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Alimentary Tract

## WHOLE-meal ancient wheat-based diet: Effect on metabolic parameters and microbiota

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## ABSTRACT

**Background & Aims:** Ancient wheat varieties are considered to be healthier than modern ones, but the data are not univocal. We investigated changes in hematochemical parameters and evaluated microbiota data before and after a set period on a diet containing a whole-meal ancient wheat mix.

**Patients and Methods:** 29 cloistered nuns were recruited. The study comprised two consecutive 30-day periods; during the first one (T1), the nuns received wheat-based foods produced with refined "modern" flour ("Simeto"); during the second one (T2) received wheat-based foods produced with an unrefined flour mix composed of "ancient" cultivars. At entry to the study (T0) and at the end of T1 and T2 hematochemical parameters and fecal microbiota and metabolome were evaluated.

**Results:** At the end of T2, there was a significant reduction in serum iron, ferritin, creatinine, sodium, potassium, magnesium, total cholesterol, LDL- and HDL-cholesterol and folic acid. Furthermore, increased the abundance of cultivable enterococci, lactic acid bacteria and total anaerobes. The ability of the gut microbiome to metabolize carbohydrates increased after the period of diet containing ancient grain products. Several volatile organic compounds increased after the one month on the diet enriched with ancient grain products.

**Conclusions:** Our data showed the beneficial effects deriving from a diet including ancient whole-meal/unrefined wheat flours.

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## 1 1. Introduction

2 Despite the fact that wheat constitutes one of the principal  
 3 calorie sources in the human diet in western countries, we are  
 4 living in an era in which there is a widespread perception that  
 5 wheat ingestion can cause health problems, with the result that  
 6 many eliminate wheat from their diet independently of whether  
 7 a clear and sure medical diagnosis has been made. Interviews and  
 8 questionnaires performed in the general population have shown an  
 9 average prevalence of self-reported symptoms caused by wheat in-  
 10 gestion of approximately 10%, ranging between 4.3 and 14.9% [1].

11 It is difficult to establish whether these self-reported symptoms  
 12 are indeed caused by emerging clinical entities or whether the  
 13 popularity of a wheat-free diet has merely been driven by social  
 14 and traditional media coverage, and the aggressive marketing by  
 15 manufacturers of gluten-free foods. In any case, in this context, in-  
 16 terest has been growing in different, "healthier" wheat varieties,  
 17 whole-meal grains and traditional baking techniques. This is be-  
 18 cause since the 1930s modern agriculture has tried to increase  
 19 yields by creating new strains and crossbreeding different wheat  
 20 and grass species ("modern" wheat). These more recent wheat  
 21 cultivars and the industrial milling technique now used, together with  
 22 the higher kneading intensities required for bread baking have  
 23 been suggested as factors determining the increasing frequency of  
 24 the wheat-related symptoms. It has also been demonstrated that  
 25 there is a great variability in the immunogenic potential of the  
 26 "ancient" and modern wheat varieties [2]. Furthermore, the recent

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27 medical literature has increasingly underlined the paramount rel- 88  
 28 evance of the role of human intestinal microbiota in maintaining 89  
 29 good health. There is growing evidence that the mutually benefi- 90  
 30 cial interactions with the microbes that comprise our commensal 91  
 31 microbiota might have been perturbed by environmental interven- 92  
 32 tions, including changes in eating habits, *i.e.* the widespread con- 93  
 33 sumption of a high-fat/low-fiber diet in adults, or changes in for- 94  
 34 mula feeding in infants [3]. 95

35 On the basis of the above considerations, in this study we eval- 96  
 36 uated the effect of a change in dietary habits, as regards the kind 97  
 37 of wheat-based foods consumed, in a “strictly controlled popula- 98  
 38 tion” of cloistered nuns. The nuns received a daily amount of mod- 99  
 39 ern refined wheat for 30 days and then a daily amount of a mix 100  
 40 of whole-meal ancient wheats. The aims of the study were to ob- 101  
 41 serve any changes in hematochemical parameters and to evaluate 102  
 42 any microbiota alterations before and after a regular diet contain- 103  
 43 ing a whole-meal ancient wheat mix. 104

## 44 2. Materials and methods

### 45 2.1. Subjects and diets

46 Twenty-nine cloistered nuns of the “Congregazione delle Suore 108  
 47 Collegine della Santa Famiglia” (Congregation of the School Sisters 109  
 48 of the Holy Family) in Palermo, Italy, consented to enter the study, 110  
 49 which was performed between October 2017 and January 2018. 111  
 50 They were all females with a mean age ( $\pm$  Standard Deviation, SD) 112  
 51 of  $53.9 \pm 20.9$  years (range 26–90 years). Supplemental File 1 sum- 113  
 52 marizes the demographic data (age), the individual measurements 114  
 53 (Body Mass Index, BMI, and waist circumference), the comorbidities 115  
 54 and any drug treatments of these subjects. None of the study 116  
 55 subjects smoked or consumed alcohol. 117

56 Supplemental File 2 shows the study design. The study com- 118  
 57 prised two 30-day periods, separated by a 2-week washout period. 119  
 58 During the first (T1), the nuns received wheat-based foods pro- 120  
 59 duced with refined flour from “Simeto” wheat (used as a modern 121  
 60 cultivar). During the wash-out they returned to their usual diet, in- 122  
 61 cluding wheat-based foods of uncontrolled origin. During the sec- 123  
 62 ond period (T2) they received wheat-based foods produced with 124  
 63 an unrefined flour mix composed in equal percentages of “Tim- 125  
 64 ilia”, “Margherita”, and “Russello” (three ancient cultivars, histori- 126  
 65 cally produced in Sicily, Italy). Supplemental File 3 presents the 127  
 66 gross composition of the flours used in the present study. The 128  
 67 menu varied from day to day, but the basic diet remained iden- 129  
 68 tical during the two 30-day periods of the study (T1 and T2): the 130  
 69 two study diets showed no differences in terms of energy intake 131  
 70 (kcal/die) or in the other nutritional values (Supplemental File 4) 132  
 71 except for fiber intake, which was approximately 3.4 g/die higher 133  
 72 in the T2 diet. The nuns received a fixed daily quantity of wheat- 134  
 73 based foods and they recorded in a diary whether these foods were 135  
 74 completely consumed or not. Three of the Authors (AD, CC, and 136  
 75 GDS) met the nuns before the beginning of the study to explain its 137  
 76 aims and to ensure adherence to the diet and to the study design; 138  
 77 the same Authors met the nuns on a weekly basis during the en- 139  
 78 tire study period to collect clinical data and clarify any doubts of 140  
 79 the participants. 141

80 The study was recorded at the Clinicaltrials.gov (registration 142  
 81 number NCT03020511 “Effects of Ancient Grains-based Diet in a 143  
 82 Closed Community”) and approved by the Ethics Committee of the 144  
 83 University of Palermo after ascertaining its compliance with the 145  
 84 standards dictated by the Declaration of Helsinki (IV Adaptation). 146

### 85 2.2. Hematochemical analysis

86 Venous blood samples were taken, after overnight fasting, at 147  
 87 entry to the study (T0), after the first 30-day period on refined

modern wheat (T1) and after the second period on a whole- 88  
 meal ancient wheat mix (T2). The following parameters were 89  
 assayed: white blood cell count, hemoglobin, serum iron, fer- 90  
 ritin, glycemia, creatinine, sodium, potassium, magnesium, cal- 91  
 cium, phosphorus, aspartate aminotransferase, alanine aminotrans- 92  
 ferase, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglyc- 93  
 erides, protein electrophoresis, vitamin D, vitamin B12, folic acid, 94  
 and glycated hemoglobin. Furthermore, at the same times, BMI and 95  
 waist circumferences were recorded. 96

### 2.3. Collection of fecal samples

Each volunteer fasted overnight, and fecal samples were col- 98  
 lected pre-prandially the following morning after the first 30-day 99  
 period on refined modern wheat (T1) and after the second peri- 100  
 od on a mix of whole-meal ancient wheats (T2). After collection, 101  
 samples were immediately mixed with Amies transport medium 102  
 (Oxoid Ltd, Basingstoke, Hampshire, England) (*ca.* 15 g, 1:1 wt/wt), 103  
 under anaerobic conditions (AnaeroGen, Oxoid Ltd, Basingstoke, 104  
 Hampshire, England) and stored at  $-80^{\circ}\text{C}$  for further metabolic 105  
 analyses. Samples diluted with Amies transport medium were also 106  
 immediately analyzed using plate counts and the Biolog-system. 107

### 2.4. Enumeration of cultivable bacteria

Fecal samples (5 g) were mixed with 45 ml sterilized physiolog- 109  
 ical solution and homogenized. Viable bacterial cells were counted 110  
 as described by De Angelis et al. [4]. The following selective me- 111  
 dia were used: Plate count agar (total anaerobes); MRS agar (*En-* 112  
*terococcus*, lactobacilli and *Leuconostoc*); Slanetz and Bartley (*En-* 113  
*terococcus*); Rogosa agar plus 1.32 mL/L of glacial acetic acid (lacto- 114  
 bacilli); M17 (*Lactococcus* and *Streptococcus*); Baird Parker (*Staphy-* 115  
*lococcus*); Wilkins-Chalgren anaerobe agar plus GN selective sup- 116  
 plements and defibrinated sheep blood (*Bacteroides*, *Porphyromonas* 117  
 and *Prevotella*); MacConkey agar No.2 (*Enterobacteriaceae*); Chro- 118  
 mocult (Merk, Darmstadt, Germany, Europe) (total coliform); GSP 119  
 agar (Sigma-Aldrich, St. Louis, MO, USA) plus penicillin-G (60 g/L) 120  
 (*Pseudomonas* and *Aeromonas*); *Bifidobacterium* agar modified (Bec- 121  
 ton Dickinson, Le Pont de Claix, SA, France) (*Bifidobacterium*). Ex- 122  
 cept for Chromocult, GSP agar, and *Bifidobacterium* Agar Modified, 123  
 all media were purchased from Oxoid Ltd. (Basingstoke, Hamp- 124  
 shire, England). 125

### 2.5. DNA extraction from stool samples and 16S rRNA metagenetic analysis

Total bacterial DNA was isolated from frozen stool samples 128  
 using the Fast DNA™ SPIN Kit for Soil (MP Kit, MP Biomedicals, 129  
 USA), according to the manufacturer’s instructions. The 16S 130  
 ribosomal RNA (rRNA) metagenetic analysis was carried out at 131  
 Genomix4Life (spin-off of the University of Salerno, Italy) using 132  
 the Illumina MiSeq platform. The V3-V4 regions of the 16S rRNA 133  
 gene were amplified to analyze diversity inside the domains of 134  
 Bacteria [5]. PCR and sequencing analyses were carried out accord- 135  
 ing to the Genomix4Life protocol. Quality control and taxonomic 136  
 assignments were performed according to the QIIME and the Ri- 137  
 bosomal Database Project Bayesian classifier in combination with 138  
 a set of custom-designed computerized pipelines implemented by 139  
 Genomix4Life to analyze the microbial communities. Taxonomic at- 140  
 tribution was carried out using a BLAST search in the NCBI 16S 141  
 rRNA sequences database [6]. The percentage of each bacterial 142  
 Operational Taxonomic Unit (OTU) was analyzed individually per 143  
 sample, providing relative abundance information based on the 144  
 numbers of reads per sample. Alpha diversity, analyzed by consid- 145  
 ering the number of observed OTUs and the Shannon diversity in- 146  
 dex, was calculated using QIIME [7]. Differences in microbial com- 147



148 munities between the two sample times were also investigated using the phylogeny-based unweighted UniFrac distance metric.

## 150 2.6. Community-level catabolic profiles

151 Carbon source utilization patterns of the fecal microbiota were  
152 assessed in triplicate using Biolog 96-well Eco micro-plates (Biolog,  
153 Inc., Hayward, CA, USA) [8]. Micro-plates contained 31 different  
154 carbon sources (carbohydrates, carboxylic acids, polymers, amino  
155 acids, amines, and miscellaneous substrates). Five grams of feces  
156 diluted with Amies transport medium (1:1) were homogenized in  
157 a bag filter with 45 mL of sterile sodium chloride [0.9% (w/v)] so-  
158 lution (Classic Mixer) to remove the solid particulate of the feces.  
159 The homogenized feces were centrifuged at 11,000 rpm for 15 min  
160 at 4 °C. The pellet was first washed with 50 mM Tris-HCl (pH 7.0),  
161 then with sterile sodium chloride [0.9% (w/v)] solution, and cen-  
162 trifuged at 11,000 rpm for 15 min at 4 °C. The cell suspension was  
163 diluted (1:10) into the sterile sodium chloride [0.9% (w/v)] solution  
164 and subsequently centrifuged at 2000 rpm for 2 min at 4 °C. The  
165 cell suspension was then diluted (1:20) into sterile chloride [0.9%  
166 (w/v)] solution and dispensed (150 µL) into each of the 96 wells  
167 of the Biolog Eco micro-plates. The micro-plates were incubated at  
168 30 °C in the dark on a slow-speed stirrer, and color development  
169 was measured at 590 nm every 24 h with a micro-plate reader (Bi-  
170 olog Microstation). Three indices were determined [9]. Shannon's  
171 diversity ( $H'$ ), indicating the substrate utilization pattern, was cal-  
172 culated as follows:  $H' = -\sum \pi \ln(\pi)$ , where  $\pi$  is the ratio of the ac-  
173 tivity of a particular substrate to the sums of activities of all sub-  
174 strates at 120 h; Substrate richness ( $S$ ), measuring the number of  
175 different substrates used, was calculated as the number of wells  
176 with a corrected absorbance greater than 0.25; Substrate evenness  
177 ( $E$ ) was defined as the equitability of activities across all utilized  
178 substrates:  $E = H'/\log S$ .

## 179 2.7. Phylogenetic investigation of communities by reconstruction of 180 unobserved states (PICRUSt) analysis

181 Phylogenetic Investigation of Communities by Reconstruction  
182 of Unobserved States (PICRUSt) analysis was carried out to pre-  
183 dict microbiota-associated biochemical pathways of gut microbiota  
184 from fecal noun samples. In detail, 16S rRNA bacteria gene se-  
185 quences were the starting point for the prediction of metabolic  
186 functions. First, a BIOM-formatted OTU table was generated using  
187 the make.biom command of the Mothur program based on  
188 a Greengenes database (May 2013 ver.; <http://greengenes.lbl.gov>).  
189 The abundance of each OTU was corrected to reflect the true bac-  
190 terial abundance by normalizing the 16S rRNA copy number for  
191 each OTU. KEGG ortholog abundances for a given OTU, table-picked  
192 against the newest version of the Greengenes database, were cal-  
193 culated by locally running the PICRUSt "predict\_metagenomes.py"  
194 script. The gene functions classified by KO were further catego-  
195 rized into KEGG pathways using the "categorize\_by\_function.py" PI-  
196 CRUSt script, which collapses thousands of predicted functions into  
197 higher categories (KEGG pathways). The enrichment of predicted  
198 KEGG pathways found in the T1 and T2 noun fecal samples (mod-  
199 ern and ancient wheat-based diets, respectively) was assessed with  
200 STAMP software83 using a two-sided Welch's  $t$ -test corrected by a  
201 Benjamini-Hochberg procedure ( $P < 0.05$ ).

## 202 2.8. Analysis of fecal volatile compounds and free amino acids

203 After preconditioning according to the manufacturer's instruc-  
204 tions, a polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber  
205 (65 µm) and a manual solid phase micro-extraction (SPME) holder  
206 (Supelco Inc., Bellefonte, PA, USA) were used. Before headspace  
207 sampling, the fiber was exposed to gas chromatography (GC) inlet

for 1 h for thermal desorption at 250 °C [10]. Three grams of fecal  
sample were placed into 10 mL glass vials and 10 µL of 4-methyl-  
2-pentanol (final concentration 33 mg/L) was added as the internal  
standard. Samples were then equilibrated for 10 min at 40 °C. SPME  
fiber was exposed to each sample for 40 min. Both the equilibra-  
tion and absorption phases were carried out with stirring. The fiber  
was then inserted into the injection port of the gas chromatograph  
for 10 min of sample desorption. GC-mass spectrometry (MS) anal-  
yses were carried out with an Agilent 7890A gas chromatograph  
(Agilent Technologies, Palo Alto, CA) coupled to an Agilent 5975C  
mass selective detector operating in electron impact mode (ion-  
ization voltage, 70 eV). A Supelcowax 10 capillary column (length  
60 m; inside diameter 0.32 mm; Supelco, Bellefonte, PA, USA) was  
used. The temperature program was: 50 °C for 1 min, followed by  
an increase at a rate of 4.5 °C/min to 65 °C, an increase at a rate  
of 10 °C/min to 230 °C, and then 230 °C for 25 min. The injector, in-  
terface and ion source temperatures were 250°, 250, and 230 °C,  
respectively. The mass-to-charge ratio interval was 30 to 350 Da  
at a rate of 2.9 scans per sec. Injection was carried out in split-  
less mode, with helium (flow rate, 1 mL/min) as the carrier gas.  
Molecules were identified based on the comparison of their re-  
tention times with those of pure compounds (Sigma-Aldrich, Mil-  
an, Italy). Identities were confirmed by searching mass spectra in  
the available databases (NIST, version 2005; Wiley, version 1996).  
Quantitative data for the compounds identified were obtained by  
interpolation of the relative area vs the internal standard area. All  
the GC-MS raw files were converted to netCDF format via Chem-  
station (Agilent Technologies, USA) and subsequently processed  
by the XCMS toolbox (<http://metlin.scripps.edu/download/>). XCMS  
software allows automatic and simultaneous retention time align-  
ment, matched filtration, peak detection, and peak matching. GC-  
MS/SPME data were organized into matrices for subsequent sta-  
tistical analysis. Total and individual FAAs from the water-soluble  
extracts were determined by a Biochrom 30 series Amino Acid An-  
alyzer (Biochrom Ltd., Cambridge Science Park, UK) as described by  
De Angelis et al. [4].

## 204 2.9. Sample size

205 Based on previous studies [11], a sample size of 26 volunteers  
206 would be sufficient to detect a difference in culturable bacteria and  
207 metabolome between the T1 and the T2 group evaluation results,  
208 with a power of 90% and a significance level of 5%. We expected  
209 an increase of 1 log cycle in the viable cell density of fecal lacto-  
210 bacilli after the diet containing ancient whole-meal grain products  
211 compared to the diet with refined modern wheat products.

## 212 2.10. Statistical analysis

213 For the hematochemical parameters, continuous variables were  
214 described as mean  $\pm$  SD if the distribution was normal, otherwise  
215 as the median and interquartile range. Differences between con-  
216 tinuous variables were assessed by paired  $t$ -test if the distribution  
217 was Gaussian; otherwise, the Wilcoxon signed-rank-test was used.

218 Culture-dependent data were obtained at least in triplicate. The  
219 analysis of variance (Student's  $t$ -test for paired, two-tailed samples)  
220 was carried out on transformed data, followed by the separation  
221 of means with Tukey's honestly significant difference (HSD), using  
222 the statistical software "Statistica" for Windows (Statistica 6.0 per  
223 Windows 1998, StatSoft, Vigonza, Italy).

## 224 3. Results

225 All the nuns fully adhered to the diet during the two 30-  
226 day periods and no changes in the kind and quantity of wheat-  
227 based foods, apart from those administered, were recorded in their

**Table 1**

Haemato-chemical parameters of Cloistered Sisters collected at baseline (T0), after the first thirty day-diet with wheat-based foods produced with a modern refined flour (T1) and after the second thirty day-diet with wheat-based foods produced with ancient unrefined flour-blend (T2).

	Patients (n=29) at baseline (T0)	Patients (n=29) after modern grain (T1)	Patients (n=28)* after ancient grain (T2)	P
<b>White Blood Cells (x mmc)</b>	6137 ± 2471	6138 ± 2371	5783 ± 1771	T0 vs T1 NS T1 vs T2 NS
<b>Hemoglobin (gr/dL)</b>	12.29 ± 1.22	12.28 ± 1.21	12.25 ± 1.41	T0 vs T1 NS T1 vs T2 NS
<b>Serum iron (mcg/dL)</b>	66.5 ± 27.2	69.5 ± 28.1	50.2 ± 23.1	T0 vs T1 NS T1 vs T2 < 0.02
<b>Ferritin (ng/mL)</b>	89 ± 202	99 ± 222	76 ± 179	T0 vs T1 NS T1 vs T2 < 0.04
<b>Glycemia (mg/dL)</b>	84.6 ± 8.5	86.1 ± 9.6	88.2 ± 7.3	T0 vs T1 NS T1 vs T2 NS
<b>Creatininine (mg/dL)</b>	0.87 ± 0.16	0.91 ± 0.16	0.75 ± 0.18	T0 vs T1 NS T1 vs T2 < 0.0001
<b>Sodium (mEq/L)</b>	139.4 ± 2.2	141 ± 3.2	135.2 ± 2.0	T0 vs T1 NS T1 vs T2 < 0.0001
<b>Potassium (mEq/L)</b>	4.47 ± 0.25	4.56 ± 0.26	4.35 ± 0.34	T0 vs T1 NS T1 vs T2 < 0.03
<b>Magnesium (mg/dl)</b>	2.16 ± 0.12	2.19 ± 0.13	2.08 ± 0.17	T0 vs T1 NS T1 vs T2 < 0.02
<b>Calcium (mg/dL)</b>	9.11 ± 0.34	9.12 ± 0.33	9.28 ± 0.38	T0 vs T1 NS T1 vs T2 < 0.02
<b>Phosphorus (mg/dL)</b>	3.42 ± 0.42	3.40 ± 0.51	3.61 ± 0.39	T0 vs T1 NS T1 vs T2 < 0.02
<b>Aspartate aminotransferase (U/L)</b>	18.2 ± 4.6	18.3 ± 4.5	20 ± 7.5	T0 vs T1 NS T1 vs T2 NS
<b>Alanine aminotransferase (U/L)</b>	13.4 ± 5.1	14.9 ± 5.3	16.1 ± 11.0	T0 vs T1 NS T1 vs T2 NS
<b>Total cholesterol (mg/dL)</b>	199.7 ± 38.6	201.6 ± 37.6	177.7 ± 29.3	T0 vs T1 NS T1 vs T2 < 0.0001
<b>LDL-cholesterol (mg/dL)</b>	108.5 ± 28.5	106.4 ± 27.4	93.2 ± 23.7	T0 vs T1 NS T1 vs T2 < 0.0001
<b>HDL-cholesterol (mg/dL)</b>	75.3 ± 18.9	76.3 ± 17.9	69.9 ± 16.9	T0 vs T1 NS T1 vs T2 < 0.0001
<b>Tryglicerides (mg/dL)</b>	73.3 ± 37.9	77.1 ± 38.9	71.1 ± 36.2	T0 vs T1 NS T1 vs T2 NS
<b>Vitamin D (ng/mL)</b>	9.5 ± 12.6	9.9 ± 11.6	9.7 ± 12.7	T0 vs T1 NS T1 vs T2 NS
<b>Vitamin B12 (pg/mL)</b>	439.8 ± 174.2	449.9 ± 182.2	427.3 ± 177.5	T0 vs T1 NS T1 vs T2 NS
<b>Folic acid (ng/mL)</b>	9.5 ± 3.9	9.7 ± 4.1	8.6 ± 2.1	T0 vs T1 NS T1 vs T2 < 0.05
<b>Glycated Hemoglobin (%)</b>	5.29 ± 0.37	5.39 ± 0.46	5.28 ± 0.35	T0 vs T1 NS T1 vs T2 NS

\* Note: one Sister not completed the 2nd sampling (T2) because she was transferred to another Sister's Congregation (drop-out).

268 dairies. One nun did not complete the 2nd sampling (T2) because  
269 she was transferred to another religious Congregation (drop-out).  
270 Periodic meetings with the Authors ensured strict adherence to the  
271 diet.

### 272 3.1. Hematochemical parameters

273 **Table 1** summarizes the mean values of the hematochemical  
274 parameters. No differences were observed between the values ob-  
275 served at baseline (T0) and those recorded at the end of the first  
276 study period (T1). At the end of the second study period (T2),  
277 when compared with the first period on modern wheat (T1), we  
278 recorded a significant reduction in serum iron ( $P=0.02$ ), ferritin  
279 ( $P=0.04$ ), creatinine ( $P=0.0001$ ), sodium ( $P=0.0001$ ), potassium  
280 ( $P=0.03$ ), magnesium ( $P=0.02$ ), total cholesterol ( $P=0.0001$ ),  
281 LDL- and HDL-cholesterol ( $P=0.0001$ , for both), and folic acid  
282 ( $P=0.05$ ). On the contrary, calcium and phosphorus levels signif-  
283 icantly increased on the ancient wheat diet ( $P=0.02$ , for both). No  
284 other statistically significant differences were found.

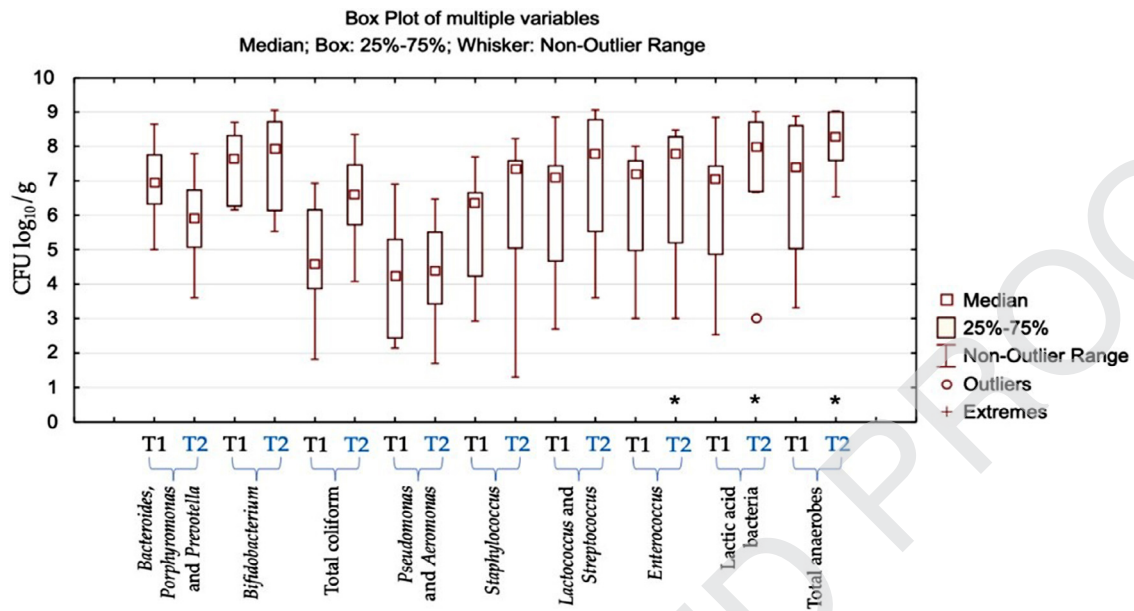
### 285 3.2. Diet containing ancient grain products affects the fecal 286 microbiota of the study group

287 **Fig. 1** shows the viable cell counts (colony-forming unit, CFU,  
288 Log<sub>10</sub>/g) of the main microbial groups found in the fecal sam-

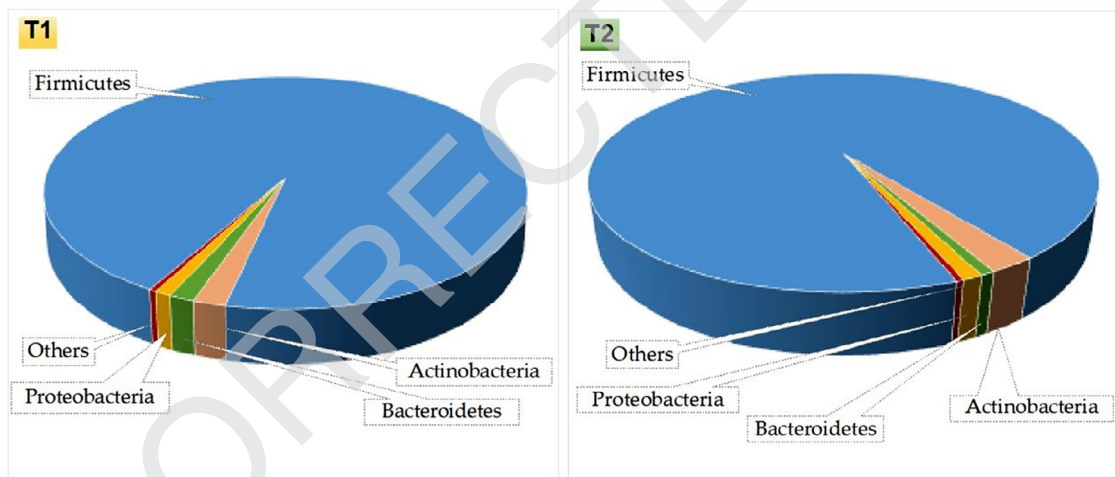
289 ples at T1 and T2. Compared to T1, the diet containing ancient  
290 grain products had an increased abundance of culturable entero-  
291 cocci, lactic acid bacteria (LABs) and total anaerobes ( $P < 0.05$ ). No  
292 statistical differences between T1 and T2 amounts were observed  
293 for *Bacteroides*, *Porphyromonas* and *Prevotella*, *Bifidobacterium*, *En-*  
294 *terobacteria*, *Pseudomonas* and *Aeromonas*, *Staphylococcus*, *Lactococ-*  
295 *cus* or *Streptococcus*.

### 296 3.3. Metagenetic analysis of the 16S rRNA genes

297 Total bacterial DNA from fecal samples of the enrolled clois-  
298 tered nuns was analyzed by sequencing the 16S rRNA gene am-  
299 plicons, resulting in  $98,399.25 \pm 34,472.14$  (mean  $\pm$  SD) reads per  
300 sample, of which  $89.54\% \pm 7.4\%$  were assigned to at least genus  
301 level. Comparing T1 vs T2, no statistical differences were observed  
302 in the number of OTUs and Shannon species diversity index. More-  
303 over, no differences were detected at the high taxonomic levels,  
304 specifically phyla (**Fig. 2**), families (**Fig. 3a** and **b**), and genera with  
305 a mean relative abundance greater than 0.1%. The main differences  
306 between the fecal microbiota of the T1 and T2 samples were de-  
307 tected at species level. Among the OTUs with a mean value of  
308 relative abundance greater than 0.1% at least for one diet, *Blautia*  
309 *wexlerae* (T1: 1.87%, T2: 4.01%;  $P=0.02$ ), *Collinsella tanakaiei* (T1:  
310 0.09%, T2: 0.16%;  $P=0.04$ ), *Atopobium fossor* (T1: 0.48%, T2: 0.75%;  
311  $P=0.041$ ) and *Slackia piriformis* (T1: 0.07%, T2: 0.14%;  $P=0.041$ )



**Fig. 1.** Counts of viable cells (CFU Log<sub>10</sub>/g) of the bacterial groups found in fecal samples of healthy females after 30 days of a diet with modern refined wheat products (T1) and after 30 days of a diet with ancient whole-meal wheat products (T2). (\**P*-value < 0.05; Student's *t*-test).



**Fig. 2.** Relative abundances (%) of total bacteria (16S rRNA gene sequences) found in fecal samples of healthy females after 30 days of a diet with modern refined wheat products (T1) and after 30 days of a diet with ancient whole-meal wheat products (T2).

312 increased after 30 days' consumption of the whole-meal ancient  
313 wheat products.

### 314 3.4. Metabolic changes in fecal microbiota as detected by biolog 315 eco-microplates and by PICRUSt analysis

316 The *H'* index and the *S* index values of the fecal microbiome  
317 were calculated (Supplemental File 5). Compared to T1, the diet  
318 containing ancient grain products produced a reduction in the *H'*  
319 and *S* indices of the fecal microbiome in the 28 healthy subjects.  
320 The *E* index confirmed the above-described significant differences  
321 (*P* < 0.05). Carbohydrates and amino acids, followed by carboxylic  
322 acids, were the organic compounds mainly utilized in all sam-  
323 ples. An opposite trend was detected between carbohydrate and  
324 amino acid utilization before and after the diet containing ancient  
325 grain products. Indeed, the ability of the gut microbiota to metabo-  
326 lize carbohydrates increased after the diet containing ancient grain  
327 products (*P* < 0.05). By contrast, the metabolism of carboxylic acids

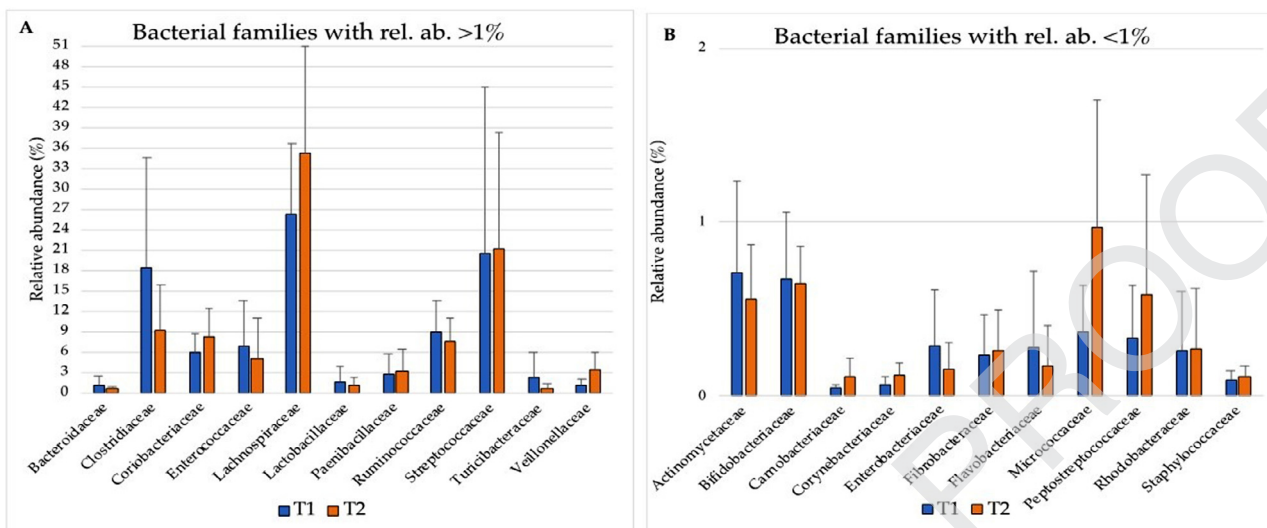
and especially of amino acids decreased. No statistical differences  
328 were found in the metabolism of polymers and amines.

329  
330 Moreover, in order to investigate how both diets influenced the  
331 microbial metabolic pathways we performed a Phylogenetic Invest-  
332 igation of Communities by Reconstruction of Unobserved States  
333 (PICRUSt) analysis. Few significant differences were found (Supple-  
334 mental File 6). Specifically, there was a significant increase in the  
335 metabolism of fructose and mannose (*P* = 0.037), of C5-branched  
336 dibasic acids (*P* = 0.044), and toluene (*P* = 0.045) after the diet with  
337 ancient grains (T2). On the other hand, no significant increases oc-  
338 curred after the diet with the modern wheat variety (T1).

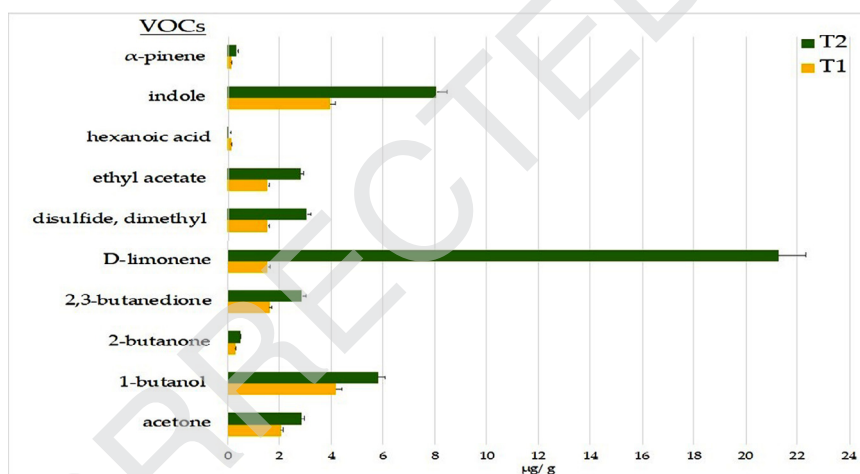
### 339 3.5. Diet containing ancient grain products affects the fecal 340 metabolome of the study group

341 Compared to baseline values, several volatile organic com-  
342 pounds (VOCs) increased after 30 days on the diet enriched  
343 with ancient grain products (*P* < 0.05) (Fig. 4). In detail, there  
344 was an increase in fecal concentrations of indole (3.94 and





**Fig. 3.** Bacterial families (16S rRNA gene sequences) with a relative abundance > 0.1% (panel A) and with a relative abundance < 0.1% (panel B) found in fecal samples of healthy females after 30 days of a diet with modern refined wheat products (T1) and after 30 days of a diet with ancient whole-meal wheat products (T2).



**Fig. 4.** Concentrations of the statistically different ( $P$ -value < 0.05; Student's  $t$ -test) volatile organic compounds (VOCs) found in fecal samples of healthy females after 30 days of a diet with modern refined wheat products (T1) and after 30 days of a diet with ancient whole-meal wheat products (T2).

345 8.03  $\mu\text{g/g}$  at T1 and T2, respectively,  $P=0.012$ ), D-limonene (1.53  
346 and 21.26  $\mu\text{g/g}$ ,  $P=0.005$ ), 1-butanol (4.17 and 5.80  $\mu\text{g/g}$ ,  $P=0.007$ ),  
347 dimethyl disulfide (1.52 and 3.05  $\mu\text{g/g}$ ,  $P=0.022$ ), alpha-pinene  
348 (0.10 and 0.33  $\mu\text{g/g}$ ,  $P=0.031$ ), ethyl acetate (1.52 and 2.78  $\mu\text{g/g}$ ,  
349  $P=0.009$ ), 2-butanone (0.25 and 0.45  $\mu\text{g/g}$ ,  $P=0.030$ ), and acetone  
350 (2.04 and 2.82  $\mu\text{g/g}$ ,  $P=0.040$ ). On the contrary, hexanoic acid de-  
351 creased after the consumption of ancient grain products (0.11 and  
352 0.04  $\mu\text{g/g}$ ,  $P=0.047$ ).

353 Total free amino acids (FAAs) were lower in the samples of sub-  
354 jects after the diet enriched with ancient grain products (T1: 11.48  
355 and T2: 8.796  $\mu\text{g/g}$ ;  $P=0.013$ ). In detail, Asp, Thr, Ser, Met, Ile, Tyr,  
356 Orn, and Arg-were found at lower concentrations (Fig. 5). Free am-  
357 monia was also lower (T1: 0.271 and T2: 0.176  $\mu\text{g/g}$ ;  $P=0.004$ ).

### 358 3.6. Correlations of serum parameters with volatile organic 359 compounds and bacterial groups

360 Correlation analysis ( $r > 0.7$ ; false discovery rate, FDR, <0.05;  
361 Supplemental File 7) showed a marked positive correlation between  
362 HDL-cholesterol levels and many culturable bacteria, except for total  
363 coliforms, and also for compounds included in "cluster B". However,  
364 a positive correlation was found between HDL-

cholesterol and all the bacterial taxa included in "cluster C" and  
LABs, which were included in "cluster D". Although LABs did not  
clearly correlate with LDL-cholesterol, there was a more definite  
negative correlation with cholesterol values as well as with phos-  
phorus. Interestingly, indole and dimethyl disulfide shared the pos-  
itive correlation with HDL-cholesterol and also a negative one with  
LDL-cholesterol, whereas only indole showed negative correlations  
with creatine calcium, and with alpha-2 and gamma globulins.  
Lastly, both the essential oils D-limonene and alpha-pinene were  
included in "cluster B", showing negative correlations principally  
associated with creatine, sodium calcium, and alpha-2 globulin.

## 4. Discussion

The positive effects of cereals on human health and blood pres-  
sure control have previously been related to a number of bioac-  
tive peptides, which may already be present in foods as natural  
components or derive from the hydrolysis of proteins by chemi-  
cal and enzymatic treatments (e.g., digestion, fermentation) [12].  
Hence, in a growing number of studies, foods and food compo-  
nents potentially active in reducing the risk of cardiovascular dis-  
ease (CVD) have been investigated [13]. Several studies have eval-

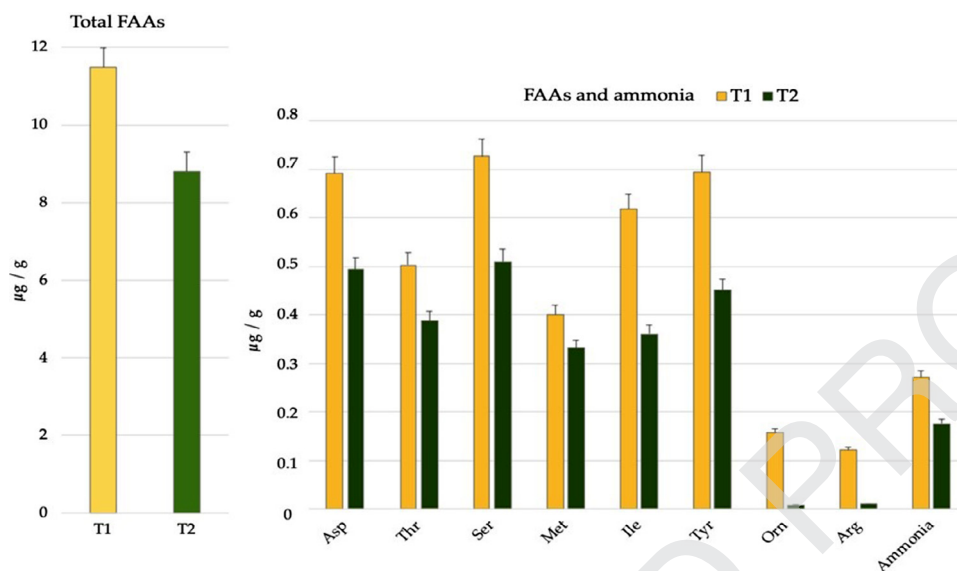


Fig. 5. Concentrations of total free amino acids (FAAs) and statistically different ( $P$ -value < 0.05; Student's  $t$ -test) FAAs and ammonia found in fecal samples of healthy females after 30 days of a diet with modern refined wheat products (T1) and after 30 days of a diet with ancient whole-meal wheat products (T2).

uated the potential functional efficacy of ancient wheats on circulatory parameters, focusing on the risk factors of oxidative stress and pro-inflammatory markers [14–16]. In this area, the ancient KAMUT® Khorasan wheat has been linked to a lower cardiovascular mortality rate in the elderly [17], whereas the Verna variety has repeatedly been shown to have significant beneficial effects on total cholesterol and LDL-cholesterol, as well as on blood glucose parameters [14,16].

It should be stressed, however, that traditional bread making was based on whole unrefined flours. Thus, the beneficial effect of ancient wheat, if real, should be evaluated in a wheat-based “ancient diet” including whole, unrefined, flours of ancient wheat varieties. For this reason, in the present study we decided to evaluate the effect of a regular diet based on an unrefined ancient wheat mix on hemato-chemical parameters and on the intestinal microbiota.

Our data on the metabolic parameters which directly affect CVD risk confirm the beneficial effects of a diet based on an unrefined ancient wheat mix. We recorded a significant reduction in serum values of total cholesterol and LDL-cholesterol of about 10–12% from the T1 value at the end of the diet with ancient wheat-based foods (T2). Interestingly, serum creatinine and sodium also decreased after the ancient wheat diet, suggesting a lower renal protein and salt load. These findings are fully in agreement with the analysis of the fecal metabolome, which showed that both total free amino acids and free ammonia were lower in samples of subjects after the diet enriched with ancient grain products. It must be underlined that these positive effects could also be due to the regular consumption of an increased quantity of dietary fiber, rather than to the qualities of the cultivars themselves (the ancient varieties versus the modern one), as the effect of fiber on human glycolipid metabolism is well known. In fact, an adequate daily fiber intake consistently reduces cholesterol levels, and thus the risk of CVD [18].

For a better understanding of the effects deriving from the change in diet, we also investigated microbiota composition. After the consumption of the unrefined ancient wheat mix, we observed small microbial variations (statistically significant) in specific OTUs rather than large shifts in gut microbiota composition. No differences were found at phylum level, whereas at bacterial family level we only detected a trend in increasing *Lachnospiraceae* abundances

and decreasing *Clostridiaceae* after the T2 diet (Fig. 3a). This could be explained by the different amount of fiber intake, even though the diet with the modern cultivar also contained an adequate daily amount of fiber. We observed also how a slight increase in fiber intake mainly increased abundances of 4 OTUs. Among these, *Blautia wexlerae* was the only taxon with a relative abundance greater than 1% after both evaluated diets (T1 and T2). Evidence has recently demonstrated the beneficial effects associated to *Lachnospiraceae*, one of the core families of the human gut microbiota, due to their markedly saccharolytic metabolism [19] and *Blautia* is one of the main taxa of this family. *Blautia wexlerae* and other species of *Blautia* have been shown to positively correlate more with vegetable macro- and micro- nutrients than with animal fats and proteins [20]. Furthermore, *Blautia wexlerae* has also been associated to a healthy microbiota in a clinical trial performed on obese versus non-obese individuals [21]. In our study, the diet with the ancient wheat mixture also improved the abundance of *Collinsella tanakaei*, whose beneficial effects are well known. In this line, Joossens et al. reported low abundances of *Collinsella* in the gut microbiota of patients with Crohn's disease [22] and for this reason different species of *Collinsella* (including *Collinsella tanakaei*) were recently used to treat patients with inflammatory bowel diseases (IBD) [23], showing promising results.

In our study, the most relevant results were observed when evaluating the metabolome and the microbial metabolic activity of the hosts by the Biolog and PICRUSt analyses. Thirty days of diet containing ancient grain products determined a reduction in  $H'$  and  $S$  indices, associated with an increased gut microbial ability to metabolize carbohydrates, particularly increasing fructose and mannose metabolism. This finding could be the typical signature of *Firmicutes* activities (e.g., enterococci and LABs), as we observed through the microbial counts of viable cells. *Firmicutes* are known to encode a lower number of metabolic pathways than *Bacteroidetes* [24] and the decreased utilization of amino acids and lower levels of both total FAAs and free ammonia found in the T2 samples could also indicate an increased abundance of metabolically active *Firmicutes*. By contrast, *Bacteroidetes* and *Proteobacteria*, which encode a large number of metabolic pathways, are mainly linked to Western diets, which are rich in animal-derived products (high fat, high protein) and low in fiber intake [25,26]. Chronic increases in both of these taxa might be a sign of an unstable gut

microbial community, as well as pathological states of the hosts [27,28]. Other studies have reported that both *Bacteroidetes* and *Firmicutes* overgrowths could be linked to an increased risk of colorectal cancer [29], suggesting that disease onset is mainly determined by strain-specific bacterial genes. In our study, we observed an increase in viable cells in *Enterococcus* and lactobacilli, both taxa of *Firmicutes*. In fact, although containing relatively few fiber-metabolizing enzymes per organism, *Firmicutes* and *Actinobacteria* are the main responders to dietary whole-meal fiber intake in a gut environment [30] due to their specialized roles. In detail, they are involved in the initiation of complex substrate degradation [31] and primarily involved in the production of short-chain fatty acids (SCFAs). Butyrate is one of the main SCFA and it represents the major energy source for bowel epithelial cells, therefore, evidence has positively correlated butyrate to healthy states [32] due to its important role in epithelial barrier integrity [33] and in the remission of IBD [34]. In a study performed on healthy subjects, 3 months of a KAMUT® Khorasan-based diet determined an increase in SCFAs and phenol compounds, as well as a slight increase in gut-health-promoting bacteria [35]. Vitaglione et al. found that a number of bacteria are involved in the release of bound phenolic compounds from dietary fiber, thereby facilitating their absorption by the host [36], and species of *Lactobacillaceae* can also be included among these [37,38]. *Enterococcus* and lactobacilli are LABs successfully used as probiotics to improve human and animal health. Recently, the genus *Lactobacillus* was reclassified [39]; however, the probiotic effects of strains previously assigned to *Lactobacillus* have been widely reported [40–42]. Some “reclassified *Lactobacillus*” strains are known to improve the bioavailability of macro- and micronutrients for the host [43], degrade gluten and lactose to reduce and even solve digestive problems related to gluten or lactose maldigestion and intolerance [44,45], produce vitamins necessary for the host (e.g., vitamins B2, B9, and B12) [46], and reduce gastrointestinal inflammation caused by pathogens [47]. LABs are also able to produce exopolysaccharides (EPS) which have beneficial effects on human health [48]. London et al. previously demonstrated the potential of EPS-producing “reclassified *Lactobacillus*” strains in therapies against hypercholesterolemia [49]. EPS-producing lactobacilli have also produced positive effects on lipid metabolism by decreasing serum triglycerides, and on total serum and liver cholesterol in mice fed with a high-fat/high-cholesterol diet [50,51]. In addition, Gunness and Gidley described how soluble dietary fibers also decrease plasma cholesterol levels via three different biological mechanisms [52]. Apart from those on lactobacilli, there are a few reports about the effectiveness of enterococcal strains as probiotics. How *Enterococcus* strains contribute to the maintenance of a healthy intestinal microbiota and to the stimulation of the immune system has been reported [53], while another study showed the potential probiotic properties of *Enterococcus faecium* via its efficacy in reducing the recovery period after acute diarrhea [54]. Interestingly, *Enterococcus* was recently also shown to have a potential contribution in reducing cholesterol levels [55,56], equal to that of *Lactobacillus* [57,58]. The cholesterol-lowering effect of some bacteria is mainly based on their bile salt hydrolase (BSH) activity [59]; *Enterococcus faecium* and *Enterococcus faecalis* strains have shown their BSH-activity in 50% and 81% of tested strains, respectively [60]. On the other hand, it is important to underline that some *Enterococcus* strains are also known to be opportunistic pathogens; indeed, they are one of the main causes of nosocomial infections [61]. Their pathogenicity derives from their antibiotic-resistant genes, often even extending to multiple antibiotic resistances; furthermore, these genes are also encoded by transferable genetic elements [62].

Interestingly, in contrast with the above-mentioned reduction in the H' and S indices linked to microbiota metabolism, we ob-

served increases in various VOCs. Fecal VOC analysis showed increased levels of 1-butanol and acetone, both metabolites that could derive from acetone-butanol-ethanol fermentation, previously associated with LABs strains [63,64]. LABs have also shown their ability to metabolize methionine in sulfur compounds, including dimethyl disulfide [65].

Other volatile compounds, i.e., D-limonene and alpha-pinene, being essential oils, could be compounds of the ancient wheat mix that we used, contained in the aleuronic layer of the unrefined *Triticum durum* flours. D-limonene has previously been detected in the leaves of *Nigella sativa* L. (black cumin) [66] and in of? *Citrus* plants such as orange, lemon, and grapefruit [67]. D-limonene has actually been used to prevent gastric diseases [68]. It is also suggested that D-limonene exerts antiproliferative effects in various cancer cell types [69]. Alpha-pinene, instead, has been found in the leaves of Chia (*Salvia hispanica*) [70], which is assuming growing importance following the (re)discovery of the positive effects that it has shown on human health [71].

On the other hand, the increased level of indole could be the result of a combination of bacterial and nutritional factors. Indole is a bacterial metabolite derived from tryptophan (Trp) metabolism [72] and animal cells cannot produce Trp. Therefore, humans rely on exogenous sources, obtained through the diet [73]. Trp can be found in various foods, such as cereals, meat, fish and fish products, legumes, seeds, nuts, milk and dairy products, and chocolate [74]. Despite the reduced utilization of protein-derived substrates by microbes, we observed a significant increase in indole levels. No evidence of differences in Trp-levels between ancient and modern wheats has been reported. Meanwhile, some lactobacilli, which encode indole-forming enzymes [75] and are able to ferment aromatic amino acids in the colon [76], could be directly responsible for the increased indole levels. Interestingly, it was recently reported that decreases in indole concentrations in the gut promote bacterial pathogenesis; by contrast, indole decreases virulence gene expression both in *Clostridium rodentium* and enterohemorrhagic *Escherichia coli* [77]. Hence, the latter findings could provide further evidence as to how intestinal microbes and their metabolites can play a direct role in health and disease.

However, the limitations of this study must be underlined. Firstly, we did not randomize the study population to receive ancient whole-meal wheat or modern wheat during the two periods: in fact, all the nuns received the modern variety during the first period and the ancient mix during the second period. This choice was made to simplify the preparation of meals and to avoid errors in administering them, but this opened up the possibility that the observed positive effects on the metabolic parameters and fecal microbiota may have been due to unknown factors other than the diet. Second, we administered whole-meal ancient flour versus refined modern flour; this made it impossible to distinguish whether the benefits were associated with the use of whole-meal flour rather than being a real advantage of the ancient wheat varieties. Future studies need to better define the relative role of ancient and whole-meal wheat flour in improving metabolic parameters and fecal microbiota. Third, our study population was composed exclusively of females, therefore the results of this study may not apply to male populations.

## 5. Conclusions

Our data showed the beneficial effects deriving from a diet based on the consumption of ancient wheat varieties, in the form of whole-meal/unrefined flours. Although further studies need to determine the respective advantages of consuming ancient wheat varieties and whole-meal/unrefined flours, we can affirm that this “ancient diet” produces beneficial effects not only on human metabolism but also on microbiota.



597 **Declaration of Competing Interest**

598 The authors declare no conflict of interest.

599 **CRedit authorship contribution statement**

600 **Antonio Carroccio**: Conceptualization, Methodology, Resources,  
601 Data curation, Writing – original draft, Writing – review & editing,  
602 Funding acquisition. **Giuseppe Celano**: Formal analysis. **Carmelo**  
603 **Cottone**: Conceptualization, Investigation, Resources. **Giuseppe Di**  
604 **Sclafani**: Conceptualization, Investigation. **Lucia Vannini**: Formal  
605 analysis. **Alberto D'Alcamo**: Investigation. **Francesco Maria Cal-**  
606 **abrese**: Formal analysis. **Pasquale Mansueto**: Investigation, Data  
607 curation, Writing – original draft, Writing – review & editing, Fund-  
608 ing acquisition. **Maurizio Soresi**: Formal analysis. **Ruggiero Fran-**  
609 **cavilla**: Formal analysis, Writing – original draft. **Maria De Angelis**:  
610 Methodology, Formal analysis, Resources, Data curation, Writing –  
611 original draft, Writing – review & editing, Funding acquisition.

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618 **Supplementary materials**

619 Supplementary material associated with this article can be  
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