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The impact of long-term dietary pattern of fecal donor on *in vitro* fecal fermentation properties of inulin†

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Although the composition of the gut microbiota is of interest, the functionality, or metabolic activity, of the gut microbiota is of equal importance: the gut microbiota can produce either harmful metabolites associated with human disease or beneficial metabolites that protect against disease. The purposes of this study were to determine the associations between dietary intake variables and fecal short and branched chain fatty acid (S/BCFA) concentrations; to determine the associations between dietary intake variables and inulin degradation, short and branched chain fatty acid (S/BCFA) production, and ammonia production during *in vitro* fecal fermentation of a highly fermentable substrate (inulin); and finally to compare results from the fermentation of inulin with those obtained in a previous report using a poorly fermentable substrate (whole wheat; Yang and Rose, *Nutr. Res.*, 2014, **34**, 749–759). Stool samples from eighteen individuals that had completed one-year dietary records were used in an *in vitro* fecal fermentation system with long-chain inulin as substrate. Few dietary intake variables were correlated with fecal S/BCFA concentrations; however, intakes of several plant-based foods, especially whole grain, dry beans, and certain vegetables that provided dietary fiber, plant protein, and B vitamins, were associated with acetate, propionate, butyrate, and total SCFA production during inulin fermentation. In contrast, intake of dairy and processed meats that provided cholesterol and little fiber, were associated with ammonia and BCFA production. Comparing results between inulin and whole wheat fermentations, significant correlations were only found for butyrate and BCFA, suggesting that regardless of the type of carbohydrate provided to the microbiota, long-term diet may have a pronounced effect on the propensity of the gut microbiota toward either beneficial metabolism (butyrate production) or detrimental metabolism (BCFA production). These results may help in the development of new dietary strategies to improve gut microbiota functionality to promote human health.

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Introduction

Long-term dietary pattern strongly affects gut microbiota composition, and diet-induced shifts in the gut microbiota composition have been associated with healthy or disease states.[1,2] However, recent studies have suggested that the predominant metabolic pathways that are active among the gut microbiota, which can also be impacted by diet,[3] may be more relevant to health than the actual composition itself.[4,5] For example, Zhang *et al.*[4] described a core gut microbiota from healthy subjects that shared the common function of producing SCFA but with different compositional profiles. Turnbaugh *et al.*[5] also showed that deviations from a core microbiome with shared metabolic pathways at the functional level, rather than at the taxonomic level, may result in a disease state. Daniel *et al.*[3] demonstrated that diet had a greater influence on the chemical metabolites produced by the gut microbiota than on the gut microbiota composition itself, suggesting that diet might exert a more pronounced impact on the gut microbiota at the functional level rather than at the compositional level.

The products of bacterial metabolism in the human gut are important to the health of the host. The gut microbiota can either produce harmful metabolites associated with disease or beneficial compounds that protect against disease.[6,7] The major products of saccharolytic fermentation by the gut microbiota, short chain fatty acids (SCFA), have been shown to regulate gene expression by binding to G protein-coupled receptors (GPR). Signaling through GPR41 and GPR43 affects a wide range of biological functions, including inflammation, appetite control, and insulin regulation.[8] For instance, SCFA have been shown to trigger the secretion of glucagon-like peptide-1,

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a hormone that results in an increase in insulin secretion and a decrease in appetite.[9] Acetate serves as an energy source for the liver and peripheral tissues and acts as a signaling molecule in metabolic pathways of gluconeogenesis and lipogenesis.[10] Propionate, a gluconeogenerator, has been linked to the inhibition of cholesterol synthesis.[11] Butyrate is the major energy source for colonocytes and possesses anti-inflammatory properties, and has been proposed to play a key role in maintaining gut homeostasis and epithelial integrity.[11,12] Butyrate has also been shown to promote energy expenditure and improve insulin sensitivity.[13] Increased butyrate and propionate have been linked to protection against diet-induced obesity as well as reduce fasting insulin and leptin levels.[14]

In contrast, branched chain fatty acids (BCFA) and ammonia, the major markers of proteolyic fermentation, are likely to be detrimental to colonic health.[15] Ammonia and BCFA may change the morphology of intestinal tissues and act as a tumor promoter in the gut.[16] Proteolyic fermentation also results in the formation of amines, which are the precursors to carcinogenic nitrosamine formation.[17]

In vitro fecal fermentation models have been used to evaluate the utilization of dietary substrates as well as production of microbial metabolites because these outcomes are difficult to measure *in vivo*. Previously, we reported that the diet of the fecal donor influences *in vitro* fermentation properties when using "pre-digested" whole wheat as a substrate.[18] We found that butyrate production in particular was correlated with fecal donor intake of many nutrients contributed by grain-, nut-, and vegetable-based foods. The study indicated that diets high in plant-based foods and high in unsaturated fats were associated with microbial metabolism that is consistent with host health.[18]

Whole wheat contains complex and poorly fermented dietary fibers (*e.g.*, cellulose and cross-linked arabinoxylan) that are generally poorly fermented (about 30%). In contrast, inulin represents a soluble type of dietary fiber, consisting of repetitive fructosyl moieties culminating in a chain-terminating glucosyl residue,[19] which can be readily fermented by the gut microbiota.[20] The objectives of this study were (1) to determine the correlations between long-term diet of the fecal donor and fecal metabolite concentrations; (2) to determine the correlations between long-term diet of the fecal donor and fermentation products arising from *in vitro* fermentation of inulin, a soluble, highly-fermentable fiber; and (3) to compare the results of inulin fermentation with those obtained in our previous study[18] on *in vitro* fermentation pre-digested whole wheat flour, a mostly insoluble and poorly-fermentable source of fiber.

Materials and methods

Subjects, dietary records, and stool sample collection

Previously, we collected dietary records and stool samples from 18 healthy subjects that had not taken antibiotics in the last six months.[18] Eleven of these subjects were female and six were male with an age range of 20–37 years. Dietary records showed that these subjects consumed varied diets that were consistent with NHANES data. Stool samples collected as described from these same individuals were used in the present study.[18] All protocols involving human subjects were approved by the University of Nebraska-Lincoln's Institutional Review Board before initiation of the study (no. 20120512624EP). All subjects gave voluntary informed consent before enrollment in this project.

Inulin substrate

HP inulin (~100%, average DP ≥ 23, Beneo, Germany) was used as the fermentation substrate for this study. Inulin was analyzed using size-exclusion chromatography[21] to confirm the absence of digestible mono- and disaccharides. No peaks were observed in the area that mono- and disaccharides elute from the column (data not shown). Therefore, the inulin was used directly in the *in vitro* system without performing *in vitro* digestion.

In vitro fermentation

In vitro fermentation of inulin substrate with the fecal inocula was carried out following our previous study[18] except the inulin substrate was not hydrated overnight because it was completely soluble. In short, 15 mg of inulin was dissolved in 1 mL of sterile fermentation medium and then inoculated with 0.1 mL of fecal slurry, prepared by blending each fecal sample separately with sterile phosphate-buffered saline (1 : 10 w/v). For each fecal sample, duplicate sample tubes for each time point (0 h and 12 h) were fermented (total 4 tubes). At each designated time point, microbial metabolism was stopped by adding 0.2 mL of 2 M KOH containing 7 mM 2-ethyl-butyrate as an internal standard for S/BCFA analysis and tubes were stored at −80 °C until analysis.

Short- and branched chain fatty acid and ammonia analysis

For analysis of fecal SCFA and BCFA concentrations, fecal samples were homogenized with sterile phosphate-buffered saline (1 : 10 w/v). The mixture was then centrifuged (10 000*g*, 5 min) and 0.4 mL of supernatant was mixed with 0.1 mL of internal standard (7 mM 2-ethylbutyrate in 2 M KOH) followed acidification, extraction into diethyl ether, and quantification by gas chromatography as described.[18]

For analysis of SCFA, BCFA, and ammonia concentrations in fermentation media, samples were thawed and centrifuged (10 000*g*, 5 min). Aliquots of the supernatant were then used for each analysis (0.5 mL for SCFA and BCFA; 0.1 mL for ammonia; 0.2 mL for inulin). Samples for SCFA and BCFA were analyzed by gas chromatography as described.[18] Samples for ammonia were assayed using the phenol hypochlorite method.[18] SCFA, BCFA, and ammonia obtained before fermentation (0 h) was subtracted from the value obtained after 12 h of fermentation to determine the concentration of each analyte produced during the fermentation only. Inulin content was measured using a commercial fructan kit (Megazyme International, Ireland) except a liquid sample (0.2 mL of

fermentation medium) was used. Inulin fermented over 12 h was calculated by subtracting the inulin content after fermentation from the inulin content before fermentation.

Statistical analyses

All statistical analyses were performed using SAS software (version 9.4; SAS Institute, Cary, NC, USA). Data for inulin fermentation and metabolite production were analyzed using ANOVA with subject as the factor followed by pairwise comparisons using the least significant difference procedure. For correlations of dietary intake of each fecal donor with metabolite production or inulin degradation during *in vitro* fermentation, Spearman correlation coefficients were computed using mean values from each subject after correcting for age and gender. Principal components analysis (PCA) was also run on the ranked dietary intake and food category data. Metabolites and inulin fermented were loaded onto the PCA biplot by correlating metabolite concentrations obtained from the subjects with their corresponding principal component loadings. Paired *t*-tests were used to compare metabolite and carbohydrate degradation between inulin (this study) and whole wheat (previous study)[18] fermentation. Pearson correlation coefficients between fermentation outcomes using inulin *versus* whole wheat substrate were also calculated.[18]

Results

Fecal metabolite concentration and correlation with dietary records

Mean fecal SCFA and BCFA were 121 μmol g^{-1} feces and 5.13 μmol g^{-1} feces, respectively (ESI Table 1†). These concentrations were similar to previous reports.[22–25] Few significant correlations were noted between fecal metabolite concentrations and dietary intake variables: butyrate was significantly correlated with cholesterol, whole grain, eggs, and soy products; iso-butyrate was significantly correlated with fructose; iso-valerate was significantly correlated with copper, total vegetables, other vegetables, and total fruit; BCFA were significantly correlated with copper, starchy vegetables not potato, and nuts and seeds.

Inulin fermentation and metabolite production

The amount of inulin fermented over 12 h of *in vitro* fermentation was determined. Individual fecal microbiotas showed differing ability to ferment the inulin (Fig. 1). The fecal microbiotas from subjects 11 and 24 were among the most able to ferment inulin, whereas the fecal microbiotas from subjects 15, 21, and 98 fermented the least.

In agreement with the percentage of inulin fermented, the SCFA production varied greatly among fecal microbiotas (Fig. 2). The total SCFA production (r = 0.70, p = 0.003) and acetate production (r = 0.67, p = 0.005) correlated with the percentage of inulin fermented. The fecal microbiota from subject 11 produced much more butyrate than any other microbiota. The fecal microbiota from subject 1 produced the most total SCFA.

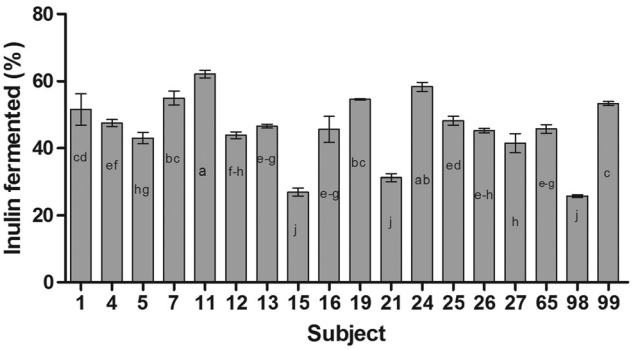

Fig. 1 Inulin fermented during 12 hours of *in vitro* fecal fermentation. Error bars show SD; bars marked with different letters are significantly different; n = 2.

As expected, unlike the SCFA data, markers of protein fermentation did not correlate with the percentage of inulin fermented (p-values ranged from 0.23 to 0.93) but did show variation among fecal microbiotas (Fig. 3). The fecal microbiota from most subjects, excluding 13 and 21, produced relatively low concentrations of BCFA. Production of these metabolites was highly correlated with ammonia production (ammonia *vs.* iso-butyrate, r = 0.76, p = 0.0006; *vs.* iso-valerate, r = 0.64, p = 0.008; *vs.* BCFA, r = 0.72, p = 0.002). When comparing between makers of saccharolytic fermentation (SCFA) and protein fermentation (BCFA), the only significant correlations were for propionate with iso-valerate (r = 0.63, p = 0.01) and BCFA (r = 0.54, p = 0.03).

Correlation between dietary records and inulin fermentation and metabolite production

The percentage of inulin fermented was only correlated with a few dietary nutrients, while many significant positive correlations between nutrient intakes were discovered for acetate, propionate, butyrate, and total SCFA production (Fig. 4). The only negative correlation for these metabolites was for cholesterol intake and butyrate production. A few significant negative correlations were also found between nutrient intakes and markers of protein fermentation: iso-valerate with polyunsaturated fat intake, and ammonia with energy, carbohydrate, starch, and thiamin intakes.

A few correlations were noted between certain food categories and inulin fermented and metabolite production during fermentation (Fig. 4). Interestingly, acetate, propionate, butyrate, and the total SCFA production carried significant positive correlations with “whole grain” intake and ammonia was negatively correlated with “whole grain” and “total grain”. Few significant correlations were found for other the food categories.

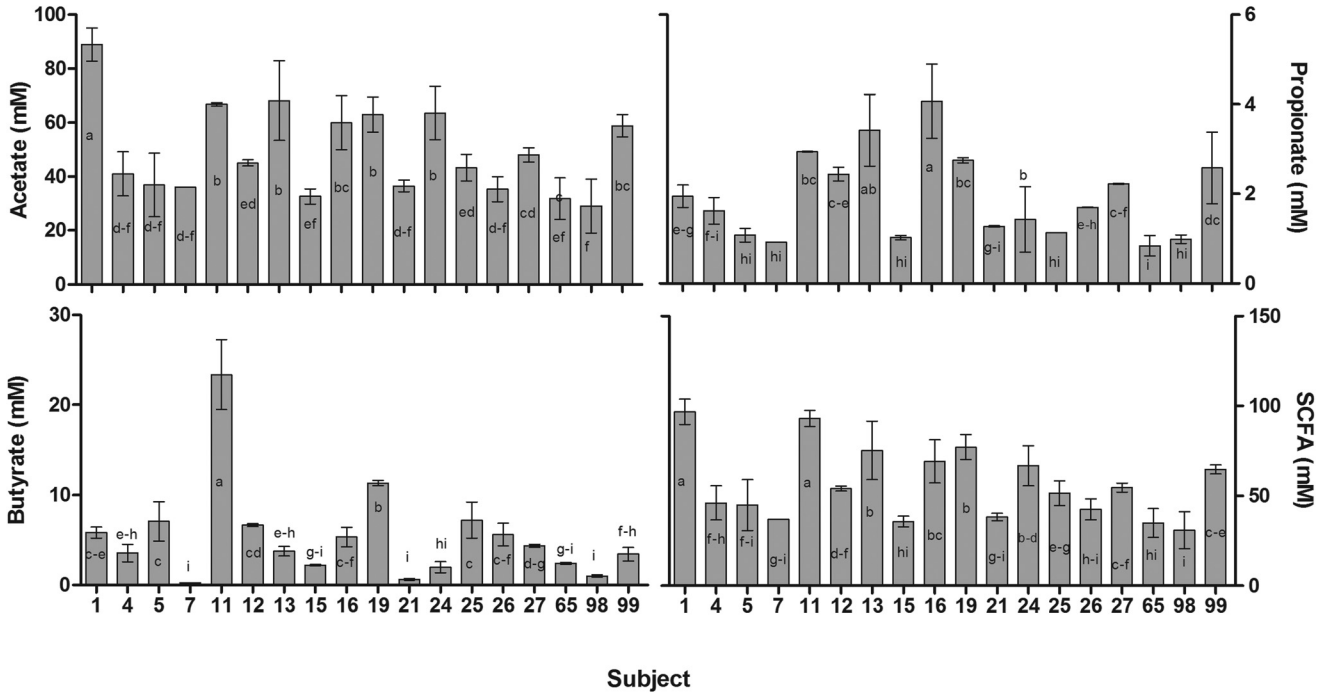

Fig. 2 Short-chain fatty acid production during 12 hours of *in vitro* fecal fermentation. Error bars show SD; bars marked with different letters are significantly different; $n = 2$; note the different scales on the y-axes.

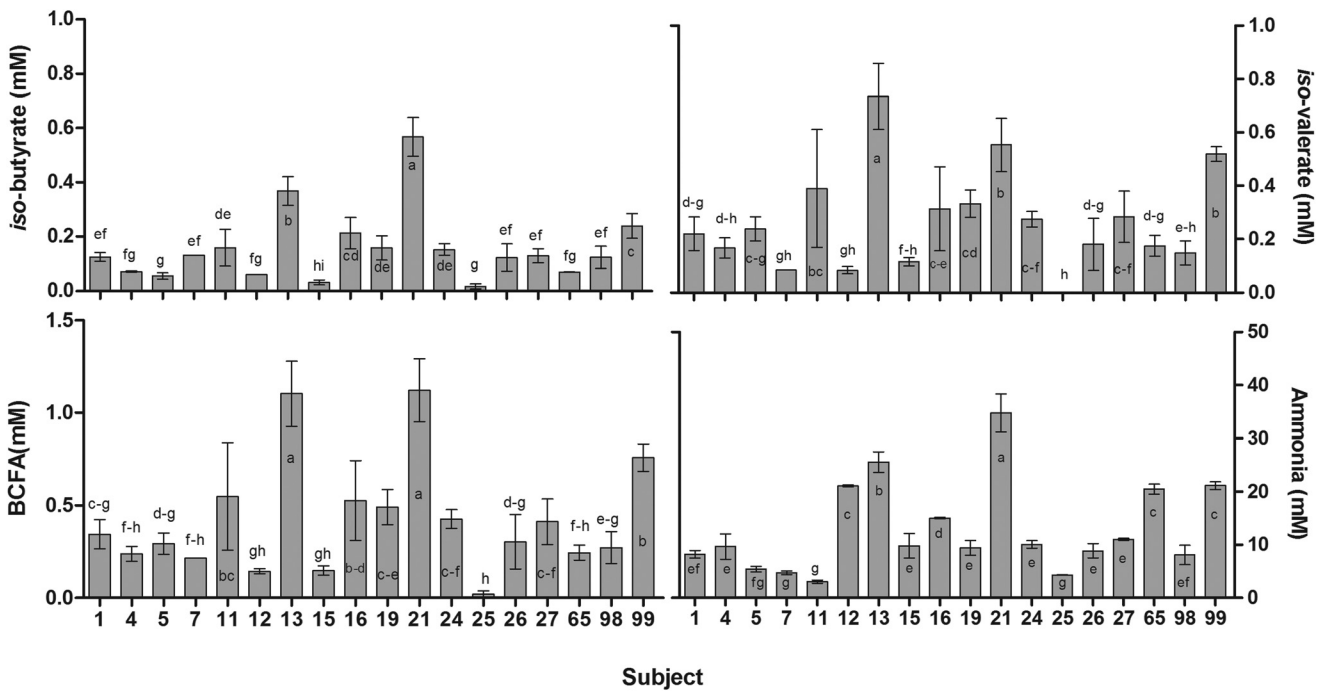

Fig. 3 Branched-chain fatty acid and ammonia production during 12 hours of *in vitro* fecal fermentation. Error bars show SD; bars marked with different letters are significantly different; $n = 2$; note the different scales on the y-axes.

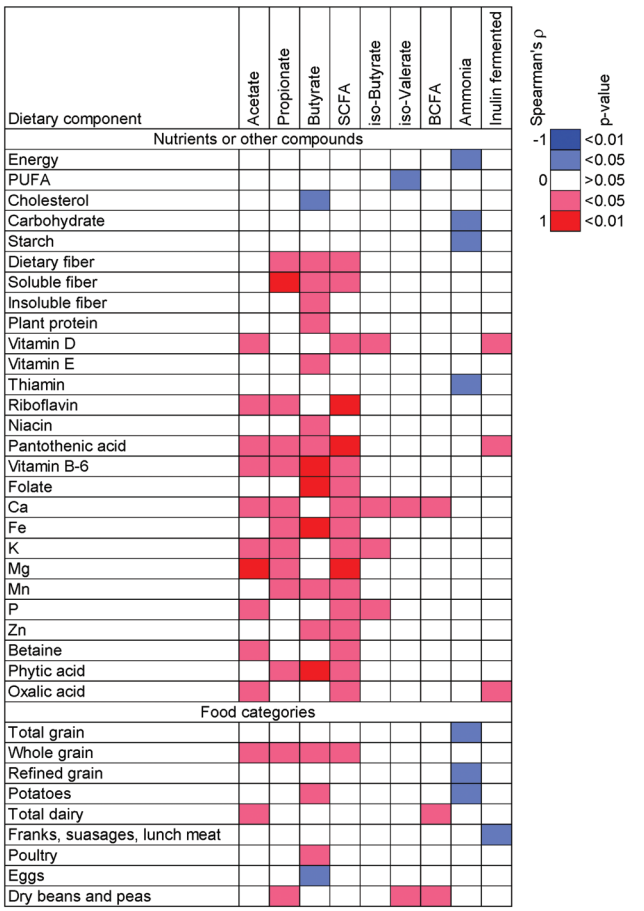

Fig. 4 Spearman ρ correlation of dietary nutrient and food category intake of the fecal donor with inulin fermented (%) and metabolite production (mm) during *in vitro* fecal fermentation; only nutrients and food categories with at least one significant correlation are shown ($p < 0.05$; $n = 18$); other nutrients and food categories analyzed included: total fat, saturated fat, trans fat, monounsaturated fat, omega-3 fatty acids, sugars, added sugar, fructose, sucrose, protein, animal protein, choline, alcohol, caffeine, vitamin A, vitamin K, vitamin B-12, vitamin C, Cu, Na, Se, total vegetables, dark green vegetables, orange and yellow vegetables, starchy vegetables, tomatoes, other vegetables, total fruit, citrus melon and berries, other fruit, fluid milk, yogurt, cheese, meat poultry and fish, beef pork veal lamb and game, fish high in omega-3 fatty acids, fish low in omega-3 fatty acids, soy products, nuts and seeds, alcoholic beverages.

Principal components analysis of dietary records with inulin fermentation and metabolite production

When performing PCA with nutrient and other compounds intakes (Fig. 5A), the first two principal components (PC) explained nearly 60% of the variation in the data set. These components separated nutrients that are generally obtained from plant foods with positive Eigenvectors on PC1 and negative Eigenvectors on PC2 (*e.g.*, dietary fiber, insoluble fiber, phytic acid, plant protein) from those nutrients that are more prevalent in animal foods with low Eigenvectors on PC1 and positive Eigenvectors on PC2 (*e.g.*, cholesterol, fat, animal protein). All SCFA carried high positive loadings on PC1 and negative loadings on PC2, indicating an intake of nutrients generally associated with plant foods were important for SCFA production from inulin. In contrast, the loadings for markers of protein fermentation (BCFA and ammonia) had negative or low PC1 loadings and positive PC2 loadings, indicating an intake of nutrients associated with animal foods were consistent with BCFA and ammonia production during inulin fermentation.

When performing PCA with food category data (Fig. 5B) the first two PC explained less of the variance in the data set (34%) and describing the meaning of each PC was more difficult compared with the nutrient data. However, the markers of protein fermentation carried negative loadings on PC1 and positive loadings on PC2. These were associated with intake of "total dairy", "fluid milk", and "franks, sausages, lunch meat". Interestingly, Eigenvectors for fresh meats such as "beef, pork, veal, lamb, game", "meat, poultry, fish", and "fish low in omega-3s" were directed in the opposite direction from "franks, sausages, lunch meat" where the BCFA and ammonia loaded. In contrast, SCFA were more consistent with intake of "dry beans and peas", "whole grain", "orange and yellow vegetables", and "tomatoes".

Comparison between fermentation outcomes using inulin as substrate and whole wheat as substrate

As mentioned, we previously reported *in vitro* fermentation properties of whole wheat substrate using fecal inocula from the same subjects as reported in this paper for inulin. On average, 46% of the inulin substrate was fermented over the 12 h of *in vitro* fermentation (Table 1), compared with only 29% of the carbohydrate fermented when using whole wheat as substrate. This demonstrates that the inulin was a more readily fermentable substrate. The inulin yielded more acetate, butyrate, and total SCFA during fermentation than the whole grain wheat, while the whole grain wheat yielded more propionate than the inulin. The average molar ratios of acetic, propionic and butyric acids after 12 h also showed the same trends when comparing between inulin and whole wheat.[18] Analysis of correlations between fermentation outcomes when using the two substrates revealed significant correlations for butyrate and BCFA production, but not for the other outcomes.

Discussion

Given the important impact of gut metabolites on human health, the first object of the present study was to determine the correlations between dietary intake variables and fecal microbial metabolite concentrations. Of the 77 dietary intake variable analyzed, only 11 showed at least one significant correlation with the microbial metabolites analyzed. Thus, we concluded that the diet of the fecal donor did not have a strong relationship to fecal microbial metabolite concentrations. This was not altogether surprising, since fecal SCFA and BCFA concentrations are not a good measure of production of these metabolites by the gut microbiota due to

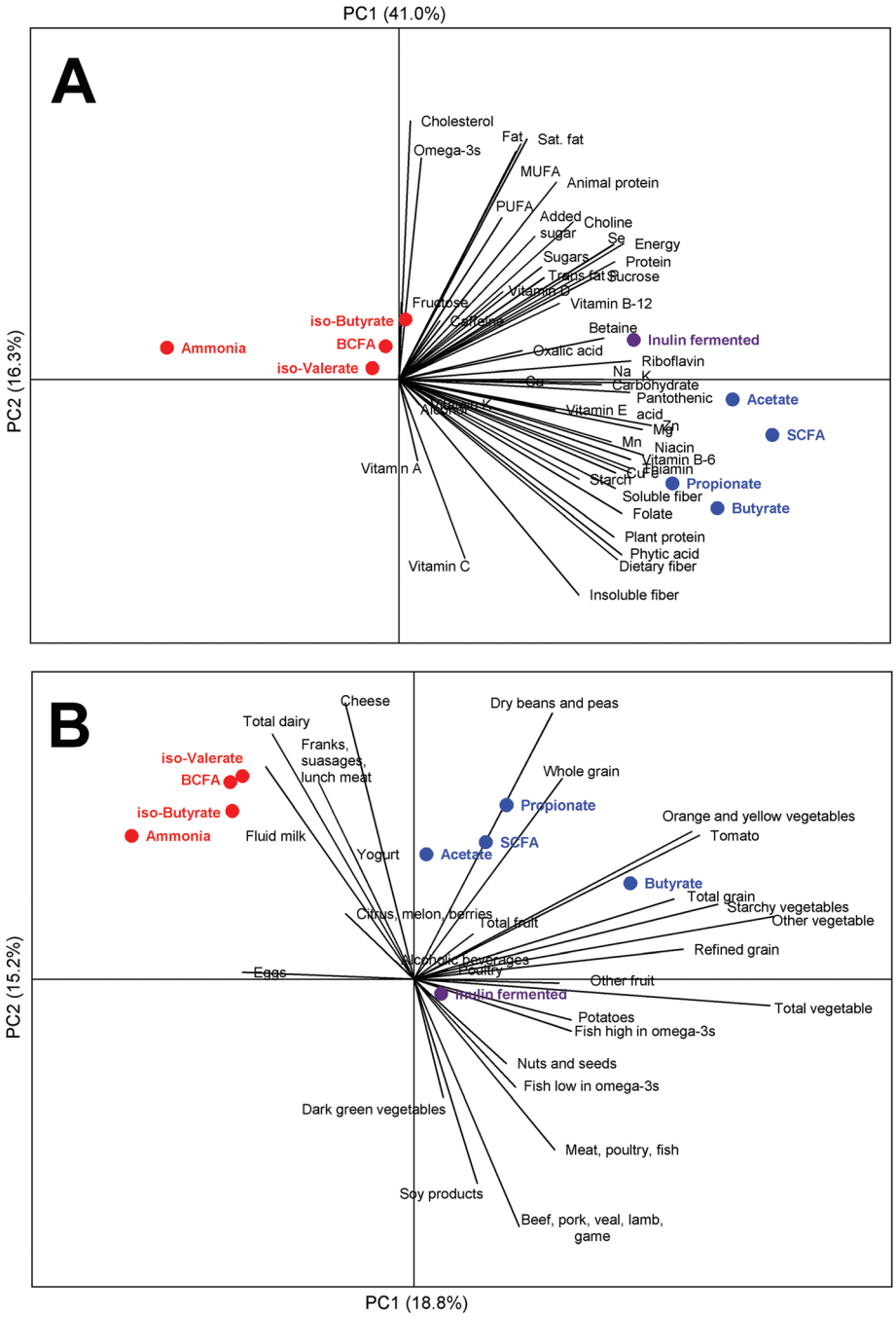

Fig. 5 Principal components biplot of nutrients (a) and food categories (b) with metabolites and inulin fermented (%) during *in vitro* fermentation.

in vivo absorption.[26] However, others have shown that dramatic dietary interventions can change the concentrations of SCFA in fecal samples in predictable ways. For instance, increases in dietary fiber intake has led to increases in fecal butyrate concentrations.[22,27] Additionally, one study reported a relationship between the long-term intake of certain dietary fiber fractions (cellulose and pectin) and foods (potatoes and apples) and fecal acetate, propionate, and butyrate concentrations in institutionalized elderly individuals.[28] We did not find such correlative relationships in the present study. This is likely because subjects did not undergo any dietary intervention and were young individuals (20–37 years) consuming widely varied diets.

Despite the lack of a clear relationship between long-term diet and fecal concentrations of SCFA and BCFA in the present study, we have previously shown that long-term diet was strongly associated with the production of these metabolites during *in vitro* fecal fermentation of predigested whole wheat

Table 1 Mean (standard error) of *in vitro* fermentation outcomes when using inulin or whole wheat as substrate and Pearson correlations (*r*) between responses obtained from individuals on the two substrates[a]

Fermentation outcome	Inulin	Wheat	*r*
Inulin or NSP fermented (%)	45.9 (2.3)	28.6 (2.1)*	−0.07
Acetate (mM)	49.2 (3.9)	34.1 (1.8)*	0.35
Propionate (mM)	1.91 (0.22)	5.51 (0.66)*	0.37
Butyrate (mM)	5.34 (1.2)	2.83 (0.60)*	0.90§
SCFA (mM)	56.4 (4.71)	42.4 (2.1)*	0.43
iso-Butyrate (mM)	0.155 (0.031)	0.173 (0.062)	0.78§
iso-Valerate (mM)	0.267 (0.044)	0.296 (0.100)	0.48§
BCFA (mM)	0.423 (0.072)	0.469 (0.158)	0.61§
Ammonia (mM)	12.8 (2.01)	11.5 (0.61)	0.31
Acetate/SCFA	0.88 (0.01)	0.81 (0.02)*	0.63§
Propionate/SCFA	0.03 (0.00)	0.13 (0.01)*	0.28
Butyrate/SCFA	0.09 (0.01)	0.07 (0.01)*	0.86§
BCFA/SCFA	0.01 (0.00)	0.01 (0.00)	0.67§

[a] Data for wheat fermentation from ref. 18; NSP, non-starch polysaccharides; SCFA, short chain fatty acids; BCFA, branched chain fatty acids; $N = 18$; *significantly different from inulin ($p < 0.05$); §significantly different from no correlation (zero).

flour in these same individuals.[18] Thus, it appears that long-term diet is not necessarily related to the concentration of microbial metabolites in the fecal samples, but instead related to the functionality of the microbiota, which was determined by performing an *in vitro* fermentation using the fecal samples.

Because our previous study assayed the functionality of the microbiota on a mostly insoluble and poorly fermentable substrate, we desired to determine if diet was related to the functionality of the gut microbiota during fermentation of a highly-fermentable substrate, inulin. During fermentation of inulin, the ability of the microbiota to produce acetate, propionate, butyrate, and total SCFA correlated with intake of many macro- and micronutrients. PCA revealed that the most important of these nutrients were those from plant foods, including whole grain, dry beans, and some types of vegetables. It is surprising that intake of these foods were associated with inulin fermentation, since they are not abundant sources of inulin (~1–3%, db).[29] However, grain-based foods, for instance, are typically the most important source of inulin in the diet because of the high consumption of these staple foods.[30] Perhaps people with high intakes of these foods had a microbiota that was more adapted to ferment the inulin and produce SCFA.

PCA also revealed a notable contrast between SCFA (markers of saccharolytic fermentation) and BCFA and ammonia (markers of proteolytic fermentation). These two groups of metabolites loaded on opposite sides of the PCA biplot, suggesting that saccharolytic and proteolytic fermentation are associated with intake of divergent diets, the former a plant-based diet and the latter an animal-based diet (especially from dairy and cured meats). The differing predominant metabolic pathways may be attributed to the difference in gut microbiota between people on these two types of diets. For instance, a high protein diet[15] or entirely animal-based diet[31] has been shown to result in a reduction in important known saccharolytic bacteria: *Roseburia*, *Eubacterium rectale*, and *Ruminococcus bromii*, accompanied by an increase in microbes more adapted to ferment protein, including *Alistipes*, *Bilophila* and *Bacteroides*, and an increase in BCFA and *N*-nitroso compounds[15] compared with the regular diet. In contrast, a high fiber diet results in increased *Prevotella* as well as butyrate producers.[2]

When comparing the metabolites produced in the present study of inulin with the previous study using digested whole wheat flour, we observed significantly more carbohydrate utilization during inulin fermentation compared with fermentation of whole wheat, indicating, as expected, that inulin was more available for fermentation than the dietary fibers in the whole wheat substrate. The differences between inulin and whole grain wheat may be due to the differences in the solubility and structure of the dietary fibers contained. For instance, inulin is more soluble than the arabinoxylans in whole wheat, which are cross-linked by phenolic groups and resistant to attack by the gut microbiota.[32] The molar ratios of SCFA in inulin and whole grain wheat fermentations were very different. Inulin was more acetogenic and butyrogenic compared with the whole grain wheat, while whole wheat was more propiogenic. This is consistent with the studies that showed inulin was more butyrogenic than wheat bran,[33] while arabinoxylan fermentation resulted in high propionate production.[34,35]

Significant correlations were found for butyrate and BCFA when using inulin *versus* whole wheat as substrate. This observation suggests that the magnitude of butyrate and BCFA production depends more on the stool donor and less on the substrate. It is generally accepted that dietary fiber substrates affect the production of microbial metabolites in *in vitro* fermentations because different bacteria vary in their capacity to utilize substrates with different structures.[36] However, our observations suggest that if the diet of the stool donor is conducive to a microbiota that favors butyrate production (or, conversely, BCFA production), high butyrate (or BCFA) production will be observed in the *in vitro* fermentation regardless of substrate (insoluble, soluble, *etc.*). This observation does not negate the value of *in vitro* fermentation systems; they are useful for comparing among substrates when using a single fecal inoculum, but when different inocula are used the magnitude of and propensity toward butyrate (or BCFA) production will vary depending on the stool donor's diet.

It is also interesting that, among the SCFA production compared between this study and the previous study using whole wheat, a significant correlation was observed only for butyrate. Butyrate producers are unique because they are highly susceptible to dietary complex carbohydrate shortages.[37] For instance, reduced dietary intake of carbohydrates results in a rapid decreased butyrate-producing bacteria (related to *Roseburia* spp. and *E. rectale*).[27] In contrast, a meal rich in indigestible carbohydrate has been shown to increase butyrate production preceding the following meal.[38]

Our study has shown that long-term intake of plant foods, especially whole grain, dry beans, and certain vegetables, is associated with acetate, propionate, butyrate, and the total SCFA production during *in vitro* fermentation of an inulin substrate, while high intake of animal foods, especially dairy foods and cured meats, is associated with markers of protein fermentation, including ammonia and BCFA production. Furthermore, the magnitude of butyrate and BCFA production during *in vitro* fermentation is dependent on diet of the fecal donor, regardless of the substrate. Thus, long-term diet may have a pronounced effect on the propensity of the gut microbiota toward saccharolytic fermentation *versus* proteolytic fermentation. Our results may help in the development of new dietary strategies to improve metabolism by the gut microbiota and thereby promote human health.

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