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RESEARCH ARTICLE

In vitro fermentation of potential prebiotic flours from natural sources: Impact on the human colonic microbiota and metabolome

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Scope: Fibers and prebiotics represent a useful dietary approach for modulating the human gut microbiome. Therefore, aim of the present study was to investigate the impact of four flours (wholegrain rye, wholegrain wheat, chickpeas and lentils 50:50, and barley milled grains), characterized by a naturally high content in dietary fibers, on the intestinal microbiota composition and metabolomic output.

Methods and results: A validated three-stage continuous fermentative system simulating the human colon was used to resemble the complexity and diversity of the intestinal microbiota. Fluorescence in situ hybridization was used to evaluate the impact of the flours on the composition of the microbiota, while small-molecule metabolome was assessed by NMR analysis followed by multivariate pattern recognition techniques. HT29 cell-growth curve assay was used to evaluate the modulatory properties of the bacterial metabolites on the growth of intestinal epithelial cells. All the four flours showed positive modulations of the microbiota composition and metabolic activity. Furthermore, none of the flours influenced the growth-modulatory potential of the metabolites toward HT29 cells.

Conclusion: Our findings support the utilization of the tested ingredients in the development of a variety of potentially prebiotic food products aimed at improving gastrointestinal health.

Keywords:
Fibers / Gut microbiota / Metabolomics / Prebiotics

1 Introduction

The microbiota is a complex and dynamic ecosystem that constantly interacts with the human metabolism, endowing the host with physiological traits that have not evolved in the host [1–3]. In particular, the intestinal microbiota can be regarded as a virtual organ able to exert a key contribution to the human energy balance, through several mechanisms: (i) extension of the host metabolic capacity to indigestible polysaccharides; (ii) regulation of fat storage; (iii) biosynthesis of essential vitamins and influence of amino acid homeostasis [3, 4]; (iv) absorption of key mineral [5]. Thus, the host can be considered a metaorganism, whose metabolism results from both the human and the collective microbial community counterparts [6].

Up-to-date metagenomic approaches have demonstrated that the human gut microbiota is composed of >1000 bacterial species that belong to only ten of the about 100 known bacterial divisions [7, 8]. Despite conservation at the highest taxonomic ranks, the intestinal microbiota is markedly individual-specific. A host-driven "top down" model for the

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Abbreviations: CMGM, culture medium for gut model; **CVD**, cardiovascular diseases; **FOS**, fructo-oligosaccharides; **GOS**, galacto-oligosaccharides; **FISH**, fluorescence in situ hybridization; **LAB**, lactic acid bacteria; **O-PLS**, orthogonal-projection to latent structure; **PCA**, principal component analysis; **PC**, principal component; **RS**, resistant starch; **TMSP**, sodium trimethylsilyl [2,2,3,3-$^2H_4$] propionate

assembly of the symbiotic microbial community and its high functional redundancy has been proposed [9, 10].

Diet is considered a major driver for changes in the compositional and functional relationship between microbiota and the host. In fact, dietary components are susceptible for metabolism by the intestinal microbial ecosystem, particularly influencing the growth and the metabolic activity of the dynamic bacterial populations thriving in the human colon [11].

To date, dietary fibers and prebiotics represent a useful dietary approach for influencing the composition of the human gut microbial community, since they are not completely metabolized by the digestive enzymes in the human small intestine. In particular, prebiotics are nondigestible food ingredients, which are fermented by the gut microbiota and beneficially affect the host stimulating growth and/or activity of specific intestinal bacteria [12]. Most common prebiotics are nondigestible galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), such as inulin and oligofructose. Inulin-type fructans are present in a range of different plants including wheat, onion, banana, garlic, leek, and agave [10]. The biological effect of prebiotics mainly depends on their influence on the gut microbiota composition and metabolism, exerted through a number of different functional properties, such as prevention of pathogen adhesion and colonization, modulation of bowel habits, regulation of lipid and glucose metabolism, and influence of the intestinal metabolome [10, 13].

Several dietary fibers, including nonstarch polysaccharides, whole-grain, cellulose, dextrans, chitins, pectins, β-glucans, and waxes have been reported to potentially provide similar beneficial effects as those of inulin-type fructans [11, 14, 15]. Very recently, it has been demonstrated that prebiotic-enriched pasta could be a useful dietary tool to manipulate gut microbiome-mediated well-being endpoint [16, 17].

In the present study, we aimed at investigating the impact of four flours characterized by a naturally high content in dietary fibers (Wholegrain rye [WGR], Nutriwheat [wholegrain wheat, NW], Pulses [chickpeas and lentils 50:50, PF], Barley milled grains [BMG]) on the human intestinal microbial ecosystem, using an in vitro three-stage continuous culture system simulating the human large intestine (colon model). The colon model used in this study provides a controlled environment that can be maintained in a steady state and that simulates the complexity and diversity of the microbiota. Therefore, it represents a relevant tool for monitoring the ecology and metabolic activities of colonic microbiota in relation to different external perturbations [18, 19].

Main bacterial groups of the fecal microbiota were evaluated during the colonic model system study using the 16S rRNA-based fluorescence in situ hybridization (FISH) approach. Potential effects of supplementation of the four flours on the microbial physiology were studied using NMR-based metabolomics in combination with multivariate pattern recognition techniques [20–22]. Finally, the ability of the metabolites of the cultural supernatants from the colonic model system, before and after the dietary supplementation, to modulate the growth of human intestinal epithelial cells was assessed using an HT29 cell-growth curve assay.

2 Materials and methods

2.1 Substrates and simulated in vitro human digestion

Four different flours (WGR, NW, PF, BMG), whose nutritional profile was characterized by official reference methods (ashes: UNI ISO 2171; proteins: UNI 10274 831/12/93 and ISO 1871 (15/12/75); total dietary fiber: AOAC 985.29; RS: AOAC 2002.02; β-glucans: AOAC 995.16 2005; FOS: AOAC 997.08; and GOS: AOAC 2001.02), were selected considering their different composition and potential functional properties (Table 1). Prior to being added into the colon model system, the flours were digested in vitro under appropriate conditions according to the procedures described by Mills et al. [23]. The selected flours (60 g) were mixed with 150 mL of sterile distilled water and homogenized in a stomacher (Seward, Worthing, UK), at high speed for 5 min. α-amylase (20 mg) was mixed with 1 mM $CaCl_2$ (6.25 mL, pH 7.0) and added to the flour solution, then incubated at 37°C for 30 min, under shaking. After the incubation, pH was adjusted to 2.0 and pepsin (2.7 g) in 0.1 M HCl (25 mL) was added, prior to a further incubation cycle, under shaking conditions, at 37°C for 2 h. Finally, bile (3.5 g) and pancreatin (560 mg) were mixed with 0.5 M $NaHCO_3$ (125 mL) at pH 7.0 and then with flour sample solution. Dialyses with membrane of 100–200 Da cut-off (Spectra/por 100–200 Da MWCO dialysis membrane, Spectrum Europe B.V., Breda, The Netherlands) were used to remove monosaccharides from the predigested flours. Ion-exchange chromatography was performed to monitor the flour digestion, thus ensuring the efficiency of the digestion procedure.

Table 1. Composition and nutritional profile of the four selected flours used in this study

Flour	Ashes	Proteins	Sugars	Total dietary fibers	Resistant starch	β-Glucans	FOS	GOS
Wholegrain rye (WGR)	1.55	8.91	1.91	18.04	<0.20	1.89	4.50	1.53
Pulses (lentils and chickpeas [PF])	3.02	22.95	1.78	15.44	2.00	<0.20	0.70	6.50
Nutriwheat (NW)	7.30	22.92	2.48	26.77	<0.20	1.22	2.3	1.0
Barley milled grains (BMG)	1.64	11.61	4.08	20.93	0.21	8.14	1.2	0.61

2.2 Three-stage continuous culture gut model system

The three-stage continuous culture model of the human colon comprised of three glass fermenters of increasing working volume, simulating the proximal (V1, 280 mL), transverse (V2, 300 mL), and distal colon (V3, 320 mL). The three fermenters connected in series were kept at 37°C, pH was maintained at 5.5 (V1), 6.2 (V2), and 6.8 (V3), and anaerobic conditions were introduced by continuously sparging with O_2-free N_2. The fermentation system was designed and validated to reproduce spatial, temporal, nutritional, and physicochemical characteristics of the microbiota in the human colon [18]. V1 was fed by means of a peristaltic pump with culture medium for gut model (CMGM), an ad hoc culture medium previously described by Macfarlane et al. and consisting of the following chemicals (g L^{-1}) in distilled water: starch, 5.0; pectin (citrus), 2.0; guar gum, 1.0; mucin (porcine gastric type III), 4.0; xylan (oatspelt), 2.0; arabinogalactan (larch wood), 2.0; inulin, 1.0; casein, 3.0; peptone water, 5.0; tryptone, 5.0; bile salts No. 3, 0.4; yeast extract, 4.5; $FeSO_4 \cdot 7H_2O$, 0.005; NaCl, 4.5; KCl, 4.5; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 1.25; $CaCl_2 \cdot 6H_2O$, 0.15; $NaHCO_3$, 1.5; cysteine, 0.8; hemin, 0.05; Tween 80, 1.0.

Human fecal samples were collected on site, kept in an anaerobic cabinet (10% H_2, 10% CO_2, 80% N_2), and used within a maximum of 15 min after collection. This experiment was carried out in duplicate using fecal samples from two different healthy volunteers (one male aged 31 and one female aged 38). None of the volunteers had received antibiotics or probiotics for at least 3 months before sampling. A 1:5 (w/w) fecal dilution in anaerobic PBS (0.1 mol L^{-1} PBS [pH 7.4]) was prepared and the samples homogenized in a stomacher (Seward) for 2 min. Each stage of the colonic model was inoculated with 100 mL fecal slurry. Total system transit time was set at 48 h, according to mean retention time of healthy individuals. Following inoculation, the colonic model was run as a batch culture for a 24-h period in order to stabilize bacterial populations prior to the initiation of medium flow. After 24 h (T0), the medium flow was initiated and the system ran for eight full volume turnovers to allow for steady state to be achieved (SS1). Taking into account the operating volume (900 mL) and the retention time (48 h) of the colonic model system, dialysis retentate of the tested flours was added daily into V1 at 1% (w/v). The tested flours were added to the system as described for a further eight volume turnovers upon which steady state 2 (SS2) was achieved. Each steady state was confirmed through sampling on three consecutive days for SCFAs and FISH analyses.

2.3 Sample collection and preparation

Samples for FISH were immediately fixed in 4% paraformaldehyde as previously described [24].

For HT29 cell growth curve analysis fermentation samples (2 mL) were taken from each vessel on three consecutive days for SS1 and SS2 and centrifuged at 12 000 × g for 15 min at room temperature. Supernatants were sterile filtered through a 0.2-μm syringe filter and frozen immediately at –20°C.

Samples for metabolomics analysis were homogenized at high speed in a mechanical homogenizer (MiniLab 8.30H, Rennie Wilmington, MA, USA) for 30–45 s, further subjected to two cycles of freezing and thawing to breakdown cell membranes and release cytoplasmic metabolites, and finally centrifuged at 10 000×g for 15 min at 4°C. Supernatants were collected and stored at –80°C until measurement.

2.4 In vitro enumeration of bacterial population by FISH

Numbers of 12 main intestinal bacterial groups, as well as total bacterial populations, were evaluated in samples from the colonic model system by FISH analysis, as previously described by Martin-Pelaez and colleagues [24]. The probes used are reported in Table 2 and were commercially synthesized and 5′-labeled with the fluorescent Cy3 dye (Sigma-Aldrich, St. Louis, MO, USA).

2.5 Modulation of HT29 cell growth by the tested flours

The influence of the colonic model supernatants, recovered before and after the supplementation of the four tested flours, on the growth and survival of the human colon carcinoma cell line HT29 was determined using the growth curve assay, as previously described by Maccaferri et al. [25]. Briefly, after allowing HT29 cells to attach for 24 h in 96-well plates, sterile filtered fermentation supernatants were added to give final concentrations of 1, 2.5, 5, and 10% (v/v) and incubated for 48 h. Growth inhibition compared to an untreated control was calculated and results are expressed as EC_{50}, which represents the effective concentration of colonic model supernatants resulting in a 50% reduction of cell number under the specified cell culture and treatment conditions compared to the growth of untreated cells.

2.6 NMR profiling

Frozen samples were thawed at room temperature and centrifuged at 16 000×g for 5 min. A total of 300 μL of a sodium phosphate buffer (70 mM Na_2HPO_4; 20% [v/v] ^2H_2O; 6.15 mM NaN_3; 6.64 mM sodium trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TMSP) [pH 7.4]) was immediately added to 300 μL of each sample, and the mixture homogenized by vortexing for 30 s. NaN_3 was added to ensure that metabolites are not generated or consumed via the action of bacteria or bacterial enzymes during the time of NMR sample preparation and NMR spectra acquisition. A total of 450 μL of this mixture was transferred into a 4.25-mm NMR tube for analysis.

Table 2. Oligonucleotide probes used in this study for FISH analysis

Target genus or group	Probe	Sequence (5′ to 3′)	Pretreatment/% Formamide	Hybridization-washing temperature (°C)
Most Bacteria	EUB338[a)]	GCTGCCTCCCGTAGGAGT	None	46–48
Most Bacteria	EUB338II[a)]	GCAGCCACCCGTAGGTGT	None	46–48
Most Bacteria	EUB338III[a)]	GCTG CCACCCGTAGGTGT	None	46–48
Atopobium, *Colinsella*, *Olsenella*, and *Eggerthella* spp.; *Cryptobacterium curtum*; *Mycoplasma equigenitalium*, and *Mycoplasma elephantis*	Ato291	GGTCGGTCTCTCAACC	None	50–50
Most *Bacteroides sensu stricto* and *Prevotella* spp.; all *Parabacteroides; Barnesiella viscericola*; and *Odoribacter splanchnicus*	Bac303	CCAATGTGGGGGACCTT	None	46–48
Most *Bifidobacterium* spp.	Bif164	CATCCGGCATTACCACCC	None	50–50
Most members of *Clostridium cluster* XIVa; *Syntrophococcus sucromutans*, [*Bacteroides*] *galacturonicus*, and [*Bacteroides*] *xylanolyticus*, *Lachnospira pectinschiza*, and *Clostridium saccharolyticum*	Erec482	GCTTCTTAGTCARGTACCG	None	50–50
Faecalibacterium prausnitzii and related sequences	Fprau655	CGCCTACCTCTGCACTAC	None	58–58
Most *Lactobacillus, Leuconostoc* and *Weissella* spp.; *Lactococcus lactis*; all *Vagococcus*, *Enterococcus*, *Melisococcus*, *Tetragenococcus*, *Catellicoccus*, *Pediococcus*, and *Paralactobacillus* spp.	Lab158	GTATTAGCAYCTGTTTCCA	Lysozyme[c)]	50–50
Most members of *Clostridium* cluster I; all members of *Clostridium* cluster II; *Clostridium tyrobutyricum*; *Adhaeribacter aquaticus*; and *Flexibacter canadensis* (family *Flexibacteriaceae*); [*Eubacterium*] *combesii* (family *Propionibacteriaceae*)	Chis150	TTATGCGGTATTAATCTYCCTTT	None	50–50
Clostridium cluster IX	Prop853	ATTGCGTTAACTCCGGCAC	None	50–50
Roseburia subcluster	Rrec584	TCAGACTTGCCG(C/T)ACCGC	None	50–50
Most Desulfovibrionales	DSV567	TACGGATTTCACTCCT	15% Formamide	46–48
Clostridium sporosphaeroides, *Ruminococcus bromii*, *Clostridium leptum*	Rbro730[b)]	TAAAGCCCAGCYAGGCCGC	Lysozyme[c)]	55–55
Ruminococcu salbus, *Ruminococcus flavefaciens*	Rfla729[b)]	AAAGCCCAGTAAGCCGCC	Lysozyme[c)]	55–55

a), b) These probes were used together in equimolar concentrations.
c) Lysozyme (100 U; 20 μL of 1 mg mL^{-1} solution of 50 000 U mg^{-1} protein).

NMR spectra for all samples were acquired using a Bruker spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 14.1 T (600.13 MHz proton Larmor frequency) equipped with a TCI CryoProbe (Bruker Biospin), an automatic tuning-matching unit, and an automatic sample changer. A BTO 2000 thermocouple served for temperature stabilization at the level of approximately 0.1 K at the sample. Before measurement, samples were kept for at least 3 min inside the NMR probehead, for temperature equilibration (27°C). For each sample, a monodimensional (1D) NMR spectrum was acquired with water peak suppression using a standard pulse sequence (Bruker terminology: noesygppr1d.comp) [26], 64 scans, 96 k data points, a spectral width of 180 28 Hz, and a relaxation delay of 4 s. Free induction decays were multiplied by an exponential function equivalent to a 1.0 Hz line-broadening factor before applying Fourier transform. Transformed spectra were corrected for phase and baseline distortions and calibrated (proton signal of TMSP at 0.00 ppm) using TopSpin (Version 2.1; Bruker BioSpin). The regions between 5.0 and 4.5 ppm, which contain the residual water signal, were removed from the subsequent analysis. Each 1D spectrum in the range between 0.2 and 10.0 ppm was segmented into 0.02-ppm chemical shift bins, and the corresponding spectral areas were integrated using AMIX software (Version 3.8.4; Bruker BioSpin) giving a total of 466 variables. The total spectral area was calculated on the remaining bins and normalization on the total area was carried out on the data prior to pattern

recognition. All metabolites of interest were then checked and their NMR signals were assigned on template 1D NMR profiles by using matching routines of AMIX 3.8.4 (Bruker BioSpin) in combination with the BBIOREFCODE (Version 2.0.0; Bruker BioSpin) reference databases and published literature [26–30].

2.7 Statistical analysis

Bacterial counts and HT29 growth modulation data were analyzed by one-way ANOVA, using Tukey's post-test analysis when the overall *p*-value of the experiment was below the value of significance ($p < 0.05$). An additional paired *t*-test was applied in order to assess the significance of results of single pairs of data. Analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Metabolomics-based data analysis was carried out using R software with scripts developed in-house. (R scripts are provided in Supporting Information). Principal component analysis (PCA) was conducted in order to observe intrinsic clusters and a general overview of the variance of the NMR profiles. Furthermore, NMR spectra were subjected to orthogonal-projection to latent structure (O-PLS) for highlighting the effects of the four different flours. O-PLS is an extension of the Partial Least Square regression method [31], featuring an integrated Orthogonal Signal Correction filter [32]. The relative concentrations of the metabolites were calculated by integrating the signals in the spectra normalized to the total area. To evaluate the effects of different flours in the three vessels, the relative concentrations of the metabolites were mean centered (separately for each vessel). Statistical significance was assessed using univariate nonparametric Wilcoxon rank-sum test between SS1 and SS2 samples of each treatment. A $p < 0.05$ was considered statistically significant.

3 Results

3.1 Impact of the flours on the human colonic microbiota

Table 3 describes the impact of the four flours on the composition of the main bacterial groups constituting the human colonic microbiota. A number of significant modifications along the study have been shown for all the tested flours. In particular, WGR mediated a significant increase in concentration of *Bifidobacterium* genus, lactic acid bacteria (LAB), and *Desulfovibrionales* spp. in all the stages of the colonic model system, whereas *Roseburia/E. rectale* group significantly decreased in V1, simulating the proximal colon, after the administration of this flour. Similarly, NW induced a significant increase in LAB in the first stage of the colonic model system, bifidobacteria in the second stage, simulating the transverse colon, and *Desulfovibrionales* spp. in the third stage, simulating the distal colon. A decrease in concentration of *Ruminococcus* spp. was further found in the whole colonic model system. While leading to an overall increase of *Bacteroides/Prevotella* and a decrease of *Roseburia/E. rectale* group in the entire colonic model system, the supplementation of PF provoked several modifications in each region of the fermentative system. In V1, there was a decrease in *E. rectale/Clostridium* cluster XIVa group and in *Atopobium* cluster, while in V2 *Clostridium* cluster IX and *Faecalibacterium prausnitzii* decreased after PF supplementation. The concentration of *F. prausnitzii* was also significantly lower in V3. The only increase in concentration was observed in *Ruminococcus* spp. in V3, after PF feeding. Finally, BMG provoked a decrease in *E. rectale/Clostridium* cluster XIVa group in the third stage of the colonic model system and *Roseburia/E. rectale* groups in the second and third stage. An increase of *Desulfovibrionales* spp. was concomitantly shown in the V1 and V2 when BMG was used as dietary flour.

3.2 HT29 growth modulation by the colonic model system supernatants

EC_{50} was used to compare the effect of colonic model supernatants, before and after the administration of the four different flours, on HT29 cell growth (Fig. 1). No significant changes between EC_{50}(SS1) and EC_{50}(SS2) were found in the all the stages composing the colonic model system, at SS1 and SS2, for any of the four tested dietary flours.

3.3 NMR analysis

The comparison of the spectra of samples from the colonic model at SS1 and SS2 samples (Table 4) demonstrated that all the tested dietary flours caused different modifications in metabolite concentrations. In particular, BMG and PF induced several changes in the metabolic profile. After the supplementation of BMG and PF, colon model metabolome was characterized by lower level of trimethylamine and higher level of acetate. In addition, PF provoked a decrease in levels of butyrate and isovalerate, while higher levels of propionate and tyrosine were found. Similar to PF, NW induced lower level of butyrate and higher level of propionate. Interestingly, WGR induced a decrease in methanol not observed with any other flour.

PCA was performed to obtain a simplified view of the variation in the data and to understand changes in composition and extent of the colonic metabolites detected by NMR in the three stages of the colon model. Two principal components (PCs) were calculated, PC1 and PC2, which explain 60.7 and 19.4% of the total variance, respectively. Scores along PC1 and PC2 showed a strong separation among the V1 samples and the V2 and V3 samples, as reported in Fig. 2 A. The outlier, found in the PCA plot, was due to the presence of a high concentration of lactate. O-PLS analysis was also performed

Table 3. Bacterial groups detected by FISH in the culture broth recovered from each stage (Vessel 1, Vessel 2, and Vessel 3) of the colonic model before (SS1) and after (SS2) the daily administration of the four dietary flours. Modifications at a confidence level of 95% ($p < 0.05$) are followed by hashkey when significantly increased or followed by an asterisk when significantly decreased between SS1 and SS2

		SS1			SS2		
		Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3
Nutriwheat (NW)	*FPrau655*	8.79 ± 0.07	8.82 ± 0.11	8.46 ± 0.11	8.66 ± 0.13	8.11 ± 0.16	7.75 ± 0.13
	Bif164	7.38 ± 0.11	7.26 ± 0.16	7.28 ± 0.14	7.70 ± 0.04	**7.61 ± 0.14#**	7.45 ± 0.08
	Lab158	7.70 ± 0.16	7.79 ± 0.18	7.85 ± 0.17	**8.23 ± 0.26#**	7.97 ± 0.18	7.72 ± 0.08
	Erec482	8.67 ± 0.09	8.71 ± 0.07	8.58 ± 0.11	8.71 ± 0.13	8.70 ± 0.15	8.56 ± 0.10
	Ato291	8.63 ± 0.12	8.53 ± 0.13	8.42 ± 0.12	8.69 ± 0.08	8.63 ± 0.12	8.39 ± 0.17
	Rrec584	7.99 ± 0.21	7.77 ± 0.07	7.87 ± 0.07	7.91 ± 0.28	7.57 ± 0.15	7.51 ± 0.16
	EUB338	9.75 ± 0.12	9.49 ± 0.07	9.38 ± 0.09	9.57 ± 0.07	9.43 ± 0.10	9.05 ± 0.06
	Bac303	8.58 ± 0.26	8.94 ± 0.07	8.41 ± 0.19	8.79 ± 0.22	8.94 ± 0.21	8.82 ± 0.11
	Rbro279/ Rfla730	8.92 ± 0.06	8.55 ± 0.19	8.30 ± 0.11	**8.38 ± 0.15***	**7.87 ± 0.08***	**7.53 ± 0.09***
	DSV567	6.49 ± 0.43	7.03 ± 0.39	6.45 ± 0.29	7.22 ± 0.16	7.50 ± 0.17	**7.83 ± 0.27#**
	Prop853	8.97 ± 0.14	8.79 ± 0.13	8.60 ± 0.11	8.82 ± 0.09	8.76 ± 0.13	8.65 ± 0.08
	Chis150	8.21 ± 0.09	8.13 ± 0.16	7.93 ± 0.23	8.15 ± 0.24	7.66 ± 0.31	7.67 ± 0.35
Whole grain rye (WGR)	*FPrau655*	8.71 ± 0.09	8.58 ± 0.07	8.24 ± 0.13	8.36 ± 0.38	8.63 ± 0.14	8.61 ± 0.15
	Bif164	7.46 ± 0.13	7.30 ± 0.12	7.26 ± 0.14	**8.54 ± 0.23#**	**8.65 ± 0.22#**	**8.71 ± 0.25#**
	Lab158	7.81 ± 0.29	7.64 ± 0.24	7.73 ± 0.19	**8.56 ± 0.19#**	**8.73 ±0.23#**	**8.52 ± 0.23#**
	Erec482	8.68 ± 0.11	8.80 ± 0.10	8.60 ± 0.14	8.04 ± 0.38	8.84 ± 0.15	8.83 ± 0.13
	Ato291	8.58 ± 0.17	8.50 ± 0.13	8.55 ± 0.13	8.88 ± 0.12	8.77 ± 0.11	8.72 ± 0.13
	Rrec584	7.88 ± 0.17	7.91 ± 0.14	7.70 ± 0.21	**7.26 ± 0.22#**	7.77 ± 0.16	7.64 ± 0.08
	EUB338	9.67 ± 0.08	9.41 ± 0.06	9.17 ± 0.17	9.61 ± 0.15	9.55 ± 0.07	9.43 ± 0.09
	Bac303	8.64 ± 0.27	8.92 ± 0.17	8.60 ± 0.16	8.78 ± 0.24	9.14 ± 0.09	8.85 ± 0.11
	Rbro279/ Rfla730	8.53 ± 0.21	8.44 ± 0.16	8.28 ± 0.18	8.29 ± 0.25	8.34 ± 0.15	7.94 ± 0.08
	DSV567	6.61 ± 0.40	6.66 ± 0.26	6.65 ± 0.15	**7.38 ± 0.37#**	**7.49 ± 0.29#**	**7.78 ± 0.28#**
	Prop853	8.80 ± 0.15	8.73 ± 0.12	8.73 ± 0.15	8.80 ± 0.17	8.95 ± 0.08	8.74 ± 0.11
	Chis150	8.18 ± 0.12	8.20 ± 0.09	8.21 ± 0.17	7.27 ± 0.37	8.35 ± 0.28	8.40 ± 0.20
Barley milled grains (BMG)	*FPrau655*	8.96 ± 0.15	8.79 ± 0.17	8.68 ± 0.12	8.79 ± 0.28	8.70 ± 0.06	8.44 ± 0.05
	Bif164	8.72 ± 0.09	8.65 ± 0.07	8.52 ± 0.08	8.74 ± 0.50	8.96 ± 0.11	8.67 ± 0.26
	Lab158	8.82 ± 0.07	8.68 ± 0.07	8.52 ± 0.05	8.63 ± 0.14	8.67 ± 0.04	8.50 ± 0.03
	Erec482	8.96 ± 0.09	8.88 ± 0.17	8.94 ± 0.06	8.58 ± 0.29	8.80 ± 0.06	**8.75 ± 0.06**
	Ato291	8.72 ± 0.04	8.82 ± 0.03	8.83 ± 0.02	8.87 ± 0.08	8.72 ± 0.07	8.60 ± 0.09
	Rrec584	8.30 ± 0.16	8.12 ± 0.08	8.14 ± 0.13	7.82 ± 0.38	**7.77 ± 0.15#**	**7.39 ± 0.10#**
	EUB338	9.38 ± 0.44	9.66 ± 0.15	9.25 ± 0.10	9.51 ± 0.17	9.37 ± 0.11	9.23 ± 0.12
	Bac303	8.34 ± 0.29	8.59 ± 0.13	8.47 ± 0.17	8.69 ± 0.25	8.93 ± 0.09	8.47 ±0.14
	Rbro279/ Rfla730	7.93 ± 0.40	8.65 ± 0.11	8.50 ± 0.18	7.64 ± 0.63	7.59 ± 0.60	8.33 ± 0.09
	DSV567	6.77 ± 0.11	6.87 ± 0.09	7.04 ± 0.09	**7.41 ± 0.19#**	**7.63 ± 0.18#**	7.54 ± 0.21
	Prop853	8.65 ± 0.16	8.80 ± 0.12	8.80 ± 0.13	8.80 ± 0.05	8.81 ± 0.09	8.56 ± 0.09
	Chis150	8.53 ± 0.07	8.57 ± 0.10	8.44 ± 0.14	8.36 ± 0.17	8.64 ± 0.08	8.49 ± 0.15
Pulses (PF)	*FPrau655*	8.86 ± 0.09	8.75 ± 0.08	8.65 ± 0.13	8.93 ± 0.06	**8.50 ± 0.05***	**8.19 ± 0.08***
	Bif164	8.05 ± 0.45	8.57 ± 0.08	8.25 ± 0.13	8.41 ± 0.19	8.89 ± 0.19	8.78 ± 0.08
	Lab158	8.73 ± 0.09	8.63 ± 0.07	8.46 ± 0.14	8.64 ± 0.04	8.61 ± 0.04	8.48 ± 0.05
	Erec482	8.99 ± 0.06	8.94 ± 0.06	8.86 ± 0.11	**8.60 ± 0.04***	8.77 ± 0.05	8.70 ± 0.05
	Ato291	8.74 ± 0.05	8.79 ± 0.06	8.76 ± 0.06	**8.87 ± 0.03***	8.93 ±0.09	8.80 ± 0.06
	Rrec584	8.61 ± 0.13	8.23 ± 0.11	8.06 ± 0.14	**7.52 ± 0.32***	**7.55 ± 0.14***	**7.46 ± 0.19***
	EUB338	9.67 ± 0.11	9.26 ± 0.08	9.26 ± 0.12	9.76 ± 0.07	9.48 ± 0.12	9.35 ± 0.07
	Bac303	8.32 ± 0.41	8.63 ± 0.13	8.53 ± 0.19	**9.45 ± 0.04#**	**9.09 ± 0.05#**	**8.95 ± 0.07#**
	Rbro279/ Rfla730	7.50 ± 0.55	8.39 ± 0.11	8.18 ± 0.11	6.25 ± 0.05	8.50 ± 0.06	**8.51 ± 0.07#**
	DSV567	7.38 ± 0.32	7.36 ± 0.27	6.88 ± 0.44	7.12 ± 0.38	6.89 ± 0.35	6.72 ± 0.34
	Prop853	8.79 ±0.10	8.83 ± 0.09	8.66 ± 0.04	8.79 ± 0.05	**8.64 ± 0.04***	8.55 ± 0.05
	Chis150	8.60 ± 0.08	8.21 ± 0.11	8.14 ± 0.13	8.19 ± 0.15	8.41 ± 0.08	8.47 ± 0.09

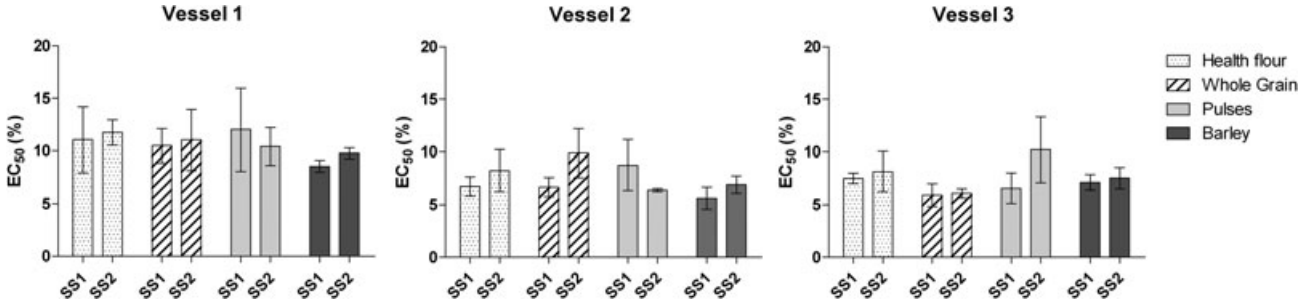

Figure 1. Growth inhibitory effect of supernatants recovered from Vessel 1, Vessel 2, and Vessel 3 of the colonic model system, before (SS1) and after (SS2) administration of the four dietary flours on HT29 carcinoma cells. Growth inhibition was assessed by coincubating HT29 cells with increasing concentration (0, 1, 2.5, 5, and 10%) of fermentation supernatants for 48 h. Results are expressed as EC50 values of SS1 and SS2 for each vessel and flour, and represent the means of four calculated EC50 concentrations ± SEM derived from two colonic models with two independent sampling times each. Measurements for each colonic model and time point were performed in triplicate.

on NMR profiles using as supervisory variable the different dietary interventions undertaken in the study (Fig. 2 B). This analysis demonstrated that the effect of PF and BMG supplementation on the colonic metabonome was higher than that resulting from administration of NW and WGR.

4 Discussion

In recent years, a new health paradigm has evolved, placing more emphasis on the beneficial aspects of diet. Although the primary role of diet is to provide nutrients to fulfill metabolic requirements, the use of foods to improve health and well-being is being increasingly accepted [33]. Very recently, the importance of diet in modulating the human gut microbiota has been discussed in the context of the "enterotype" theory, which categorized human gut microbiota in three clusters identifiable by the variation in the levels of one of three genera: *Bacteroides*, *Prevotella*, and *Ruminococcus*, respectively [34]. Wu et al. demonstrated that fat and fiber intakes are associated with particular bacterial groups, and that enterotypes are determined by long-term dietary habits [35].

Dietary fibers and prebiotics can be included in a wide range of foods, such as bakery, dairy, and beverage products. In particular, prebiotics are oligosaccharides or more complex saccharides that are selectively metabolized by some

Table 4. *p*-values resulting from the comparison between metabolite concentrations in the colonic model before (SS1) and after (SS2) the daily administration of the four different dietary flours. For each flour, comparison was made on the basis of results deriving from NMR spectra of two colonic models, at SS1 and SS2

Compound	Chemical shift (multiplicity) and assignment	Pulses flour (PF)		Barley milled grains (BMG)		Nutriwheat (NW)		Wholegrain rye (WG)	
Acetate	1.918 (t) CH_3	↑	**4.33×10^{-3}**	↑	**2.16×10^{-3}**	↓	8.18×10^{-1}	↑	5.89×10^{-1}
Alanine	1.485 (d) CH_3	↑	6.49×10^{-2}	↓	6.99×10^{-1}	↓	6.99×10^{-1}	↑	5.89×10^{-1}
Butyrate	1.563 (m) CH_2	↓	**2.16×10^{-3}**	↓	2.40×10^{-1}	↓	**8.66×10^{-3}**	↑	6.99×10^{-1}
Ethanol	1.186 (t) CH_3	↑	6.99×10^{-1}	↑	3.10×10^{-1}	↓	3.10×10^{-1}	↓	5.89×10^{-1}
Formate	8.460 (s) CH_3	↓	6.99×10^{-1}	↓	8.18×10^{-1}	↓	9.37×10^{-1}	↑	1.32×10^{-1}
Fumarate	6.523 (s) CH_3	↑	6.99×10^{-1}	↑	3.94×10^{-1}	↑	1.00×10^{-0}	↑	2.40×10^{-1}
Isoleucine	1.011 (d) CH_3	↑	3.10×10^{-1}	↓	1.32×10^{-1}	↑	3.10×10^{-1}	↑	9.37×10^{-1}
Isovalerate	2.058 (d) CH_2	↓	**2.60×10^{-2}**	↑	9.37×10^{-1}	↓	9.37×10^{-1}	↑	5.89×10^{-1}
Lactate	1.350 (d) CH_3	↑	6.99×10^{-1}	↑	1.00×10^{-0}	↓	3.94×10^{-1}	↑	2.40×10^{-1}
Leucine	0.965 (t) CH_3	↑	3.94×10^{-1}	↓	6.99×10^{-1}	↑	2.40×10^{-1}	↑	8.18×10^{-1}
Lysine	3.032 (t) CH_2	↑	1.32×10^{-1}	↑	9.37×10^{-1}	↑	**2.16×10^{-3}**	↓	6.99×10^{-1}
Methanol	3.366 (s) CH_3	↑	1.80×10^{-1}	↓	3.94×10^{-1}	↓	5.89×10^{-1}	↓	**2.16×10^{-3}**
Phenylalanine	7.438 (m) CH	↑	6.49×10^{-2}	↑	3.10×10^{-1}	↑	1.32×10^{-1}	↑	1.00×10^{-0}
Propionate	1.059 (t) CH_3	↑	**2.60×10^{-2}**	↓	1.00×10^{-0}	↑	**2.16×10^{-3}**	↓	3.10×10^{-1}
Trimethylamine	2.876 (s) CH_3	↓	**4.11×10^{-2}**	↓	**8.66×10^{-3}**	↓	1.80×10^{-1}	↓	1.80×10^{-1}
Tyrosine	6.905 (m) CH	↑	**4.11×10^{-2}**	↑	3.10×10^{-1}	↑	1.32×10^{-1}	↓	3.94×10^{-1}
Uracil	5.810 (d) CH	↑	9.37×10^{-1}	↑	5.89×10^{-1}	↓	1.52×10^{-2}	↓	8.18×10^{-1}
Valine	0.995 (d) CH_3	↑	6.99×10^{-1}	↓	6.49×10^{-2}	↑	9.37×10^{-1}	↓	8.18×10^{-1}

Significant modifications are highlighted in bold.
s, singlet; d, doublet; t, triplet; m, multiplet.

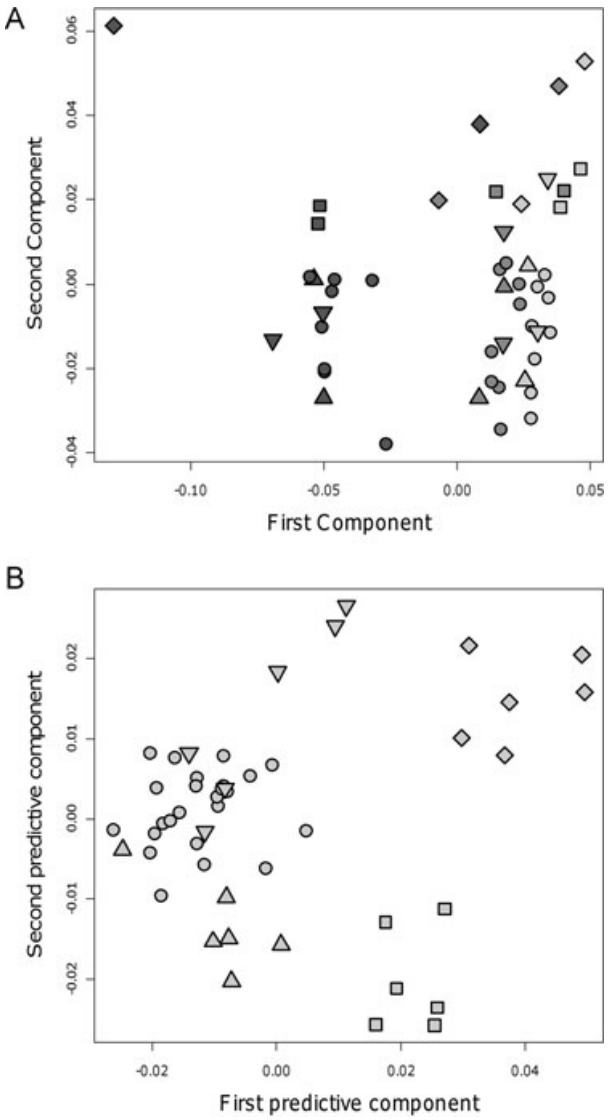

Figure 2. Multivariate analysis of ^{1}H-NMR spectra of fecal samples. (A) PCA scores plot colored according to the vessel: dark gray, Vessel 1; gray, Vessel 2; and light gray, Vessel 3. (B) O-PLS scores plot discriminating the effects of different prebiotics. Circle, SS1; square, PF; rhombus, BMG; triangle, NW; inverted triangle, WGR.

commensal groups of bacteria, including those considered to be beneficial for the host, thus impacting on the gut microbiota composition and functional activity [13, 36].

Cereal grains can contain naturally occurring oligosaccharides such as galactosyl derivatives of sucrose stachyose and raffinose and fructosyl derivatives of sucrose. Furthermore, cereal grains and pulses contain dietary fiber that encompasses a heterogeneous range of complex polysaccharides that are not substantially digested in the small intestine and pass through to the colon. Therefore, dietary fiber constitutes a potential and not yet fully explored source of prebiotics from cereals and legumes [37].

In the perspective of designing foods from natural sources and more effective dietary strategies for human health promotion, here we studied the putative prebiotic potential of four flours, which are naturally rich in fiber, by assessing their impact on the human intestinal microbial ecosystem.

The four tested flours belong to two different categories, cereal grain flours (namely wholegrain rye, wholegrain wheat, and barley milled grains), and pulses flour, which are not yet evaluated for their prebiotic potential. In particular, the two wholegrain flours, one deriving from rye and one from wheat, contain naturally high concentrations of GOS and FOS, while the pulses flour, composed by a mix of lentils and chickpeas 50:50, is particularly rich in GOS and resistant starch (RS). Finally, the barley milled grains flour is a rich source for β-glucans.

β-glucans are components of dietary cereals that are becoming increasingly recognized as functional ingredients in food and drink products [38]. Mixed-linkage β-glucans, present at high levels in the BMG flour, have been demonstrated to evoke a range of metabolic and physiological responses, as lowering cholesterol levels and insulin responses [38–40]. In the present study, an increase in acetate, following BMG administration in the colon model system, has been demonstrated. Our results support the hypothesis that, through a modulation of the gut microbiota and the subsequent production of different amounts of SCFAs, barley could exert a beneficial role for the host. This positive modulation is also supported by Hughes et al. [41], who evaluated the in vitro fermentation of barley-derived β-glucans by the human fecal microbiota, demonstrating a significant modification in SCFAs profile, mainly propionate. Notably, we also demonstrated a decrease in the concentration of trimethylamine, a precursor of the trimethylamine N-oxide. This harmful compound, produced by commensal inhabitants of the intestinal microbiota, has been demonstrated to play a role in the atherogenesis [42].

Pulses contain a number of bioactive substances including enzyme inhibitors, lectins, phytates, oligosaccharides, and phenolic compounds. These polyphenolic compounds consist mainly of tannins, phenolic acids, and flavonoids [43]. Phenolics are metabolized by the gut microbiota, affecting intestinal health [44]. In addition, chickpeas, which contain significant levels of oligosaccharides, nonstarch polysaccharides, RS, and resistant protein can exert a not yet fully explored prebiotic potential [45]. Here, we demonstrated that PF induces an extensive modulation of the colonic metabolome, provoking an increase in concentration of acetate and propionate, as well as a decrease of butyrate and branched-chain fatty acid isovalerate. This metabolic shift can be explained by the overall increase of *Bacteroides/Prevotella* species, known to be primary propionate and acetate producers, which are representing a considerable share of the intestinal microbiota resulting from the supplementation with PF. Conversely, the decrease of major butyrate producers as *Clostridium* cluster IV and XIVa can be related to the decrease of butyrate [46]. Notably, propionate is a major microbial fermentation

metabolite in the human gut with well-known health effects not only at colon level, but also in a broader human body context. In fact, propionate is thought to lower lipogenesis and serum cholesterol level, as well as to play a role in weight control by stimulating satiety [47].

Wholegrain has been extensively studied for its beneficial effects and several epidemiological studies have shown its protective activity against cancer, diabetes, obesity, and cardiovascular disease (CVD) [48, 49]. However, little is known about peculiar modulation of the colonic microbiota related to wholegrain rye and wheat. In the present study, we have demonstrated that WGR impacts the colonic microbiota, eliciting an increase in concentration of bifidobacteria, lactobacilli, and Desulfovibrionales, while a decrease in *Roseburia* genus was observed. Similarly, NW caused significant yet less-pronounced increase of bifidobacteria, LAB, and Desulfovibrionales in comparison with WGR, whereas a decrease of ruminococci was further demonstrated. These data are in accordance with those of Costabile et al. [14], who for the first time broadly investigated the impact of wholegrain on the human colonic microbiota. In particular, these authors found significant increases in lactobacilli/enterococci after the ingestion of either wheat bran or wholegrain but in *Bifidobacterium* spp. only after wholegrain consumption. Concerning the results presented in this study about the bifidogenic effect of WGR and NW, it should be highlighted that basal level of bifidobacteria detected at SS1 in the colonic model systems fed with these two flours was lower than that assessed at SS1 for BMG and PF. Since a masking effect at high concentration of bifidobacteria has been suggested by human trials [50], it could be hypothesized a similar effect also in the present in vitro study for BMG and PF. However, previous results obtained using the same colon model approach did not encounter any difficulty in detecting significant modulation of bacterial groups even at concentration higher than 8.0 log cells mL^{-1} [25]. Therefore, the results here presented about the ability of NW and WGR in modulating the concentration of bifidobacteria and lactobacilli can be considered a valuable specific characteristic of these flours.

While the health-promoting effects of LAB and *Bifidobacterium* spp. within the human gut microbiota are well established, the detrimental role of sulphate-reducing bacteria, i.e., Desulfovibrionales, has been suggested, recognizing this bacterial group as a putative causative agent of ulcerative colitis [51]. However, very recent publications suggest that Desulfovibrionales are not directly associated with major health issues. Hildebrandt et al. [52] demonstrated that, irrespectively of the phenotype, a high-fat diet was leading to an increased concentration of Proteobacteria, mainly accountable to Desulfovibrionales, but this modification of the gut microbiota was not associated to major health outputs. Furthermore, higher numbers of Desulfovibrionales were detected in healthy children compared to malnourished ones [53]. Similarly, colorectal cancer patients had significantly lower numbers of *Desulfovibrio* spp. than healthy individuals, as recently reported in a review by Kinross et al. [54]. These controversial results require further studies, in order to better understand the questionable role of Desulfovibrionales in the human gut ecology. Therefore, modification of sulphate-reducing bacteria assessed in the present study, in response to the supplementation of NW, WGR, and BMG, are challenging to be interpreted in terms of physiological relevance for the host.

The supplementation of wholegrain-based flours (WGR and NW) induced modulation of the colonic metabolome. Indeed, we demonstrated a decrease in the concentration of methanol, a compound that has been previously demonstrated to increase in a number of pathological conditions, especially in subjects affected by *Campylobacter jejuni* and ulcerative colitis [55]. A decrease in methanol concentration related to a modulation of the colonic microbiota toward a more bifidogenic structure has been reported in literature. In fact, a decrease in this alcohol has been associated with positive and bifidogenic modulation of the colonic microbiota of patients affected by Crohn's diseases after treatment with the antibiotic rifaximin [25]. Conversely, no significant changes in concentrations of the SCFA acetic, propionic, butyric, or caproic acids were observed in response to WGR administration.

NMR analysis of the colon model metabolome following the NW administration depicted a different scenario. NW is mediating a decrease of butyrate and an increase of propionate and the essential amino acid lysine. Notably, the decrease in concentration of butyrate could be explained by a depletion of *Ruminococcus* spp. after the supplementation of the wholegrain wheat flour, since ruminococci are well known to produce butyrate [56].

Growth inhibition of colon carcinoma cells, differentiation, and apoptosis are thought to be mechanisms by which dietary fiber exerts a chemopreventive effect in the colon [57]. However, the administration of all the four flours from natural sources did not influence the growth-modulatory potential of the supernatants recovered from the three stages of the colonic model system. Since it has been demonstrated that the concentration of butyrate in fermentation supernatants from colonic model systems was directly associated with their growth inhibitory potential [58], our observations in the HT29 growth assay are in accordance with the NMR results showing that the concentration of butyrate in the supernatants did not change or even decreased after administration of the flours. In conclusion, wholegrain rye and wheat, pulses, and barley milled grain flours showed peculiar and positive modulations of the intestinal microbiota composition and its small molecule metabolome. Therefore, our results could support the utilization of these ingredients in the development of a variety of potentially prebiotic food products aimed at improving gastrointestinal health.

The authors have declared no conflict of interest.

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