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

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Running title: Whole-grain barley and human fecal microbiota

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ABSTRACT

This study aimed at comparing the fecal microbiota and metabolome of 26 healthy subjects before (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 barley β-glucans. Metabolically active bacteria were analyzed through pyrosequencing of the gene 16S rRNA and community-level catabolic profiles. Pyrosequencing data showed that *Clostridiaceae* (*Clostridium orbiscindens*, *Clostridium* sp.), *Roseburia hominis*, and *Ruminococcus* sp. increased while other *Firmicutes* and *Fusobacteria* decreased in HSB compared to HS fecal samples. Community-level catabolic profiles were the lowest in HSB. Compared to HS, cultivable lactobacilli increased in HSB fecal samples while *Bacteroides, Porphyromonas* and *Prevotella*, *Enterobacteriaceae*, total coliforms, and *Pseudomonas, Alcaligenes* and *Aeromonas* decreased. Metabolome analyses were performed using amino acid analyzer and gas-chromatography mass spectrometry-solid-phase micro-extraction. A marked increase of short chain fatty acids (SCFA) 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-glucans 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.

INTRODUCTION

Whole-grain barley and oat, and some dry-milled bran grain products were authorized to be used 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 fibers. European Food Safety Authority recognized that the "regular consumption of oat β-glucans can actively lower/reduce blood LDL-cholesterol and total cholesterol (3). In addition to reduction 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 metabolic syndrome were well documented (4, 5). The minimum dose of 3 g/day of β-glucans is recommended to get positive effects on human health (1, 3, 6).

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, digestion, and absorption of molecules, including glucose, dietary cholesterol and bile acids (7, 8); (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 improved insulin sensitivity (10, 11); and iv) inhibited hepatic synthesis of cholesterol by acetate 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 metabolically active microbes at the colon level (14). Saccharolytic fermentation was associated with the synthesis of short-chain fatty acids (SCFA) (acetate, propionate and butyrate), intermediate metabolites such as succinate, acrylate, lactate, formate and ethanol, and small final molecules (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-butyric, iso-valeric and 2-methylbutyric acids), free amino acids (FAA) and some potentially toxic metabolites (phenols, indoles, ammonia and amines) (14, 16, 17). The type of colonic fermentation is mainly depends on the type of microbiota and fermentable substrate availability (17). Based on

65 the key role for human healthy, the intestinal microbiota was acknowledged as a metabolic organ
66 (18). The major part of the current research on novel functional foods is moved towards the
67 selection and characterization of prebiotics (e.g. inulin, fructo-oligosaccharides and galacto-
68 oligosaccharides), which are not digested by human gastrointestinal enzymes but selectively
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
72 bifidobacteria was shown by *in vitro* studies (20, 21) and animal experiments (22, 23).
73 Nevertheless, other studies that also used animal models did not show significant effects of β-
74 glucans on lactobacilli and/or bifidobacteria (24, 25). Unfortunately, human clinical challenges that
75 have dealt with the prebiotic effect of β-glucans are rather scarce. Barley β-glucans increased the
76 cell density of colonic bifidobacteria on older healthy subjects (26). A pilot study with
77 polypectomized patients showed no significant effect of β-glucans on the fecal microbiota and the
78 concentration of SCFA (27). The evidence that β-glucans positively influence the human intestinal
79 microbiota is still insufficient or difficult to interpret, and additional studies are needed to fill this
80 gap (5).
81 This study compared the fecal microbiota and metabolome of healthy subjects before (HS) and after
82 (HSB) two months of daily administration of durum wheat flour and whole-grain barley Pasta
83 containing the minimum recommended intake (3 g) of β-glucans. The fecal microbiota was
84 characterized through integrated approaches, which were based on culture-independent and -
85 dependent methods.

86 **MATERIALS AND METHODS**

87 **Subjects**

88 The study was carried out in accordance with the Helsinki Declaration (IV Adaptation) and
89 European Guidelines for Good Clinical Practice. The protocol of the study was approved by the

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.

Feeding regime

Pasta Granoro Cuore Mio (Granoro srl, Corato, BA) was used in this study. Pasta was made by 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% of dry matter; carbohydrate, 69% of dry matter; fat, 2% of dry matter; total fibres and β-glucans, 7% and 3% of dry matter, respectively. Before starting with the administration of 100 g Pasta Granoro Cuore Mio Pasta containing 3 g of barley β-glucans (Granoro srl), each volunteer was instructed to follow the usual diet, including 100 g of Pasta every day for two months. Before and after the 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 frequency of food" and the "component intake" data, respectively (Table 1), by using official Italian food composition databases (INRAN, http://nut.entecra.it/646/tabelle_di_composizione_degli_alimenti.html and IEO, http://www.bda-ieo.it/uk/index.aspx). All volunteers confirmed that not remarkable changes occurred in meals and medication during the two months of treatment. Additionally, a questionnaire aimed at evaluating intestinal effects (bloating, meteorism, constipation, satiety, diarrhea, reflux and flatulence) was administered (26). Gastrointestinal symptoms were graded from 0 (no symptoms) to 3 (severe symptoms) (26). Before the treatment with durum wheat and whole-grain barley Pasta, colesterolemia, body mass index, fecal microbiota and metabolome were determined.

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.

Collection of fecal samples

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

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).

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

Sequencer (454 Life Sciences, Branford, CT, USA). Primers forward 28F: GAGTTTGATCNTGGCTCAG and reverse 519R: GTNTTACNGCGGCKGCTG, based upon the V1–V3 region (*Escherichia coli* position 27–519) of the 16 S rRNA gene, were used (30). The bTEFAP procedures were performed based upon RTL protocols http://www.researchandtesting.com (Research and Testing Laboratories, Lubbock, TX). Raw sequence data were screened, trimmed and filtered with default settings, using the QIIME pipeline version 1.4.0 (http://qiime.sourceforge.net). 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. Sequences are available at the http://www.researchandtesting.com/docs. FASTA sequences for each sample, without chimeras, were evaluated using BLASTn against the database derived from GenBank (http://ncbi.nlm.nih.gov) (32).

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 high-quality 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 Chao1 richness estimator: $S_{chao1} = S_{obs} + \frac{n1(n1-1)}{2(n2+1)}$

where n_i is the number of OTU with abundance i.

Second, overall diversity (which is determined by both richness and evenness, the distribution of abundance among distinct taxa) was expressed as Shannon Diversity. Shannon diversity (*H'*) is calculated using: $H' = - \sum_{i=1}^{R} piln\ (pi)$

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, using the vegan (35), labdsv (36), DESeq2 (37), and phyloseq (38) packages.

Community-level catabolic profiles (CLCP)

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 sources (carbohydrates, carboxylic acids, polymers, amino acids, amines, and miscellaneous substrates) in triplicate. Five grams of feces diluted with Amies Transposrt medium (1:1) were homogenized in a filter bag with 45 mL of sterile sodium chloride (0.9% (wt/vol)) solution (Classic Blender) to remove solid particulate of feces. The homogenized feces were centrifuged at 11.000 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 was diluted (1:10) into the sterile sodium chloride solution and, subsequently, centrifuged at 2000 RPM for 2 min at 4°C. The cellular suspension was diluted (1:20) into sterile chloride solution and dispensed (150 µL) into each of the 96 wells of the Biolog Eco micro-plates. The micro-plates were

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

196 $$H' = - \Sigma p_i \ln (p_i) \qquad (1)$$

197 where p_i is the ratio of the activity of a particular substrate to the sums of activities of all substrates
198 at 120 h. Substrate richness (*S*), measuring the number of different substrates used, was calculated
199 as the number of wells with a corrected absorbance higher than 0.25. Substrate evenness (*E*) was
200 defined as the equitability of activities across all utilized substrates:

201 $$E = H'/\log S \qquad (2)$$

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,
207 plus 1.32 ml/l of glacial acetic acid (*Lactobacillus*); M17 (*Lactococcus* and *Streptococcus*); Baird
208 Parker (*Staphylococcus*); Wilkins-Chalgren anaerobe agar, plus GN selective supplements and
209 sheep blood defibrinated (*Bacteroides*, *Porphyromonas* and *Prevotella*); MacConkey agar No2
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
213 Claix, SA, France); and Hoyle medium (*Corynebacterium*). Except for *Bifidobacterium* agar
214 modified, Chromocult and GSP agar, all media were purchased by Oxoid Ltd (Basingstoke,
215 Hampshire, England).

216 **Fecal concentration of free amino acids**

FAA of fecal samples were analyzed through the Biochrom 30 series amino acid analyzer (Biochrom Ltd., Cambridge Science Park, England) with a sodium cation-exchange column (20 by 0.46 cm (inner diameter)). A mixture of amino acids at known concentrations (Sigma Chemical Co., Milan, Italy) was added with cysteic acid, methionine sulfoxide, methionine sulfone, tryptophan, 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 the samples at 4°C for 1 h, and centrifuging at 15,000 x *g* for 15 min. The supernatant was filtered through a 0.22-µm-pore-size filter and when necessary diluted, with sodium citrate (0.2 M, pH 2.2) loading buffer. Amino acids were post-column derivatized with ninhydrin reagent and detected by 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

After preconditioning, according to the manufacturer's instructions, a polydimethylsiloxane/Divinylbenzene 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 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 µl of 4-methyl-2-pentanol (final concentration of 33 mg/l), 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 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-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) 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, and helium (flow rate, 1 ml/min) was used as the carrier gas. Molecules were identified based on 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 areas versus the internal standard area (42). All the GC-MS/SPME 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 matrix for subsequent statistical analysis.

Statistical analysis

Culture dependent data were obtained at least in triplicates. The analysis of variance (ANOVA) was carried out on transformed data followed by separation of means with Tukey's HSD, using the 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 differences between HS and HSB samples, GC/MS/SPME data were analyzed by Canonical discriminant Analysis of Principal Coordinates (41). The total variance obtained in the principal 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% 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 identified (43). Metabolically active bacterial families/genera and fecal volatile compounds were

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

Nucleotide sequence accession number

The sequence data were submitted to the Sequence Read Archive database of the National Center for Biotechnology Information under accession no. PRJNA290897.

RESULTS

Diet and clinical evaluation

In this study, the diet was monitored for two months before starting the administration of durum wheat flour and whole-grain barley Pasta containing the minimum recommended intake (3 g) of β-glucans. This was done to ascertain that every volunteer consumed equivalent amounts of carbohydrates, total protein and fat. The only statistically ($P<0.05$) difference in the diet, before and after administration of durum wheat flour and whole-grain barley Pasta, was the amount of total 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 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 used as an internal control to validate the adherence of each volunteer to the diet and, consequently, the consistency of the study.

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

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). According to alpha diversity values, the three phylogeny-based beta-diversity measures did not 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 statistical test indicated no significant (P=0.199) differences between the microbial diversity of HS 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 (*Actinobacteria, Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Lentisphaerae*, *Proteobacteria* and *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) differences were found for the phyla relative abundances between HS and HSB. The only exception was the phylum *Fusobacteria*, which was higher in HS compared to HSB (average relative abundance of 3.06 *vs* 1.85%, P=0.019).

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.

OTU correlation

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 correlations were always found (e.g., *Coriobacteriaceae* and *Streptococcaceae*; *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 showed an increased number of positive correlations in HSB compared to HS.

Community level catabolic profiles and cultivable bacteria

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 statistical significance (equitability) of the H' and S index values, confirmed the above-described significant ($P < 0.05$) differences.

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 *Lactobacillus* was lower than those found for HSB. Other significant ($P<0.05$) differences concerned the number of presumptive *Bacteroides*, *Porphyromonas* and *Prevotella*, *Enterobacteria*, total coliforms and *Pseudomonas, Alcaligenes* and *Aeromonas*, which were the lowest in HSB fecal samples.

The fecal metabolome

Compared to HS, fecal samples of HSB had lower ($P<0.05$) levels of some FAA (Pro, Trp, Thr, and Arg) and metabolites from the catabolism of FAA (γ-amino butyric acid) (Fig. S5). The levels of 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 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

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

346 **OTU-metabolite correlations**

347 Correlations between metabolically active bacterial families/genera and metabolome data (FAA and
348 VOC) were found (Fig. 6). *Coriobacteriaceae*, *Streptococcaceae*, *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
351 *Ruminococcus* and Tpr, γ-amino-butyric acid, acetic and butanoic acids. Hexanoic acid was also
352 positively correlated with *Parabacteroides*, *Clostridiaceae* and *Clostridium*. *Bacteroidaceae*,
353 *Bacteroides* and *Alcaligenaceae* were positively correlated with Tpr, acetic, butanoic, and
354 propanoic acids, NH_3, indole, Arg, acetohenone and benzaldheyde. *Roseburia* and *Lachnospiraceae*
355 showed various positive correlations, including Thr, γ-amino-butyric acid, acetic acid, butanoic
356 acid, NH_3, 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
363 decreased with dietary supplementation of β-glucans from sea cucumber (*Apostichopus japonicas*)
364 (44) and mirror carp (*Cyprinus carpio*) (45). The phylogenetic composition of the analyzed samples
365 confirms that the Firmicutes and Bacteroidetes phyla constitute the most abundant bacterial OTUs

366 of human intestinal microbiota. The composition of the main bacterial phyla (*Firmicutes* and
367 *Bacteroidetes*) within the enrolled volunteers was variously affected, without an unique statistically
368 significant trend. According to the 16S rRNA gene-based high-throughput sequencing, wide
369 variation among individuals was found (46, 47). *Clostridiaceae* (*Clostridium orbiscindens*,
370 *Clostridium* sp.), *Roseburia hominis* and *Ruminococcus* sp. increased following the intervention.
371 Previously, it was shown that barley and oat β-glucans induced clostridial cluster IX populations
372 and *Clostridium histolyticum* subgroup during *in vitro* fermentation by human fecal microbiota (48).
373 On the contrary, oat β-glucans did not favor the growth of *Clostridium* sp. in SHIME and C57BL/6J
374 mice models (21, 24, 49). However, several discrepancies between the different studies could be
375 due to the different models and methods used. *Faecalibacterium prausnitzii, Faecalibacterium* sp.
376 and *Dialister invisus* decreased following the diet intervention with barley β-glucans. A similar
377 trend was found for the genus *Fusobacterium*, belonging to *Fusobacteria*. Based on OTU
378 correlations, β-glucans negatively impacted on bacterial interactions.

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
383 that diet intervention with whole-grain barley markedly decreased the total number of fecal
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
386 Hughes et al. (48), cultivable presumptive thermophilic and mesophilic *Lactobacillus* increased
387 following diet intervention with barley β-glucans. No positive effects were observed regarding the
388 genus *Bifidobacterium*. A randomized, placebo-controlled, double-blind human intervention trial
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

detected only in subjects older than 50 years. The administration of 3 g/day of β-glucans to 33 polypectomized patients showed no effects on the fecal cultivable lactobacilli and bifidobacteria (27). An increased level of lactobacilli was found in animal models after barley or oat containing diet, especially using high-viscosity β-glucans (22, 23, 24). At the same time, oat product based diet or β-glucans from *Laminaria digitata*, *Laminaria hyperborea* and *Saccharomyces cerevisiae* did not increase the levels of lactobacilli in pigs and rats (25, 50). Taken together, these results suggested 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 prior treatment might be responsible for the different response to β-glucans enriched diet. Presumptive cultivable *Bacteroides, Porphyromonas* and *Prevotella* decreased following the diet 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* 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 feeding with products enriched of oat fiber (50). A barley reach diet was associated with reduced 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 (27). In *vitro* and animal trials support that *Enterobacteriaceae* could not utilize β-glucans (20, 25, 50). According to in *vitro* and animal trials, this study showed a significant reduction of cultivable *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) were not effective to decrease total cultivable coliforms or *Escerichia coli* (26). First, this study showed that cell density of cultivable *Pseudomonas, Alcaligenes* and *Aeromonas* markedly decreased after the diet intervention with Pasta enriched of barley β-glucans.

In *vitro* and animal trials support that high barley β-glucans consumption is associated with a significant increase of the synthesis of SCFA (46, 49, 50). This study confirmed the previous GC-

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418 MS data showing a noticeable increase of 2-methyl-propanoic acid, acetic acid, butanoic (butyric)
419 acid and propanoic (propionic) acid after the diet intervention with durum wheat flour and whole-
420 grain barley Pasta. SCFA induce positive gastrointestinal and systemic effects (51). SCFA are also
421 important modulators of the host immune function (52). Acetate is used as energy source for liver
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
425 islet cells of rats (55). Butyric acid serves as the main energy source for colonocytes and protects
426 from inflammation (56, 57). Butyrate affects also the regulation of apoptosis and cellular
427 proliferation, resulting in reduced risk of colon cancer (58). BCFA (e.g., iso-butyrate and iso-
428 valerate) were not affected by whole-grain barley, indicating that the highest synthesis of SCFA
429 was obtained without increasing undesirable protein fermentation (44). Previously, it was
430 demonstrated that the intake of dietary fibers or symbiotic foods (e.g., fructooligosaccharides,
431 *Lactobacillus helveticus* and *Bifidobacterium longum*) leads to a modulation of the gut metabolic
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.

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

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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).

650 **FIG 6** Significant correlations between metabolically active bacterial OTU (family and genus
651 level) and after free amino acids and volatile organic compounds after two months of diet
652 intervention with durum wheat flour and whole-grain barley Pasta. The colors of the scale bar
653 denote the nature of the correlation, with 1 indicating a perfectly positive correlation (read) and -1
654 indicating a perfectly negative correlation (green) between two microbial families or genera. Only
655 significant correlations (FDR<0.05) are shown.
656
657

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.

Basic characteristics	**HS**	**HSB**
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^c
LDL Cholesterol (mg/dl)	107.4±25.2^b	93.25±24.5^c
HDL Cholesterol (mg/dl)	62.48±16.63^a	62.41±16.56^a
Weekly cumulative frequency of food	**HS**	**HSB**
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

Fruits	93[a]	96[a]
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]

661 [a–c]Values within a row with different superscript letters are significantly different (P<0.05).

662

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

Phylum	Family	Avg (%) HS*	Avg (%) HSB	p-value 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

664 *Each healthy subject was analysed before (HS) and after (HSB) two months of diet intervention
665 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
667 (P<0.05) differences between fecal samples of HS and HSB.

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669

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

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 42°C)	6.61[b] (4.60 – 8.93)	7.53[a] (4.93 – 9.16)
Lactobacillus (mesophilus 25°C)	6.61[b] (3.26 – 8.98)	7.38[a] (4.46 – 9.28)
Enterococcus	7.05[a] (5.36 – 8.55)	7.29[a] (3.90 – 9.19)
Lactococcus and *Streptococcus*	7.74[a] (5.36 – 8.55)	7.57[a] (4.25 – 9.26)
Staphylococcus	5.68[a] (2.84 – 8.11)	6.14[a] (4.38 – 8.08)
Bacteroides, Porphyromonas and *Prevotella*	9.09[a] (3.00 – 9.92)	5.17[b] (2.52 – 6.44)
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 *Aeromonas*	6.42[a] (1.97 – 7.95)	3.68[b] (1.00 – 4.81)
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)

671 *Each healthy subject was analysed before (HS) and after (HSB) two months of diet intervention
672 with 100 g/day of durum wheat and whole-grain barley pasta containing 3% (wt/wt) of β-glucans.
673 [a–b]Values within a row with different superscript letters are significantly different ($P<0.05$).

674

Figure 1

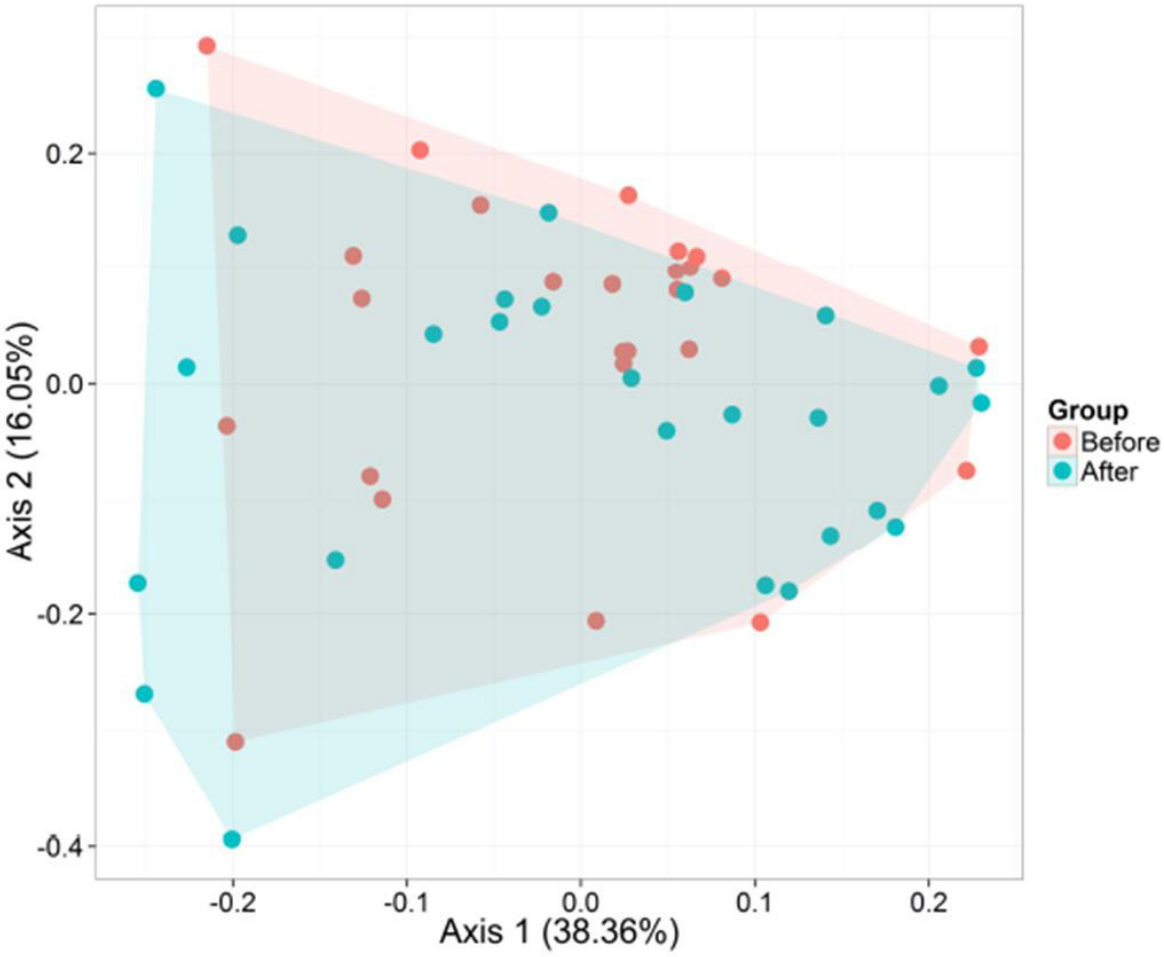


Figure 2

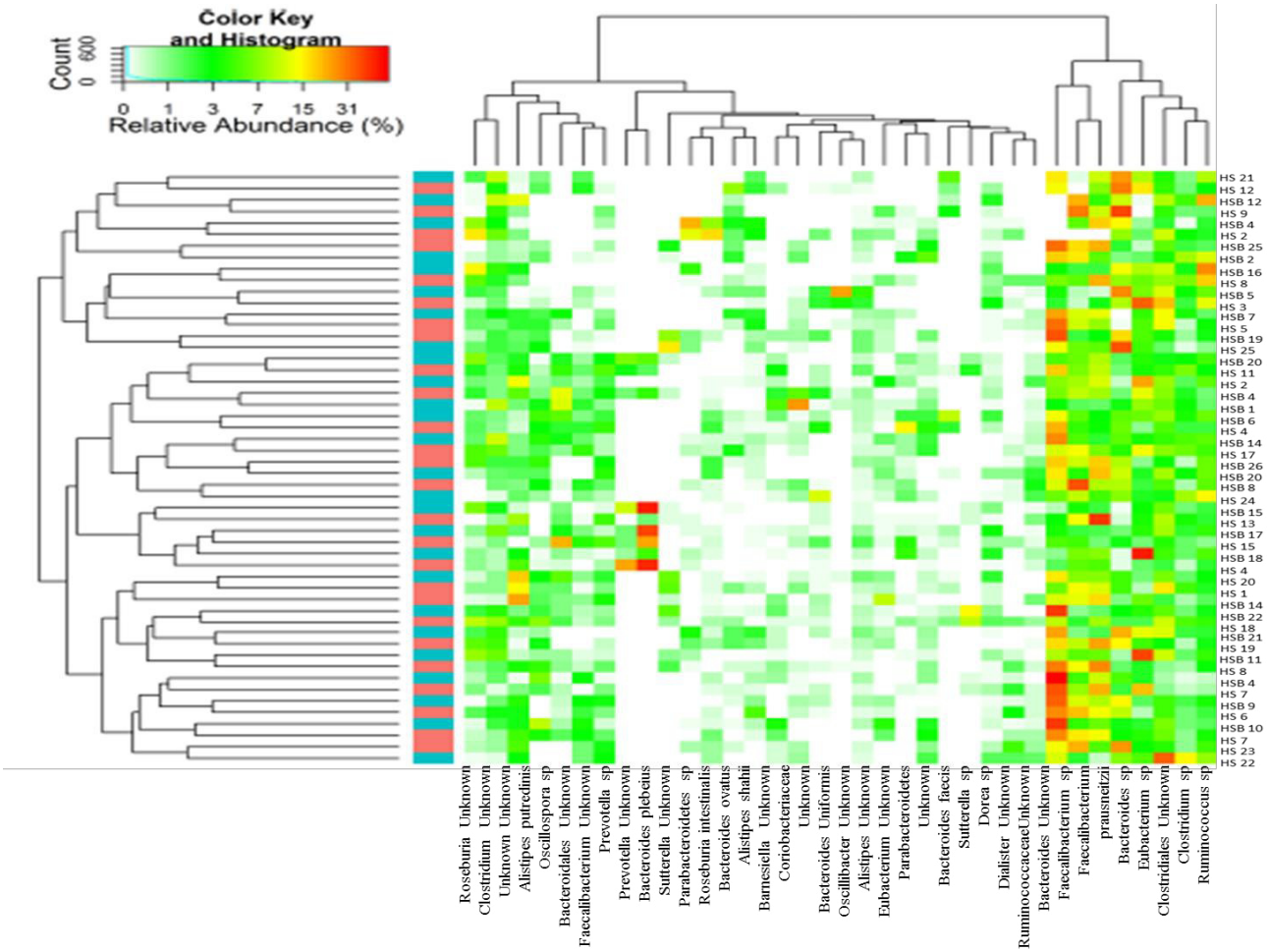


Figure 3

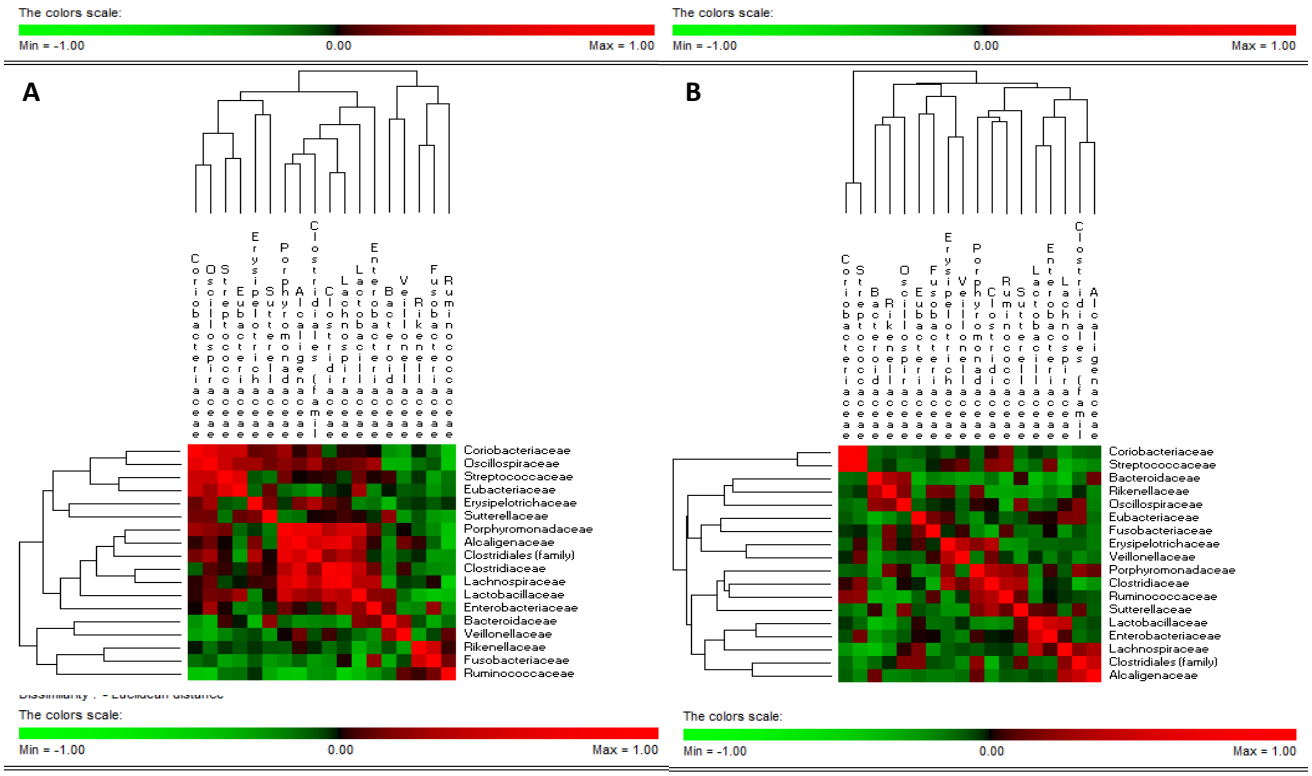


Figure 4

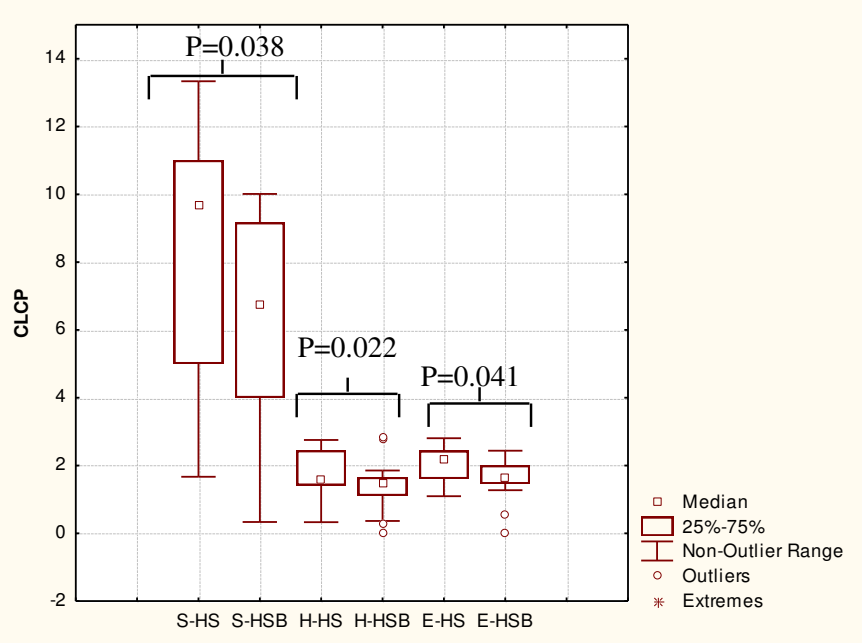
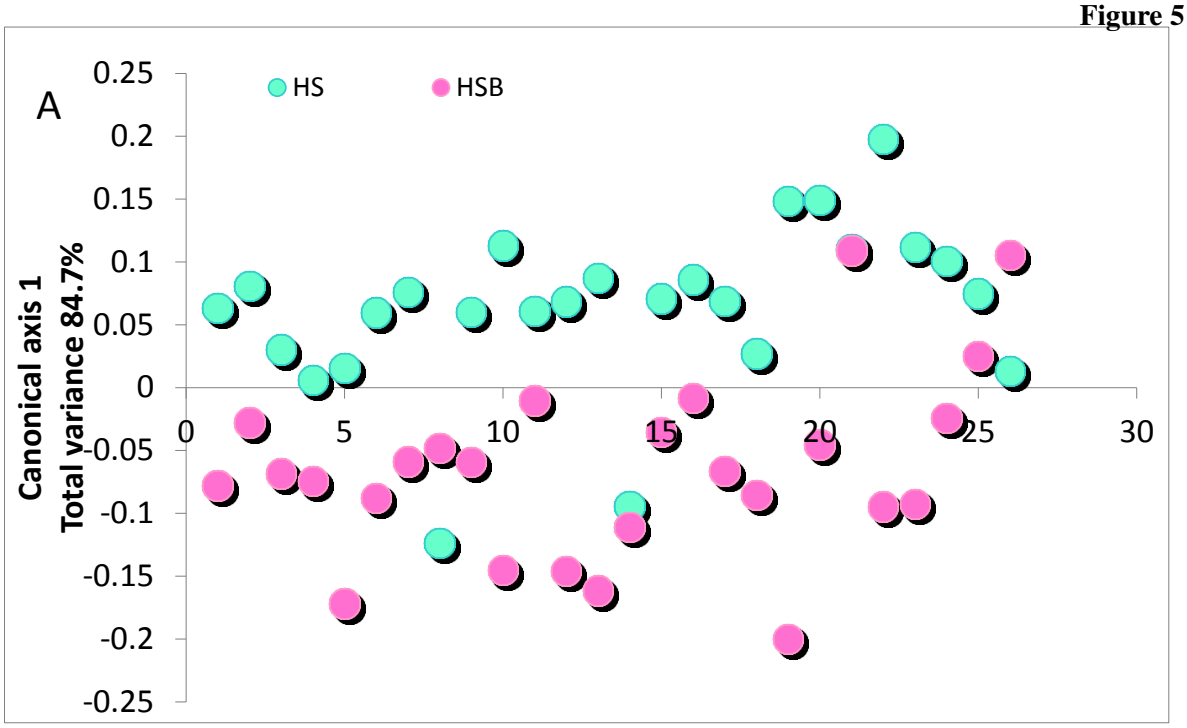


Figure 5



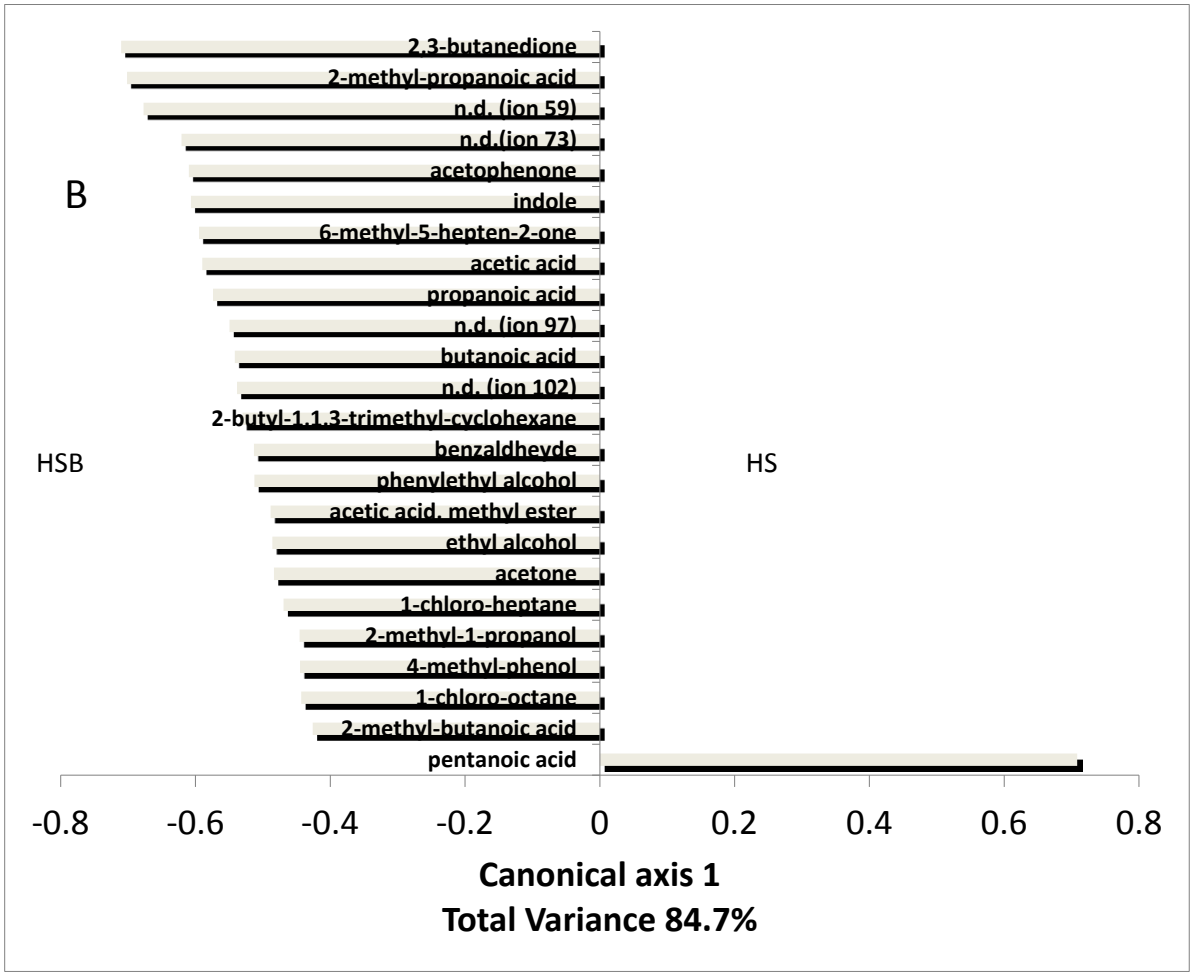


Figure 6