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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.

1. Introduction

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

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

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medical literature has increasingly underlined the paramount relevance of the role of human intestinal microbiota in maintaining good health. There is growing evidence that the mutually beneficial interactions with the microbes that comprise our commensal microbiota might have been perturbed by environmental interventions, including changes in eating habits, *i.e.* the widespread consumption of a high-fat/low-fiber diet in adults, or changes in formula feeding in infants [3].

On the basis of the above considerations, in this study we evaluated the effect of a change in dietary habits, as regards the kind of wheat-based foods consumed, in a "strictly controlled population" of cloistered nuns. The nuns received a daily amount of modern refined wheat for 30 days and then a daily amount of a mix of whole-meal ancient wheats. The aims of the study were to observe any changes in hematochemical parameters and to evaluate any microbiota alterations before and after a regular diet containing a whole-meal ancient wheat mix.

2. Materials and methods

2.1. Subjects and diets

Twenty-nine cloistered nuns of the "Congregazione delle Suore Collegine della Santa Famiglia" (Congregation of the School Sisters of the Holy Family) in Palermo, Italy, consented to enter the study, which was performed between October 2017 and January 2018. They were all females with a mean age (± Standard Deviation, SD) of 53.9 ± 20.9 years (range 26–90 years). Supplemental File 1 summarizes the demographic data (age), the individual measurements (Body Mass Index, BMI, and waist circumference), the comorbidities and any drug treatments of these subjects. None of the study subjects smoked or consumed alcohol.

Supplemental File 2 shows the study design. The study comprised two 30-day periods, separated by a 2-week washout period. During the first (T1), the nuns received wheat-based foods produced with refined flour from "*Simeto*" wheat (used as a modern cultivar). During the wash-out they returned to their usual diet, including wheat-based foods of uncontrolled origin. During the second period (T2) they received wheat-based foods produced with an unrefined flour mix composed in equal percentages of "*Timilia*", "*Margherito*", and "*Russello*" (three ancient cultivars, historically produced in Sicily, Italy). Supplemental File 3 presents the gross composition of the flours used in the present study. The menu varied from day to day, but the basic diet remained identical during the two 30-day periods of the study (T1 and T2): the two study diets showed no differences in terms of energy intake (kcal/die) or in the other nutritional values (Supplemental File 4) except for fiber intake, which was approximately 3.4 g/die higher in the T2 diet. The nuns received a fixed daily quantity of wheat-based foods and they recorded in a diary whether these foods were completely consumed or not. Three of the Authors (AD, CC, and GDS) met the nuns before the beginning of the study to explain its aims and to ensure adherence to the diet and to the study design; the same Authors met the nuns on a weekly basis during the entire study period to collect clinical data and clarify any doubts of the participants.

The study was recorded at the Clinicaltrials.gov (registration number NCT03020511 "Effects of Ancient Grains-based Diet in a Closed Community") and approved by the Ethics Committee of the University of Palermo after ascertaining its compliance with the standards dictated by the Declaration of Helsinki (IV Adaptation).

2.2. Hematochemical analysis

Venous blood samples were taken, after overnight fasting, at entry to the study (T0), after the first 30-day period on refined modern wheat (T1) and after the second period on a whole-meal ancient wheat mix (T2). The following parameters were assayed: white blood cell count, hemoglobin, serum iron, ferritin, glycemia, creatinine, sodium, potassium, magnesium, calcium, phosphorus, aspartate aminotransferase, alanine aminotransferase, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, protein electrophoresis, vitamin D, vitamin B12, folic acid, and glycated hemoglobin. Furthermore, at the same times, BMI and waist circumferences were recorded.

2.3. Collection of fecal samples

Each volunteer fasted overnight, and fecal samples were collected pre-prandially the following morning after the first 30-day period on refined modern wheat (T1) and after the second period on a mix of whole-meal ancient wheats (T2). After collection, samples were immediately mixed with Amies transport medium (Oxoid Ltd, Basingstoke, Hampshire, England) (*ca.* 15 g, 1:1 wt/wt), under anaerobic conditions (AnaeroGen, Oxoid Ltd, Basingstoke, Hampshire, England) and stored at −80 °C for further metabolic analyses. Samples diluted with Amies transport medium were also immediately analyzed using plate counts and the Biolog-system.

2.4. Enumeration of cultivable bacteria

Fecal samples (5 g) were mixed with 45 ml sterilized physiological solution and homogenized. Viable bacterial cells were counted as described by De Angelis et al. [4]. The following selective media were used: Plate count agar (total anaerobes); MRS agar (*Enterococcus*, lactobacilli and *Leuconostoc*); Slanetz and Bartley (*Enterococcus*); Rogosa agar *plus* 1.32 mL/L of glacial acetic acid (lactobacilli); M17 (*Lactococcus* and *Streptococcus*); Baird Parker (*Staphylococcus*); Wilkins-Chalgren anaerobe agar *plus* GN selective supplements and defibrinated sheep blood (*Bacteroides*, *Porphyromonas* and *Prevotella*); MacConkey agar No.2 (*Enterobacteriaceae*); Chromocult (Merk, Darmstadt, Germany, Europe) (total coliform); GSP agar (Sigma-Aldrich, St. Louis, MO, USA) *plus* penicillin-G (60 g/L) (*Pseudomonas* and *Aeromonas*); *Bifidobacterium* agar modified (Becton Dickinson, Le Pont de Claix, SA, France) (*Bifidobacterium*). Except for Chromocult, GSP agar, and *Bifidobacterium* Agar Modified, all media were purchased from Oxoid Ltd. (Basingstoke, Hampshire, England).

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

Total bacterial DNA was isolated from frozen stool samples using the Fast DNA™ SPIN Kit for Soil (MP Kit, MP Biomedicals, USA), according to the manufacturer's instructions. The 16S ribosomal RNA (rRNA) metagenetic analysis was carried out at Genomix4Life (spin-off of the University of Salerno, Italy) using the Illumina MiSeq platform. The V3-V4 regions of the 16S rRNA gene were amplified to analyze diversity inside the domains of Bacteria [5]. PCR and sequencing analyses were carried out according to the Genomix4Life protocol. Quality control and taxonomic assignments were performed according to the QIIME and the Ribosomal Database Project Bayesian classifier in combination with a set of custom-designed computerized pipelines implemented by Genomix4Life to analyze the microbial communities. Taxonomic attribution was carried out using a BLAST search in the NCBI 16S rRNA sequences database [6]. The percentage of each bacterial Operational Taxonomic Unit (OTU) was analyzed individually per sample, providing relative abundance information based on the numbers of reads per sample. Alpha diversity, analyzed by considering the number of observed OTUs and the Shannon diversity index, was calculated using QIIME [7]. Differences in microbial com-

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

2.6. Community-level catabolic profiles

Carbon source utilization patterns of the fecal microbiota were assessed in triplicate using Biolog 96-well Eco micro-plates (Biolog, Inc., Hayward, CA, USA) [8]. Micro-plates contained 31 different carbon sources (carbohydrates, carboxylic acids, polymers, amino acids, amines, and miscellaneous substrates). Five grams of feces diluted with Amies transport medium (1:1) were homogenized in a bag filter with 45 mL of sterile sodium chloride [0.9% (w/v)] solution (Classic Mixer) to remove the solid particulate of the feces. The homogenized feces were centrifuged at 11,000 rpm for 15 min at 4 °C. The pellet was first washed with 50 mM Tris–HCl (pH 7.0), then with sterile sodium chloride [0.9% (w/v)] solution, and centrifuged at 11,000 rpm for 15 min at 4 °C. The cell suspension was diluted (1:10) into the sterile sodium chloride [0.9% (w/v)] solution and subsequently centrifuged at 2000 rpm for 2 min at 4 °C. The cell suspension was then diluted (1:20) into sterile chloride [0.9% (w/v)] solution and dispensed (150 µL) into each of the 96 wells of the Biolog Eco micro-plates. The micro-plates were incubated at 30 °C in the dark on a slow-speed stirrer, and color development was measured at 590 nm every 24 h with a micro-plate reader (Biolog Microstation). Three indices were determined [9]. Shannon's diversity (H'), indicating the substrate utilization pattern, was calculated as follows: H'=-$\Sigma\ \pi \ln$ (pi), where π is the ratio of the activity of a particular substrate to the sums of activities of all substrates at 120 h; Substrate richness (S), measuring the number of different substrates used, was calculated as the number of wells with a corrected absorbance greater than 0.25; Substrate evenness (E) was defined as the equitability of activities across all utilized substrates: $E = H'/\log S$.

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

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was carried out to predict microbiota-associated biochemical pathways of gut microbiota from fecal noun samples. In detail, 16S rRNA bacteria gene sequences were the starting point for the prediction of metabolic functions. First, a BIOM-formated OTU table was generated using the make.biom command of the Mothur program based on a Greengenes database (May 2013 ver.; http://greengenes.lbl.gov). The abundance of each OTU was corrected to reflect the true bacterial abundance by normalizing the 16S rRNA copy number for each OTU. KEGG ortholog abundances for a given OTU, table-picked against the newest version of the Greengenes database, were calculated by locally running the PICRUSt "predict_metagenomes.py" script. The gene functions classified by KO were further categorized into KEGG pathways using the "categorize by function.py" PICRUSt script, which collapses thousands of predicted functions into higher categories (KEGG pathways). The enrichment of predicted KEGG pathways found in the T1 and T2 noun fecal samples (modern and ancient wheat-based diets, respectively) was assessed with STAMP software83 using a two-sided Welch's *t*-test corrected by a Benjamini-Hochberg procedure ($P < 0.05$).

2.8. Analysis of fecal volatile compounds and free amino acids

After preconditioning according to the manufacturer's instructions, a polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (65 μm) and a manual solid phase micro-extraction (SPME) holder (Supelco Inc., Bellefonte, PA, USA) were used. Before headspace 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 and10 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 equilibration 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) analyses 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 (ionization 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, interface 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 splitless mode, with helium (flow rate, 1 mL/min) as the carrier gas. Molecules were identified based on the comparison of their retention times with those of pure compounds (Sigma-Aldrich, Milan, 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 Chemstation (Agilent Technologies, USA) and subsequently processed by the XCMS toolbox (http://metlin.scripps.edu/download/). XCMS software allows automatic and simultaneous retention time alignment, matched filtration, peak detection, and peak matching. GC-MS/SPME data were organized into matrices for subsequent statistical analysis. Total and individual FAAs from the water-soluble extracts were determined by a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, UK) as described by De Angelis et al. [4].

2.9. Sample size

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

2.10. Statistical analysis

For the hematochemical parameters, continuous variables were described as mean ± SD if the distribution was normal, otherwise as the median and interquartile range. Differences between continuous variables were assessed by paired *t*-test if the distribution was Gaussian; otherwise, the Wilcoxon signed-rank-test was used.

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

3. Results

All the nuns fully adhered to the diet during the two 30-day periods and no changes in the kind and quantity of wheat-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*
White Blood Cells (x mmc)	6137 ± 2471	6138 ± 2371	5783 ± 1771	T0 *vs* T1 NS T1 *vs* T2 NS
Hemoglobin (gr/dL)	12.29 ± 1.22	12.28 ± 1.21	12.25 ± 1.41	T0 *vs* T1 NS T1 *vs* T2 NS
Serum iron (mcg/dL)	66.5 ± 27.2	69.5 ± 28.1	50.2 ± 23.1	T0 *vs* T1 NS T1 *vs* T2 < 0.02
Ferritin (ng/mL)	89 ± 202	99 ± 222	76 ± 179	T0 *vs* T1 NS T1 *vs* T2 < 0.04
Glycemia (mg/dL)	84.6 ± 8.5	86.1 ± 9.6	88.2 ± 7.3	T0 *vs* T1 NS T1 *vs* T2 NS
Creatininine (mg/dL)	0.87 ± 0.16	0.91 ± 0.16	0.75 ± 0.18	T0 *vs* T1 NS T1 *vs* T2 <0.0001
Sodium (mEq/L)	139.4 ± 2.2	141 ± 3.2	135.2 ± 2.0	T0 *vs* T1 NS T1 *vs* T2 < 0.0001
Potassium (mEq/L)	4.47 ± 0.25	4.56 ± 0.26	4.35 ± 0.34	T0 *vs* T1 NS T1 *vs* T2 < 0.03
Magnesium (mg/dl)	2.16 ± 0.12	2.19 ± 0.13	2.08 ± 0.17	T0 *vs* T1 NS T1 *vs* T2 < 0.02
Calcium (mg/dL)	9.11 ± 0.34	9.12 ± 0.33	9.28 ± 0.38	T0 *vs* T1 NS T1 *vs* T2 < 0.02
Phosphorus (mg/dL)	3.42 ± 0.42	3.40 ± 0.51	3.61 ± 0.39	T0 *vs* T1 NS T1 *vs* T2 < 0.02
Aspartate aminotransferase (U/L)	18.2 ± 4.6	18.3 ± 4.5	20 ± 7.5	T0 *vs* T1 NS T1 *vs* T2 NS
Alanine aminotransferase (U/L)	13.4 ± 5.1	14.9 ± 5.3	16.1 ± 11.0	T0 *vs* T1 NS T1 *vs* T2 NS
Total cholesterol (mg/dL)	199.7 ± 38.6	201.6 ± 37.6	177.7 ± 29.3	T0 *vs* T1 NS T1 *vs* T2 < 0.0001
LDL-cholesterol (mg/dL)	108.5 ± 28.5	106.4 ± 27.4	93.2 ± 23.7	T0 *vs* T1 NS T1 *vs* T2 < 0.0001
HDL-cholesterol (mg/dL)	75.3 ± 18.9	76.3 ± 17.9	69.9 ± 16.9	T0 *vs* T1 NS T1 *vs* T2 < 0.0001
Tryglicerides (mg/dL)	73.3 ± 37.9	77.1 ± 38.9	71.1 ± 36.2	T0 *vs* T1 NS T1 *vs* T2 NS
Vitamin D (ng/mL)	9.5 ± 12.6	9.9 ± 11.6	9.7 ± 12.7	T0 *vs* T1 NS T1 *vs* T2 NS
Vitamin B12 (pg/mL)	439.8 ± 174.2	449.9 ± 182.2	427.3 ± 177.5	T0 *vs* T1 NS T1 *vs* T2 NS
Folic acid (ng/mL)	9.5 ± 3.9	9.7 ± 4.1	8.6 ± 2.1	T0 *vs* T1 NS T1 *vs* T2 < 0.05
Glycated Hemoglobin (%)	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).

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

3.1. Hematochemical parameters

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

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

Fig. 1 shows the viable cell counts (colony-forming unit, CFU, Log10/g) of the main microbial groups found in the fecal samples at T1 and T2. Compared to T1, the diet containing ancient grain products had an increased abundance of culturable enterococci, lactic acid bacteria (LABs) and total anaerobes ($P < 0.05$). No statistical differences between T1 and T2 amounts were observed for *Bacteroides, Porphyromonas* and *Prevotella*, *Bifidobacterium*, *Enterobacteria*, *Pseudomonas* and *Aeromonas, Staphylococcus*, *Lactococcus* or *Streptococcus*.

3.3. Metagenetic analysis of the 16S rRNA genes

Total bacterial DNA from fecal samples of the enrolled cloistered nuns was analyzed by sequencing the 16S rRNA gene amplicons, resulting in 98,399.25 ± 34,472.14 (mean ± SD) reads per sample, of which 89.54% ± 7.4% were assigned to at least genus level. Comparing T1 *vs* T2, no statistical differences were observed in the number of OTUs and Shannon species diversity index. Moreover, no differences were detected at the high taxonomic levels, specifically phyla (Fig. 2), families (Fig. 3a and b), and genera with a mean relative abundance greater than 0.1%. The main differences between the fecal microbiota of the T1 and T2 samples were detected at species level. Among the OTUs with a mean value of relative abundance greater than 0.1% at least for one diet, *Blautia wexlerae* (T1: 1.87%, T2: 4.01%; $P=0.02$), *Collinsella tanakaei* (T1: 0.09%, T2: 0.16%; $P=0.04$), *Atopobium fossor* (T1: 0.48%, T2: 0.75%; $P=0.041$) and *Slackia piriformis* (T1: 0.07%, T2: 0.14%; $P=0.041$)

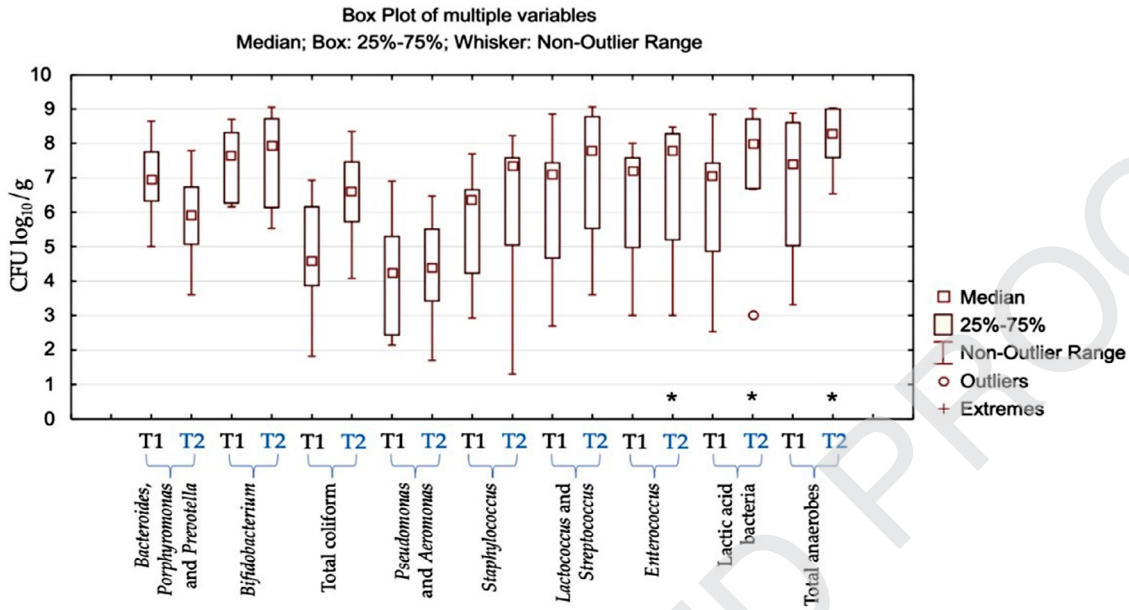

Fig. 1. Counts of viable cells (CFU Log10/g) of the bacterial groups found in fecal samples of healthy females after 30 days of a diet with modern refined wheat products (T1) and after 30 days of a diet with ancient whole-meal wheat products (T2). (*P-value < 0.05; Student's t-test).

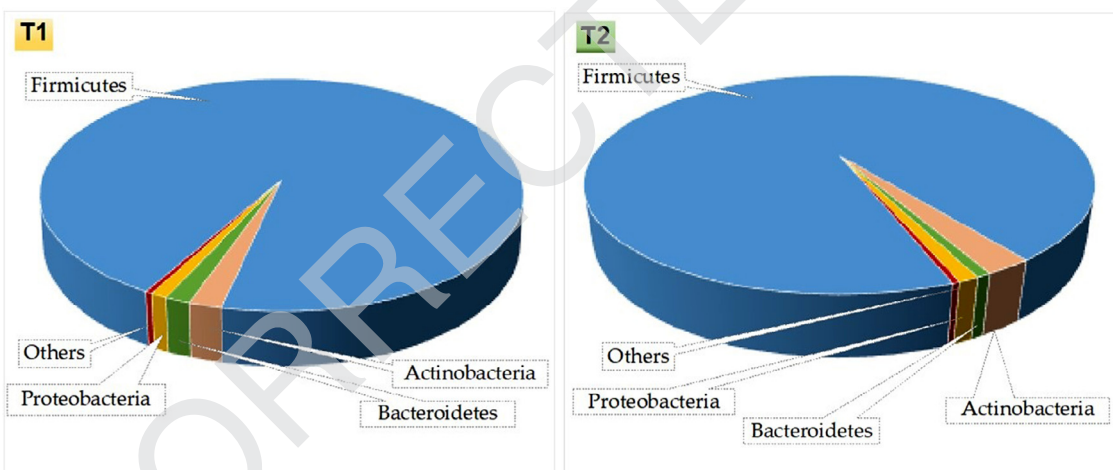

Fig. 2. Relative abundances (%) of total bacteria (16S rRNA gene sequences) found in fecal samples of healthy females after 30 days of a diet with modern refined wheat products (T1) and after 30 days of a diet with ancient whole-meal wheat products (T2).

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

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

The H' index and the S index values of the fecal microbiome were calculated (Supplemental File 5). Compared to T1, the diet containing ancient grain products produced a reduction in the H' and S indices of the fecal microbiome in the 28 healthy subjects. The E index confirmed the above-described significant differences ($P < 0.05$). Carbohydrates and amino acids, followed by carboxylic acids, were the organic compounds mainly utilized in all samples. An opposite trend was detected between carbohydrate and amino acid utilization before and after the diet containing ancient grain products. Indeed, the ability of the gut microbiota to metabolize carbohydrates increased after the diet containing ancient grain products ($P < 0.05$). By contrast, the metabolism of carboxylic acids and especially of amino acids decreased. No statistical differences were found in the metabolism of polymers and amines.

Moreover, in order to investigate how both diets influenced the microbial metabolic pathways we performed a Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis. Few significant differences were found (Supplemental File 6). Specifically, there was a significant increase in the metabolism of fructose and mannose ($P = 0.037$), of C5-branched dibasic acids ($P = 0.044$), and toluene ($P = 0.045$) after the diet with ancient grains (T2). On the other hand, no significant increases occurred after the diet with the modern wheat variety (T1).

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

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

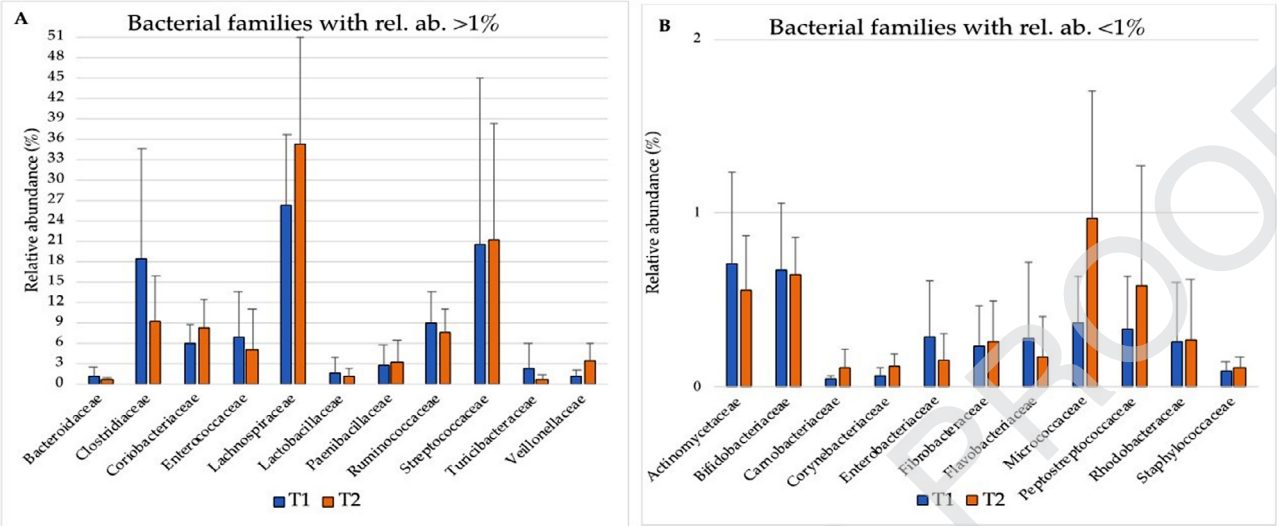

Fig. 3. Bacterial families (16S rRNA gene sequences) with a relative abundance > 0.1% (panel *A*) and with a relative abundance < 0.1% (panel B) found in fecal samples of healthy females after 30 days of a diet with modern refined wheat products (T1) and after 30 days of a diet with ancient whole-meal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

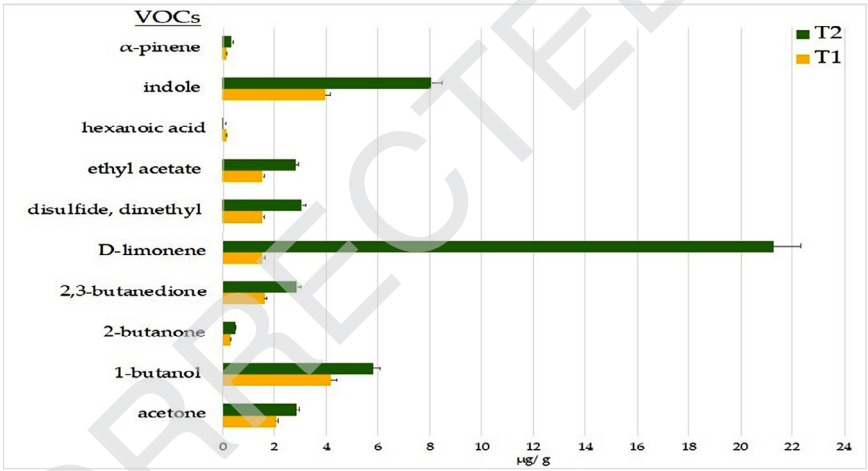

Fig. 4. Concentrations of the statistically different (P-value < 0.05; Student's *t*-test) volatile organic compounds (VOCs) found in fecal samples of healthy females after 30 days of a diet with modern refined wheat products (T1) and after 30 days of a diet with ancient whole-meal wheat products (T2).

8.03 µg/g at T1 and T2, respectively, $P = 0.012$), D-limonene (1.53 and 21.26 µg/g, $P = 0.005$), 1-butanol (4.17 and 5.80 µg/g, $P = 0.007$), dimethyl disulfide (1.52 and 3.05 µg/g, $P = 0.022$), alpha-pinene (0.10 and 0.33 µg/g, $P = 0.031$), ethyl acetate (1.52 and 2.78 µg/g, $P = 0.009$), 2-butanone (0.25 and 0.45 µg/g, $P = 0.030$), and acetone (2.04 and 2.82 µg/g, $P = 0.040$). On the contrary, hexanoic acid decreased after the consumption of ancient grain products (0.11 and 0.04 µg/g, $P = 0.047$).

Total free amino acids (FAAs) were lower in the samples of subjects after the diet enriched with ancient grain products (T1: 11.48 and T2: 8.796 µg/g; $P = 0.013$). In detail, Asp, Thr, Ser, Met, Ile, Tyr, Orn, and Arg-were found at lower concentrations (Fig. 5). Free ammonia was also lower (T1: 0.271 and T2: 0.176 µg/g; $P = 0.004$).

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

Correlation analysis ($r > 0.7$; false discovery rate, FDR, <0.05; Supplemental File 7) showed a marked positive correlation between HDL-cholesterol levels and many culturable bacteria, except for total coliforms, and also for compounds included in "cluster B". However, 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 phosphorus. Interestingly, indole and dimethyl disulfide shared the positive 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 pressure control have previously been related to a number of bioactive peptides, which may already be present in foods as natural components or derive from the hydrolysis of proteins by chemical and enzymatic treatments (*e.g.*, digestion, fermentation) [12]. Hence, in a growing number of studies, foods and food components potentially active in reducing the risk of cardiovascular disease (CVD) have been investigated [13]. Several studies have eval-

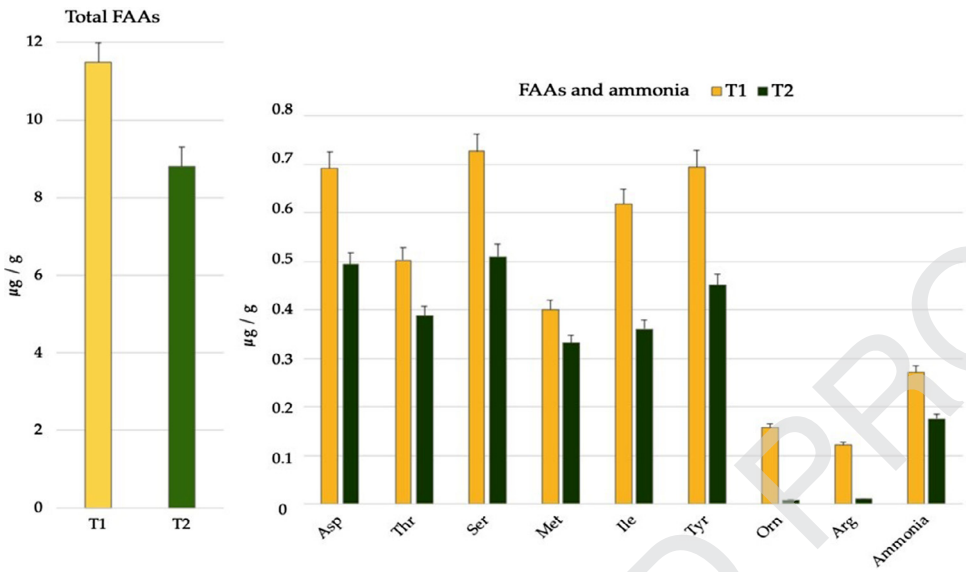

Fig. 5. Concentrations of total free amino acids (FAAs) and statistically different (P-value < 0.05; Student's *t*-test) FAAs and ammonia found in fecal samples of healthy females after 30 days of a diet with modern refined wheat products (T1) and after 30 days of a diet with ancient whole-meal wheat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

Declaration of Competing Interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Antonio Carroccio: Conceptualization, Methodology, Resources, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition. **Giuseppe Celano:** Formal analysis. **Carmelo Cottone:** Conceptualization, Investigation, Resources. **Giuseppe Di Sclafani:** Conceptualization, Investigation. **Lucia Vannini:** Formal analysis. **Alberto D'Alcamo:** Investigation. **Francesco Maria Calabrese:** Formal analysis. **Pasquale Mansueto:** Investigation, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition. **Maurizio Soresi:** Formal analysis. **Ruggiero Francavilla:** Formal analysis, Writing – original draft. **Maria De Angelis:** Methodology, Formal analysis, Resources, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.dld.2021.04.026.

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