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Effect of whole-grain barley on the human fecal microbiota and metabolome

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1	The role of whole-grain barley on human fecal microbiota and metabolome
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21 ABSTRACT

This study aimed at comparing the fecal microbiota and metabolome of 26 healthy subjects before 22 23 (HS) and after (HSB) two months of diet intervention based on the administration of durum wheat flour and whole-grain barley Pasta containing the minimum recommended daily intake (3 g) of 24 barley β -glucans. Metabolically active bacteria were analyzed through pyrosequencing of the gene 25 16S rRNA and community-level catabolic profiles. Pyrosequencing data showed that 26 Clostridiaceae (Clostridium orbiscindens, Clostridium sp.), Roseburia hominis, and Ruminococcus 27 sp. increased while other Firmicutes and Fusobacteria decreased in HSB compared to HS fecal 28 samples. Community-level catabolic profiles were the lowest in HSB. Compared to HS, cultivable 29 lactobacilli increased in HSB fecal samples while Bacteroides, Porphyromonas and Prevotella, 30 Enterobacteriaceae, total coliforms, and Pseudomonas, Alcaligenes and Aeromonas decreased. 31 Metabolome analyses were performed using amino acid analyzer and gas-chromatography mass 32 33 spectrometry-solid-phase micro-extraction. A marked increase of short chain fatty acids (SCFA) 34 such as 2-methyl-propanoic acid, acetic, butyric and propionic acids was found in HSB with respect to HS fecal samples. Durum wheat flour and whole-grain barley Pasta containing 3% of barley β -35 glucans appeared to be effective in the modulation of the composition and the metabolic pathways 36 37 of the intestinal microbiota, leading to an increased level of SCFA.

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39 INTRODUCTION

40 Whole-grain barley and oat, and some dry-milled bran grain products were authorized to be used 41 according to the health claim "decrease the risk of coronary heart disease" by Food and Drug Administration (1, 2). Whole barley and oat flours contain β -glucans which are soluble dietary 42 fibers. European Food Safety Authority recognized that the "regular consumption of oat β -glucans 43 can actively lower/reduce blood LDL-cholesterol and total cholesterol (3). In addition to reduction 44 45 of cholesterol, the positive associations between the consumption of β -glucans and the reduction/prevention of cardiovascular diseases, and reduction of glycemia, insulin resistance and 46 metabolic syndrome were well documented (4, 5). The minimum dose of 3 g/day of β -glucans is 47 recommended to get positive effects on human health (1, 3, 6). 48

49 Several mechanisms were described for explaining the hypocholesterolemic effect of β -glucans: (i) increased viscosity at the level of the small intestine and, consequently, slowed gastric emptying, 50 digestion, and absorption of molecules, including glucose, dietary cholesterol and bile acids (7, 8); 51 52 (ii) decreased enter-hepatic bile acids by binding at intestinal level with subsequent increased use of cholesterol for bile acids synthesis (9, 10); (iii) reduced synthesis of hepatic cholesterol due to the 53 54 improved insulin sensitivity (10, 11); and iv) inhibited hepatic synthesis of cholesterol by acetate 55 and propionate, which are produced by colonic fermentation of β -glucans (12, 13). Saccharolytic and proteolytic fermentations are the major fermentation processes, which are carried-out by 56 metabolically active microbes at the colon level (14). Saccharolytic fermentation was associated 57 with the synthesis of short-chain fatty acids (SCFA) (acetate, propionate and butyrate), intermediate 58 59 metabolites such as succinate, acrylate, lactate, formate and ethanol, and small final molecules 60 (hydrogen, methane and carbon dioxide) (15). Proteolytic fermentation was associated with the synthesis of SCFA (acetate, propionate and butyrate) and branched-chain fatty acid (BCFA) (iso-61 butyric, iso-valeric and 2-methylbutyric acids), free amino acids (FAA) and some potentially toxic 62 metabolites (phenols, indoles, ammonia and amines) (14, 16, 17). The type of colonic fermentation 63 64 is mainly depends on the type of microbiota and fermentable substrate availability (17). Based on 3

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the key role for human healthy, the intestinal microbiota was acknowledged as a metabolic organ 65 (18). The major part of the current research on novel functional foods is moved towards the 66 selection and characterization of prebiotics (e.g. inulin, fructo-oligosaccharides and galacto-67 oligosaccharides), which are not digested by human gastrointestinal enzymes but selectively 68 69 stimulate the growth and/or activity of GRAS bacteria that may improve host health (19). Indeed, a 70 prebiotic effect of β -glucans towards the intestinal microbiota was also hypothesized (5). In 71 particular, the positive effect of β -glucans on the growth of beneficial intestinal lactobacilli and bifidobacteria was shown by in vitro studies (20, 21) and animal experiments (22, 23). 72 Nevertheless, other studies that also used animal models did not show significant effects of β -73 glucans on lactobacilli and/or bifidobacteria (24, 25). Unfortunately, human clinical challenges that 74 have dealt with the prebiotic effect of β -glucans are rather scarce. Barley β -glucans increased the 75 cell density of colonic bifidobacteria on older healthy subjects (26). A pilot study with 76 77 polypectomized patients showed no significant effect of β -glucans on the fecal microbiota and the concentration of SCFA (27). The evidence that β -glucans positively influence the human intestinal 78 79 microbiota is still insufficient or difficult to interpret, and additional studies are needed to fill this 80 gap (5).

This study compared the fecal microbiota and metabolome of healthy subjects before (HS) and after (HSB) two months of daily administration of durum wheat flour and whole-grain barley Pasta containing the minimum recommended intake (3 g) of β -glucans. The fecal microbiota was characterized through integrated approaches, which were based on culture-independent and dependent methods.

86 MATERIALS AND METHODS

87 Subjects

The study was carried out in accordance with the Helsinki Declaration (IV Adaptation) and European Guidelines for Good Clinical Practice. The protocol of the study was approved by the Applied and Environmental Microbiology Institutional Review Board of the Azienda Ospedaliero-Universitaria Consorziale Policlinico of Bari, Italy (Authorization nr. 1570/2014). Written consents were obtained from all volunteers. One groups of Caucasian HS (15 female and 11 male), aged between 28 and 57 years, were enrolled in the study (Table 1). Exclusion criteria were history of gastro-intestinal disease, diabetes, cardiovascular diseases, hyperlipidemia and consume of alcohol. Volunteers were not treated with antibiotics and/or functional foods (probiotics and/or prebiotics) for at least three months before recruitment and sampling.

97 Feeding regime

Pasta Granoro Cuore Mio (Granoro srl, Corato, BA) was used in this study. Pasta was made by 98 99 using a mixture of durum wheat flour (75%) and whole-grain barley flour (25%). The gross composition of Pasta Granoro Cuore Mio was as follows: moisture, 11%; protein (N x 5.70), 11% 100 101 of dry matter; carbohydrate, 69% of dry matter; fat, 2% of dry matter; total fibres and β -glucans, 7% 102 and 3% of dry matter, respectively. Before starting with the administration of 100 g Pasta Granoro 103 Cuore Mio Pasta containing 3 g of barley β -glucans (Granoro srl), each volunteer was instructed to 104 follow the usual diet, including 100 g of Pasta every day for two months. Before and after the 105 dietary treatment, a food frequency questionnaire and a 24-h recall questionnaire were administered to each volunteer by a dietitian. These two tools allowed to extrapolate the "weekly cumulative 106 107 frequency of food" and the "component intake" data, respectively (Table 1), by using official Italian 108 food composition databases (INRAN, IEO, 109 http://nut.entecra.it/646/tabelle di composizione degli alimenti.html and http://www.bdaieo.it/uk/index.aspx). All volunteers confirmed that not remarkable changes occurred in meals and 110 medication during the two months of treatment. Additionally, a questionnaire aimed at evaluating 111 intestinal effects (bloating, meteorism, constipation, satiety, diarrhea, reflux and flatulence) was 112 113 administered (26). Gastrointestinal symptoms were graded from 0 (no symptoms) to 3 (severe 114 symptoms) (26). Before the treatment with durum wheat and whole-grain barley Pasta, 115 colesterolemia, body mass index, fecal microbiota and metabolome were determined.

116 Determination of blood cholesterol

The level of total cholesterol was measured on the Siemens Dimension RxL Max by using the Siemens enzymatic methods (Siemens Medical Solution Diagnostics, Tarrytown, NY). LDL- and HDL-cholesterol level was estimated by using the Friedewald equation (28) for value below 300 mg/dl.

121 Collection of fecal samples

Each volunteer had fasted overnight, and fecal sample was collected in the morning pre-prandial. 122 Fecal samples were collected on three different days of the same week. After collection, samples 123 were immediately mixed with RNA later (Sigma-Aldrich, St. Louis, MO, USA) (ca. 5 g, 1:2 wt/vol) 124 or Amies Transport medium (Oxoid LTD, Basingstoke, Hampshire, England) (ca. 15 g, 1:1 wt/wt), 125 under anaerobic conditions (AnaeroGen, Oxoid LTD, Basingstoke, Hampshire, England). Fecal 126 samples suspended in RNA later were stored at -80°C for further RNA and metabolomic analyses. 127 128 Samples diluted with Amies Transport medium were immediately subjected to plate counts and analysis by Biolog system. 129

130 RNA extraction from fecal samples

An aliquot of ca. 200 mg of fecal sample diluted in RNA later was used for RNA extraction with the Stool total RNA purification kit (Norgen Biotek Corp., Ontario, Canada, USA). Quality and concentration of RNA extracts were determined using 1% agarose-0.5X TBE gels and spectrophotometric measurements at 260, 280 and 230 nm through the NanoDrop ND-1000 Spectrophotometer. Total RNA extracted (ca. 2.5 µg) was transcribed to cDNA using random examers and the Tetro cDNA synthesis kit from Bioline (Bioline USA Inc, Tanunton, MA, USA), according to the manufacturer's instructions (29).

138 Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) and data analyses

For each volunteer, the three cDNA samples were pooled and used for bTEFAP analysis. Pooled samples cDNA were analyzed for each subject. bTEFAP was performed by Research and Testing Laboratories (Lubbock, TX), according to standard laboratory procedures and using the 454 FLX 142 Sequencer (454 Life Sciences, Branford, CT, USA). Primers forward 28F: GAGTTTGATCNTGGCTCAG and reverse 519R: GTNTTACNGCGGCKGCTG, based upon the 143 V1-V3 region (Escherichia coli position 27-519) of the 16 S rRNA gene, were used (30). The 144 bTEFAP procedures were performed based upon RTL protocols http://www.researchandtesting.com 145 (Research and Testing Laboratories, Lubbock, TX). Raw sequence data were screened, trimmed and 146 147 filtered with default settings, using the QIIME pipeline version 1.4.0 (http://qiime.sourceforge.net). 148 Chimeras were excluded by using the B2C2 (http://www.researchandtesting.com/B2C2.html) (31). Sequences less than 250 bp were removed. The average length of the sequences was 484 bp. 149 150 Sequences are available at the http://www.researchandtesting.com/docs. FASTA sequences for each 151 sample, without chimeras, were evaluated using BLASTn against the database derived from GenBank (http://ncbi.nlm.nih.gov) (32). 152

153 Bioinformatics and data analysis

The sequences were first clustered into Operational Taxonomic Unit (OTU) clusters with 97% identity (3% divergence), using USEARCH (33). To determine the identities of bacteria, sequences were first queried, using a distributed BLASTn.NET algorithm (33) against the database of highquality 16S bacterial sequences that derived from NCBI. Database sequences were characterized as high quality based on criteria, which were originally described by Ribosomal Database Project (RDP, v10.28) (34).

Alpha diversity (rarefaction, Good's coverage, Chao1 richness and Shannon diversity indices) and beta diversity measures were calculated and plotted using QIIME. Diversity was examined from two perspectives. First, overall richness (i.e., number of distinct organisms present within the microbiome) (alpha diversity), was expressed as the number of OTU, and was quantified using the

164 Chaol richness estimator:
$$S_{chaol} = S_{obs} + \frac{M(n-2)}{2(n^2+1)}$$

165 where n_i is the number of OTU with abundance *i*.

- 166 Second, overall diversity (which is determined by both richness and evenness, the distribution of 167 abundance among distinct taxa) was expressed as Shannon Diversity. Shannon diversity (H') is
- 168 calculated using: $H' = -\sum_{i=1}^{R} piln(pi)$
- 169 where *R* is richness and *pi* is the relative abundance of the *i*th OTU.
- Measures of diversity were screened for group differences using an analysis of variance (ANOVA). Multivariate differences among groups were evaluated with "Permutational Multivariate Analysis of Variance Using Distance Matrices," function adonis (35). For ADONIS, distances among samples were calculated using un-weighted or weighted UniFrac, and then an ANOVA-like simulation was conducted to test for group differences. In addition, multivariate differences were assessed using "Analysis of Similarities," function anosim (35). Sequence data were processed using a Research and Testing pipeline that is described at
- http://www.researchandtesting.com/docs/Data_Analysis_Methodology.pdf. Spearman correlations
 were computed between OTU and metabolite concentration. All analyses were conducting in R,
- 179 using the vegan (35), labdsv (36), DESeq2 (37), and phyloseq (38) packages.
- 180 Community-level catabolic profiles (CLCP)

181 Carbon source utilization patterns of the fecal microbiota were assessed using Biolog 96-well Eco micro-plates (Biolog, Inc., Hayward, CA, USA) (39). Micro-plates contained 31 different carbon 182 sources (carbohydrates, carboxylic acids, polymers, amino acids, amines, and miscellaneous 183 substrates) in triplicate. Five grams of feces diluted with Amies Transposrt medium (1:1) were 184 homogenized in a filter bag with 45 mL of sterile sodium chloride (0.9% (wt/vol)) solution (Classic 185 186 Blender) to remove solid particulate of feces. The homogenized feces were centrifuged at 11.000 187 RPM for 15 min at 4°C. The pellet was washed with 50 mM Tris-HCl (pH 7.0), then with sterile sodium chloride solution and centrifuged at 11.000 RPM for 15 min at 4°C. The cellular suspension 188 was diluted (1:10) into the sterile sodium chloride solution and, subsequently, centrifuged at 2000 189 RPM for 2 min at 4°C. The cellular suspension was diluted (1:20) into sterile chloride solution and 190 191 dispensed (150 µL) into each of the 96 wells of the Biolog Eco micro-plates. The micro-plates were 8

incubated at 37° C in the dark, under anaerobic conditions and slowly stirring. The color development was measured at 590 nm every 24 h with a micro-plate reader (Biolog Microstation). Three indices were determined (40). Shannon's diversity (*H'*), indicating the substrate utilization pattern, was calculated as follows:

$$H' = -\Sigma p_i \ln (p_i) \qquad (1)$$

where p_i 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 higher than 0.25. Substrate evenness (*E*) was defined as the equitability of activities across all utilized substrates:

$$E = H' / \log S \qquad (2)$$

202 Enumeration of cultivable bacteria

203 Diluted fecal samples (20 g) were mixed with 80 ml sterilized physiological solution and 204 homogenized. Counts of viable bacterial cells were carried out as described by De Angelis et al. 205 (41). The following selective media were used: Wilkins-Chalgren anaerobe agar (total anaerobes); 206 MRS agar (Enterococcus and Lactobacillus); Slanetz and Bartley (Enterococcus); Rogosa agar, plus 1.32 ml/l of glacial acetic acid (Lactobacillus); M17 (Lactococcus and Streptococcus); Baird 207 208 Parker (Staphylococcus); Wilkins-Chalgren anaerobe agar, plus GN selective supplements and sheep blood defibrinated (Bacteroides, Porphyromonas and Prevotella); MacConkey agar No2 209 210 (Enterobacteriaceae); Chromocult (total coliform) (Merk, Darmstadt, Germany, Europe); GSP agar 211 (Sigma-Aldrich, St. Louis, MO, USA), plus penicillin-G (60 g/l) (Pseudomonas, Alcaligenes and 212 Aeromonas); Bifidobacterium agar modified (Bifidobacterium) (Becton Dickinson, Le Pont de Claix, SA, France); and Hoyle medium (Corynebacterium). Except for Bifidobacterium agar 213 214 modified, Chromocult and GSP agar, all media were purchased by Oxoid Ltd (Basingstoke, 215 Hampshire, England).

216 Fecal concentration of free amino acids

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FAA of fecal samples were analyzed through the Biochrom 30 series amino acid analyzer 217 (Biochrom Ltd., Cambridge Science Park, England) with a sodium cation-exchange column (20 by 218 0.46 cm (inner diameter)). A mixture of amino acids at known concentrations (Sigma Chemical Co., 219 Milan, Italy) was added with cysteic acid, methionine sulfoxide, methionine sulfone, tryptophan, 220 221 ornithine, glutamic acid, and γ -amino-butyric acid and used as standard. Proteins and peptides in the fecal samples were precipitated by addition of 5% (vol/vol) cold solid sulfosalicylic acid, holding 222 the samples at 4°C for 1 h, and centrifuging at 15,000 x g for 15 min. The supernatant was filtered 223 through a 0.22-µm-pore-size filter and when necessary diluted, with sodium citrate (0.2 M, pH 2.2) 224 225 loading buffer. Amino acids were post-column derivatized with ninhydrin reagent and detected by 226 absorbance at 440 (proline and hydroxyproline) or 570 (all the other amino acids) nm.

Gas-chromatography mass spectrometry-solid-phase microextraction (GC-MS/SPME) analysis of fecal volatile compounds

manufacturer's 229 After preconditioning, according to the instructions, а 230 polydimethylsiloxane/Divinylbenzene fiber (65 µm) and a manual solid phase micro-extraction 231 (SPME) holder (Supelco Inc., Bellefonte, PA, USA) were used. Before headspace sampling, the 232 fiber was exposed to GC inlet for 1 h for thermal desorption at 250°C. Three grams of fecal sample were placed into 10 ml glass vials and added of 10 ul of 4-methyl-2-pentanol (final concentration of 233 33 mg/l), as the internal standard. Samples were then equilibrated for 10 min at 40°C. SPME fiber 234 was exposed to each sample for 40 min. Both equilibration and absorption phases were carried out 235 236 with stirring. The fiber was then inserted into the injection port of the gas chromatograph for 10 min of sample desorption. GC-MS analyses were carried out with an Agilent 7890A gas chromatograph 237 238 (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 239 (length, 60 m; inside diameter, 0.32 mm; Supelco, Bellefonte, PA) was used. The temperature 240 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 241

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a rate of 10°C/min to 230°C, and then 230°C for 25 min. The injector, interface, and ion source 242 temperatures were 250, 250, and 230°C, respectively. The mass-to-charge ratio interval was 30 to 243 350 Da at a rate of 2.9 scans per sec. Injection was carried out in splitless mode, and helium (flow 244 rate, 1 ml/min) was used as the carrier gas. Molecules were identified based on comparison of their 245 retention times with those of pure compounds (Sigma-Aldrich, Milan, Italy). Identities were 246 247 confirmed by searching mass spectra in the available databases (NIST, version 2005; Wiley, version 248 1996). Quantitative data for the compounds identified were obtained by interpolation of the relative areas versus the internal standard area (42). All the GC-MS/SPME raw files were converted to 249 250 netCDF format via Chemstation (Agilent Technologies, USA) and subsequently processed by the XCMS toolbox (http://metlin.scripps.edu/download/). XCMS software allows automatic and 251 simultaneous retention time alignment, matched filtration, peak detection, and peak matching. GC-252 253 MS/SPME data were organized into matrix for subsequent statistical analysis.

254 Statistical analysis

255 Culture dependent data were obtained at least in triplicates. The analysis of variance (ANOVA) was 256 carried out on transformed data followed by separation of means with Tukey's HSD, using the 257 statistical software Statistica for Windows (Statistica 6.0 per Windows 1998, StatSoft, Vigonza, Italy). Letters indicate significant different groups (P < 0.05) by Tukey's test. In order to identify 258 differences between HS and HSB samples, GC/MS/SPME data were analyzed by Canonical 259 discriminant Analysis of Principal Coordinates (41). The total variance obtained in the principal 260 261 coordinates used to perform the CAP was higher than 80% for all the samples. Significance testing was carried out using 999 permutations. The correctly performed permutation test assigns ca. 90% 262 263 of the samples. Moreover, models based on projection on latent structures (PLS) in its discriminant (DA) version were built based on the normalized concentration of the significant molecules 264 identified (43). Metabolically active bacterial families/genera and fecal volatile compounds were 265

analyzed by Principal Component Analysis (PCA) using the statistical software Statistica for
Windows (Statistica 6.0 per Windows 1998, StatSoft).

268 Nucleotide sequence accession number

269 The sequence data were submitted to the Sequence Read Archive database of the National Center

270 for Biotechnology Information under accession no. PRJNA290897.

271 RESULTS

272 Diet and clinical evaluation

In this study, the diet was monitored for two months before starting the administration of durum 273 wheat flour and whole-grain barley Pasta containing the minimum recommended intake (3 g) of β -274 275 glucans. This was done to ascertain that every volunteer consumed equivalent amounts of 276 carbohydrates, total protein and fat. The only statistically (P<0.05) difference in the diet, before and 277 after administration of durum wheat flour and whole-grain barley Pasta, was the amount of total 278 fibers (Table 1). All volunteers well tolerated barley flour including β -glucans and no statistical (P>0.05) effects regarding bloating, abdominal pain score, flatulence, frequency or consistency of 279 280 evacuations of feces were observed (Table S1). The observed reduction of non-HDL cholesterol confirmed the European Food Safety Authority healthy claim. The estimation of cholesterol was 281 used as an internal control to validate the adherence of each volunteer to the diet and, consequently, 282 the consistency of the study. 283

Richness and diversity of the fecal microbiota based on 16S rRNA gene sequencing data analysis

Total metabolically active bacteria from fecal samples of healthy subjects before (HS) and after (HSB) administration of durum wheat flour and whole-grain barley Pasta were analyzed by pyrosequencing of 16S rRNA gene. After quality control, pyrosequencing analysis yielded 156,563 total bacterial reads sequences with a mean of 3010 (ranged from 1146 to 5621) for sample. The metabolically active bacterial community was analyzed by rarefaction curves (Fig. S1), estimated 12 Operational taxonomic units (OTU), richness estimator (Chao 1) and diversity index (Shannon). The Good's Estimated Sample Coverage was ca. 97%. The mean number of estimated OTU of 127.19 *vs* 132 (P=0.605) for HS and HSB, respectively. According to OTU values, the mean values of Chao1 (152 *vs* 160.8) and Shannon index (3.27 *vs* 3.3) values were not significantly different (P=0.466; P=0.794 for Chao1 and Shannon index, respectively) between HS and HSB (Fig. S2).

296 According to alpha diversity values, the three phylogeny-based beta-diversity measures did not 297 show clear separation between the microbiota composition of HS and HSB in weighted and unweighted UniFrac distance principle coordinate analysis plots (Fig. 1; Fig. S3). Besides, Adonis 298 299 statistical test indicated no significant (P=0.199) differences between the microbial diversity of HS 300 and HSB. However, ANOSIM results, which were based on weighted UniFrac testing for multivariate difference, differed (P=0.04; TestStat 0.05) between HS and HSB. Overall, seven phyla 301 302 (Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Lentisphaerae, Proteobacteria and 303 Verrucomicrobia) and one candidate division (TM7) were identified (Fig. S4). However, Firmicutes and Bacteroidetes represented more than 85% of all 16S rRNA sequences. No significant (P>0.05) 304 305 differences were found for the phyla relative abundances between HS and HSB. The only exception 306 was the phylum Fusobacteria, which was higher in HS compared to HSB (average relative abundance of 3.06 vs 1.85%, P=0.019). 307

According to alpha and beta-diversity and considering the 35 most dominant OTU (representing 95% of the total microbiota) of all fecal samples, HS and HSB volunteers were variously distributed (Fig. 2). Compared to fecal microbiota of HS, only few statistically (P<0.05) differences were detected (Table 2). HSB samples showed the highest relative abundance of *Clostridiaceae* (*Clostridium orbiscindens*; *Clostridium* sp.), *Lachnospiraceae* (*Roseburia hominis*), *Ruminococcus* sp. Other *Ruminococcaceae* (*Faecalibacterium prausnitzii* and *Faecalibacterium* sp.), *Dialister invisus* and *Fusobacteriaceae* (*Fusobacterium*) were the lowest in HSB.

315 **OTU correlation**

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correlations

323 Community level catabolic profiles and cultivable bacteria

were

always

found

showed an increased number of positive correlations in HSB compared to HS.

324 The substrate utilization pattern (H' index) and substrate richness (S index) values were calculated (Fig. 4). Compared to HS, the H and S indices of HSB decreased. The E index, which measures the 325 statistical significance (equitability) of the H' and S index values, confirmed the above-described 326 327 significant (P < 0.05) differences.

OTU correlation was investigated considering family- (Fig. 3A,B) or genus-level (Fig. 3C,D)

taxonomic assignments and significant correlations at FDR<0.050. Several family positive

Porphyromonadaceae and Clostridiaceae). Other positive correlations were lost in HSB compared

to HS (e.g. Eubacteriaceae with Coriobacteriaceae, Oscillospiraceae and Streptococcaceae).

Similar trend was detected also at genus-level. The only exception was for Bacteroides, which

Coriobacteriaceae

and

Streptococcaceae;

(e.g.,

328 Selective media were used to enumerate cultivable bacteria (Table 3). Compared to HS, HSB showed a lower (P=0.05) number of total anaerobes. The median values of presumptive 329 330 Lactobacillus was lower than those found for HSB. Other significant (P<0.05) differences concerned the number of presumptive Bacteroides, Porphyromonas and Prevotella, Enterobacteria, 331 332 total coliforms and Pseudomonas, Alcaligenes and Aeromonas, which were the lowest in HSB fecal 333 samples.

334 The fecal metabolome

335 Compared to HS, fecal samples of HSB had lower (P<0.05) levels of some FAA (Pro, Trp, Thr, and 336 Arg) and metabolites from the catabolism of FAA (γ -amino butyric acid) (Fig. S5). The levels of 337 some volatile organic compounds (VOC), which was detected in fecal samples of HS, markedly differed in HSB (Fig. 5A; Fig. S6). Compared to HS, fecal samples of HSB showed increased 338 339 content of several short chain fatty acids (SCFA) (2-methyl-propanoic acid, acetic acid, butanoic acid and propanoic acid) (Fig. 5B). Pentanoic acid was the only SCFA found at the highest level in 340

HS. The other statistically significant differences for VOC regarded the levels of phenylethyl
alcohol, benzaldheyde, indole, 2,3-butanedione, 6-methyl-5-hepten-2-one and acetophenone, which
were the highest in HSB. FAA and GC-MS/SPME data were also analyzed using the Principal
Component Analysis (PCA). The discrimination of fecal samples between HS and HSB was evident
(Fig. S7).

346 **OTU-metabolite correlations**

Correlations between metabolically active bacterial families/genera and metabolome data (FAA and 347 VOC) were found (Fig. 6). Coriobacteriaceae, Streptococcaceae, 348 Faecalibacterium, 349 Ruminococcaceae and Ruminococcus were positively correlated with hexanoic and propanoic acids. 350 Other positive correlations were found for *Ruminococcaceae* and Tpr and γ -amino-butyric acid, and *Ruminococcus* and Tpr, γ -amino-butyric acid, acetic and butanoic acids. Hexanoic acid was also 351 352 positively correlated with Parabacteroides, Clostridiaceae and Clostridium. Bacteroidaceae, Bacteroides and Alcaligenaceae were positively correlated with Tpr, acetic, butanoic, and 353 354 propanoic acids, NH₃, indole, Arg, acetohenone and benzaldheyde. Roseburia and Lachnospiraceae 355 showed various positive correlations, including Thr, γ -amino-butyric acid, acetic acid, butanoic 356 acid, NH₃, indole, propanoic acid, phenyl ethyl alcohol and 2,3-butanedione.

357 DISCUSSION

358 This is one of the few studies showing the effects of durum wheat flour and whole-grain barley 359 Pasta on the human fecal microbiota and metabolome, using a meta-omics approach. As shown 360 through pyrosequencing analysis, the intervention with durum flour wheat and whole-grain barley 361 Pasta, resulting in the ingestion of 3 g/day of β -glucans, did not affect the values of alpha and beta 362 diversity. Previously, high-throughput sequencing techniques revealed that alpha diversity decreased with dietary supplementation of β -glucans from sea cucumber (*Apostichopus japonicas*) 363 (44) and mirror carp (*Cyprinus carpio*) (45). The phylogenetic composition of the analyzed samples 364 365 confirms that the Firmicutes and Bacteroidetes phyla constitute the most abundant bacterial OTUs

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379 The positive bacterial interactions also decreased during dietary supplementation with β -glucans 380 from sea cucumber (44). In agreement, the community level catabolic profiles showed decreased 381 substrate utilization pattern (H' index) and Shannon index after diet intervention with barley β -382 glucans. All these results indicated a low metabolic diversity. Culture-dependent methods showed that diet intervention with whole-grain barley markedly decreased the total number of fecal 383 384 anaerobic cultivable bacteria. Decreased levels of cultivable anaerobes were also found during in 385 vitro fermentation of oat β -glucans by human fecal slurry (20). According to the *in vitro* study of Hughes et al. (48), cultivable presumptive thermophilic and mesophilic Lactobacillus increased 386 following diet intervention with barley β -glucans. No positive effects were observed regarding the 387 genus Bifidobacterium. A randomized, placebo-controlled, double-blind human intervention trial 388 389 showed that cultivable fecal lactobacilli of 26 healthy volunteers were not affected by 0.75 g of 390 barley β -glucans (26). The same study showed a statistically significant increase of the cell density 391 of bifidobacteria. After grouping volunteers according to age, a significant bifidogenic effect was 16

of human intestinal microbiota. The composition of the main bacterial phyla (Firmicutes and

Bacteroidetes) within the enrolled volunteers was variously affected, without an unique statistically

significant trend. According to the 16S rRNA gene-based high-throughput sequencing, wide

variation among individuals was found (46, 47). Clostridiaceae (Clostridium orbiscindens,

Clostridium sp.), Roseburia hominis and Ruminococcus sp. increased following the intervention.

Previously, it was shown that barley and oat β -glucans induced clostridial cluster IX populations

and *Clostridium histolyticum* subgroup during *in vitro* fermentation by human fecal microbiota (48).

On the contrary, oat β -glucans did not favor the growth of *Clostridium* sp. in SHIME and C57BL/6J

mice models (21, 24, 49). However, several discrepancies between the different studies could be

due to the different models and methods used. Faecalibacterium prausnitzii, Faecalibacterium sp.

and *Dialister invisus* decreased following the diet intervention with barley β -glucans. A similar

trend was found for the genus Fusobacterium, belonging to Fusobacteria. Based on OTU

correlations, β-glucans negatively impacted on bacterial interactions.

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393 polypectomized patients showed no effects on the fecal cultivable lactobacilli and bifidobacteria 394 (27). An increased level of lactobacilli was found in animal models after barley or oat containing 395 diet, especially using high-viscosity β -glucans (22, 23, 24). At the same time, out product based diet 396 or β -glucans from Laminaria digitata, Laminaria hyperborea and Saccharomyces cerevisiae did not 397 increase the levels of lactobacilli in pigs and rats (25, 50). Taken together, these results suggested 398 that the prebiotic potential of whole-grain barley/ β -glucans was not always reproducible *in vivo*. The complex biochemical interactions and antagonistic activities within the intestinal microbiota 399 prior treatment might be responsible for the different response to β -glucans enriched diet. 400 401 Presumptive cultivable Bacteroides, Porphyromonas and Prevotella decreased following the diet 402 intervention with barley β -glucans. Overall, abundance of *Prevotella* is higher in humans which consume more vegetable based diet (14). During in vitro fermentation, Prevotella-Bacteroides 403 404 increased only with low molecular weight β -glucans (48). Cultivable *Bacteroides* decreased in SHIME model after oat bran feeding (21), while no significant differences were found in rats after 405 406 feeding with products enriched of oat fiber (50). A barley reach diet was associated with reduced 407 cultivable Bacteroides in the intestinal tract of rats (22). According to this study, cultivable *Bacteroides* were negatively affected in polypectomized patients after intake of barley β -glucans 408 (27). In vitro and animal trials support that Enterobacteriaceae could not utilize β -glucans (20, 25, 409 410 50). According to in *vitro* and animal trials, this study showed a significant reduction of cultivable 411 Enterobacteriaceae and total coliforms. Similar trend was found in administration of 3 g/day of β glucans to 33 polypectomized patients (27). On the contrary, small amounts of β -glucans (0.75) 412 were not effective to decrease total cultivable coliforms or Escerichia coli (26). First, this study 413 414 showed that cell density of cultivable Pseudomonas, Alcaligenes and Aeromonas markedly 415 decreased after the diet intervention with Pasta enriched of barley β -glucans. 416 In vitro and animal trials support that high barley β -glucans consumption is associated with a

detected only in subjects older than 50 years. The administration of 3 g/day of β -glucans to 33

417 significant increase of the synthesis of SCFA (46, 49, 50). This study confirmed the previous GC-

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MS data showing a noticeable increase of 2-methyl-propanoic acid, acetic acid, butanoic (butyric) 418 acid and propanoic (propionic) acid after the diet intervention with durum wheat flour and whole-419 grain barley Pasta. SCFA induce positive gastrointestinal and systemic effects (51). SCFA are also 420 important modulators of the host immune function (52). Acetate is used as energy source for liver 421 422 and peripheral tissues, and acts as signaling molecules in gluconeogenesis and lipogenesis (53). 423 Propionate serves as precursor for gluconeogenesis and it reduces the synthesis of hepatic 424 cholesterol (54). Propionate was also associated with a decrease of insulin secretion in pancreatic islet cells of rats (55). Butyric acid serves as the main energy source for colonocytes and protects 425 from inflammation (56, 57). Butyrate affects also the regulation of apoptosis and cellular 426 427 proliferation, resulting in reduced risk of colon cancer (58). BCFA (e.g., iso-butyrate and isovalerate) were not affected by whole-grain barley, indicating that the highest synthesis of SCFA 428 was obtained without increasing undesirable protein fermentation (44). Previously, it was 429 demonstrated that the intake of dietary fibers or symbiotic foods (e.g., fructooligosaccharides, 430 Lactobacillus helveticus and Bifidobacterium longum) leads to a modulation of the gut metabolic 431 432 activities with an increase of SCFA (59-61). Previously, in vitro data shows that barley and oat β -433 glucans or other oligosaccharides support the growth of *Clostridia* strains which synthesize acetate 434 and butyrate (62, 63). Bacteroides strains are propionate producers by the succinate pathway (64) 435 and they were positively correlated with propionic acid.

This study highlighted some *in vivo* effects of whole-grain barley towards fecal microbiota and metabolome. Whole-grain barley appeared to be effective in the modulation of the composition and the metabolic pathways of the intestinal microbiota, leading to an increased level of SCFA.

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624 Figure legends

FIG 1 Principle Coordinate Analysis (PCoA) of metabolically active bacteria. PCoA was based on weighted UniFrac analysis of all 16S rRNA gene sequences found on fecal samples of healthy subject before (HS) and after two months of diet intervention (HSB) with durum wheat flour and whole-grain barley Pasta.

FIG 2 Heatmap summarizing the relative abundance of the 35 most dominant species in RNA samples directly extracted from healthy subjects before (HS) and after (HSB) two months of diet intervention with durum wheat flour and whole-grain barley Pasta. The color key defines the percentages of OTU in the samples.

FIG 3 Significant correlations between family (A, B) and genus (C, D) bacterial OTU before (A, C) and after (B, D) two months of diet intervention with durum wheat flour and whole-grain barley Pasta. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (read) and -1 indicating a perfectly negative correlation (green) between two microbial families or genera. Only significant correlations (FDR<0.05) are shown.

FIG 4 Community level catabolic profiles. CLCP indices (utilization pattern substrate (H'), substrate richness (S), and equitability (E)) of the fecal microbiota of healthy subjects before (HS) and after (HSB) two months of diet intervention with durum wheat flour and whole-grain barley Pasta. Data are the means of three independent experiments (n 3). The center line of each box represents the median, and the top and bottom of the box represent the 75th and 25th percentiles of the data, respectively. The top and bottom of the error bars represent the 5th and 95th percentiles of the data, respectively. The circles in each box plot extend to the outliers of the data.

FIG 5 Score (A) and loading coefficient (B) plots of Canonical Discriminant Analysis of Principal Coordinates (CAP) of volatile organic metabolites in feces of healthy subjects before (HS) and after (HSB) diet intervention with durum wheat flour and whole-grain barley Pasta. Compounds significantly associated with the feces of HSB (negative axis) or HS samples (positive axis) Data are the means of three independent experiments (n = 3).

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FIG 6 Significant correlations between metabolically active bacterial OTU (family and genus level) and after free amino acids and volatile organic compounds after two months of diet intervention with durum wheat flour and whole-grain barley Pasta. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (read) and -1 indicating a perfectly negative correlation (green) between two microbial families or genera. Only significant correlations (FDR<0.05) are shown.

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658	TABLE 1 . Basic characteristics and diet of volunteers. Each healthy subject was analyzed before
659	(HS) and after (HSB) two months of diet intervention with 100 g/day of durum wheat and whole-
660	grain barley pasta containing 3% (wt/wt) of β -glucans.

	HS	HSB	
Basic characteristics		пэр	
Age (years)	39±9	39±9	
Male (%)	46	46	
Body Mass Index (kg/m ²)	22.6±3 ^a	22.6±3 ^a	
Total Cholesterol (mg/dl)	183.8±30.2 ^b	173.25±27.4°	
LDL Cholesterol (mg/dl)	107.4±25.2 ^b	93.25±24.5°	
HDL Cholesterol (mg/dl)	62.48±16.63 ^a	62.41±16.56 ^a	
Weekly cumulative	HS	HSB	
frequency of food			
Milk	50 ^a	50 ^a	
Pasta	100 ^a	0^{b}	
Pasta β-Glucan	0^{a}	100 ^b	
Bread	97 ^a	100^{a}	
Meats	85 ^a	84 ^a	
Cured meats	73 ^a	68 ^a	
Fish	77 ^a	81 ^a	
Dairy Products	81 ^a	77 ^a	
Eggs	89 ^a	84 ^a	
Legumes	77 ^a	76 ^a	
Leafy vegetables	100 ^a	100^{a}	

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Fruits

Yogurt	8^{a}	8 ^a	
Sweets	59 ^a	57 ^a	
Components Intake	HS	HSB	
Water (g)	717.0±274.6 ^a	853.4±512.7 ^a	
Protein (g)	69.9±29.5 ^a	77.6±41.7 ^a	
Lipid (g)	58.3±31.3 ^a	68.7±42.2 ^a	
Carbohydrate (g)	199.8±73.9 ^a	237.4±101.6 ^a	
Starch (g)	130.1±54.9 ^a	151.1±70.3 ^a	
Soluble sugars (g)	58.7±27.9 ^a	73.4±37.6 ^a	
Alcohol (g)	7.9±13.3 ^a	6.5±12.1 ^a	
Total fiber (g)	13.4±8.2 ^a	22.0±11.7 ^b	
Energy (kcal)	1613.1±609.6 ^a	1894.3±908.9 ^a	
Sodium (mg)	1233.3±886.5 ^a	1498.5±1180.4 ^a	
Potassium (mg)	2091.7±888.6 ^a	2408.5±1433.0 ^a	
Iron (mg)	11.7±20.7 ^a	8.3±6.1 ^a	
Calcium (mg)	446.2±274.6 ^a	533.0±339.8 ^a	
Phosphorus (mg)	986.1±705.8 ^a	940.6625.7 ^a	
Thiamine (mg)	0.7±0.3 ^a	0.9±0.8 ^a	
Riboflavin (mg)	0.9±0.4 ^a	1.0±0.8 ^a	
Niacin (mg)	11.5±6.3 ^a	12.78.5 ^a	
Vit. C (mg)	64.6±39.7 ^a	179.3±340.3 ^a	
Vit. E (mg)	5.0±8.9 ^a	5.8±12.1 ^a	
Values within a row with differ	ent superscript letters are signific	eantly different (P<0.05)	

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^{a-c}Values within a row with different superscript letters are significantly different (P<0.05).
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				r
		HS*	HSB	S/SB
Firmicutes	Clostridiaceae	5.26	8.08	0.019
	Eubacteriaceae	8.827	5.135	0.229
	Ruminococcaceae	31.55	23.37	0.023
Fusobacteria	Fusobacteriaceae	3.06	1.85	0.019
	Specie			
Firmicutes	Clostridium orbiscindens	0.22	0.68	0.045
Firmicutes	Clostridium sp.	2.95	4.69	0.048
	Roseburia hominis	0.01	0.12	0.047
Firmicutes	Faecalibacterium prausnitzii	12.27	6.08	0.004
	Faecalibacterium sp.	11.29	6.32	0.016
	Ruminococcus sp.	5.60	8.82	0.047
Firmicutes	Dialister invisus	0.52	0.13	0.034
Fusobacteria	Fusobacterium sp.	3.06	1.85	0.019

663 **TABLE 2**. Pyrosequencing data summary.

*Each healthy subject was analysed before (HS) and after (HSB) two months of diet intervention

with 100 g/day of durum wheat and whole-grain barley pasta containing 3% (wt/wt) of β -glucans.

666 Relative abundance (average value, Avg %) of predominant bacterial taxa, showing significant

(P < 0.05) differences between fecal samples of HS and HSB.

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Microbial group	Mean no. of cultivable cells, log CFU/ml (range)		
	HS*	HSB	
Total anaerobes	9.80 ^a (9.33 – 9.93)	8.42 ^b (4.98 – 9.81)	
Lactobacillus (thermophilus	6.61 ^b (4.60 - 8.93)	7.53 ^a (4.93 – 9.16)	
42°C)			
Lactobacillus (mesophilus	6.61 ^b (3.26 – 8.98)	7.38 ^a (4.46 – 9.28)	
25°C)			
Enterococcus	7.05 ^a (5.36 – 8.55)	7.29 ^a (3.90 – 9.19)	
Lactococcus and	7.74 ^a (5.36 – 8.55)	7.57 ^a (4.25 – 9.26)	
Streptococcus			
Staphylococcus	5.68 ^a (2.84 – 8.11)	6.14 ^a (4.38 – 8.08)	
Bacteroides, Porphyromonas	9.09 ^a (3.00 – 9.92)	5.17 ^b (2.52 – 6.44)	
and Prevotella			
Enterobacteriaceae	7.07 ^a (5.11 – 9.27)	6.11 ^b (4.19 – 7.14)	
Total Coliforms	6.94 ^a (1.91 – 8.93)	$6.48^{b}(1.00-5.32)$	
Pseudomonas, Alcaligenes and	6.42 ^a (1.97 – 7.95)	$3.68^{b}(1.00-4.81)$	
Aeromonas			
Bifidobacterium	7.16 ^a (4.33 – 9.80)	6.90 ^a (4.19 – 9.09)	
Corynebacterium	4.93 ^a (1.74 – 7.68)	5.35 ^a (2.00 - 7.17)	

670 **TABLE 3**. Fecal cultivable bacteria of the main microbial groups.

*Each healthy subject was analysed before (HS) and after (HSB) two months of diet intervention

with 100 g/day of durum wheat and whole-grain barley pasta containing 3% (wt/wt) of β-glucans.

 $^{a-b}$ Values within a row with different superscript letters are significantly different (P<0.05).





Figure 2

Count 0 600



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Figure 4



AEM



0.25

0.2

0.15

-0.15

0

Canonical axis 1

А

O HS

5

HSB

10`

15

20



Figure 5

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Figure 6

