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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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1	RESEARCH
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3	Does intake of bread supplemented with wheat germ have a preventive role on
4	cardiovascular disease risk? A randomised, placebo-controlled, crossover trial.
5	
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22	Keywords: wheat germ; lipid profile; glucose profile; randomised controlled trial;
23	cardiovascular risk.
24	
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26 ABSTRACT

28	Objective: Our aim was elucidating the role of germ in primary prevention of
29	cardiovascular events and we selected a staple food for supplementation. Accordingly,
30	the effects of daily consumption of refined bread having 6 g of wheat germ were
31	evaluated in fasting cholesterol and triglycerides, fasting and postprandial glucose,
32	insulin sensitivity and CRP. This germ supplementation corresponds to 6-fold increase
33	in the global mean consumption of germ and, importantly, it preserves the sensory
34	proprieties of refined bread which is crucial for consumer's acceptance.
35	Design: Fifty-five healthy adults were recruited in a randomised, double-blinded
36	crossover trial with 15-week follow-up, comprising a 2-week run-in, two intervention
37	periods of 4 weeks/each and a 5-week washout period. Fasting venous blood samples
38	were collected at the end of each stage. Postprandial glucose was measured at different
39	time points. The effect of daily intake of wheat germ-enriched bread was compared with
40	control bread.
41	Results: Study subjects had mean age of 34 y and BMI between 19 and 38 kg/m ² .
42	Among the 52 subjects, 15% and 4% of them were borderline-high and high fasting
43	total plasma cholesterol, respectively. Two participants had high fasting total plasma
44	triglycerides. We observed that daily intake of 6 g wheat germ had no significant effect
45	on cholesterol and triglycerides levels, on postprandial glucose response, and on insulin
46	sensitivity. No effect was also observed for the subgroups of participants who
47	completed the outcome measures and complied with the daily bread intake ($n = 47$).
48	Conclusions: The absence of alterations on lipid and glucose profiles suggests that
49	germ up to 6 g/day, may have no preventive effect on CVD risk. However, it is

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3 of 36		BMJ Open
	50 51	important to investigate other food vehicles that can accommodate higher doses of wheat germ, in future studies.
	52	Trial registration number: NCT02405507.
	53	
	54	Strengths and limitations of this study:
	55	 This study followed the best practices for designing, conducting and reporting
	56	clinical trials to support health claims on food products, namely random
	57	allocation, double blinding, reporting methods to measure and maximise
	58	compliance.
	59	 We used validated outcomes which are considered beneficial physiological
	60	effects for human health.
	61	• To the best of our knowledge, this is the largest study to assess the impact of
	62	germ intake in human subjects.
	63	• Although compliance was high, it is uncertain whether this is due to over-
	64	reporting, since there is no biomarker for wheat germ intake.
	65	 A longer intervention period would be desired for evaluating an effect on
	66	lipoprotein cholesterol, nonetheless could have a major impact on loss to follow-
	67	up in this crossover study.
	68	
		3

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69 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

Page 5 of 36

BMJ Open

(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, placebocontrolled 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 BMJ Open: first published as 10.1136/bmjopen-2018-023662 on 17 January 2019. Downloaded from http://bmjopen.bmj.com/ on June 13, 2025 at Agence Bibliographique de Enseignement Superieur (ABES) . Protected by copyright, including for uses related to text and data mining, Al training, and similar technologies.

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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

134	Participants
135	Fifty-five healthy volunteer subjects were recruited from the Porto metropolitan area in
136	northern Portugal through public advertisements in the University and Faculty websites,
137	and in online newspapers. The detailed study protocol has been previously
138	described.[33] Briefly, volunteers were invited to visit our Research Unit (CINTESIS)
139	for a physical exam and a brief questionnaire about their medical history and
140	background diet in order to check their eligibility to participate in the study. Inclusion
141	criteria included subjects age 18 to 60 years old, non-diabetic, and non-smoker. The
142	exclusion criteria included the use of medication / dietary supplements, potentially
143	interfering with this trial, not willing to avoid prebiotics and probiotics for the duration
144	of the study, and change of dietary habits within the 4-week prior to screening (for
145	instance, to start a diet high in fibre). In the protocol manuscript, we described sample
146	size calculations; 40 participants were required to allow for an 80% power and 95%
147	confidence level.[33]
148	
149	The study protocol was approved by the Health Ethics Committee of São João Hospital
150	Centre and the Ethics Committee of the Faculty of Medicine of the University of Porto,
151	and all participants provided written informed consent. The clinical trial was conducted
152	from June 2015 to October 2016, in accordance with ethical principles of the
153	Declaration of Helsinki, international law and Good Clinical Practice guidelines. This
154	study is registered in ClinicalTrials.gov database, reference NCT02405507, and
155	followed the CONSORT reporting guidelines (online supplementary table S1).
156	
157	Participant and public involvement

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Page 8 of 36

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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.

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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 (5week). 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

183	wheat germ intake. Participants were randomly assigned into two intervention groups
184	(ratio 1:1) using a computer-generated allocation sequence by a statistician not involved
185	in recruitment and intervention delivery. The intervention arm comprised the daily
186	consumption of wheat bread (100 g) supplemented with wheat germ (6 g), whereas the
187	control arm involved the daily intake of wheat bread (100 g) without any
188	supplementation. The bread provided to participants replaced their usual bread intake
189	during meals, namely during breakfast or afternoon snack.
190	
191	The participants and the research team were blinded to the study breads
192	(intervention/control). In this regard, we previously tested the best formula that masked
193	wheat germ supplementation, in terms of bread texture, volume and flavour. Moreover,
194	the bread was delivered to each participant in opaque bags with a label code (A/B); only
195	the outsourced company responsible for bread production (Padaria Ribeiro Lda.,
196	Portugal) knew the correspondence code. The unblinding was performed after the
197	statistical analysis had been completed.
198	
199	Bread formulation
200	Control breads were prepared by mixing 6 kg refined wheat flour (Germen S.A.,
201	Portugal) with 3.3 L water, 97 g of salt, 60 g of bread improver mix (Germen, S.A.,
202	Portugal), and 180 g of baker's yeast. The mixture then fermented for 15 min at room
203	temperature. Dough was divided and molded into pieces (123 g each), and then placed
204	in a fermentation chamber with 80% relative humidity for 60 minutes at 30°C. Finally,
205	baking was done at 190 °C during 20 min. Preparation of intervention bread was similar
206	to control bread; 480 g of refined wheat flour was replaced by raw wheat germ (Germen

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208	
209	Nutrient composition of control and intervention breads was analysed by Silliker
210	Portugal, S.A. (Mérieux Nutrisciences Corporation).
211	
212	Blood sampling and analysis
213	The outcomes variables were measured on study participants under 12 hours overnight
214	fasting conditions, at the end of 1) run-in, 2) first intervention, 3) washout and 4) second
215	intervention. Accordingly, a venous blood sample was collected by venipuncture into
216	serum separator tubes (BD Vacutainer SST II Advance, Becton, Dickinson and
217	Company). For measuring glycated haemoglobin (HbA1c), blood was collected into
218	tubes containing K ₂ EDTA (BD Vacutainer; Becton, Dickinson and Company). All
219	venous blood samples were centrifuged at $1377 \times g$ for 10 min at room temperature
220	(CompactStar CS4; VWR), within 30 minutes after collection. A serum aliquot was
221	immediately stored in a special cool transport container (at $< -10^{\circ}$ C) for insulin
222	quantification. All biological samples were shipped to an outsourced certified medical
223	laboratory (Clínica Laboratorial de Guimarães, S.A., Portugal) under refrigerated
224	conditions. Analysis was performed within 24 hours after collection. Fasting glucose,
225	total cholesterol and triglycerides were measured using specific enzymatic colorimetric
226	methods, whereas LDL and HDL cholesterol were quantified by the
227	elimination/catalase method. Serum CRP concentrations were measured by latex-
228	enhanced immunoturbidimetric assay. These biochemical quantifications were done
229	with the automatic analyser ADVIA 1800 (Siemens Healthcare Diagnostics). Insulin
230	was determined by chemiluminescent microparticle immunoassay method using the
231	automatic analyser Architect i2000 (Abbott Laboratories). The insulin resistance was

232	estimated using the homeostasis model assessment for insulin resistance (HOMA-
233	IR):[34, 35]
234	(fasting insulin (μ U/mL) × [fasting glucose (mg/dL) ÷ 18.01]) / 22.5
235	
236	Regarding the postprandial glucose, four capillary blood samples were obtained by
237	finger prick sampling using disposable lancet devices (Glucocard MX; Arkray) and a
238	glucose meter. Postprandial glycaemia was measured in the fasting state (0) and at 30,
239	60 and 120 minutes after intervention or control bread intake. The postprandial glucose
240	response was expressed as the incremental area under curve (IAUC), by using the
241	trapezoidal rule ignoring the area below the fasting baseline, as previously
242	described.[36]
243	
244	Statistical Analysis
245	Statistical analysis was performed using SPSS version 23 software (SPSS Inc., Chicago,
246	IL, USA). Data from all participants who were randomly assigned and completed an
247	initial assessment were included in the intention-to-treat statistical analysis.
248	Additionally, an analysis was also performed including the participants who adhered to
249	the study protocol only, namely those that completed the outcome measures and
250	complied with the daily bread intake (assessed by daily questionnaire). Numerical data
251	are expressed as means \pm SD, and treatment effects with 95% CI. Statistical significance
252	was set at a 2-sided P value of 0.05. However, we also decided to perform an
253	adjustment for multiple comparison because of the multiple primary outcomes analysed;
254	thus the type 1 error and significance level associated with any individual variable
255	difference took into account all comparisons performed and were ruled significant after

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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

274 Baseline characteristics

275	Of 68 subjects screened to assess eligibility to participate in the study protocol, 55 were
276	randomly assigned, and 52 completed an initial assessment and were included in the
277	statistical analysis (figure 1). Eight individuals dropped out: three did not wish to
278	continue the study for unspecified reasons, three declined to participate due to personal
279	reasons (see figure 1), and the last two because of unrelated illness (gastroparesis and
280	pneumonia). Study participants were healthy men and women, with a mean age of 34
281	years (range: 18–59 years), and BMI (in kg/m ²) between 19 and 38 (34 normal weight,
282	14 overweight and 4 obese). All participants had normal fasting glucose and normal
283	glucose tolerance. At baseline, forty-two had normal fasting total plasma cholesterol (<
284	200 mg/dL), 8 had borderline-high fasting total plasma cholesterol (200-239 mg/dL)
285	and 2 had high fasting total plasma cholesterol (> 240 mg/dL). Fifty participants had
286	normal fasting total plasma triglycerides (< 150 mg/dL) and two had high fasting total
287	plasma triglycerides (200-499 mg/dL). Test results for haemoglobin and for liver
288	function showed no evidence of ill health. Baseline characteristics of the 52 study
289	participants that were included in the intention-to-treat statistical analysis are listed in
290	table 1.

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	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
3 4 5 6	¹ Mean ± SD. CRP, C-reactive protein; HbA1c, glyc. lipoprotein; HOMA-IR, homeostasis model assessmi incremental area under the curve: LDL, low-density	nent for	r insulin resistance; IAUC,
7	Participant compliance		
8	Daily questionnaires revealed good compliance	to the	e study protocol. The daily
9	consumption of bread was assessed and the ave	rage c	compliance was $92.1\% \pm 9.3$ and
0	did not vary depending of bread type. Intervention	ion an	d control arm had the same
1	percentage of compliance: $92.2\% \pm 11.1$ and 92	2.0% ±	= 10.0, respectively ($P = 0.920$).
2	None of the participants reported adverse effect	S.	
)3			
)4	Composition of intervention and control brea	ads	
)5	The content of protein, and dietary fibre was high	gher ir	n the wheat germ-enriched bread
)6	(9.6% and 7.5%, respectively), whereas control	bread	had a higher content of

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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 alphalinolenic acid was 73.3% and 41.7% higher in the intervention bread, respectively (table
2).

312 Table 2 Chemical composition of wheat germ-enriched and control breads per

313 100 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

315 Blood lipids

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316 There was no statistically significant difference between wheat germ-enriched and

317 control breads, after 4-week intake, for cholesterol (total, LDL and HDL) and serum

to beet eview only

318 triglycerides (table 3).

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

outcomes

		Wheat g	erm-e	enriched bread				Contr	ol bread		1	Effect of wheat gern	\mathbf{n}^2
) 2	Ν	Baseline ¹	Ν	Post-Intervention ¹	P value within group	Ν	Baseline ¹	Ν	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
$\begin{array}{r} 322 \\ 323 \\ 324 \end{array}^{1} Mean \pm SD. \\ resistance; IA \\ ^{2} Intervention \end{array}$	UC, in effect tion-se	ncremental area ur s were analysed us	nder t sing l	the curve: LDL, lo inear mixed mode	noglobin; w-density l for repea	lipop ted r	protein. neasures with com	npour	tein; HOMA-IR, ho nd symmetry as cov vention effect was 2	meostasis variance str	ructure.		lin

⁴The 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.

Page 18 of 36

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330	No difference was also observed for participants who completed the outcome measures
331	and complied with the daily bread intake ($n = 47$; total cholesterol: $P = 0.797$, HDL
332	cholesterol: $P = 0.996$, LDL cholesterol: $P = 0.665$, and triglycerides: $P = 0.762$). The
333	same result was obtained when participants with normal fasting total plasma cholesterol
334	and triglycerides are analysed (n = 41; total cholesterol: $P = 0.981$, HDL cholesterol: P
335	= 0.413, LDL cholesterol: $P = 0.833$, and triglycerides: $P = 0.718$). The results were not
336	statistically different when participants with borderline or high fasting total plasma
337	cholesterol were included (n = 10; total cholesterol: $P = 0.432$, HDL cholesterol: $P =$
338	0.170, LDL cholesterol: $P = 0.781$, and triglycerides: $P = 0.508$). Statistical analysis of
339	blood lipid outcomes showed no interaction between intervention and sequence,
340	potentially excluding a carryover effect.
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342	
	Glucose metabolism and C-reactive protein
343	Glucose metabolism and C-reactive protein There were no significant changes in postprandial glucose peak values (at 30 minutes)
343 344	
	There were no significant changes in postprandial glucose peak values (at 30 minutes)
344	There were no significant changes in postprandial glucose peak values (at 30 minutes) after 4-week consumption of wheat germ-enriched bread (-5.13 \pm 3.03 mg/dL; P =
344 345	There were no significant changes in postprandial glucose peak values (at 30 minutes) after 4-week consumption of wheat germ-enriched bread (-5.13 \pm 3.03 mg/dL; P = 0.097) or control bread (2.35 \pm 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
344 345 346	There were no significant changes in postprandial glucose peak values (at 30 minutes) after 4-week consumption of wheat germ-enriched bread (-5.13 \pm 3.03 mg/dL; P = 0.097) or control bread (2.35 \pm 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
344 345 346 347	There were no significant changes in postprandial glucose peak values (at 30 minutes) after 4-week consumption of wheat germ-enriched bread (-5.13 \pm 3.03 mg/dL; P = 0.097) or control bread (2.35 \pm 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
344 345 346 347 348	There were no significant changes in postprandial glucose peak values (at 30 minutes) after 4-week consumption of wheat germ-enriched bread (-5.13 \pm 3.03 mg/dL; P = 0.097) or control bread (2.35 \pm 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.

- 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

1		
2 3	354	protocol were analysed (n = 47; IAUC: P = 0.597 , Insulin: P = 0.709 , HOMA-IR: P =
4 5	355	0.597, and CRP: P = 0.959).
6 7	356	
8 9	357	The intervention-sequence interaction was significant for fasting glucose (-5.73 [-10.00;
10 11		
12 13	358	-1.46], P=0.010) and for HbA1c (0.15 [0.07; 0.23], P<0.001), revealing the existence of
14 15	359	a carryover effect in these two outcomes. HbA1c reflects the average blood glucose
16	360	level in previous 2 to 3 months.[38]
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DISCUSSION

364	Several authors argue that diet should be seen as a key component of overall CVD
365	prevention and health management care plan.[47-49] It is well known that corrective
366	dietary interventions can positively alter lipid profile, blood pressure, BMI, endothelial
367	function, insulin sensitivity, and several inflammatory markers, not only for individuals
368	with genetic predisposition for CVD[50] but also for general population.[3, 4, 35, 48] In
369	order to correctly inform consumers and food and nutrition policy makers about the
370	benefits of supplementing food products with whole grain ingredients, there is an urgent
371	need for clarifying the differences (whether any) between whole-grains and bran or
372	germ individually. First, adding bran or germ individually may not have identical
373	physiologic benefits as the whole grain; and secondly, these two fractions alone may
374	have distinct physiological effects on cardiovascular-health promotion when compared
375	with the whole-grain.[52]
376	with the whole-grain.[52]
376 377	In the present study, we evaluated the health effects of wheat germ intake in 52 healthy
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377 378 379	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
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377 378 379 380 381	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
377 378 379 380 381 382	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
377 378 379 380 381 382 383	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
377 378 379 380 381 382 383 383	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

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

389

390 Germ of wheat was chosen due to its potential to reduce blood cholesterol and 391 triglycerides concentrations, as suggested in previous studies. [23, 24] The proposed 392 mechanisms for lowering plasma cholesterol include a) the inhibition of pancreatic 393 lipase activity by soluble proteins present on wheat germ; [39] b) the reduction in 394 triglyceride lipolysis; [40] and c) reduction in cholesterol absorption by the endogenous 395 wheat germ phytosterols.[41] However, the existing evidence regarding the lipid-396 lowering properties of wheat germ from previous studies is controversial. First, 397 reduction of total cholesterol and LDL was only evaluated in hypercholesterolemic 398 individuals after diet supplementation with 20 g/day for 4-week period. Second, daily 399 ingestion of 30 g wheat germ supplement for 4-week markedly decreases (-39%) plasma triglycerides in hypertriglyceridemic individuals, whereas no reduction was observed in 400 401 the normotriglyceridemic subgroup.[23] 402 403 In our study, we enrolled a moderately large and heterogeneous sample of participants 404 which is representative of general population, with normal, borderline-high and high 405 fasting total plasma cholesterol and triglycerides. The statistical power of this study is 406 higher than preceding studies and for that reason, smaller differences could be detected 407 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. 408 409 However, the inexistence of wheat germ effects reported here does not necessarily 410 disprove the potential cholesterol- and triglyceride-lowering effect described in previous studies. In particular, because in those studies, wheat germ was consumed in higher 411

Page 22 of 36

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412	doses and by individuals at risk. In our opinion, a dose-response analysis for wheat
413	germ intake and lipid profile should be investigated in the future, for general population
414	and high-risk groups.
415	
416	Effect of wheat germ in improving postprandial glycaemia or glucose metabolism was
417	not demonstrated in our study, in accordance with previous studies. Earlier studies
418	addressing the long-term impact of wheat germ on blood lipids of hypercholesterolemic
419	individuals did not observe any significant variation on fasting glucose, insulin,
420	fructosamine and HbA1c;[24] and one single study with six healthy participants showed
421	that wheat germ had no effect on postprandial glucose and insulin concentrations.[43]
422	Even so, we decided to investigate the impact of wheat germ on glucose metabolism
423	because it was recently suggested that intake of alpha-linolenic acid has been associated
424	with lower insulin resistance[44] and our wheat germ enriched-bread contains more
425	41.7% of alpha-linolenic than control bread (table 2).
426	
427	Up until now, the beneficial effect of wheat germ in lowering blood cholesterol and
428	triglycerides was only demonstrated when used as a diet supplementation with 20 g[24]
429	or 30 g/day[23] during 4-week periods. Incorporation of such high proportions of wheat
430	germ in food products, without impairing their nutritional profiles, is difficult since
431	germ changes their sensory proprieties. Our study is the first interventional study
432	evaluating the effect of wheat germ in higher doses than the global mean consumption
433	of germ and higher than the germ present in the recommended doses of whole grains in
434	the diet, in a randomised, double-blinded, placebo-controlled clinical trial using a
435	functional food product, and not as a dietary supplement. More importantly, the
436	nutritional status (table 2), texture and flavour in the wheat germ-enriched bread were

437	not compromised (online supplementary table S2). The aim was to develop a product
438	that could reduce CVD risk, whilst maintaining the sensory characteristics of white
439	refined-bread. Importantly, besides allowing blinding and the analysis of the effect of
440	added germ individually, this no-difference is crucial for consumer's acceptance if this
441	is envisioned as a long-term goal.
442	
443	Our study followed the best practices for designing, conducting and reporting clinical
444	trials to support health claims on food products, namely random allocation, double
445	blinding, reporting methods to measure and maximise compliance. The strength of the
446	current study also includes the use of validated outcomes which are considered
447	beneficial physiological effects for human health.[33] Finally, to our knowledge, this is
448	the largest study to assess the impact of germ intake in human subjects and, also
449	important, targets the general population instead of a high-risk group.
450	
451	The limitations of this study include the duration of the intervention period. Although 4-
452	week is considered the minimal intervention for evaluating an effect on lipoprotein
453	cholesterol, 8-week would be more desirable; however, such intervention period in a
454	crossover study could have a major impact on loss to follow-up. Secondly, the absence
455	of a biomarker specific for germ intake is also a limitation; adherence was monitored
456	through daily self-reported questionnaire and, though compliance with the study
457	protocol was optimal, it is uncertain whether there was over-reporting. Lastly, we
458	decided not to collect information about diet and physical activity levels during the
459	study in order to avoid changes in general participants' lifestyle and dietary patterns;
460	and this could be seen as a limitation.
461	

Page 24 of 36

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462	In summary, our goal was to demonstrate the effect of a staple food supplemented with
463	wheat germ in its maximal concentration without compromise its sensory properties.
464	However, the intake of 6 g/day of wheat germ does not contribute to reduce the
465	cardiovascular risk factors: plasma triglycerides, total cholesterol, LDL cholesterol, or
466	increase the HDL cholesterol levels, or even improve glycaemic control, in a generally
467	healthy normal population. In order to justify an enrichment of food products with
468	wheat germ as a public health approach to prevent CVD, the beneficial effects of wheat
469	germ on human health should be investigated in other food vehicles that can
470	accommodate higher doses of germ. Chemical and sensory proprieties of biscuits,
471	noodles and cakes supplemented with increasing amounts of wheat germ (up to 30%)
472	have been recently analysed and improved.[31, 55, 56] These technological advances let
473	us to envisage that other food products functionalised with higher wheat germ content
474	can be developed. Future studies following our clinical trial design are needed to
475	elucidate if high amounts of daily intake of wheat germ are effective in reducing CVD
476	risk.
477	
478	
479	Abbreviations CRP, C-reactive protein; CVD, cardiovascular disease; HDL, high-density
480	lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; IAUC,
481	incremental area under curve; LDL, low-density lipoprotein.
482	
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486	

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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 curr	ent
study are not publicly available because the ethics committee only allowed the use of the da	ata in
the context of the present research project, however anonymised partial datasets or summar	ies
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). to beet terien only

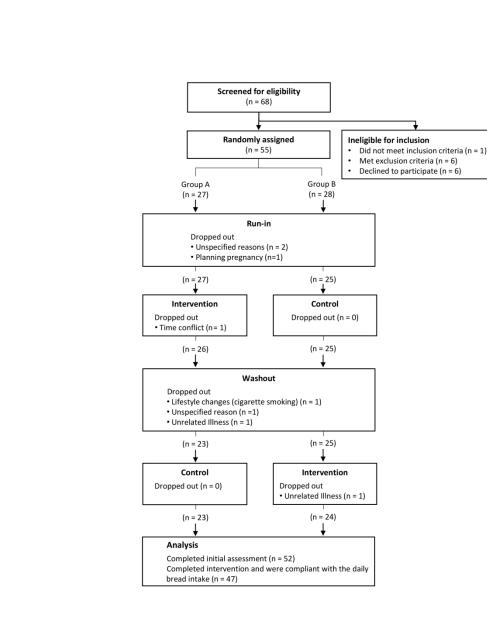


Figure 1 Flow chart of participants through the study.

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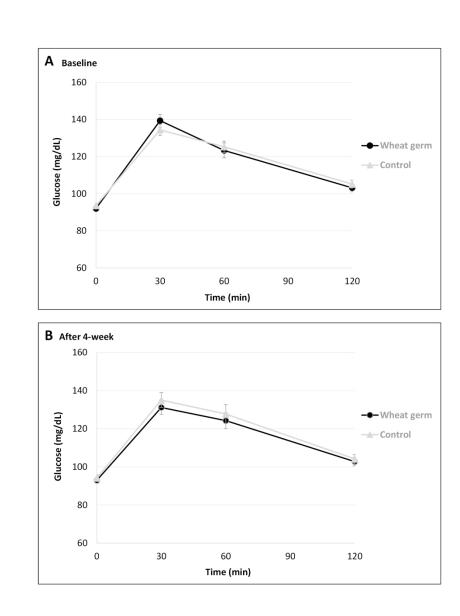


Figure 2 Mean \pm SEM postprandial glucose concentrations in response to a 100 g wheat germ-enriched bread or control bread, at baseline (A), and after 4-week intervention (B). No significant effect in the incremental area under the curve (IAUC) was observed between wheat germ-enriched and control breads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

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1 2 3 4 5 6 7	ONLINE SUPPLEMENTARY TABLES
8 9 10 11 12 13 14 15 16	
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Supplementary	Table	BMJ Open BMJ Open S1. CONSORT 2010 checklist ¹ BMJ Open BMJ O	
Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract		for	
	1a	Identification as a randomised trial in the title Structured summary of trial design, methods, results, and conclusions (for specific structured see CONSORT for abstracts)	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific and ance 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		ata ata	
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation rates	7,8
	3b	Important changes to methods after trial commencement (such as eligibility arteria), 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, Biclouding how and when they were actually administered	8,9
Outcomes	ба	Completely defined pre-specified primary and secondary outcome measures and building how and	
		when they were assessed	8
	6b	Any changes to trial outcomes after the trial commenced, with reasons How sample size was determined	none
Sample size	7a	How sample size was determined	7
	7b		not applicable
Randomisation:		When applicable, explanation of any interim analyses and stopping guideline is in the second stopping	
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 siz	9
Allocation concealment	9	Mechanism used to implement the random allocation sequence (such as sequentized)	
mechanism		containers), describing any steps taken to conceal the sequence until intervention were assigned	9, 10
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and very assigned	
		participants to interventions 9	9, 10

Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care	
8		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	13a	For each group, the numbers of participants who were randomly assigned, rectanded	
strongly recommended)		treatment, and were analysed for the primary outcome	13, 14
	13b	For each group, losses and exclusions after randomisation, together with rea	13, 14
Recruitment	14a	Dates defining the periods of recruitment and follow-up	7
	14b	Why the trial ended or was stopped	not app
Baseline data	15	A table showing baseline demographic and clinical characteristics for each groups	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,
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	not app
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 ONSORT for harms)	not app
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if regvant, multiplicity of	
			23
Generalisability	21	analyses Generalisability (external validity, applicability) of the trial findings	20, 24
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and con detain other relevant	
_		evidence s a	21, 22,
Other information		Age	
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
		ormation, visit: www.consort-statement.org.	

Supplementary Table S2. Sensory evaluation of wheat germ-enriched bread versus control bread¹

	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

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

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

³1 - 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.

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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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Complete List of Authors:	Rosário, André; Universidade do Porto Faculdade de Medicina, Centre for Health Technology and Services Research (CINTESIS); Universidade do Porto Faculdade de Medicina, Department of Community Medicine, Information and Health Decision Sciences (MEDCIDS) Pinheiro, Helder; NOVA University of Lisbon NOVA Medical School, Nutrition & Metabolism; Hospital Curry Cabral, Infectious Diseases Service Marques, Cláudia; NOVA University of Lisbon NOVA Medical School, Centre for Health Technology and Services Research (CINTESIS); NOVA University of Lisbon NOVA Medical School, Nutrition & Metabolism Teixeira, José; University of Minho, Center of Biological Engineering Calhau, Conceição; NOVA University of Lisbon NOVA Medical School , Centre for Health Technology and Services Research (CINTESIS); NOVA University of Lisbon NOVA Medical School, Nutrition & Metabolism Teixeira, José; University of Minho, Center of Biological Engineering Calhau, Conceição; NOVA University of Lisbon NOVA Medical School , Centre for Health Technology and Services Research (CINTESIS); NOVA University of Lisbon NOVA Medical School, Nutrition & Metabolism Azevedo, Lus Filipe ; Universidade do Porto Faculdade de Medicina, Centre for Health Technology and Services Research (CINTESIS); Universidade do Porto Faculdade de Medicina, Centre for Health Technology and Services Research (CINTESIS); Universidade do Porto Faculdade de Medicina, Department of Community Medicine, Information and Health Decision Sciences (MEDCIDS)
Primary Subject Heading :	Nutrition and metabolism
Secondary Subject Heading:	Public health
Keywords:	wheat germ, lipid profile, glucose profile, randomised controlled trial, cardiovascular risk



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4 5 6	2	
7 8	3	Does intake of bread supplemented with wheat germ have a preventive role on
9 10 11	4	cardiovascular disease risk markers in healthy volunteers? A randomised,
12 13	5	controlled, crossover trial.
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16 17 18	7	André Moreira-Rosário ^{1,2*} , Helder Pinheiro ^{3,4} , Cláudia Marques ^{1,3} , José A Teixeira ⁵ ,
19 20	8	Conceição Calhau ^{1,3} , and Luís F Azevedo ^{1,2}
21 22	9	
23 24 25	10	¹ Centre for Health Technology and Services Research (CINTESIS), Portugal.
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33 34	14	Portugal.
35 36	15	⁴ Infectious Diseases Service, Hospital Curry Cabral, Lisbon, Portugal.
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49 50	21	Portugal. Telephone number: +351 225 513 622. E-mail: andrerosario@med.up.pt.
51 52	22	
53 54 55	23	Keywords: wheat germ; lipid profile; glucose profile; randomised controlled trial;
56 57	24	cardiovascular risk.
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Page 2 of 37

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27 ABSTRACT

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Objective: Intake of whole grains is associated with a reduced risk of cardiovascular 29 30 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 31 32 cardiovascular events and therefore, a staple food was selected for 6 g of germ 33 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 34 consumer's acceptance. 35 **Design:** Randomised, double-blinded, crossover, controlled clinical trial with 15-week 36 37 follow-up comprising a 2-week run-in, two intervention periods of 4 weeks/each and a 38 5-week washout period. 39 Setting: A single centre in the north of Portugal. Participants: 55 eligible healthy adults (mean age of 34 years and BMI between 19 and 40 41 38 kg/m^2) were randomly assigned. 42 **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). 43 44 **Outcomes:** Changes in fasting cholesterol and triglycerides, fasting and postprandial 45 glucose, insulin sensitivity and CRP. **Results:** We observed no significant effect of daily intake of wheat germ on cholesterol 46 47 and triglycerides levels, on postprandial glucose response, and on insulin sensitivity. Incremental area under curve (IAUC) glucose and homeostasis model assessment for 48 49 insulin resistance (HOMA-IR) did not change, suggesting that 6 g of wheat germ have 50 no effect on glucose metabolism. No effect was also observed in the subgroup of 51 participants who complied with the protocol (n = 47).

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52	Conclusions: The absence of alterations on lipid and glucose profiles suggests that
53	germ up to 6 g/day, may have no preventive effect on CVD risk. However, it is
54	important to investigate other food vehicles that can accommodate higher doses of
55	wheat germ, in future studies.
56	Trial registration number: NCT02405507.
57	
58	Strengths and limitations of this study:
59	 This study followed the best practices for designing, conducting and reporting
60	clinical trials to support health claims on food products, namely random
61	allocation, double blinding, reporting methods to measure and maximise
62	compliance.
63	 We used validated outcomes which are considered beneficial physiological
64	effects for human health.
65	• To the best of our knowledge, this is the largest study to assess the impact of
66	germ intake in human subjects.
67	 Although compliance was high, it is uncertain whether this is due to over-
68	reporting, since there is no biomarker for wheat germ intake.
69	• A longer intervention period would be desired for evaluating an effect on
70	lipoprotein cholesterol, nonetheless could have a major impact on loss to follow-
71	up in this crossover study.
72	

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73 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

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98 (1g/day average ranging from 0.2 to 2.9 g/day),[17-19] while the intervention studies used
99 a daily supplementation of 20 or 30 g/day during 4-week period. Specifically, these
100 clinical trials showed that intake of raw wheat germ can reduce cholesterol and
101 triglycerides in rats[20-22] and also in hypercholesterolemic and hypertriglyceridemic
102 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

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2 3 4	122	impact on CVD metabolic risk factors of daily consumption of 100 g of wheat white-
5 6	123	bread enriched with 6 g of wheat germ during 4-week, is here presented.
7 8 9 10 11 12 13 14 15 16 17 18 19 20 122 23 24 25 26 7 28 29 30 31 22 33 4 5 36 37 38 9 40 142 43 44 56 57 8 9 60	124	

METHODS

126 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

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2 3 4	149	study is registered in ClinicalTrials.gov database, reference NCT02405507, and
4 5 6	150	followed the CONSORT reporting guidelines (online supplementary table S1).
7 8	151	
9 10 11	152	Patient and Public Involvement
12 13	153	Before recruitment, volunteers that did not participate in the study were invited to be
14 15 16	154	involved in the development of the bread formula used in this trial. They had no role in
17 18	155	setting the research question, the outcome measures, the design, or implementation of
19 20	156	the study. We included inputs from the participants of the study, namely any burden
21 22 23	157	associated with the procedures, throughout the follow-up in order to optimise their
23 24 25	158	involvement and compliance. Participants were involved in the sensory evaluation of
26 27	159	the study breads, as well as in the recruitment process by encouraging others to
28 29 30	160	participate. Upon publication, participants will be informed of the results of this study
30 31 32	161	through direct email.
33 34	162	
35 36	163	Study design and intervention
37 38 39	164	Our study was a 15-week, randomised, double-blinded, crossover, controlled clinical
40 41	165	trial. The trial comprised four stages: a run-in period (2-week), two crossover
42 43	166	intervention periods (4-week each), and a washout period between interventions (5-
44 45 46	167	week). At the end of each stage, blood samples for measurement of plasma cholesterol
40 47 48	168	(total, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL)),
49 50	169	triglycerides, C-reactive protein (CRP), and postprandial glucose were collected from
51 52 53	170	each study participant as primary outcomes. Blood samples were also collected for
54 55	171	measurement of fasting glucose and insulin as secondary outcomes (online
56 57	172	supplementary figure S1). Primary and secondary outcomes related with gastrointestinal
58 59 60	173	discomfort were also evaluated in this trial, however they will be reported later.

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175	Participants were instructed not to change their physical activity levels, maintain their
176	dietary habits and do not consume any food or dietary product supplemented with germ
177	during the study. Compliance to the study protocol (daily consumption of bread) was
178	monitored through daily self-reported questionnaire, since there is no biomarker for
179	wheat germ intake. Participants were randomly assigned into two intervention groups
180	(ratio 1:1) using a computer-generated allocation sequence by a statistician not involved
181	in recruitment and intervention delivery. The intervention arm comprised the daily
182	consumption of wheat bread (100 g) supplemented with wheat germ (6 g), whereas the
183	control arm involved the daily intake of wheat bread (100 g) without any
184	supplementation. The bread provided to participants replaced their usual bread intake
185	during meals, namely during breakfast or afternoon snack.
186	
187	The participants and the research team were blinded to the study breads
188	(intervention/control). In this regard, we previously tested the best formula that masked
189	wheat germ supplementation, in terms of bread texture, volume and flavour. Moreover,
190	the bread was delivered to each participant in opaque bags with a label code (A/B); only
191	the outsourced company responsible for bread production (Padaria Ribeiro Lda.,
192	Portugal) knew the correspondence code. The unblinding was performed after the
193	statistical analysis had been completed.
194	
195	Bread formulation
196	Control breads were prepared by mixing 6 kg refined wheat flour (Germen S.A.,
197	Portugal) with 3.3 L water, 97 g of salt, 60 g of bread improver mix (Germen, S.A.,
198	Portugal), and 180 g of baker's yeast. The mixture then fermented for 15 min at room

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temperature. Dough was divided and molded into pieces (123 g each), and then placed 199 200 in a fermentation chamber with 80% relative humidity for 60 minutes at 30°C. Finally, baking was done at 190 °C during 20 min. Preparation of intervention bread was similar 201 202 to control bread; 480 g of refined wheat flour was replaced by raw wheat germ (Germen 203 S.A., Portugal) only. 204 205 Nutrient composition of control and intervention breads was analysed by Silliker 206 Portugal, S.A. (Mérieux Nutrisciences Corporation). 207 **Blood sampling and analysis** 208 209 The outcomes variables were measured on study participants under 12 hours overnight 210 fasting conditions, at the end of 1) run-in, 2) first intervention, 3) washout and 4) second 211 intervention. Accordingly, a venous blood sample was collected by venipuncture into 212 serum separator tubes (BD Vacutainer SST II Advance, Becton, Dickinson and 213 Company). For measuring glycated haemoglobin (HbA1c), blood was collected into 214 tubes containing K₂EDTA (BD Vacutainer; Becton, Dickinson and Company). All venous blood samples were centrifuged at $1377 \times g$ for 10 min at room temperature 215 216 (CompactStar CS4; VWR), within 30 minutes after collection. A serum aliquot was 217 immediately stored in a special cool transport container (at $< -10^{\circ}$ C) for insulin quantification. All biological samples were shipped to an outsourced certified medical 218 219 laboratory (Clínica Laboratorial de Guimarães, S.A., Portugal) under refrigerated 220 conditions. Analysis was performed within 24 hours after collection. Fasting glucose, 221 total cholesterol and triglycerides were measured using specific enzymatic colorimetric 222 methods, whereas LDL and HDL cholesterol were quantified by the elimination/catalase method. Serum CRP concentrations were measured by latex-223

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224	enhanced immunoturbidimetric assay. These biochemical quantifications were done
225	with the automatic analyser ADVIA 1800 (Siemens Healthcare Diagnostics). Insulin
226	was determined by chemiluminescent microparticle immunoassay method using the
227	automatic analyser Architect i2000 (Abbott Laboratories). The insulin resistance was
228	estimated using the homeostasis model assessment for insulin resistance (HOMA-
229	IR):[35, 36]
230	(fasting insulin (μ U/mL) × [fasting glucose (mg/dL) ÷ 18.01]) / 22.5
231	
232	Regarding the postprandial glucose, four capillary blood samples were obtained by
233	finger prick sampling using disposable lancet devices (Glucocard MX; Arkray) and a
234	glucose meter. Postprandial glycaemia was measured in the fasting state (0) and at 30,
235	60 and 120 minutes after intervention or control bread intake. The postprandial glucose
236	response was expressed as the incremental area under curve (IAUC), by using the
237	trapezoidal rule ignoring the area below the fasting baseline, as previously
238	described.[37]
239	
240	Statistical Analysis
0.4.1	
241	Statistical analysis was performed using SPSS version 23 software (SPSS Inc., Chicago,
241 242	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
242	IL, USA). Data from all participants who were randomly assigned and completed an
242 243	IL, USA). Data from all participants who were randomly assigned and completed an initial assessment were included in the intention-to-treat statistical analysis.
242 243 244	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
242 243 244 245	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
242 243 244 245 246	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

Page 13 of 37

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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]

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RESULTS

271	
272	Baseline characteristics
273	Of 68 subjects screened to assess eligibility to participate in the study protocol, 55 were
274	randomly assigned: 27 to the intervention-control sequence and 28 to the control-
275	intervention sequence. Fifty-two participants completed an initial assessment and were
276	included in the statistical analysis (figure 1). Eight individuals dropped out: three did
277	not wish to continue the study for unspecified reasons, three declined to participate due
278	to personal reasons (see figure 1), and the last two because of unrelated illness
279	(gastroparesis and pneumonia). Study participants were healthy men and women, with a
280	mean age of 34 years (range: 18–59 years), and BMI (in kg/m ²) between 19 and 38 (34
281	normal weight, 14 overweight and 4 obese). All participants had normal fasting glucose
282	and normal glucose tolerance. At baseline, forty-two had normal fasting total plasma
283	cholesterol (< 200 mg/dL), 8 had borderline-high fasting total plasma cholesterol (200-
284	239 mg/dL) and 2 had high fasting total plasma cholesterol (> 240 mg/dL). Fifty
285	participants had normal fasting total plasma triglycerides (< 150 mg/dL) and two had
286	high fasting total plasma triglycerides (200-499 mg/dL). Test results for haemoglobin
287	and for liver function showed no evidence of ill health. Baseline characteristics of the
288	52 study participants that were included in the intention-to-treat statistical analysis are
289	listed in table 1.
290	

	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
292 293 294 295	¹ Mean \pm SD. CRP, C-reactive protein; HbA1c, lipoprotein; HOMA-IR, homeostasis model ass incremental area under the curve: LDL, low-det	essment for	insulin resistance; IAUC
296	Participant compliance		
297	Daily questionnaires revealed good complia	ance to the	study protocol. The da
298	consumption of bread was assessed and the	average co	mpliance was 92.1%
299	did not vary depending of bread type. Interv	vention and	control arm had the s
300	percentage of compliance: $92.2\% \pm 11.1$ and	d 92.0% ±	10.0, respectively (P =
301	None of the participants reported adverse et	ffects.	
302			
	Composition of intervention and control	breads	
303			
303 304	The content of protein, and dietary fibre wa	s higher in	the wheat germ-enric

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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 alphalinolenic 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

312 100 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

1 2	
3 315 4	There was no statistically significant difference between wheat germ-enriched and
5 316	control breads, after 4-week intake, for cholesterol (total, LDL and HDL) and serum
7 8 317 9	triglycerides (table 3).
9 10 318 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60	

]	Total cholesterol, mg/dL	N	Wheat	germ-						n 17 ng fo			
]	Total cholesterol, mg/dL	N			enriched bread				Contro	bl bread us mu		Effect of wheat ger	$\overline{\mathbf{m}^2}$
]	Total cholesterol, mg/dL		Baseline ¹	Ν	Post-Intervention ¹	P value within group	Ν	Baseline ¹	Ν	s regiment Post-Interibited	P value within group	Effect (95% CI)	P valu betwee grouj
1		52	175.17 ± 31.82	50	172.98 ± 32.4	0.147	48	174.00 ± 32.17	48	to 170.00 ±€ Swi	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 ± a13@70a	0.012	0.14 (-1.83, 2.12)	0.880
	LDL cholesterol, mg/dL	52	97.31 ± 26.07	50	96.92 ± 26.22	0.853	48	97.23 ± 25.79	48	95.21 ± a.70	0.356	1.74 (-3.80, 7.28)	0.53
	Triglycerides, mg/dL	52	85.13 ± 54.67	50	83.8 ± 42.62	0.565	48	81.10 ± 49.96	48	82.48 ± 1 9	0.763	-3.60 (-16.28, 9.08)	0.57
	Glucose, mg/dL ³	27	84.22 ± 6.82	26	81.27 ± 7.32	0.011	25	83.48 ± 6.13	25		0.012	-0.24 (-3.28, 2.81)	0.87
]	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)	0.62
]	HOMA-IR	51	1.46 ± 0.68	50	1.44 ± 0.67	0.973	47	1.43 ± 0.78	48	1.54 ± 3.38 0	0.541	0.12 (-0.49, 0.26)	0.52
1	HbA1c, % ⁴	27	5.18 ± 0.31	26	5.26 ± 0.30	0.004	24	5.16 ± 0.15	24	5.21 ± ± 16	0.002	0.027 (-0.03, 0.09)	0.380
]	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 ± 3 101 47	0.812	-221.13 (-901.34, 459.08)	0.52
	CRP, mg/dL	52	0.13 ± 0.21	50	0.18 ± 0.42	0.374	48	0.12 ± 0.17	48	0.25 ± g. 57	0.093	-0.07 (-0.26, 0.13)	0.48
826	resistance; IAUC, incr ² Intervention effects w ³ The intervention-sequ second sequence.	reme vere a uence uence was t	ntal area under the analysed using lin e interaction was s e interaction was s used in the analys	e curv near m signif signif	ve: LDL, low-densi nixed model for rep ficant (-5.73 [-10.00 ficant (0.15 [0.07, 0	ty lipopro eated me 0, -1.46], .23]; P <	otein. asure P = 0 0.001	s with compoun .010), the interv	d sym entior	imetry as covariant n effect wab 2.67 i fect was -0:04 in the	nce structure n the first ne first sea	sequence and -3.66 in the quence and 0.12 in the	

Page 19 of 37

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2 3 4	331	No difference was also observed for those participants who completed the outcome
5 6	332	measures and complied with the daily bread intake between wheat germ-enriched bread
7 8 9	333	and control bread (n = 47; total cholesterol: $P = 0.797$, HDL cholesterol: $P = 0.996$,
9 10 11	334	LDL cholesterol: $P = 0.665$, and triglycerides: $P = 0.762$). The same result was obtained
12 13	335	when participants with normal fasting total plasma cholesterol and triglycerides are
14 15	336	analysed (n = 41; total cholesterol: $P = 0.981$, HDL cholesterol: $P = 0.413$, LDL
16 17 18	337	cholesterol: $P = 0.833$, and triglycerides: $P = 0.718$). The results were not statistically
19 20	338	different when participants with borderline or high fasting total plasma cholesterol were
21 22	339	included (n = 10; total cholesterol: P = 0.432, HDL cholesterol: P = 0.170, LDL
23 24 25	340	cholesterol: $P = 0.781$, and triglycerides: $P = 0.508$). Statistical analysis of blood lipid
26 27	341	outcomes showed no interaction between intervention and sequence, potentially
28 29	342	excluding a carryover effect.
30 31 32	343	
33 34	344	Glucose metabolism and C-reactive protein
35 36	345	There were no significant changes in postprandial glucose peak values (at 30 minutes)
37 38	346	after 4-week consumption of wheat germ-enriched bread (-5.13 \pm 3.03 mg/dL; P =
39 40 41	347	0.097) or control bread ($2.35 \pm 3.73 \text{ mg/dL}$; P = 0.531), and neither between
42 43	348	interventions ($P = 0.182$). Blood glucose response curves for 2 hours after the intake of
44 45	349	intervention and control breads are shown in figure 2.
46 47 48	350	
49 50	351	Comparing with control, wheat germ-enriched bread had no significant effect on IAUC
51 52	352	glucose, fasting insulin or HOMA-IR (table 3), suggesting that 6 g of wheat germ did
53 54 55	353	not improve postprandial glycaemia or glucose metabolism. No differences in CRP
55 56 57	354	were observed between wheat germ-enriched and control breads. CRP and glucose
58 59	355	results are not statistically different when only participants who adhered to the study
60		

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

Page 22 of 37

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

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416 avoid changes in general participants' lifestyle and dietary patterns; and this could be417 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

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Page 24 of 37

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> 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

465 cardiovascular risk factors: plasma triglycerides, total cholesterol, LDL cholesterol, or

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466	increase the HDL cholesterol levels, or even improve glycaemic control, in a generally
467	healthy normal population. In order to justify an enrichment of food products with
468	wheat germ as a public health approach to prevent CVD, the beneficial effects of wheat
469	germ on human health should be investigated in other food vehicles that can
470	accommodate higher doses of germ. Chemical and sensory proprieties of biscuits,
471	noodles and cakes supplemented with increasing amounts of wheat germ (up to 30%)
472	have been recently analysed and improved.[32, 47, 48] These technological advances let
473	us to envisage that other food products functionalised with higher wheat germ content
474	can be developed. Future studies following our clinical trial design are needed to
475	elucidate if high amounts of daily intake of wheat germ are effective in reducing CVD
476	risk.
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479	Abbreviations CRP, C-reactive protein; CVD, cardiovascular disease; HDL, high-density
480	lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; IAUC,
481	incremental area under curve; LDL, low-density lipoprotein.
482	
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486	
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493	Competing interests None declared.
494	
495	Contributors The authors' responsibilities were as follows – AMR was partially responsible
496	for study design, statistical analysis plan, carrying out the trial, manuscript writing and final
497	revision. HP was partially responsible for study design, carrying out the trial, manuscript
498	writing and final revision. CM collaborated in the study design, data collection and final
499	revision of the manuscript. JAT collaborated in the study design and final revision of the
500	manuscript. CC was responsible for the general coordination of the project, study design,
501	manuscript writing and final revision. LFA was responsible for the general coordination of the
502	project, study design, statistical analysis plan, manuscript writing and final revision.
503	
504	Data sharing statement The full datasets generated during and/or analysed during the current
505	study are not publicly available because the ethics committee only allowed the use of the data in
506	the context of the present research project, however anonymised partial datasets or summaries
507	of the data are available from the corresponding author on reasonable request.
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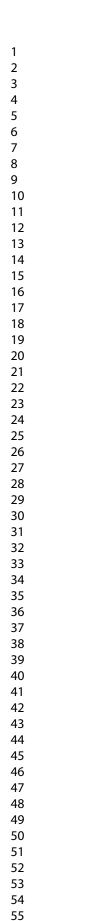
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4 5 67 6	9 Figures:							
7 68 8	Figure 1 Flow chart of participants through the study.							
9 68 10	Figure 2 Mean ± SEM postprandial glucose concentrations in response to a 100 g wheat germ-							
11 12 68	2 enriched bread or control bread, at baseline (A), and after 4-week intervention (B). No							
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16 68 17	4 germ-enriched and control breads ($P = 0.524$).							
17 18 68 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60	germ-enriched and control breads (P = 0.524).							

Page 32 of 37

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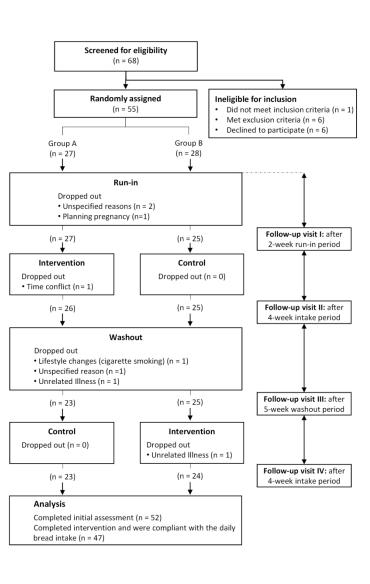


Figure 1 Flow chart of participants through the study.

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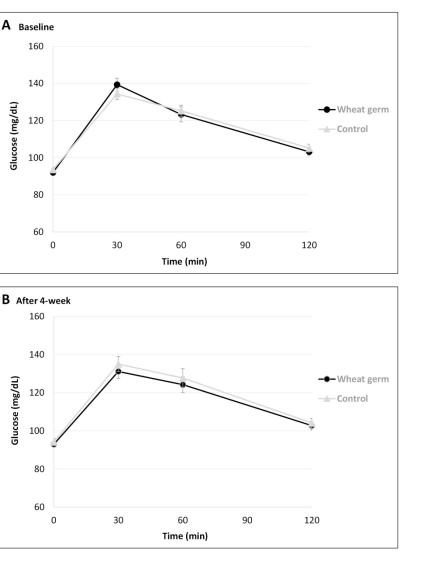


Figure 2 Mean \pm SEM postprandial glucose concentrations in response to a 100 g wheat germ-enriched bread or control bread, at baseline (A), and after 4-week intervention (B). No significant effect in the incremental area under the curve (IAUC) was observed between wheat germ-enriched and control breads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

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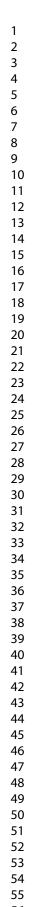


nlamontary Table S1. CONSORT 2010 checklist C

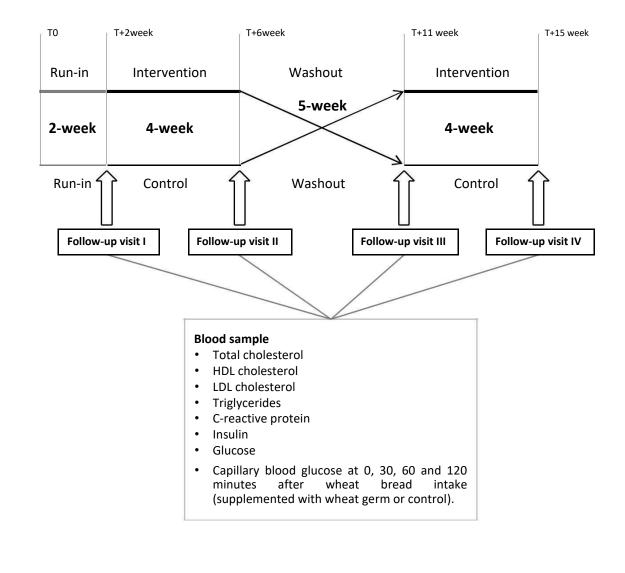
Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract		for	
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific endance see CONSORT for	
		abstracts)	3,4
Introduction		d nen D	
Background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

ge 35 of 37		BMJ Open G en		
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	_10	
	116	If relevant, description of the similarity of interventions	10	
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outco	12, 13	
	12b	Methods for additional analyses, such as subgroup analyses and adjusted anadyses	12, 13	
Results				
Participant flow (a diagram is	13a	For each group, the numbers of participants who were randomly assigned, received intended		
strongly recommended)		treatment, and were analysed for the primary outcome and a domain of the primary outcome	14, 15	
	13b	For each group, losses and exclusions after randomisation, together with rea	14, 15	
Recruitment	14a	For each group, losses and exclusions after randomisation, together with reasons of the periods of recruitment and follow-up Why the trial ended or was stopped	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 and	15	
Numbers analysed	16	For each group, number of participants (denominator) included in each anal		
		analysis was by original assigned groups	14	
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estanded 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 a dust data analyses,		
		distinguishing pre-specified from exploratory	19, 20	
Harms	19	All important harms or unintended effects in each group (for specific guidance see	not applicable	
Discussion	20			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if refevant, multiplicity of	22	
	21	analyses characteristic and the second	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 congoder of the relevant	22.22.24	
		evidence a	22, 23, 24	
Other information		Registration number and name of trial registry		
Registration	23		4	
Protocol	24	Where the full trial protocol can be accessed, if available Sources of funding and other support (such as supply of drugs), role of funders	8, 29	
Funding	25		25	
1From CONSORT Group. For m	ore info	For peer review only - http://bmjopen.bmj.com/site/about/guidelines.xhtml		3

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Supplementary Figure S1. Schedule of assessments



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Supplementary Table S2. Sensory evaluation of wheat germ-enriched bread versus control bread¹

	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

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

²1 - Very much disliked, 2 - much disliked, 3 - disliked, 4 - neither liked nor disliked, 5 - liked, 6 - liked a lot, 7 - very much liked. ³1 - dislike extremely, 2 - dislike v

- 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.