiovascular disease; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; IAUC, incremental area under curve; LDL, low-density lipoprotein.

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Competing interests None declared**.**

Contributors The authors' responsibilities were as follows – AMR was partially responsible for study design, statistical analysis plan, carrying out the trial, manuscript writing and final revision. HP was partially responsible for study design, carrying out the trial, manuscript writing and final revision. CM collaborated in the study design, data collection and final revision of the manuscript. JAT collaborated in the study design and final revision of the manuscript. CC was responsible for the general coordination of the project, study design, manuscript writing and final revision. LFA was responsible for the general coordination of the project, study design, statistical analysis plan, manuscript writing and final revision.

Data sharing statement The full datasets generated during and/or analysed during the current study are not publicly available because the ethics committee only allowed the use of the data in the context of the present research project, however anonymised partial datasets or summaries of the data are available from the corresponding author on reasonable request.

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721

Figures:

Figure 1 Flow chart of participants through the study.

Figure 2 Mean ± SEM postprandial glucose concentrations in response to a 100 g wheat germ-enriched bread or control bread, at baseline (A), and after 4-week intervention (B). No significant effect in the incremental area under the curve (IAUC) was observed between wheat germ-enriched and control breads (P = 0.524).

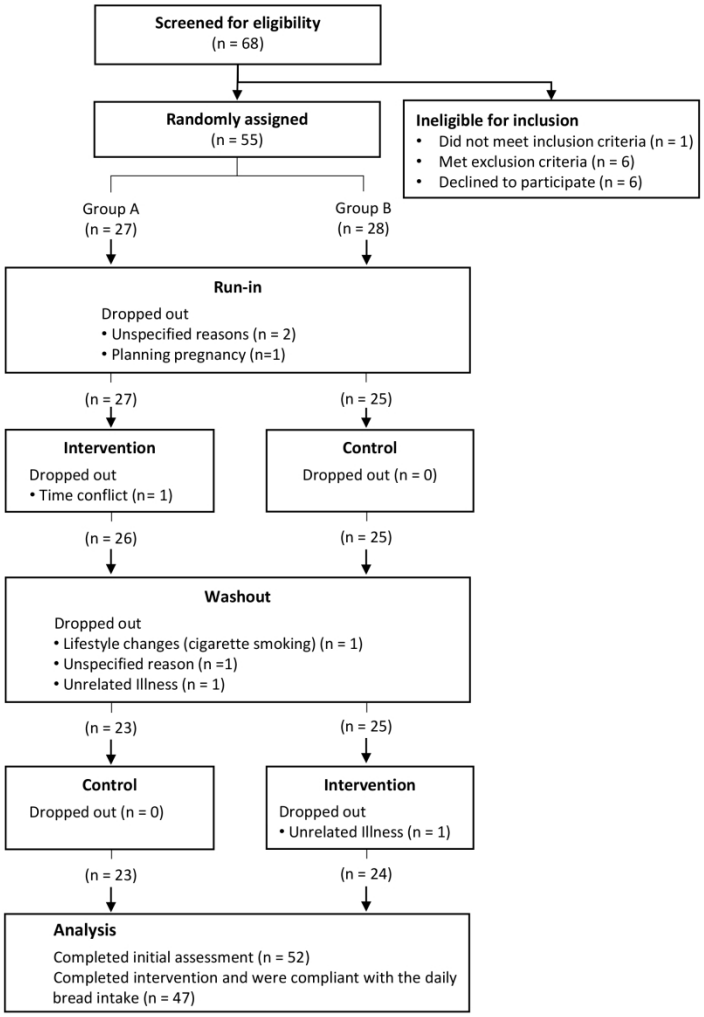
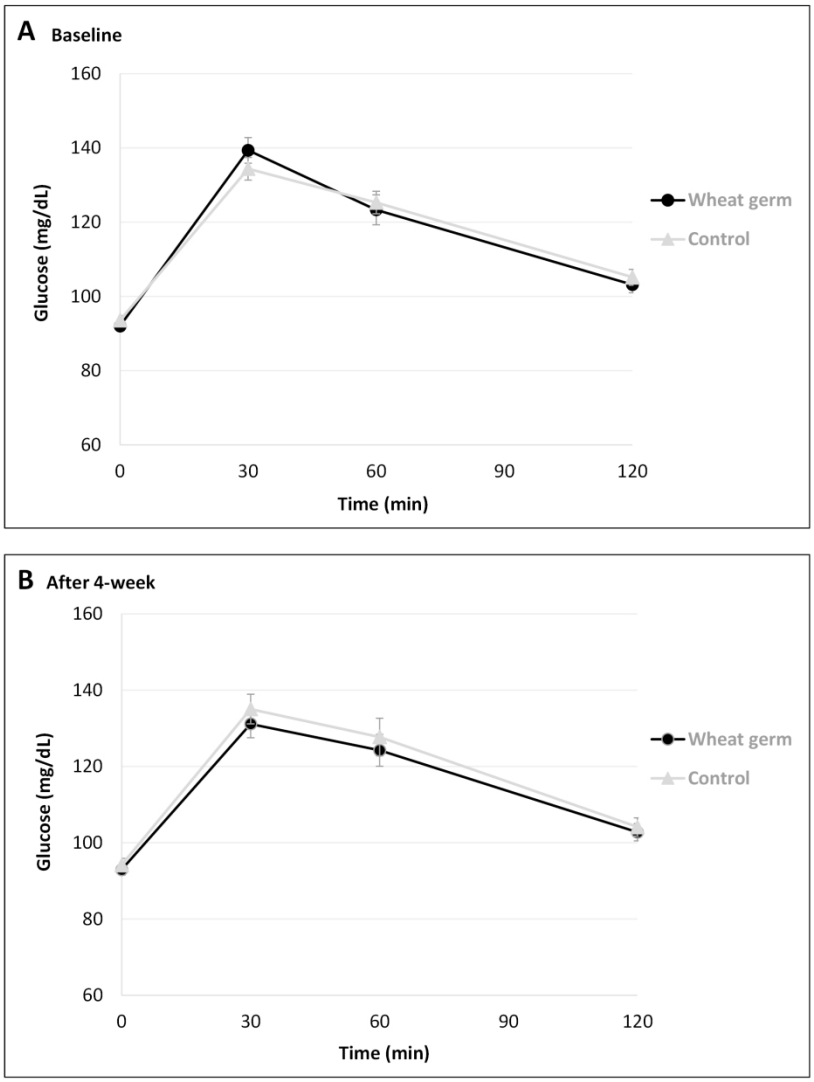


Figure 1 Flow chart of participants through the study.

199x250mm (300 x 300 DPI)



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199x250mm (300 x 300 DPI)

ONLINE SUPPLEMENTARY TABLES

Supplementary Table S1. CONSORT 2010 checklist[1]

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4, 5, 6
	2b	Specific objectives or hypotheses	6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	7, 8
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	none
Participants	4a	Eligibility criteria for participants	7
	4b	Settings and locations where the data were collected	7
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	8, 9
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	8
	6b	Any changes to trial outcomes after the trial commenced, with reasons	none
Sample size	7a	How sample size was determined	7
	7b	When applicable, explanation of any interim analyses and stopping guidelines	not applicable
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	9
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	9
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	9, 10
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	9, 10

Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	9
	11b	If relevant, description of the similarity of interventions	9
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	11, 12
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	11, 12
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	13, 14
	13b	For each group, losses and exclusions after randomisation, together with reasons	13, 14
Recruitment	14a	Dates defining the periods of recruitment and follow-up	7
	14b	Why the trial ended or was stopped	not applicable
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	14
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	13, 14
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	16, 17, 18
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	not applicable
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	18, 19
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	not applicable
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	23
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	20, 24
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	21, 22, 23
Other information			
Registration	23	Registration number and name of trial registry	3
Protocol	24	Where the full trial protocol can be accessed, if available	7
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	25

[1]From CONSORT Group. For more information, visit: www.consort-statement.org.

Supplementary Table S2. Sensory evaluation of wheat germ-enriched bread versus control bread[1]

	Wheat germ-enriched bread	Control bread
Flavour[2]	4.70 ± 1.53	4.76 ± 1.50
Texture[2]	4.12 ± 1.83	4.58 ± 1.71
Global impression[3]	5.73 ± 2.08	6.12 ± 2.27

[1]All values are mean differences ± SD, n = 33. There were no significant differences between the groups based on the Wilcoxon signed ranks test.
[2]1 - Very much disliked, 2 - much disliked, 3 - disliked, 4 - neither liked nor disliked, 5 - liked, 6 - liked a lot, 7 - very much liked.
[3]1 - dislike extremely, 2 - dislike very much, 3 - dislike moderately, 4 - dislike slightly, 5 - neither like nor dislike, 6 - like slightly, 7 - like moderately, 8 - like very much and 9 liked extremely.

BMJ Open

Does intake of bread supplemented with wheat germ have a preventive role on cardiovascular disease risk markers in healthy volunteers? A randomised, controlled, crossover trial.

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Primary Subject Heading:	Nutrition and metabolism
Secondary Subject Heading:	Public health
Keywords:	wheat germ, lipid profile, glucose profile, randomised controlled trial, cardiovascular risk

RESEARCH

Does intake of bread supplemented with wheat germ have a preventive role on cardiovascular disease risk markers in healthy volunteers? A randomised, controlled, crossover trial.

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Keywords: wheat germ; lipid profile; glucose profile; randomised controlled trial; cardiovascular risk.

Word count: **4131**

ABSTRACT

Objective: Intake of whole grains is associated with a reduced risk of cardiovascular disease (CVD). This evidence is also strong for bran alone, but findings about germ are conflicting. Our aim was to elucidate the role of germ in primary prevention of cardiovascular events and therefore, a staple food was selected for 6 g of germ supplementation. This corresponds to 6-fold increase in the global mean consumption of germ, while preserves the sensory proprieties of refined bread which is crucial for consumer's acceptance.

Design: Randomised, double-blinded, crossover, controlled clinical trial with 15-week follow-up comprising a 2-week run-in, two intervention periods of 4 weeks/each and a 5-week washout period.

Setting: A single centre in the north of Portugal.

Participants: 55 eligible healthy adults (mean age of 34 years and BMI between 19 and 38 kg/m^2) were randomly assigned.

Interventions: The study consisted of two intervention periods including daily intake of refined wheat bread enriched with 6 g of wheat germ and control (non-enriched bread).

Outcomes: Changes in fasting cholesterol and triglycerides, fasting and postprandial glucose, insulin sensitivity and CRP.

Results: We observed no significant effect of daily intake of wheat germ on cholesterol and triglycerides levels, on postprandial glucose response, and on insulin sensitivity. Incremental area under curve (IAUC) glucose and homeostasis model assessment for insulin resistance (HOMA-IR) did not change, suggesting that 6 g of wheat germ have no effect on glucose metabolism. No effect was also observed in the subgroup of participants who complied with the protocol (n = 47).

Conclusions: The absence of alterations on lipid and glucose profiles suggests that germ up to 6 g/day, may have no preventive effect on CVD risk. However, it is important to investigate other food vehicles that can accommodate higher doses of wheat germ, in future studies.

Trial registration number: NCT02405507.

Strengths and limitations of this study:

- This study followed the best practices for designing, conducting and reporting clinical trials to support health claims on food products, namely random allocation, double blinding, reporting methods to measure and maximise compliance.
- We used validated outcomes which are considered beneficial physiological effects for human health.
- To the best of our knowledge, this is the largest study to assess the impact of germ intake in human subjects.
- Although compliance was high, it is uncertain whether this is due to over-reporting, since there is no biomarker for wheat germ intake.
- A longer intervention period would be desired for evaluating an effect on lipoprotein cholesterol, nonetheless could have a major impact on loss to follow-up in this crossover study.

INTRODUCTION

Cardiovascular diseases (CVD) remain the leading cause of mortality in the world, having accounted for 15 million of deaths in 2015.[1] Risk factors for CVD are well-identified and they include smoking, type 2 diabetes or high glucose levels, hypertension and elevated cholesterol levels.[2] Diet is therefore an important modifiable risk factor for CVD and, within the diet, some dietary components may have an important preventive role.[3-5] In this regard, cereal whole grains are a promising protective measure due to emerging evidence of an inverse association between their intake and CVD risk as shown by recent systematic reviews and meta-analyses.[5-8] The role of whole grains in reducing CVD risk is broader: 1) by improving glucose metabolism through better postprandial glucose and insulin responses;[9] and 2) by reductions in plasma cholesterol levels.[10] The health benefits associated with whole grains intake seems to be mediated by their high content in plant-derived redox-active compounds that may activate anti-oxidant pathways and thereby have anti-inflammatory proprieties.[11]

Cereal whole grains distinguish from refined grains by the presence of bran and germ fractions. These two fractions accumulate higher amounts of protective bioactive compounds, such as fibres, micronutrients, vitamins and phytochemicals. CVD prevention has been associated with bran intake,[7, 12-15] but findings about germ are conflicting.[7, 16] Recent systematic reviews addressing prospective studies reported no inverse association between germ intake and CVD risk, in contrast with prior clinical trials involving high-risk groups. But these two types of studies report different amounts of germ intake. The meta-analyses of the prospective studies reported a low germ intake

(1g/day average ranging from 0.2 to 2.9 g/day),[17-19] while the intervention studies used a daily supplementation of 20 or 30 g/day during 4-week period. Specifically, these clinical trials showed that intake of raw wheat germ can reduce cholesterol and triglycerides in rats[20-22] and also in hypercholesterolemic and hypertriglyceridemic humans.[23, 24]

Dietary guidelines around the world recommend 85 g daily intake of whole grains,[25] which contains nearly 2.6 g of germ. However, their daily consumption is far below the recommendations and consumers prefer highly refined products.[26-28] In fact, supplementation with whole-grain ingredients is therefore an elegant way to overcome consumer's preferences while contributes for public health, as long as these ingredients are indeed beneficial. Clarification of the physiological effects of germ is needed. However, fortification of food products with germ is challenging because germ becomes rancid very rapidly due to high content in unsaturated lipids together with lipases and lipoxygenases,[29-31] and it also negatively affects the sensory properties of the final food product.[32] Thus, in order to address these specificities, germ stabilization[33] is necessary immediately after milling to inhibit enzymatic rancidity, while the percentage of germ in the final product should be tested whether long-term consumer acceptance is desired.

In this context, we designed a randomised, double-blinded, crossover, controlled clinical trial targeting the general population, in order to evaluate the physiological and metabolic effects of germ intake in a dose higher than the amount reported in the previous prospective studies, wherein no preventive CVD effect was demonstrated. The

impact on CVD metabolic risk factors of daily consumption of 100 g of wheat white-bread enriched with 6 g of wheat germ during 4-week, is here presented.

METHODS

Participants

Fifty-five healthy volunteer subjects were recruited from the Porto metropolitan area in northern Portugal through public advertisements in the University and Faculty websites, and in online newspapers. The detailed study protocol has been previously described.[34] Briefly, volunteers were invited to visit our Research Unit (CINTESIS) for a physical exam and a brief questionnaire about their medical history and background diet in order to check their eligibility to participate in the study. Inclusion criteria included subjects age 18 to 60 years old, non-diabetic, and non-smoker. The exclusion criteria included the use of medication / dietary supplements, potentially interfering with this trial, not willing to avoid prebiotics and probiotics for the duration of the study, and change of dietary habits within the 4-week prior to screening (for instance, to start a diet high in fibre). In the protocol manuscript, we described sample size calculations. Briefly, we calculated the sample size for each individually primary outcome, taking into account the difference in mean change from baseline and the respective standard deviation. The sample size of the study was determined by the outcome that required the highest number of participants. Accordingly, 40 participants were required to allow for an 80% power and 95% confidence level.[34]

The study protocol was approved by the Health Ethics Committee of São João Hospital Centre and the Ethics Committee of the Faculty of Medicine of the University of Porto, and all participants provided written informed consent. The clinical trial was conducted from June 2015 to October 2016, in accordance with ethical principles of the Declaration of Helsinki, international law and Good Clinical Practice guidelines. This

study is registered in ClinicalTrials.gov database, reference NCT02405507, and followed the CONSORT reporting guidelines (online supplementary table S1).

Patient and Public Involvement

Before recruitment, volunteers that did not participate in the study were invited to be involved in the development of the bread formula used in this trial. They had no role in setting the research question, the outcome measures, the design, or implementation of the study. We included inputs from the participants of the study, namely any burden associated with the procedures, throughout the follow-up in order to optimise their involvement and compliance. Participants were involved in the sensory evaluation of the study breads, as well as in the recruitment process by encouraging others to participate. Upon publication, participants will be informed of the results of this study through direct email.

Study design and intervention

Our study was a 15-week, randomised, double-blinded, crossover, controlled clinical trial. The trial comprised four stages: a run-in period (2-week), two crossover intervention periods (4-week each), and a washout period between interventions (5-week). At the end of each stage, blood samples for measurement of plasma cholesterol (total, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL)), triglycerides, C-reactive protein (CRP), and postprandial glucose were collected from each study participant as primary outcomes. Blood samples were also collected for measurement of fasting glucose and insulin as secondary outcomes (online supplementary figure S1). Primary and secondary outcomes related with gastrointestinal discomfort were also evaluated in this trial, however they will be reported later.

Participants were instructed not to change their physical activity levels, maintain their dietary habits and do not consume any food or dietary product supplemented with germ during the study. Compliance to the study protocol (daily consumption of bread) was monitored through daily self-reported questionnaire, since there is no biomarker for wheat germ intake. Participants were randomly assigned into two intervention groups (ratio 1:1) using a computer-generated allocation sequence by a statistician not involved in recruitment and intervention delivery. The intervention arm comprised the daily consumption of wheat bread (100 g) supplemented with wheat germ (6 g), whereas the control arm involved the daily intake of wheat bread (100 g) without any supplementation. The bread provided to participants replaced their usual bread intake during meals, namely during breakfast or afternoon snack.

The participants and the research team were blinded to the study breads (intervention/control). In this regard, we previously tested the best formula that masked wheat germ supplementation, in terms of bread texture, volume and flavour. Moreover, the bread was delivered to each participant in opaque bags with a label code (A/B); only the outsourced company responsible for bread production (Padaria Ribeiro Lda., Portugal) knew the correspondence code. The unblinding was performed after the statistical analysis had been completed.

Bread formulation

Control breads were prepared by mixing 6 kg refined wheat flour (Germen S.A., Portugal) with 3.3 L water, 97 g of salt, 60 g of bread improver mix (Germen, S.A., Portugal), and 180 g of baker's yeast. The mixture then fermented for 15 min at room

temperature. Dough was divided and molded into pieces (123 g each), and then placed in a fermentation chamber with 80% relative humidity for 60 minutes at 30ºC. Finally, baking was done at 190 °C during 20 min. Preparation of intervention bread was similar to control bread; 480 g of refined wheat flour was replaced by raw wheat germ (Germen S.A., Portugal) only.

Nutrient composition of control and intervention breads was analysed by Silliker Portugal, S.A. (Mérieux Nutrisciences Corporation).

Blood sampling and analysis

The outcomes variables were measured on study participants under 12 hours overnight fasting conditions, at the end of 1) run-in, 2) first intervention, 3) washout and 4) second intervention. Accordingly, a venous blood sample was collected by venipuncture into serum separator tubes (BD Vacutainer SST II Advance, Becton, Dickinson and Company). For measuring glycated haemoglobin (HbA1c), blood was collected into tubes containing K_2EDTA (BD Vacutainer; Becton, Dickinson and Company). All venous blood samples were centrifuged at 1377 × g for 10 min at room temperature (CompactStar CS4; VWR), within 30 minutes after collection. A serum aliquot was immediately stored in a special cool transport container (at < -10°C) for insulin quantification. All biological samples were shipped to an outsourced certified medical laboratory (Clínica Laboratorial de Guimarães, S.A., Portugal) under refrigerated conditions. Analysis was performed within 24 hours after collection. Fasting glucose, total cholesterol and triglycerides were measured using specific enzymatic colorimetric methods, whereas LDL and HDL cholesterol were quantified by the elimination/catalase method. Serum CRP concentrations were measured by latex-

enhanced immunoturbidimetric assay. These biochemical quantifications were done with the automatic analyser ADVIA 1800 (Siemens Healthcare Diagnostics). Insulin was determined by chemiluminescent microparticle immunoassay method using the automatic analyser Architect i2000 (Abbott Laboratories). The insulin resistance was estimated using the homeostasis model assessment for insulin resistance (HOMA-IR):[35, 36]

(fasting insulin (μU/mL) × [fasting glucose (mg/dL) ÷ 18.01]) / 22.5

Regarding the postprandial glucose, four capillary blood samples were obtained by finger prick sampling using disposable lancet devices (Glucocard MX; Arkray) and a glucose meter. Postprandial glycaemia was measured in the fasting state (0) and at 30, 60 and 120 minutes after intervention or control bread intake. The postprandial glucose response was expressed as the incremental area under curve (IAUC), by using the trapezoidal rule ignoring the area below the fasting baseline, as previously described.[37]

Statistical Analysis

Statistical analysis was performed using SPSS version 23 software (SPSS Inc., Chicago, IL, USA). Data from all participants who were randomly assigned and completed an initial assessment were included in the intention-to-treat statistical analysis. Additionally, an analysis was also performed including the participants who adhered to the study protocol only, namely those that completed the outcome measures and complied with the daily bread intake (assessed by daily questionnaire). Numerical data are expressed as means ± SD, and treatment effects with 95% CI. Statistical significance was set at a 2-sided P value of 0.05. However, we also decided to perform an

adjustment for multiple comparison because of the multiple primary outcomes analysed; thus the type 1 error and significance level 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).[38]

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. A linear mixed model for repeated measures, with compound symmetry as the 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 variables. In order to account for between subject variability and to adjust for any nonspecific 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 estimated intervention effect for each sequence and in these cases, we only used the first period of the crossover trial in the analysis, following Pocock's recommendations.[39]

RESULTS

Baseline characteristics

Of 68 subjects screened to assess eligibility to participate in the study protocol, 55 were randomly assigned: 27 to the intervention-control sequence and 28 to the control-intervention sequence. Fifty-two participants completed an initial assessment and were included in the statistical analysis (figure 1). Eight individuals dropped out: three did not wish to continue the study for unspecified reasons, three declined to participate due to personal reasons (see figure 1), and the last two because of unrelated illness (gastroparesis and pneumonia). Study participants were healthy men and women, with a mean age of 34 years (range: 18–59 years), and BMI (in kg/m^2) between 19 and 38 (34 normal weight, 14 overweight and 4 obese). All participants had normal fasting glucose and normal glucose tolerance. At baseline, forty-two had normal fasting total plasma cholesterol (< 200 mg/dL), 8 had borderline-high fasting total plasma cholesterol (200-239 mg/dL) and 2 had high fasting total plasma cholesterol (> 240 mg/dL). Fifty participants had normal fasting total plasma triglycerides (< 150 mg/dL) and two had high fasting total plasma triglycerides (200-499 mg/dL). Test results for haemoglobin and for liver function showed no evidence of ill health. Baseline characteristics of the 52 study participants that were included in the intention-to-treat statistical analysis are listed in table 1.

Table 1 Baseline characteristics of study participants included in the analysis[1]

Characteristics	*N*	Value
Sex, n		
Male		16
Female		36
Age, years	*52*	33.67 ± 11.69
Body weight, kg	*52*	66.69 ± 11.84
BMI, kg/m^2	*52*	23.98 ± 3.98
Total cholesterol, mg/dL	*52*	173.73 ± 32.17
HDL cholesterol, mg/dL	*52*	60.71 ± 15.04
LDL cholesterol, mg/dL	*52*	96.15 ± 26.33
Triglycerides, mg/dL	*52*	84.40 ± 56.42
Glucose, mg/dL	*52*	83.87 ± 6.44
Insulin, µU/mL	*50*	7.15 ± 3.56
HOMA-IR	*50*	1.49 ± 0.79
HbA1c, %	*51*	5.17 ± 0.25
IAUC glucose, mg.min/dL	*50*	3322.24 ± 2086.88
CRP, mg/dL	*52*	0.12 ± 0.18

[1]Mean ± SD. CRP, C-reactive protein; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; IAUC, incremental area under the curve: LDL, low-density lipoprotein.

Participant compliance

Daily questionnaires revealed good compliance to the study protocol. The daily consumption of bread was assessed and the average compliance was 92.1% ± 9.3 and did not vary depending of bread type. Intervention and control arm had the same percentage of compliance: 92.2% ± 11.1 and 92.0% ± 10.0, respectively (P = 0.920). None of the participants reported adverse effects.

Composition of intervention and control breads

The content of protein, and dietary fibre was higher in the wheat germ-enriched bread (9.6% and 7.5%, respectively), whereas control bread had a higher content of

carbohydrate and starch (5.5% and 6.0%, respectively). The content of fat and energy are comparable in both breads. As expected, the quantity of total phytosterols and alpha-linolenic acid was 73.3% and 41.7% higher in the intervention bread, respectively (table 2).

Table 2 Chemical composition of wheat germ-enriched and control breads per 100 g product weight

	Wheat germ-enriched bread	Control bread
Energy, kJ	1154.7	1182.8
Protein, g	9.7	8.8
Fat, g	5.4	5.5
Carbohydrate, g	44.4	47.0
Dietary fibre, g	4.3	4.0
Total sugar, g	3.4	3.4
Starch, g	40.8	43.4
Total phytosterols, mg	52.0	30.0
Moisture, g	34.5	32.9
Ash, g	1.7	1.9
Fatty acid, % of total fatty acids		
14:0	0.2	0.0
16:0	18.9	20.7
16:1	3.3	3.7
18:0	2.5	4.6
18:1n–9	14.9	16.0
18:1n–7	1.2	1.1
18:2n-6	52.9	49.8
20:0	0.2	0.0
18:3n–3	5.1	3.6
20:1n–9	0.8	0.5

Blood lipids

There was no statistically significant difference between wheat germ-enriched and control breads, after 4-week intake, for cholesterol (total, LDL and HDL) and serum triglycerides (table 3).

Table 3 The effect of 4-week wheat germ-enriched bread versus control bread on cardiovascular, inflammatory and metabolic risk markers

	Wheat germ-enriched bread					Control bread					Effect of wheat germ[2]		
	N	Baseline[1]	*N*	Post-Intervention[1]	*P* value within group	*N*	Baseline[1]	*N*	Post-Intervention[1]	*P* value within group	Effect	(95% CI)	*P* value between group
Total cholesterol, mg/dL	*52*	175.17 ± 31.82	*50*	172.98 ± 32.4	0.147	*48*	174.00 ± 32.17	*48*	170.00 ± 32.79	0.100	1.20	(-4.54, 6.95)	0.675
HDL cholesterol, mg/dL	*52*	60.92 ± 15.13	*50*	59.38 ± 16.07	0.010	*48*	60.65 ± 15.21	*48*	58.38 ± 14.77	0.012	0.14	(-1.83, 2.12)	0.886
LDL cholesterol, mg/dL	*52*	97.31 ± 26.07	*50*	96.92 ± 26.22	0.853	*48*	97.23 ± 25.79	*48*	95.21 ± 27.0	0.356	1.74	(-3.80, 7.28)	0.530
Triglycerides, mg/dL	*52*	85.13 ± 54.67	*50*	83.8 ± 42.62	0.565	*48*	81.10 ± 49.96	*48*	82.48 ± 49.0	0.763	-3.60	(-16.28, 9.08)	0.571
Glucose, mg/dL[3]	*27*	84.22 ± 6.82	*26*	81.27 ± 7.32	0.011	*25*	83.48 ± 6.13	*25*	80.68 ± 7.0	0.012	-0.24	(-3.28, 2.81)	0.876[5]
Insulin, µU/mL	*51*	7.00 ± 3.08	*50*	7.04 ± 2.83	0.729	*47*	6.84 ± 3.45	*48*	7.32 ± 3.75	0.524	-0.39	(-2.03, 1.24)	0.629
HOMA-IR	*51*	1.46 ± 0.68	*50*	1.44 ± 0.67	0.973	*47*	1.43 ± 0.78	*48*	1.54 ± 0.88	0.541	0.12	(-0.49, 0.26)	0.529
HbA1c, %[4]	*27*	5.18 ± 0.31	*26*	5.26 ± 0.30	0.004	*24*	5.16 ± 0.15	*24*	5.21 ± 0.16	0.002	0.027	(-0.03, 0.09)	0.380[5]
IAUC glucose, mg.min/dL	*51*	3399.58 ± 2095.88	*48*	3017.74 ± 1959.09	0.166	*46*	3379.91 ± 2149.45	*44*	3334.59 ± 2101.47	0.812	-221.13	(-901.34, 459.08)	0.524
CRP, mg/dL	*52*	0.13 ± 0.21	*50*	0.18 ± 0.42	0.374	*48*	0.12 ± 0.17	*48*	0.25 ± 0.57	0.093	-0.07	(-0.26, 0.13)	0.481

[1]Mean ± SD. CRP, C-reactive protein; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; IAUC, incremental area under the curve: LDL, low-density lipoprotein.

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

[3]The intervention-sequence interaction was significant (-5.73 [-10.00, -1.46], P = 0.010), the intervention effect was 2.07 in the first sequence and -3.66 in the second sequence.

[4]The intervention-sequence interaction was significant (0.15 [0.07, 0.23]; P < 0.001), the intervention effect was -0.04 in the first sequence and 0.12 in the second sequence.

[5]Only the first period was used in the analysis due the existence of carryover effect. Differences in changes within and between groups were compared by using the paired and unpaired t-test, respectively.

No difference was also observed for those participants who completed the outcome measures and complied with the daily bread intake between wheat germ-enriched bread and control bread (n = 47; total cholesterol: P = 0.797, HDL cholesterol: P = 0.996, LDL cholesterol: P = 0.665, and triglycerides: P = 0.762). The same result was obtained when participants with normal fasting total plasma cholesterol and triglycerides are analysed (n = 41; total cholesterol: P = 0.981, HDL cholesterol: P = 0.413, LDL cholesterol: P = 0.833, and triglycerides: P = 0.718). The results were not statistically different when participants with borderline or high fasting total plasma cholesterol were included (n = 10; total cholesterol: P = 0.432, HDL cholesterol: P = 0.170, LDL cholesterol: P = 0.781, and triglycerides: P = 0.508). Statistical analysis of blood lipid outcomes showed no interaction between intervention and sequence, potentially excluding a carryover effect.

Glucose metabolism and C-reactive protein

There were no significant changes in postprandial glucose peak values (at 30 minutes) after 4-week consumption of wheat germ-enriched bread (-5.13 ± 3.03 mg/dL; P = 0.097) or control bread (2.35 ± 3.73 mg/dL; P = 0.531), and neither between interventions (P = 0.182). Blood glucose response curves for 2 hours after the intake of intervention and control breads are shown in figure 2.

Comparing with control, wheat germ-enriched bread had no significant effect on IAUC glucose, fasting insulin or HOMA-IR (table 3), suggesting that 6 g of wheat germ did not improve postprandial glycaemia or glucose metabolism. No differences in CRP were observed between wheat germ-enriched and control breads. CRP and glucose results are not statistically different when only participants who adhered to the study

protocol were analysed (n = 47; IAUC: P = 0.597, Insulin: P = 0.709, HOMA-IR: P = 0.597, and CRP: P = 0.959).

The intervention-sequence interaction was significant for fasting glucose (-5.73 [-10.00; -1.46], P=0.010) and for HbA1c (0.15 [0.07; 0.23], P<0.001), revealing the existence of a carryover effect in these two outcomes. Therefore, the crossover design was not considered and only the first period was used in the analysis, following Pocock's recommendations.[39] Nevertheless, no differences in fasting glucose or HbA1c between wheat germ-enriched and control breads were observed. HbA1c reflects the average blood glucose level in previous 2 to 3 months.[40]

DISCUSSION

In the present study, we evaluated the health effects of wheat germ intake in 52 healthy individuals. Blood lipids (cholesterol and triglycerides), fasting and postprandial glucose, CRP and insulin were evaluated during 15-week in a randomised crossover design. The results presented show that the intake of 6 g/day of wheat germ for 4-week, has no effect on cholesterol and triglycerides levels, on glucose metabolism, namely in IAUC glucose, and on insulin sensitivity (HOMA-IR). Thus, our findings strongly suggest that wheat germ by itself has no impact on the cardiovascular risk factors evaluated and thus, a beneficial physiological effect of wheat germ up to 6 g/day, by decreasing the risk of CVD in normal adults, is unlikely. Six grams of wheat germ intake per day corresponds to 2.4-fold increase in the germ present in the daily recommendation for whole grains intake and to a 6-fold increase in the global mean consumption of germ.

In this study, we elucidated the role of germ in the primary prevention of cardiovascular events and for this, we tested and developed a wheat germ-enriched bread without compromising the nutritional status (table 2), texture and flavour (online supplementary table S2). Importantly, besides allowing blinding and the analysis of the effect of added germ individually, this no-difference is crucial for consumer's acceptance if this is envisioned as a long-term goal. Bread was chosen as vehicle for germ intake because it is a staple food, major contributor to carbohydrates intake, whereas the wheat is globally a staple grain. Up until now, the beneficial effect of wheat germ in lowering blood cholesterol and triglycerides was only demonstrated when used as a diet supplementation with 20 g[24] or 30 g/day[23] during 4-week periods. Incorporation of

such high proportions of wheat germ in food products, without impairing their nutritional profiles, is difficult since germ changes their sensory proprieties. Our study is the first interventional study evaluating the effect of wheat germ in higher doses than the global mean consumption of germ and higher than the germ present in the recommended doses of whole grains. This intervention is a randomised, double-blinded, controlled clinical trial that uses a functional food product as supplementation vehicle, and not a dietary supplement. Our study followed the best practices for designing, conducting and reporting clinical trials to support health claims on food products, namely random allocation, double blinding, reporting methods to measure and maximise compliance. The strength of the current study also includes the use of validated outcomes which are considered to be beneficial physiological effects for human health.[34] Finally, to our knowledge, this is the largest study to assess the impact of germ intake in human subjects and importantly, targets the general population instead of a high-risk group. The limitations of this study include the duration of the intervention period. Although 4-week is considered the minimal intervention for evaluating an effect on lipoprotein cholesterol, 8-week would be more desirable; however, such intervention period in a crossover study could have a major impact on loss to follow-up. Secondly, the absence of a biomarker specific for germ intake is also a limitation; adherence was monitored through daily self-reported questionnaire and, though compliance with the study protocol was optimal, it is uncertain whether there was over-reporting. Thirdly, the presence of carryover effects in the fasting glucose and HbA1c reduced the statistical power for these two outcomes, since the crossover design was not considered and only the first period was analysed. Lastly, we decided not to collect information about diet and physical activity levels during the study in order to

avoid changes in general participants' lifestyle and dietary patterns; and this could be seen as a limitation.

In order to correctly inform consumers and food and nutrition policy makers about the benefits of supplementing food products with whole grain ingredients, there is an urgent need for clarifying the differences (whether any) between whole-grains and bran or germ individually. First, adding bran or germ individually may not have identical physiologic benefits as the whole grain; and secondly, these two fractions alone may have distinct physiological effects on cardiovascular-health promotion when compared with the whole-grain.[41] Germ of wheat was chosen due to its potential to reduce blood cholesterol and triglycerides concentrations, as suggested in previous studies.[23, 24] The proposed mechanisms for lowering plasma cholesterol include a) the inhibition of pancreatic lipase activity by soluble proteins present on wheat germ;[42] b) the reduction in triglyceride lipolysis;[43] and c) reduction in cholesterol absorption by the endogenous wheat germ phytosterols.[44] However, the existing evidence regarding the lipid-lowering properties of wheat germ from previous studies is controversial. First, reduction of total cholesterol and LDL was only evaluated in hypercholesterolemic individuals after diet supplementation with 20 g/day for 4-week period. Second, daily ingestion of 30 g wheat germ supplement for 4-week markedly decreases (-39%) plasma triglycerides in hypertriglyceridemic individuals, whereas no reduction was observed in the normotriglyceridemic subgroup.[23]

In our study, we enrolled a moderately large and heterogeneous sample of participants which is representative of general population, with normal, borderline-high and high fasting total plasma cholesterol and triglycerides. The statistical power of this study is

higher than preceding studies and for that reason, smaller differences could be detected if they indeed existed. Thus, it is not plausible that the absence of any statistical significance on the wheat germ-induced changes is due to the lack of statistical power. However, the inexistence of wheat germ effects reported here does not necessarily disprove the potential cholesterol- and triglyceride-lowering effect described in previous studies. In particular, because in those studies, wheat germ was consumed in higher doses and by individuals at risk. In our opinion, a dose-response analysis for wheat germ intake and lipid profile should be investigated in the future, for general population and high-risk groups.

Effect of wheat germ in improving postprandial glycaemia or glucose metabolism was not demonstrated in our study, in accordance with previous studies. Earlier studies addressing the long-term impact of wheat germ on blood lipids of hypercholesterolemic individuals did not observe any significant variation on fasting glucose, insulin, fructosamine and HbA1c;[24] and one single study with six healthy participants showed that wheat germ had no effect on postprandial glucose and insulin concentrations.[45] Even so, we decided to investigate the impact of wheat germ on glucose metabolism because it was recently suggested that intake of alpha-linolenic acid has been associated with lower insulin resistance[46] and our wheat germ enriched-bread contains more 41.7% of alpha-linolenic than control bread (table 2).

In summary, our goal was to demonstrate the effect of a staple food supplemented with wheat germ in its maximal concentration without compromise its sensory properties. However, the intake of 6 g/day of wheat germ does not contribute to reduce the cardiovascular risk factors: plasma triglycerides, total cholesterol, LDL cholesterol, or

increase the HDL cholesterol levels, or even improve glycaemic control, in a generally healthy normal population. In order to justify an enrichment of food products with wheat germ as a public health approach to prevent CVD, the beneficial effects of wheat germ on human health should be investigated in other food vehicles that can accommodate higher doses of germ. Chemical and sensory proprieties of biscuits, noodles and cakes supplemented with increasing amounts of wheat germ (up to 30%) have been recently analysed and improved.[32, 47, 48] These technological advances let us to envisage that other food products functionalised with higher wheat germ content can be developed. Future studies following our clinical trial design are needed to elucidate if high amounts of daily intake of wheat germ are effective in reducing CVD risk.

Abbreviations CRP, C-reactive protein; CVD, cardiovascular disease; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; IAUC, incremental area under curve; LDL, low-density lipoprotein.

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Competing interests None declared**.**

Contributors The authors' responsibilities were as follows – AMR was partially responsible for study design, statistical analysis plan, carrying out the trial, manuscript writing and final revision. HP was partially responsible for study design, carrying out the trial, manuscript writing and final revision. CM collaborated in the study design, data collection and final revision of the manuscript. JAT collaborated in the study design and final revision of the manuscript. CC was responsible for the general coordination of the project, study design, manuscript writing and final revision. LFA was responsible for the general coordination of the project, study design, statistical analysis plan, manuscript writing and final revision.

Data sharing statement The full datasets generated during and/or analysed during the current study are not publicly available because the ethics committee only allowed the use of the data in the context of the present research project, however anonymised partial datasets or summaries of the data are available from the corresponding author on reasonable request.

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Figures:

Figure 1 Flow chart of participants through the study.

Figure 2 Mean ± SEM postprandial glucose concentrations in response to a 100 g wheat germ-enriched bread or control bread, at baseline (A), and after 4-week intervention (B). No significant effect in the incremental area under the curve (IAUC) was observed between wheat germ-enriched and control breads (P = 0.524).

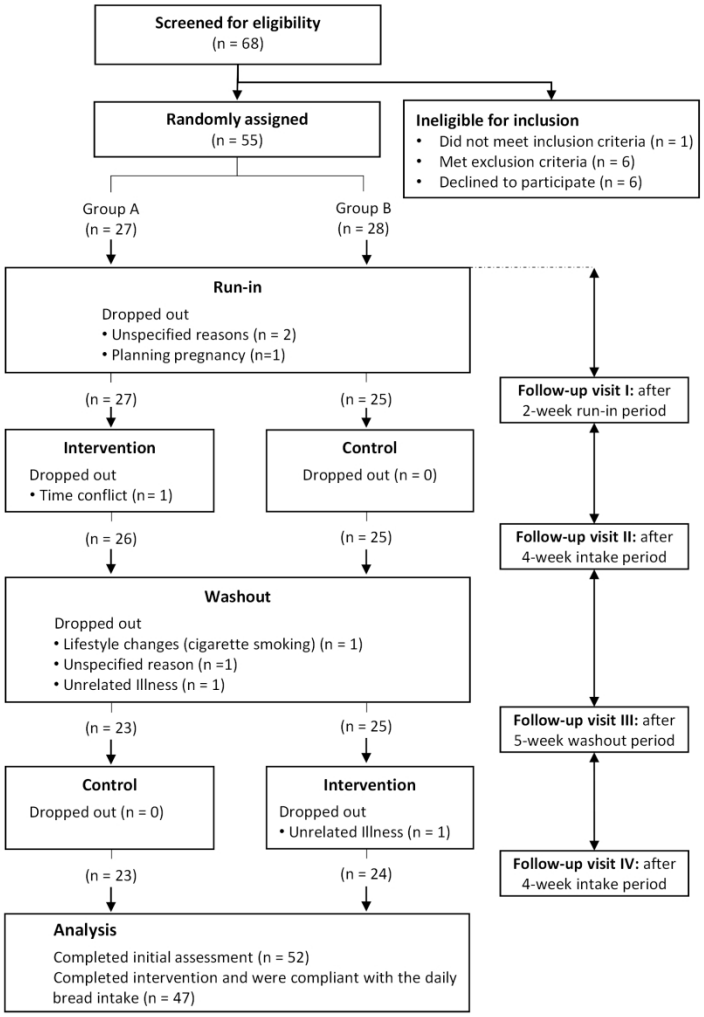

Figure 1 Flow chart of participants through the study.

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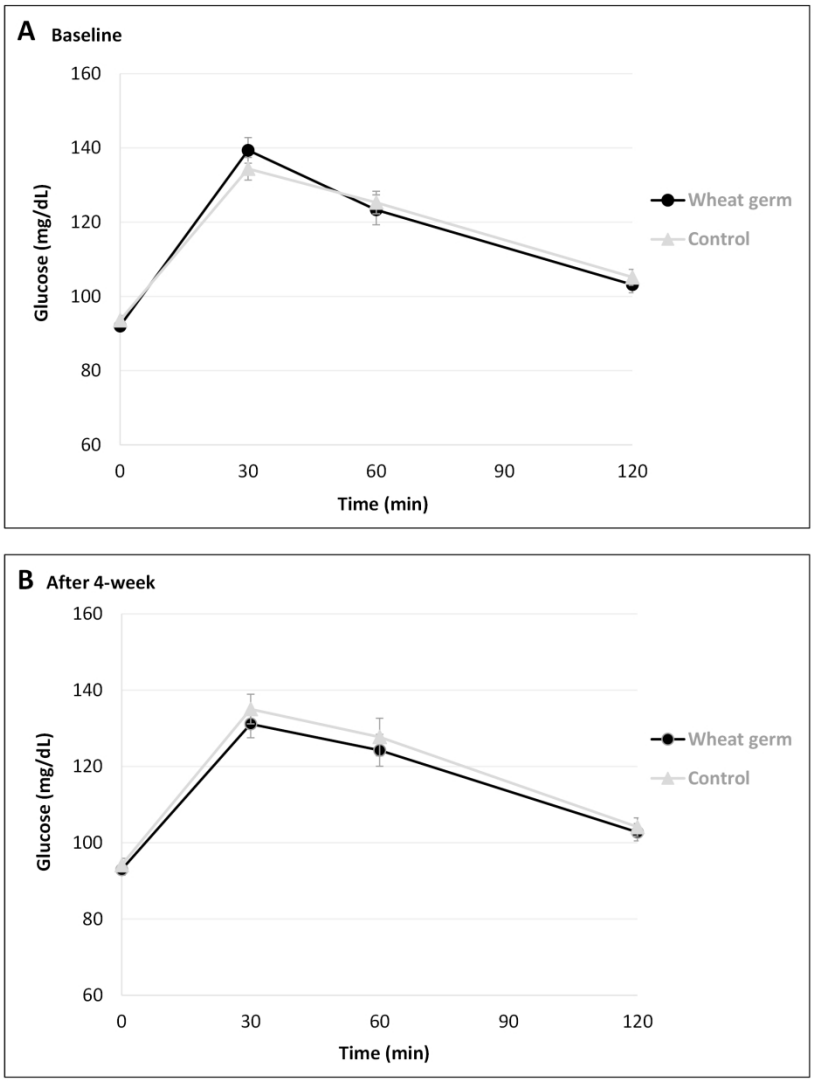

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199x250mm (300 x 300 DPI)

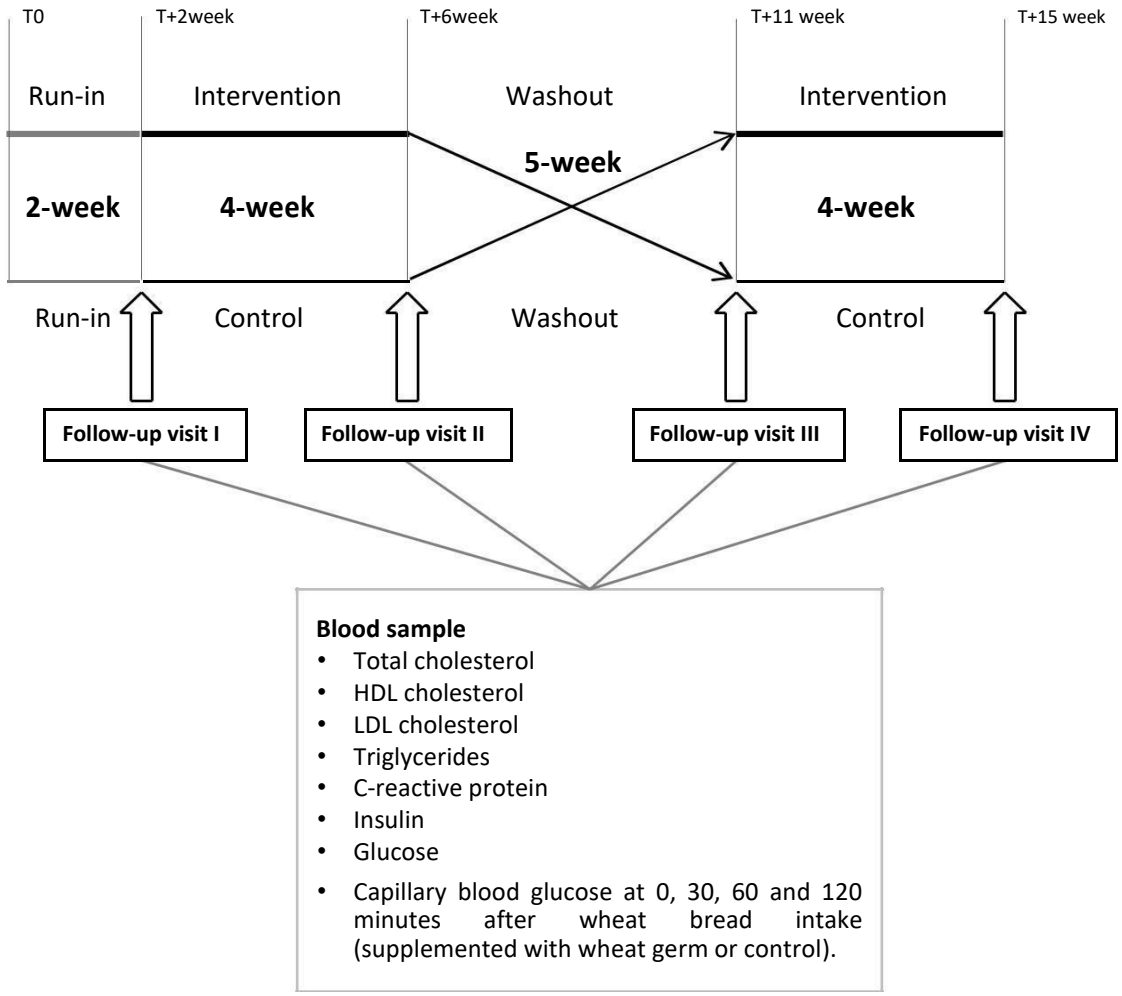
Supplementary Table S1. CONSORT 2010 checklist[1]

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	3, 4
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	5, 6, 7
	2b	Specific objectives or hypotheses	6, 7
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	8, 9
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	none
Participants	4a	Eligibility criteria for participants	8
	4b	Settings and locations where the data were collected	8
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	9, 10
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	9
	6b	Any changes to trial outcomes after the trial commenced, with reasons	none
Sample size	7a	How sample size was determined	8
	7b	When applicable, explanation of any interim analyses and stopping guidelines	not applicable
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	10
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	10
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	10
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	10

Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	10
	11b	If relevant, description of the similarity of interventions	10
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	12, 13
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	12, 13
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	14, 15
	13b	For each group, losses and exclusions after randomisation, together with reasons	14, 15
Recruitment	14a	Dates defining the periods of recruitment and follow-up	8
	14b	Why the trial ended or was stopped	not applicable
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	15
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	14
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	17, 18, 19
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	not applicable
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	19, 20
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	not applicable
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	22
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	21, 24, 25
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	22, 23, 24
Other information			
Registration	23	Registration number and name of trial registry	4
Protocol	24	Where the full trial protocol can be accessed, if available	8, 29
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	25

[1]From CONSORT Group. For more information, visit: www.consort-statement.org.

Supplementary Figure S1. Schedule of assessments



Supplementary Table S2. Sensory evaluation of wheat germ-enriched bread versus control bread[1]

	Wheat germ-enriched bread	Control bread
Flavour[2]	4.70 ± 1.53	4.76 ± 1.50
Texture[2]	4.12 ± 1.83	4.58 ± 1.71
Global impression[3]	5.73 ± 2.08	6.12 ± 2.27

[1] All values are mean differences ± SD, n = 33. There were no significant differences between groups based on the Wilcoxon signed ranks test.

[2] 1 - Very much disliked, 2 - much disliked, 3 - disliked, 4 - neither liked nor disliked, 5 - liked, 6 - liked a lot, 7 - very much liked.

[3] 1 - dislike extremely, 2 - dislike very much, 3 - dislike moderately, 4 - dislike slightly, 5 - neither like nor dislike, 6 - like slightly, 7 - like moderately, 8 - like very much and 9 liked extremely.