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Müller, Mattea; Hermes, Gerben D.A.; Canfora, Emanuel E.; Smidt, Hauke; Masclee, Ad A.M. et al

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RESEARCH ARTICLE | *Microbiome and Host Interactions*

Distal colonic transit is linked to gut microbiota diversity and microbial fermentation in humans with slow colonic transit

Mattea Müller,[1]* Gerben D. A. Hermes,[2]* Emanuel E. Canfora,[1] Hauke Smidt,[2] Ad A. M. Masclee,[3] Erwin G. Zoetendal,[2] and Ellen E. Blaak[1]

[1]*Department of Human Biology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre+, Maastricht, The Netherlands;* [2]*Laboratory of Microbiology, Wageningen University & Research, Wageningen, The Netherlands; and* [3]*Department of Internal Medicine, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre+, Maastricht, The Netherlands*

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Müller M, Hermes GD, Canfora EE, Smidt H, Masclee AA, Zoetendal EG, Blaak EE. Distal colonic transit is linked to gut microbiota diversity and microbial fermentation in humans with slow colonic transit. *Am J Physiol Gastrointest Liver Physiol* 318: G361–G369, 2020. First published December 23, 2019; doi:10.1152/ajpgi.00283.2019.—Longer colonic transit time and hard stools are associated with increased gut microbiota diversity. Here, we investigate to what extent quantitative measures of (segmental) colonic transit time were related to gut microbiota composition, microbial metabolites, and gut-related parameters in a human cross-sectional study. Using radiopaque markers, (segmental) colonic transit time (CTT) was measured in 48 lean/overweight participants with long colonic transit but without constipation. Fecal microbiota composition was determined using 16S rRNA gene amplicon sequencing. Associations between gastrointestinal transit (segmental CTT and stool frequency and consistency), microbiota diversity and composition, microbial metabolites [short-chain fatty acids (SCFA), branched-chain fatty acids, and breath hydrogen], habitual diet, and gut-related host parameters [lipopolysaccharide-binding protein (LBP) and fecal calprotectin] were investigated using univariate and multivariate approaches. Long descending (i.e., distal) colonic transit was associated with increased microbial α-diversity but not with stool consistency. Using unweighted and weighted UniFrac distance, microbiota variation was not related to (segmental) CTT but to demographics, diet, plasma LBP, and fecal calprotectin. Bray–Curtis dissimilarity related only to stool consistency. Rectosigmoid and descending colonic transit were negatively associated with fecal SCFA and plasma acetate, respectively. This study suggests that the distal colon transit may affect not only microbiota diversity but also microbial metabolism.

NEW & NOTEWORTHY We extend previous findings showing that long distal colonic transit time influences microbial diversification and fermentation, whereas stool consistency is related to microbiota composition in humans with a long colonic transit. This study puts the importance of the (distal) colonic site in microbiota ecology forward, which should be considered in future therapeutic studies targeting, for instance, short-chain fatty acid production to improve metabolic health.

gastrointestinal transit; gut microbiota; short-chain fatty acids; stool consistency

INTRODUCTION

The gut microbiota is involved in important physiological processes including nutrient digestion, stimulation of mucosal immunity, and protection against pathogens (26). Alterations of the human gut microbiota have been associated with a plethora of pathologies, including inflammatory bowel diseases, obesity, and type 2 diabetes mellitus (T2DM) (21, 33). Products of microbial metabolism are suggested to partly mediate the interaction between the gut microbiota and the host metabolism (47). In the proximal colon, the gut microbiota preferably ferments indigestible carbohydrates yielding the short-chain fatty acids (SCFA) acetate, butyrate, and propionate (7). In the more distal colon, carbohydrates are gradually depleted, causing a switch to microbial protein fermentation that yields a variety of end products such as branched-chain fatty acids (BCFA), phenolic compounds, amines, and ammonia (48). SCFA may beneficially affect body weight regulation and insulin sensitivity, whereas some of the toxic protein catabolites may have detrimental effects on gut health (8).

To interpret the implications of microbiota alterations in the context of diseases, it is important to understand what drives microbial composition and microbial metabolism in the healthy state. Recent population-wide microbiome studies have substantiated the evidence for many host-related covariates such as age, sex, and medication (15, 52). Additionally, stool consistency [via self-reported Bristol stool scores (BSS)] has been identified as an additional covariate explaining microbiota variation (15). Stool consistency is moderately correlated with quantitative measures of gastrointestinal (GI) transit, i.e., radiopaque marker method and scintigraphy (37). Therefore, it is widely used as a noninvasive estimate of GI transit time. Hard stool was associated with increased microbiota diversity in the Flemish Gut Flora Project and increased microbial richness in a small study in healthy women (15, 42). However, microbiota diversity was not related to stool consistency in the Dutch LifeLines Cohort (38). Most consistently, the family *Oxalobacteraceae* and genera *Methanobrevibacter*, *Akkermansia*, *Butyricimonas*, and *Desulfovibrio* were related to hard stools, whereas *Roseburia* and *Blautia* were associated with softer

* M. Müller and G. D. A. Hermes contributed equally to this work.
Address for reprint requests and other correspondence: E. E. Blaak, Dept. of Human Biology, NUTRIM School for Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre+, P.O. Box 616, 6200 Maastricht, The Netherlands (e-mail: e.blaak@maastrichtuniversity.nl).

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stool in these studies (15, 38, 42). Only one study has used quantitative measures of GI transit and reported a positive correlation between microbiota diversity and an increased urinary proteolytic profile associated with longer GI transit (36). Moreover, the relationship between GI transit and the gut microbiota composition may be bidirectional because both in vitro and rodent models have shown that microbial metabolites stimulated colonic muscle contractility and peristalsis (e.g., SCFA and hydrogen) or inhibited colonic muscle contractility (e.g., methane) (3, 35, 39). There is further evidence that gut microbiota composition as well as microbial fermentation may differ depending on transit time and colonic site (13, 31, 51). Nevertheless, there is little human data on the interaction between (segmental) colonic transit, gut microbiota composition, and microbial fermentation. The objective of the present study was to investigate the associations between (segmental) colonic transit and the gut microbiota composition and diversity in a cross-sectional human study. We analyzed the fecal microbiota composition of normoglycemic, lean-to-overweight participants. Stool consistency, fecal SCFA, BCFA, plasma SCFA and breath hydrogen, and gut-related host parameters [i.e., lipopolysaccharide-binding protein (LBP) and fecal calprotectin] were analyzed. We hypothesized that (segmental) colonic transit influences fecal microbiota diversity, composition, and fermentation. First, we investigated associations between microbial α-diversity and GI transit parameters using a univariate approach. Second, we assessed whether GI transit parameters, age, sex, body mass index (BMI), and dietary intake as well as SCFA, BCFA, and gut-related host parameters were linked to microbial composition using multivariate approaches. Lastly, we investigated whether (segmental) colonic transit time was related to microbial fermentation, i.e., fecal SCFA, BCFA, and plasma SCFA.

PARTICIPANTS AND METHODS

Study population. This cross-sectional analysis included the baseline data of 48 healthy, normoglycemic lean-to-overweight Caucasian women and men aged 20–55 yr prior to participating in a human intervention study at the Maastricht University Medical Centre+ (MUMC+) aiming to improve colonic transit time by means of a prebiotic fiber supplementation (29). Normoglycemia was determined by oral glucose tolerance test (OGTT) according to the diagnostic criteria of the American Diabetes Association, 2010 (1). Initial recruitment was based on a defecation frequency of ≤4 per week. Participants with colonic transit time >35 h determined by radiopaque marker method were included. Exclusion criteria were related to outcomes of the interventional study (29): two or more symptoms of constipation according to ROME III criteria, diagnosis of T2DM, prior abdominal surgery, cardiovascular or gastrointestinal diseases, or the use of antibiotics, laxatives, prebiotics, or probiotics 3 mo before the study or during study participation. Participants did not use β-blockers, lipid- or glucose-lowering medication, or corticosteroids. All participants gave written consent before inclusion. The study protocol was approved by the MUMC+ Medical Ethical Committee and was conducted in accordance with the Declaration of Helsinki (revised version, October 2008). During an initial screening, the participant's medical history and anthropometrics were assessed and an OGTT was performed. On *clinical investigation day 1* (CID1), (segmental) colonic transit was measured. A week after CID1, participants came fasted to the university for blood sampling and to deliver stool samples for CID2.

Segmental and total colonic transit time. On 6 consecutive days, participants ingested 10 radiopaque markers (Colonic Transit, P. & A. Mauch, Münchenstein, Switzerland) in the morning. On *day 7*, abdominal radiographs were taken in the morning of CID1, 24 h after ingestion of the last marker. Based on the visible number of markers on the abdominal X-ray, total colonic transit time was calculated as follows: sum of markers on X-ray/[24 (time in hours between ingestions) × 10 (daily dosage of markers)]. Segmental transit times of ascending, descending, and rectosigmoid colon were assessed using anatomical landmarks as follows: ascending colonic transit time was calculated by the sum of markers in the area between an imaginary line right of the vertebral spinous processes from the fifth lumbar vertebra to the pelvic outlet. Descending colonic transit time was calculated by the sum of markers in the area between an imaginary line left of the vertebral spinous processes and the imaginary line above the fifth lumbar vertebra and the left anterior superior iliac crest. Rectosigmoid transit time was calculated by the sum of markers in the area under the imaginary line from the pelvic brim on the right to the superior iliac crest on the left (Fig. 1) (5).

Dietary intake and stool consistency and frequency. Participants were instructed by a dietician to weigh and document food intake on two weekdays and one weekend day. Energy and macronutrient intake were analyzed using the Dutch food composition database (Nederlands Voedingsstoffenbestand 2016/5.0, National Institute for Public Health and Environment, Ministry of Health, Welfare and Sport, The Hague, The Netherlands). Participants completed a 7-day gastrointestinal questionnaire reporting on stool frequency and stool consistency using the Bristol stool scale (BSS).

Blood sampling and biochemical analysis. One week after CID1, participants were instructed to come to the university after an overnight fast (10–12 h) for CID2. After a cannula was inserted into the antecubital vein, blood samples were taken during the fasted state. Blood was collected in prechilled EDTA tubes (0.2 mol/L EDTA; Sigma, Dorset, UK) for SCFA and LBP. SCFA in plasma were

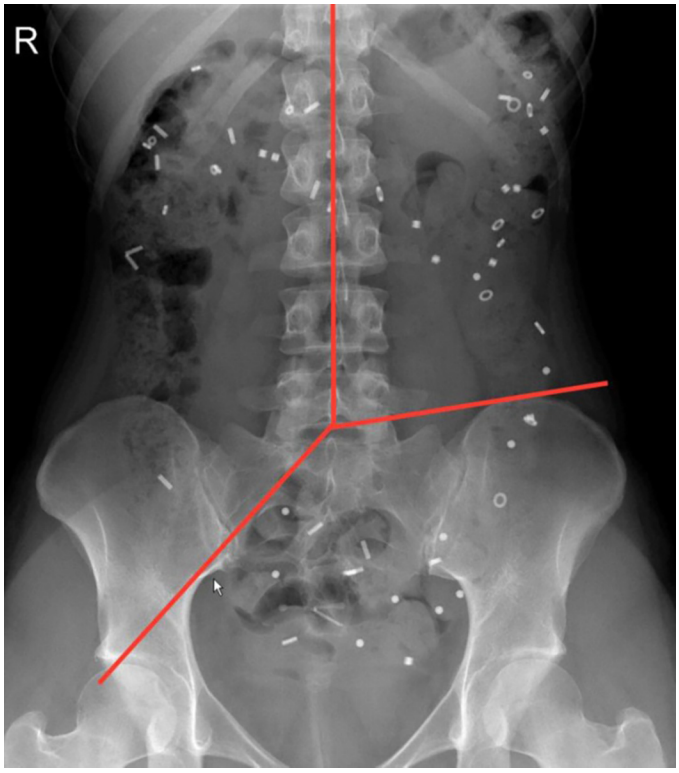

Fig. 1. Example of segmental colonic transit assessment via radiopaque marker method. Red lines depict anatomical landmarks distinguishing ascending, descending, and rectosigmoid colon. Markers counted within these areas are used to calculate segmental colonic transit according to Metcalf et al. (27). R, right side.

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measured using liquid chromatography-mass spectrometry, as reported previously (41). For the detection of LBP, plates (Greiner Mocrolon 600 high binding; Sigma Aldrich, St. Louis, MO) were coated with polyclonal anti-human LBP antibodies. Diluted plasma samples (1:5,000) and a standard dilution series with recombinant LBP were added to the plate. Detection occurred with a biotinylated polyclonal rabbit anti-human LBP IgG, followed by peroxidase-conjugated streptavidin and substrate. The detection limit for the LBP assay was 200 pg/mL.

Fecal short- and branched-chain fatty acids and calprotectin. Fecal samples were collected at home and stored in the participants' freezer at −20°C, transported on dry ice, and stored on arrival at the university at −80°C on CID1. Fecal SCFA (acetate, propionate, butyrate, valerate, caproate) and BCFA (isobutyrate, isovalerate) were measured by gas chromatography-mass spectrometry (Dr. Stein and Colleague Medical Laboratory, Mönchengladbach, Germany), as described previously (18). Fecal calprotectin was determined using a commercial enzyme-linked immunosorbent assay (BÜHLMANN fCAL, Basel, Switzerland).

Microbiota composition. DNA was isolated from 0.25 g feces with repeated bead beating followed by automated isolation and purification using a Maxwell 16 Tissue LEV Total RNA Purification kit (Promega, Madison, WI). The V4 region of the 16S rRNA gene was amplified with a double barcoded primer pair 515F (5′-GTGCCAGCMGCCGCGGTAA)-806R (5′-GGACTACHVGGGTWTCTAAT) as previously described (34). Each sample was amplified in triplicate using Phusion hot start II high fidelity polymerase (Thermo Scientific). The 40-μL PCR reactions contained 28.4 μL nucleotide-free water (Promega, Madison, WI), 0.4 μL of 2 U/μL polymerase, 8 μL of 5X Phusion Green HF Buffer, 0.8 μL of 10-μM stock solutions of each of the barcoded forward (515F) and reverse (806R) primers, 0.8 μL of 10-mM 2-deoxynucleotide 5′-triphosphates (dNTPs; Promega), and 0.8 μL of template DNA. Cycling conditions were as follows: reactions were held at 98°C for 30 s with amplification proceeding for 25 cycles at 98°C for 10 s, 50°C for 10 s, 72°C for 10 s, and a final extension of 7 min at 72°C. PCR products were checked for correct size on a 1% agarose gel and subsequently combined and purified with the CleanPCR kit (CleanNA, Alphen aan den Rijn, The Netherlands). Purified PCR products were quantified with Qubit using the dsDNA BR Assay kit (Invitrogen, Carlsbad, CA), and a composite sample for sequencing was created by combining equimolar amounts of amplicons (200 ng) from the individual samples. The resulting library was sent to GATC Biotech AG for 2X150nt sequencing on an Illumina Hiseq2500 instrument. Sequence analysis was performed in NG-Tax using default settings (34). Paired-end libraries were demultiplexed using read pairs with perfectly matching barcodes. Amplicon sequence variants (ASV) were picked as follows: sequences were ordered by abundance per sample, and reads were considered valid when their cumulative abundance was ≤0.1%. Taxonomy was assigned using the SILVA reference database version 128 (6). ASVs are defined as individual sequence variants rather than a cluster of sequence variants with a shared similarity above a specified threshold such as operational taxonomic units.

The total bacterial counts in samples was assessed by quantitative PCR (qPCR). The qPCR measurements were performed in triplicates in 25-μL reactions in an iQ5 iCycler (Bio-Rad, Veenendaal, The Netherlands) using the iQ SYBR Green Supermix kit (Bio-Rad), with universal bacterial primers targeting the 16S rRNA gene: Eub341F(5-CCTACGGGAGGCAGCAG-3′), Eub534R (5′-ATTACCGCGGCTGCTGGC-3′) (30). The thermal cycling conditions included an initial DNA denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. Standard curves for the assays were prepared using serial dilutions of purified full-length PCR amplicons amplified with universal primers 27F (5′-GTTTGATCCTGGCTCAG) - 1492R (5′-GGTTACCTTGTTACGACTT) from fecal genomic DNA. Gene copy numbers were calculated per gram of feces.

Statistical analysis. Normality of data was assessed with the Gaussian distribution and Shapiro-Wilk test procedure, and not normally distributed variables were square root-transformed. Significant Spearman correlations ($P < 0.05$) between colonic and segmental colonic transit time with microbiota-related variables were included in multiple linear regression analysis. Age, sex, BMI, and protein and fiber intake were identified as covariates (i.e., changing standardized β-coefficient of the simple linear regression by more than 10%) of the association colonic transit, SCFA and BCFA, and therefore included as covariates in the linear regression. Multicollinearity was checked using variance inflation factor index, which was <1.5 for all analyzed variables. Statistical analyses were performed using SPSS 22.0 (IBM, Armonk, NY).

For the gut microbiota composition, all analyses were performed in R version 3.4. Distance-based redundancy analysis (db-RDA) with pairwise weighted UniFrac, unweighted UniFrac distance, and Bray Curtis dissimilarity were performed to determine the multivariate effects of the environmental variables on the microbiota composition using the capscal*e* function from the vegan package (32). RDA is a technique summarizing the linear relationships between a set of variables, i.e., gut microbiota composition explained by a set of explanatory variables, or host variables. Missing values were imputed using the K-nearest neighbor algorithm as implemented in the bnstruct package (17). All environmental variables were scaled (mean set to zero) before the RDA was performed. To determine which set of environmental variables resulted in the most parsimonious model for each distance matrix, we performed forward and reverse automatic stepwise model selection for constrained ordination methods using permutation tests with the ordistep function from the vegan package, which bases the term choice on Akaike's information criterion. For robustness, we performed the same automatic model selection using the ordiR2step function, which only performs forward model building and bases term choice solely on adjusted R^2 and P value. Both methods were in concordance with regard to the selection of significant terms. To determine the overlap and relationship in microbiota variation explained by the significant environmental variables, we used variation partitioning using the varpart function as implemented in vegan. Inverse Simpson, Shannon, ASV richness, and Faith's phylogenetic diversity were calculated to define microbial α-diversity for each participant as implemented in vegan and picante (22). To determine whether α-diversity was dependent on sequencing depth or absolute bacterial count, we calculated correlations with each diversity metric and the sequencing depth and absolute bacterial count, respectively. Based on normality of the data, we used Spearman or Pearson correlation.

UniFrac and unweighted UniFrac, which are based on the phylogenetic relatedness of the ASVs, were used as estimators of microbial β-diversity between the participants. Whereas weighted UniFrac considers the abundance of each ASV, unweighted UniFrac provides equal weight to all ASVs, thereby focusing on presence or absence of low abundance ASVs. Additionally, we calculated Bray-Curtis dissimilarity, which maximizes the pairwise distance between individual samples between 0 and 1 (0 meaning two sites have the same composition, i.e., sharing all the species, and 1 meaning two sites do not share any species). Univariate partial Spearman correlations (corrected for age and sex) were calculated using the ppcor package, and multiple testing correction was performed using the Benjamini–Hochberg procedure (23). Corrected P values (Q values) of < 0.1 were considered significant.

RESULTS

Descending colonic transit correlates with microbiota α-diversity. In total, 42 participants were included in this analysis (Table 1). Sequencing-based microbiota profiling was

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Table 1. *Participants characteristics*

	Mean ± SD/Median [25th−75th]	Range
Sex, male/female	11/31	
Age, yr	36 ± 12	20–54
Body mass index, kg/m^2	24.5 ± 2.8	19–31
Fasting glucose, mmol/L	4.9 ± 0.2	4.2–5.6
OGTT 2 h glucose, mmol/L	4.8 ± 1.2	2.1–7.2
Gastrointestinal transit		
Colonic transit, h	76.8 [60–91.2]	40–132
Ascending colonic transit, h	19.0 [10.6–29.8]	3.4–70.6
Descending transit, h	20.2 [10.6–34.6]	1.0–73.0
Rectosigmoid transit, h	32.2 [22.6–46.0]	3.4–80.2
Bristol stool score	3.0 [2.0–4.0]	1–6
Stool frequency, stool/week	4.0 [3.0–5.0]	0–11
Dietary intake		
Total energy intake, kcal/day	1,930 ± 521	1,563–2,163
Fat, E%	35.9 ± 6.5	30.5–40.2
Saturated fat, E%	13.1 ± 3.7	7.7–24.3
Protein intake, E%	16.5 ± 3.4	9.1–23.2
Carbohydrate intake, E%	44.1 ± 6.2	31.8–57.1
Fiber intake, g/day	20.3 ± 6.6	6.4–34.5
Gut-related host parameters		
Fecal calprotectin, μg/g	49.1 [24.3–94.9]	82.6–366.1
Plasma LBP, pg/ml	14.7 [10.0–17.6]	11.8–78.5
Microbiota-related metabolites		
Fasting breath H_2, ppm	6.0 [2.0–11.5]	7–25
Total plasma SCFA, mmol/L	99.6 [73.1–129]	11.7–183.7
Plasma acetate, mmol/L	91.5 [66.9–122.4]	2.7–175.3
Plasma propionate, mmol/L	2.8 [1.9–3.3]	0.78–5.6
Plasma butyrate, mmol/L	4.3 [2.9–5.3]	0–6.7
Total fecal SCFA, μmol/g	67.1 [46.0–86.5]	24.5–152.5
Fecal acetate, μmol/g	39.0 [29.3–49.6]	13.4–102.8
Fecal propionate, μmol/g	11.0 [7.1–15.1]	2.8–36.2
Fecal butyrate, μmol/g	8.5 [5.4–13.5]	0.6–33.6
Fecal valerate, μmol/g	1.5 [0.7–2.4]	0.1–8.4
Fecal caproate, μmol/g	1.1 [0.4–1.7]	0.1–5.0
Fecal isobutyrate, μmol/g	1.8 [1.1–2.6]	0.1–5.4
Fecal isovalerate, μmol/g	2.9 [1.6–4.2]	0.5–8.9

n = 42 participants. E%; energy percent; LBP, lipopolysaccharide-binding protein; OGTT, oral glucose tolerance test; SCFA, short-chain-fatty acids; ppm, parts per million.

successful for n = 42 participants and yielded 470,928 ± SD 171,136 reads per sample. Spearman rank correlations showed that increased α-diversity was significantly associated with longer descending colonic transit time but not to total colonic transit time, BSS, or stool frequency (Fig. 2). These associations remained after correction for age and sex using partial Spearman correlations (Supplemental Table S1; all supplemental material is available at https://doi.org/10.6084/m9.figshare.9861134.v6). Importantly, technical covariates as well as absolute bacterial counts did not have an impact on α-diversity estimates (Supplemental Table S2).

Age, sex, diet, and stool consistency, but not colonic transit, are associated with microbiota variation. Furthermore, we analyzed whether GI transit parameters as well as host parameters were linked to microbiota composition using distance-based redundancy analysis (db-RDA). We included (segmental) colonic transit time, stool consistency, age, sex, BMI, dietary intake, and microbial and gut-related parameters as listed in Table 1. We first selected the most parsimonious model using automatic model selection. Subsequently, to determine the relationship between these variables as well as their effect on the microbiota composition, we calculated their simple effects (i.e., the effect of the variable in isolation) and their conditional effects, i.e., the effect of the variable when all other significant variables were kept constant (Supplemental Table S3). Weighted UniFrac-based db-RDA showed that age, sex, and total fat intake were significantly related to microbiota variation. Total fat intake was inversely correlated with the relative abundance of *Bifidobacterium*. Age correlated mainly with the relative abundance of *Prevotella 9* and *Methanobrevibacter*, and inversely with *Bifidobacterium* and two *Blautia* ASVs (Fig. 3*A*). Using unweighted UniFrac-based db-RDA, microbiota variation was explained by age, plasma LBP, protein intake, and fecal calprotectin (Fig. 3*B*). Total protein intake and fecal calprotectin correlated with the presence of *Blautia* and *Bifidobacterium* ASVs. Consistent with their relative abundance in weighted UniFrac, age correlated with the presence of *Methanobrevibacter* and was inversely correlated with the presence of *Blautia* and *Bifidobacterium* ASVs. Thus, not only the presence of these ASVs is associated with age but also their relative abundance. Using Bray-Curtis dissimilarity, only BSS significantly explained microbiota variation. BSS correlated with ASVs from the genus *Faecalibacterium*, *Prevotella 9*, and an unclassified member of the *Lachnospiraceae* family (Fig. 3*C*). Other GI transit parameters did not significantly explain microbiota variation using any of the metrics. Univariate testing of single ASVs or genera with single GI transit parameter partial Spearman rank correlations adjusted for age and sex showed no significant associations after adjustment for multiple testing (Supplemental Table S4).

Segmental colonic transit is associated with plasma acetate and fecal SCFA. Next, we determined the relationship between the microbial metabolites listed in Table 1 and colonic and segmental colonic transit time. Multiple linear regression analysis showed that fecal butyrate, propionate, valerate, and caproate were consistently negatively associated with rectosigmoid transit time before and also after adjustment for age, sex, BMI, protein, and fiber intake (Table 2). Fasting plasma acetate was negatively associated with descending colonic transit. Additionally, fecal caproate was negatively associated with total colonic and ascending transit. Fecal BCFA isovalerate and isobutyrate were not related to (segmental) colonic transit time.

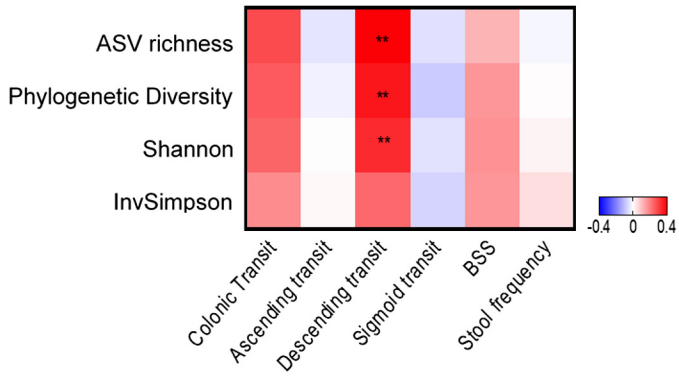

Fig. 2. Descending colonic transit is linked to microbial α-diversity in healthy humans. Spearman correlation heat map of microbiota α-diversity indices and gastrointestinal transit parameters in n = 42 participants. **$P < 0.05$. ASVs, amplicon sequencing variants; BSS, Bristol stool score.

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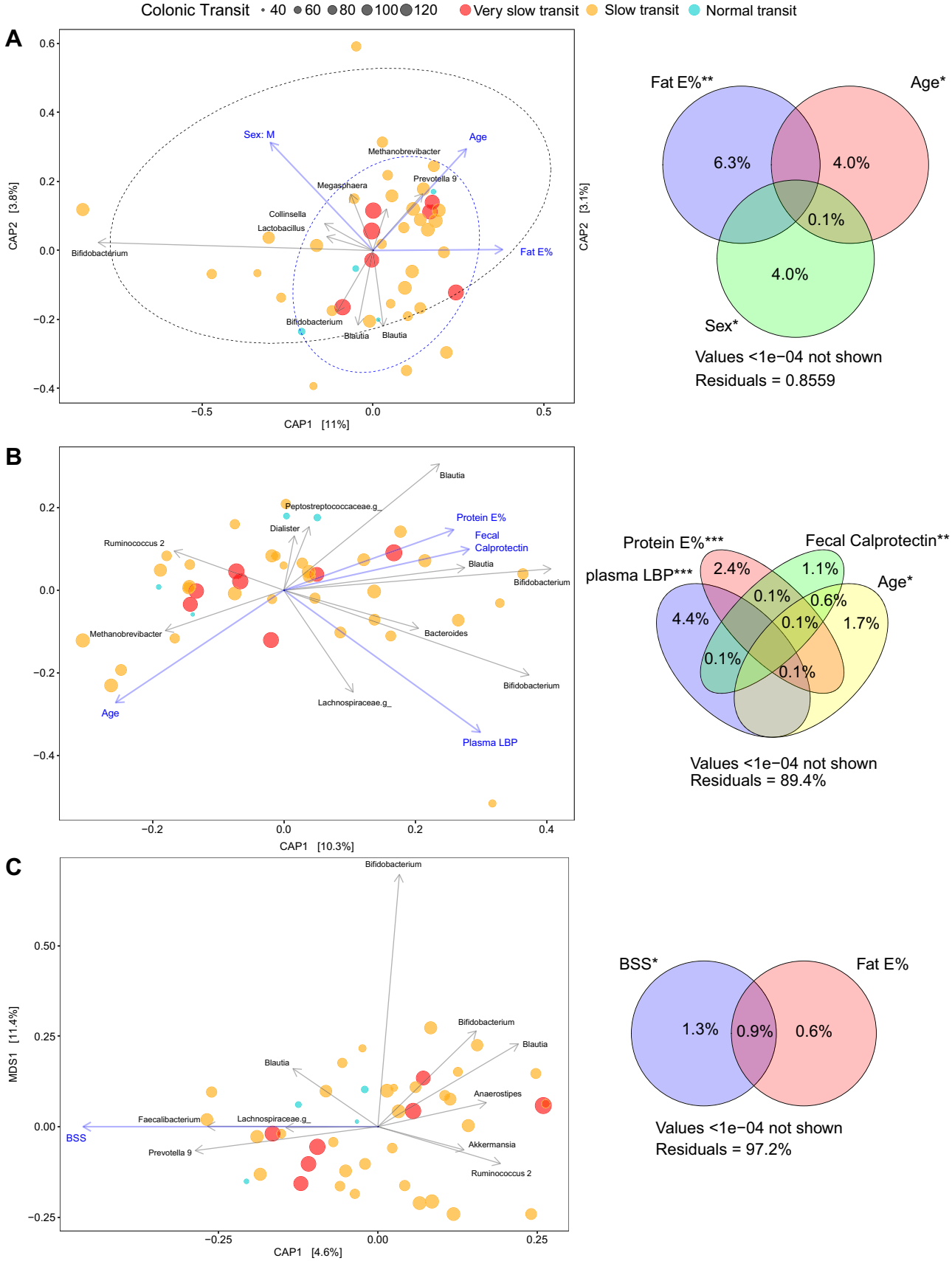

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Table 2. *Linear regression coefficients between colonic and segmental colonic transit and SCFA*

	Colonic Transit		Ascending Transit		Descending Transit		Rectosigmoid Transit	
	Std β adj	*P*	Std β adj	*P*	Std β adj	*P*	Std β adj	*P*
Plasma acetate	−0.207 ± 0.291	0.205	−0.03 ± 0.322	0.830	−0.346 ± 0.076	**0.028***	−0.042 ± 0.268	0.804
Fecal propionate	−0.245 ± 0.118	0.129	−0.036 ± 0.132	0.829	−0.271 ± 0.097	0.092	−0.356 ± 0.103	**0.028***
Fecal butyrate	−0.322 ± 0.121	0.085	0.001 ± 0.136	0.999	−0.251 ± 0.101	0.111	−0.431 ± 0.102	**0.012***
Fecal valerate	−0.265 ± 0.060	0.090	−0.022 ± 0.067	0.892	−0.220 ± 0.050	0.162	−0.485 ± 0.049	**0.001***
Fecal caproate	−0.414 ± 0.050	**0.007***	−0.261 ± 0.057	0.107	−0.347 ± 0.042	**0.025***	−0.428 ± 0.045	**0.006***

β, standardized β coefficient + standard error of coefficient of plasma acetate and fecal short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA) as dependent variables in a multiple regression analysis adjusted for age, sex, body mass index, and protein and fiber intake. *P* value represents significance of coefficient. *$P < 0.05$.

DISCUSSION

Stool consistency as a proxy of GI transit is an important covariate of the fecal microbiota. However, little is known about the interaction between colonic transit time, microbiota composition, and microbial metabolites. Here, we extend previous findings with an objective measurement of GI transit (quantitative radiopaque marker methods) as well as self-reported stool consistency and show that (more distal) colonic transit is linked to microbial α-diversity and fermentation and that microbiota composition was mostly explained by demographics, dietary fat, and protein intake instead of GI transit parameters in healthy participants. We further show that technical covariates or bacterial counts did not affect the observations and biological interpretation of α-diversity estimates.

GI transit affects most of the longitudinal colonic gradients, including nutrient availability and colonic content consistency, which together might greatly influence (spatial) microbiota composition (33). Here, we showed that increased α-diversity was related to longer descending colonic transit but not to stool consistency. Nutrient availability may have been the key driver for this observation because a longer distal colonic transit time may be accompanied by a greater and/or prolonged depletion of fermentable carbohydrates (especially with Western diets low in dietary fiber) in the descending colon. Carbohydrate depletion in turn promotes diversification by challenging bacteria to switch to other less efficient substrate sources, i.e., proteins, which attenuates the growth of fast-growing species and favors the growth of slow-growing, more specialized competitors (44). Furthermore, microbiota variation using weighted UniFrac (placing emphasis on microbial abundance) was explained by sex, age, and fat intake, whereas with unweighted UniFrac (also emphasizing the effect of less abundant species), plasma LBP, protein intake, fecal calprotectin, and age significantly explained microbiota variation. Self-reported stool consistency was the only significant explanatory variable related to Bray–Curtis dissimilarity (only taking abundances into account), which is consistent with previous population-wide studies (15, 19). Nevertheless, in contrast to a study by Roager et al. (36), colonic transit was not related to microbiota composition measured by weighted UniFrac. Colonic transit was much longer in the present study (median 72 h) as compared with Roager et al. (median 16.5 h), which suggests different compositional dynamics in short compared with longer colonic transit. In general, even if significant, the associations with phenotypical data possessed generally low effect sizes and substantial unexplained variance, which is also observed in large population-wide studies (15).

Age and sex are known covariates of gut microbiota (21a), and plasma LBP and fecal calprotectin as systemic and local inflammatory markers have been previously reported to be related to microbiota variation (45, 52). However, the effect of total fat and protein intake on microbiota composition is less clear. In the present study, total fat intake was strongly inversely associated with *Bifidobacterium*, consistent with data of animal high-fat diet models (9, 50) and humans (16, 46). Lipid degradation by microbiota under the anoxic conditions of the gut is not well characterized, yet dietary fat may act via different mechanisms on microbiota abundance, e.g., via antimicrobial and proinflammatory effects, and/or via bile acid metabolism (49). In the present study, total protein intake and age were explanatory host variables for unweighted UniFrac variation, as observed previously in a cross-sectional analysis in healthy young adults (14). Although proteins can be used as energy substrates in different bacterial pathways (8), excessive protein fermentation is assumed to be detrimental for gut health due to production of toxic catabolites such as ammonia, amines, and phenols (48). However, in the present study, total protein intake was moderately positively correlated with gut-health-related *Bifidobacterium* and *Blautia*. Furthermore, we observed that age and *Methanobrevibacter* presence and relative abundance were positively correlated, which is in line with other reports observing a relationship between age and methanogens (12, 28). Yet little is known about the influence of the habitual diet on methanogens; only one study reported a positive correlation between dairy intake and methanogens in children (40). Furthermore, consistent with data from healthy females (43), *Blautia* was inversely correlated to the abundance of *Methanobrevibacter*, which is likely driven by substrate competition because both can use hydrogen as an energy source (11).

Fig. 3. Redundancy-based analysis (RDA) of microbiota β-diversity, gastrointestinal transit, and host variables. RDA model was constrained by total colonic transit, stool consistency, age, sex, body mass index, dietary intake [total energy intake, dietary fat, saturated fat, carbohydrate, protein, and fiber intake] microbiota-related metabolites [fecal and plasma short-chain and branded-chain fatty acids), and gut-related parameters (plasma lipopolysaccharide-binding protein (LBP), fecal calprotectin] in $n = 42$ participants. Weighted UniFrac (*A*), unweighted UniFrac (*B*), and Bray–Curtis (*C*) with the respective Venn diagram explaining the nonredundant and redundant explanatory variance of the respective model. *$P < 0.05$, **$P < 0.025$, ***$P < 0.001$. BSS Bristol stool score. E%, percentage of energy intake.

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In the present study, longer rectosigmoid transit time was associated with low concentrations of fecal SCFA. In line with this, studies with relatively low numbers of healthy participants reported that lower fecal SCFA was associated with longer colonic transit time, as measured by fecal excretion of indigestible markers (13, 24). Longer rectosigmoid transit may exacerbate low-nutrient conditions and attenuated microbial energy metabolism, as reflected by lower SCFA concentrations in feces observed in the present study. Therefore, we suggest that the duration that fecal waste resides in the rectosigmoid influences fecal SCFA excretion by attenuating microbial fermentation and/or by increasing the time for absorption. Despite often being used as fermentation markers, fecal SCFA concentrations reflect the net result of absorption, production, and bacterial cross-feeding (4). Based on our data, fecal SCFA should be considered as most representative for the SCFA production and/or absorption in the very distal colon and may not necessarily reflect production and/or absorption of other more proximal colonic sites. To the best of our knowledge, this is the first study investigating the effect of GI transit on plasma SCFA. Plasma acetate, but not plasma butyrate or propionate, was negatively associated with descending colonic transit time. SCFA absorption into the circulation is highest in the distal colon (31); hence, acetate absorption into the circulation may to some extent be determined by colonic transit time. However, we cannot exclude other confounding factors, including a delayed peak in plasma SCFA due to long GI transit as well as endogenous acetate production and utilization.

Finally, we note that self-reported stool consistency and the more objective radiopaque measure of colonic transit time were not correlated. Despite being widely used as a surrogate marker for GI transit in population studies, correlations between stool consistency and colonic transit time determined by radiopaque markers, scintigraphy, and wireless capsules are only moderate, with more inconsistent results for healthy adults compared with constipated patients (20, 25, 37). Nevertheless, rather than estimating colonic transit per se, stool consistency may be a more accurate reflection of colonic water absorption and colonic peristaltic mixing, which are also factors influencing gut microbiota composition, as shown in in vitro simulators of the human colon (2).

The strength of the present study is the detailed phenotyping of study participants regarding GI transit parameters because we evaluated GI transit with quantitative radiopaque marker methods as well as self-reported stool consistency. The major limitation of this study is its cross-sectional design, limiting causal implications.

To conclude, we demonstrate that distal colonic transit time was related to microbial α-diversity, fecal SCFA, and plasma acetate, whereas microbiota composition was related to age, sex, and dietary intake and only to a minor extent to stool consistency. We extend current knowledge suggesting that the distal colon transit may affect not only microbiota diversity but also microbial metabolism. Thus, when targeting the microbial fermentation to improve the host health, the colonic site of fermentation should be considered in future interventional studies. Further studies looking at microbial functionality are warranted to disentangle the interactions between (site-specific) GI transit time and gut microbiota.

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DISCLAIMERS

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.M. conceived and designed research; M.M. performed experiments; M.M. and G.D.H. analyzed data; M.M., G.D.H., E.E.C., E.G.Z., and E.E.B. interpreted results of experiments; M.M. and G.D.H. drafted manuscript; G.D.H. prepared figures; G.D.H., E.E.C., H.S., A.A.M., E.G.Z., and E.E.B. edited and revised manuscript; H.S., A.A.M., E.G.Z., and E.E.B. approved final version of manuscript.

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