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^{1}H NMR-Based Identification of Intestinally Absorbed Metabolites by Ussing Chamber Analysis of the Rat Cecum

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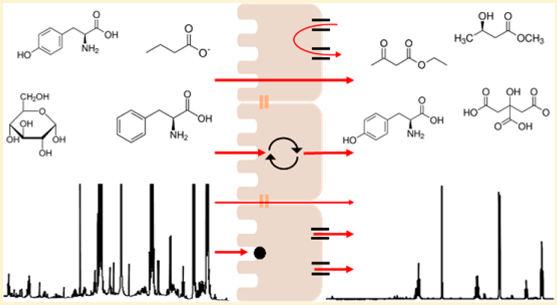
Supporting Information

ABSTRACT: The large intestine (cecum and colon) is a complex biochemical factory of vital importance to human health. It plays a major role in digestion and absorption by salvaging nutrients from polysaccharides via fermentation initiated by the bacteria that comprise the gut microbiome. We hypothesize that the intestinal epithelium absorbs a limited number of luminal metabolites with bioactive potential while actively excluding those with toxic effects. To explore this concept, we combined ^{1}H NMR detection with Ussing chamber measurements of absorptive transport by rat cecum. Numerous metabolites transported across the epithelium can be measured simultaneously by ^{1}H NMR, a universal detector of organic compounds, alleviating the need for fluorescent or radiolabeled compounds. Our results demonstrate the utility of this approach to delineate the repertoire of fecal solutes that are selectively absorbed by the cecum and to determine their transport rates.



The colon is vitally important to human health, containing trillions of beneficial bacteria, also known as the gut microbiome, that help harvest energy, synthesize vitamins, exclude pathogenic invaders, and modulate the immune system.[1,2] The gut microbiome is incredibly complex and dynamic. An unbalanced microbiome can disturb physiological homeostasis and stimulate or contribute to health problems, including obesity,[3−5] diabetes,[6,7] colon cancer,[8,9] cardiovascular disease,[10,11] allergies,[12,13] autism,[14−16] irritable bowel disease (IBD), and Crohn's disease.[17,18] How the gut microbiome communicates chemically with its host remains unclear. There is firm evidence for a two-way dialogue across the intestinal epithelium, a thin sheet of polarized cells that physically separates the gut lumen from the body, regulates the chemical and physical environment of the intestinal lumen (and thereby shapes bacterial colonization and growth), and functions as a gatekeeper that absorbs beneficial microbial metabolites while excluding potential toxins.[19] Although the mechanistic links between the metabolome and human health remain poorly understood, some components of the metabolome, including the short chain fatty acids acetate, butyrate and propionate, are known to exert beneficial effects by serving as favored metabolic fuels and as regulators of cellular gene expression.[20]

In this study, we explore the use of the Ussing chamber, an apparatus used to measure transport and barrier functions of living epithelial tissue, in combination with ^{1}H NMR spectroscopy to characterize the absorption of microbial metabolites by the rat cecum, a specialized region of the rodent large intestine that functions as a fermentation chamber. Cecal tissue, removed from the rat, was stripped of seromuscular layers and mounted between two chambers representing luminal (mucosal or apical) and serosal (basolateral) compartments.[21] Ussing chamber experiments have been previously used in the study of colonic transport with a variety of approaches employed for the detection of transported metabolites including, fluorescence labeling,[22] enzyme-linked immunosorbent assay (ELISA),[23] radioisotope tracers,[24,25] and high-performance liquid chromatography (HPLC) with detection by ultraviolet (UV),[26−28] diode array detectors (DAD),[29] and MS/MS.[30] Each of these analytical methods has limitations. Addition of a fluorescent tag changes the structure of a compound and can greatly affect the transport rates of small molecule metabolites, and radio-labeled compounds can be costly and limited in their availability. An additional limitation of using fluorescent- or radio-labeled compounds is the challenge of studying the simultaneous transport of multiple metabolites. Although antibody-based ELISA offers good sensitivity, it is highly specific for the targeted analyte. The separation provided by HPLC-UV or -DAD allows for the analysis of multiple metabolites, but these compounds must contain a detectable chromophore. HPLC-UV or -DAD methods also require that the identity of a metabolite is known, and that standards are available to obtain calibration plots for quantitation. HPLC-MS/MS offers good sensitivity, but is best suited for targeted analyses because of the optimization of sample preparation and separation methods

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required; for example, HPLC-MS/MS is often used in Ussing chamber studies of drug absorption.[31,32]

Applications of nuclear magnetic resonance spectroscopy (NMR) in metabolomics and metabolite profiling continue to grow due to its ability to analyze a wide variety of compounds in a single experiment and without destruction of sample.[33,34] ^{1}H NMR provides a comprehensive overview of the sample components facilitating both identification of unknown metabolites and quantification of compounds relative to a single internal standard.[35,36] In this work, we make use of these advantages by exploring the use of ^{1}H NMR spectroscopy for analysis of metabolite transport in Ussing chamber experiments.

EXPERIMENTAL SECTION

Intestinal Tissue Preparation. Female Sprague−Dawley rats (190−250 g) were purchased from Taconic Biosciences (Rensselaer, NY) and housed at 23 ± 1 °C on a 12:12-h light-dark cycle with free access to water and a standard rodent diet. All animal protocols were approved by the University of California Riverside Institutional Animal Care and Use Committee. Experiments reported in this study used rat cecum, the predominant site of microbial fermentation along the distal intestine. Rats were euthanized by gradual displacement of air with CO_2. The cecum was isolated, opened along the mesenteric border, and rinsed in an ice-cold Parson's solution comprised of: 110 mM NaCl, 25 mM $NaHCO_3$, 4 mM KCl, 2 mM Na_2HPO_4, 1.25 mM $CaCl_2$, 1 mM $MgSO_4$, and 12 mM glucose, saturated with 5% CO_2/95% O_2. The osmolarity of all solutions was adjusted to match that of rat cecal stool fluid (305 mOsm) by the addition of NaCl or water. The cecum was pinned, mucosal-side down, onto an ice-cold Sylgard silicone tray and divided into equal halves. To preserve viability and minimize intrinsic neural influences, the wall of the cecum was stripped of its outer serosa and muscle layers by dissection under a stereomicroscope to obtain a conventional mucosa-submucosa preparation.[21]

Measurement of Metabolite Transport. Absorptive solute flux, transmucosal potential difference (V_T), transmucosal electrical resistance (TER), and short-circuit current (I_{sc}) across the isolated rat cecum were measured using an Ussing chamber technique,[21] with minor modifications. The stripped mucosa-submucosa preparation was mounted on small pins across an oblong aperture (5 × 22 mm; Physiologic Instruments P2252, San Diego, CA) with an exposed tissue area of 1.0 cm^2, and incubated in an Ussing chamber (Physiologic Instruments EM-CSYS-2). Chambers were maintained at 37 °C by heated water jackets and continuously mixed by gas lift with 5% CO_2/95% O_2. To suppress prostanoid influences, indomethacin (1 μM) was included in all serosal solutions, added from a 64 mM stock solution prepared in deuterated DMSO (Cambridge Isotope Laboratories, Tewksbury, MA); the use of deuterated DMSO precluded the DMSO resonance from obscuring metabolite NMR signatures. V_T was measured through 170 mM KCl agar bridges connected to a pair of calomel electrodes and monitored with a voltage clamp amplifier (Physiological Instruments VCC-MC2). The mucosa was maintained in an "open circuit" mode except during periodic (every 5 min) measurements of TER to monitor tissue integrity: V_T was clamped briefly (15 s) at 0 mV by applying a current (I_{sc}) through a pair of Ag/AgCl electrodes kept in contact with the luminal and serosal solutions via 170 mM KCl agar bridges. TER (ohm·cm^2) was calculated using Ohm's law after recording the change in I_{sc} evoked by a 2 mV pulse. At the beginning of each experiment, both sides of the tissue were incubated with Parson's solution for 30 min to establish a basal steady-state condition. To initiate transport, the luminal solution was replaced with 2 mL of prewarmed rat cecal stool fluid or a solution containing physiological concentrations of short chain fatty acids (SCFA) including acetate, butyrate and propionate (SCFA solution; defined below), and the serosal fluid was replaced with 2 mL of fresh Parson's solution. At consecutive 45 min intervals, the luminal and serosal solutions (2 mL) were harvested and replaced at the same rate to avoid subjecting the tissue to hydrostatic pressure gradients. In a series of preliminary time course experiments with SCFA or stool fluid, we established that the most rapidly transported solutes (acetate, butyrate, propionate) accumulated in the serosal solution at a constant rate for up to 90 min, affirming that the tissue remained viable and that retrograde fluxes (in the serosal-to-luminal direction) of absorbed SCFAs were negligible over this interval. Accordingly, the rate of serosal accumulation (μmol/cm^2·h) was compared over two successive 45 min intervals to obtain an average value. In a series of experiments designed to evaluate the potential effects of contaminating bacteria, tissues were preincubated for 45 min in Parson's solution containing the broad-spectrum antibiotics metronidazole (50 μg/mL) and streptomycin (100 μg/mL).

Evaluation of Tissue Viability. At the conclusion of each measurement, the tissue was restored to baseline conditions by incubation in bilateral Parson's solution for ≥10 min. Forskolin (10 μM) was added to the serosal chamber to evoke electrogenic Cl^- secretion, recorded as the peak positive I_{sc} after 10 min. Tissues exhibiting signs of functional impairment, that is, low electrical resistance (≤30 ohm·cm^2), weak secretory I_{sc} response (≤35 μA/cm^2), or significant differences (≥30%) in solute transport over the first (45 min) and second (90 min) intervals, were excluded from analyses.

Luminal Solutions. "SCFA Solution" was formulated to resemble native rat cecal stool fluid with respect to major electrolytes (98.5 mM Na^+, 45 mM K^+, 20 mM Mg^{2+}, 2.5 mM Ca^{2+}, 59 mM Cl^-, 3 mM SO_4^{2-}), major short chain fatty acids (59.5 mM acetate, 43 mM butyrate, 18 mM propionate), pH (6.50) and osmolarity (305 mOsm). Native stool fluid was isolated from rat ceca immediately after CO_2 euthanasia. Stool from this segment has the highest fluid content and microbial fermentative capacity.[37] Microbes and solids were removed by 4 successive cycles of centrifugation (15 500 × g, 7 min) and filtration (0.45 μm). The pellet of stool solids was rinsed with SCFA Solution (1/2 pellet volume), which was then combined with undiluted stool fluid, resulting in small dilution (<30%) of all solutes except major SCFAs and electrolytes. To exclude the potential influence of variance in stool fluid composition, stool fluids from 13 rats pooled (11 mL; pH = 5.70 ± 0.21) and stored at −70 °C. Before use, aliquots were thawed and clarified by centrifugation.

^{1}H NMR Analysis of Ussing Chamber Samples. A 65 μL aliquot of deuterated 50 mM sodium phosphate buffer in D_2O (pD 7.4) containing 0.35 or 0.45 mM 3-(trimethylsilyl)-propane-1-sulfonic acid (DSS-d_6, Cambridge Isotope Laboratories, Tewksbury, MA) as a chemical shift and quantitation reference, and 0.2 mM ethylenediaminetetraacetic acid-d_{16} (EDTA-d16, Sigma-Aldrich, St. Louis, MO) was added to a 5 mm NMR tube (Wilmad 535-pp or equivalent) containing 585 μL of solution removed from the Ussing chamber. The DSS concentration was determined in a separate NMR experiment relative to the concentration of the primary standard potassium

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Table 1. Metabolites Quantified in Ussing Chamber Samples with the Number of Experimental Replicates Indicated by n[a]

		luminal (μM)		serosal (μM)		
metabolites	^{1}H NMR chemical shifts (ppm)	stool fluid (n = 6)	SCFA	stool fluid (n = 10)	SCFA (n = 10)	Parson's (n = 6)
3-hydroxybutyrate	1.18 (d)*, 2.29 (m), 2.39 (m), 4.13 (m)	ND		8.24 ± 4.33	18.4 ± 5.77	1.13 ± 0.38
acetate	1.91 (s)*	91000 ± 19100	62500	1360 ± 492	818 ± 161	4.35 ± 1.33
acetoacetate	2.27 (s)*, 3.34 (s)	177 ± 54.3		6.25 ± 5.46	12.1 ± 9.26	ND
alanine	1.47 (d)*, 3.78 (q)	1020 ± 376		26.1 ± 11.1	20.2 ± 7.86	28.2 ± 1.33
butyrate	0.88 (t)*, 1.55 (m), 2.15 (t)	33100 ± 6150	43000	683 ± 229	500 ± 114	ND
choline	3.19 (s)*, 3.52 (m), 4.07 (m)	56.3 ± 20.7		7.83 ± 1.92	6.00 ± 2.90	8.37 ± 2.67
citrate	2.54 (d)*, 2.65 (d)	ND		4.71 ± 3.28	5.24 ± 2.89	2.92 ± 0.51
formate	8.44 (s)*	223 ± 60.8		4.34 ± 2.14	2.87 ± 1.08	3.04 ± 1.12
hydrocinnamate	2.48 (t), 2.88 (t)*, 7.31 (m)	164 ± 63.9		NQ	ND	ND
isoleucine	0.93 (t), 1.00 (d)*, 1.25 (m), 1.46 (m), 1.97 (m), 3.66 (d)	279 ± 111		3.79 ± 1.86	2.04 ± 0.82	2.14 ± 0.59
lactate	1.32 (d)*, 4.10 (q)	1633 ± 417		451 ± 189	408 ± 119	469 ± 171
lysine	1.47 (m), 1.72 (m). 1.90 (m), 3.20 (t)*, 3.74 (t)	761 ± 191		16.3 ± 6.91	4.13 ± 1.26	4.57 ± 1.00
niacin	7.51 (dd), 8.24 (m), 8.60 (dd), 8.93 (d)*	28.5 ± 4.87		NQ	ND	ND
oxaloacetate	2.37 (s)*	ND		5.53 ± 4.62	5.26 ± 2.37	ND
phenylalanine	3.20 (m), 3.98 (dd), 7.32 (d), 7.36 (m)*, 7.42 (m)	212 ± 86.3		2.07 ± 1.06	1.21 ± 0.73	1.72 ± 0.42
propionate	1.05 (t)*, 2.17 (m)	18200 ± 3890	18000	292 ± 115	211 ± 44.5	ND
pyruvate	2.36 (s)*	ND		3.57 ± 2.24	4.37 ± 2.09	12.4 ± 3.33
succinate	2.39 (s)*	1490 ± 516		5.99 ± 1.91	5.37 ± 2.37	7.42 ± 3.36
tyrosine	3.05 (dd), 3.19 (dd), 3.93 (dd), 6.89 (m)*, 7.18 (m)	254 ± 102		3.07 ± 1.18	1.47 ± 0.41	1.97 ± 0.46
tyramine	2.82 (t), 3.12 (t), 6.75 (d)*, 7.12 (d)	117 ± 31.5		NQ	ND	ND
valine	0.98 (d)*, 1.02 (d), 2.26 (m), 3.60 (d)	448 ± 143		5.38 ± 2.38	3.60 ± 1.01	4.21 ± 1.01

[a]Luminal: Concentrations (μM) of metabolites initially present in the luminal chamber of the Ussing chamber. Serosal: Concentrations of metabolites measured in the corresponding serosal solution. Some resonances were not within the limits of detection (ND), or were detectable at values below the limits of quantitation (NQ; <10:1 S/N).

hydrogen phthalate (KHP, minimum 99.95% purity; Sigma-Aldrich) as described by Larive et al.[38]

^{1}H NMR spectra were acquired at 298 K using a Bruker Avance NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a BBI probe and operating at 599.52 MHz. A 90° pulse was applied and the solvent resonance was suppressed by excitation sculpting (Bruker pulse sequence zgesgp).[39] Spectra were acquired into 32768 points with coaddition of 4096 transients, 128 dummy scans, and a relaxation delay of 0.05 s and acquisition time of 2.38 s.

^{1}H NMR Analysis of Stool Fluid. A 150 μL aliquot of deuterated 50 mM sodium phosphate buffer in D_2O (pD 7.4) containing 0.45 mM DSS-d_6 and 0.2 mM EDTA-d_{16} was added to a 3 mm NMR tube (New Era, NE-H5/3-Br) containing 50 μL of filtered cecal stool fluid. Samples of cecal stool fluid were removed from 6 rats, with each sample obtained from a different rat. ^{1}H NMR spectra were acquired at 298 K using a Bruker Avance III NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a TCI CryoProbe and operating at 700.23 MHz. Water suppression was conducted using 1D NOESY (noesypr1d) with presaturation during the 120 ms mixing time. Spectra were acquired into 32768 points with coaddition of 256 transients, 32 dummy scans, and a relaxation delay of 15.0 s and acquisition time of 2.03 s.

^{1}H NMR Processing Parameters. All spectra were preprocessed using Bruker Topspin 3.2 for initial phasing and chemical referencing to DSS (0.000 ppm). Further processing was performed using Mnova 10.0 (Mestrelab Research, Bajo, Spain). Free induction decays (FIDs) were apodized by multiplication by an exponential decay equivalent to 1.0 Hz line broadening, zero-filled to 131072 points, manually phased and 2.31 Hz (Whittaker smoothing) baseline correction applied. Assignments of ^{1}H NMR resonances were performed by comparison with the Human Metabolome Database (hmdb.ca),[40] Chenomx,[41] and various literature reports,[2,18,42,43] with assignments confirmed using an in-house spectral library of authentic metabolite standards measured under similar conditions to those employed in this work. The chemical shift assignments of the metabolites quantified in this study are summarized in Table 1.

NMR is a well-established method for quantitative analysis, having the advantage that a single standard at known concentration can be used to quantify all the components in a complex mixture, provided care is taken to account for the longitudinal or transverse (T_1) relaxation times of the interrogated nuclei.[35] The experimental parameters used in the measurement of T_1 relaxation times and the equations used for metabolite quantitation are described in the Supporting Information.

Each 45 min time interval constitutes a replicate (n), with Ussing chamber analyses being performed on 6 (n = 12) rats for luminal stool fluid, 5 rats (n = 10) for luminal SCFA solution, and 3 rats (n = 6) for luminal Parson's solution. Once metabolite concentrations were determined, statistical analyses were performed using SPSS Statistics software (IBM, Armonk, New York, USA). The Tukey fences method using interquartile range (IQR) values[44] was applied to the data sets for each sample type to determine the presence of outliers, and it was determined that 1 rat (n = 2) was an outlier among 10 of the 18 metabolites and was removed from the data set. This removal resulted in 5 rats (n = 10) for the analyses of stool fluid, 5 rats (n = 10) for the SCFA solution, and 3 rats (n = 6) for the Parson's solution (Table 1). Statistical differences between the 3 sample types (stool fluid, SCFA solution, and Parson's

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solution) were calculated using a one-way ANOVA plus post hoc Tukey test.[45,46]

RESULTS AND DISCUSSION

The Ussing chamber serves as an indispensable tool for measuring the transport and diffusion of ions, nutrients, and drugs across the epithelial tissues of various organs and species.[47−50] The Ussing chamber is comprised of two hemichambers separated by a transporting epithelium and its underlying mucosal layer (Figure 1). By convention, the epithelium is oriented with its mucosal surface facing the left luminal hemichamber and opposing surface facing the right serosal hemichamber. This arrangement allows experimental control over both luminal and serosal environments and the ability to measure the rates at which molecules traverse the tissue in the absorptive (luminal-to-serosal) and secretory (serosal-to-luminal) directions.

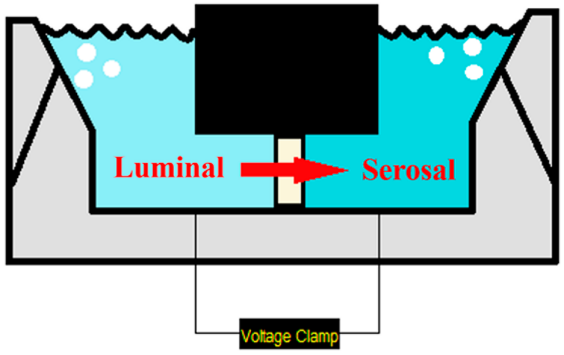

Figure 1. Diagram of the Ussing chamber used to measure rates of absorptive transport and tissue metabolism by excised rat cecum.

To identify the subset of microbial metabolites that are afforded access to the circulating blood via intestinal absorption, understand metabolite transport behaviors and effectively simulate the gut environment in Ussing chamber experiments, it is important to evaluate transport from samples of stool fluid that recapitulate the complex milieu of metabolites available for potential transport and metabolism in vivo. Many prior studies have been limited in their ability to simultaneously measure the transport of multiple solutes due to the analytical methods employed.[22−25,30] In this study, we investigate the utility of ^{1}H NMR to simultaneously measure the absorptive transport of diverse components of the luminal metabolome under conditions that recapitulate the native cecal lumen. The use of ^{1}H NMR as a primary detection method afforded several advantages, including (i) quantification of diverse organic species without the use of labels; (ii) the use of a single internal standard without the need for standards for each compound or calibration plots;[51] (iii) identification of new compounds in the serosal solution derived from the biochemical transformation of molecules during transit through the epithelial tissue; and (iv) determination of relative absorptive transport rates by direct comparison of NMR spectra.

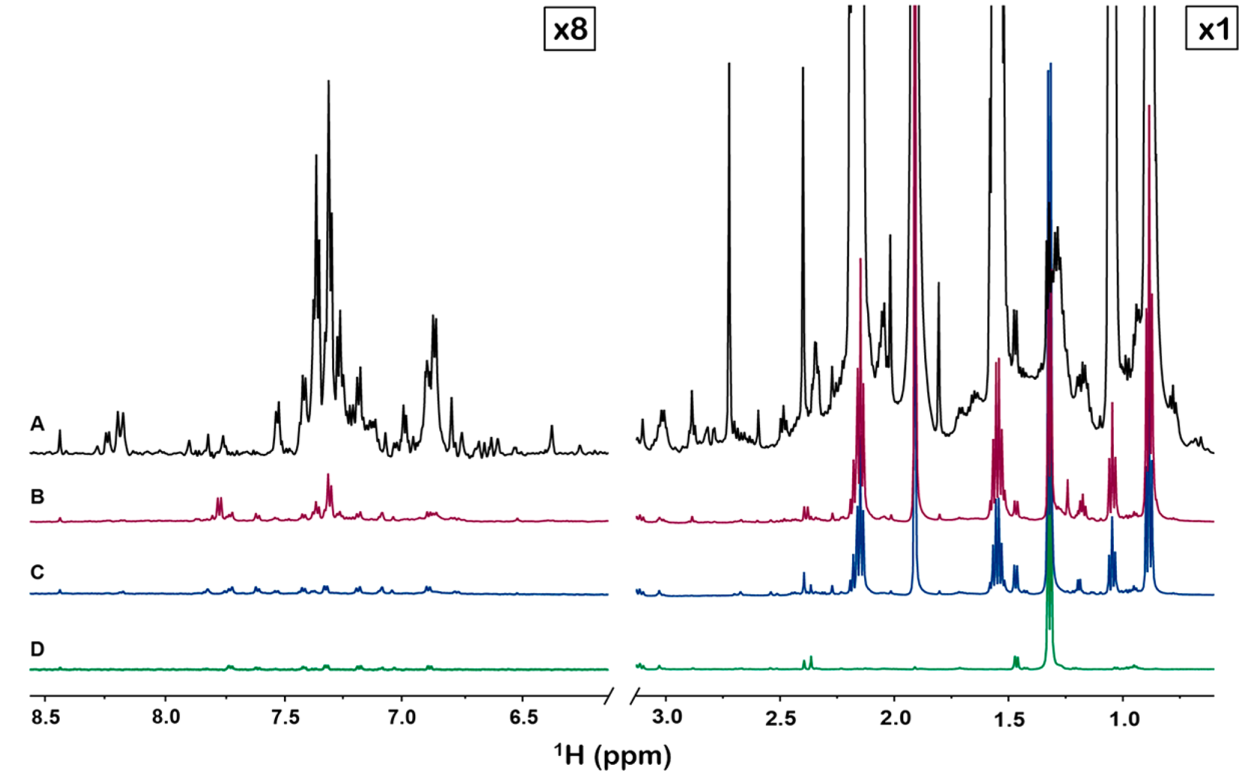

Figure 2. ^{1}H NMR spectra of (A) cecal stool fluid, (B) Ussing chamber serosal chamber sample with cecal stool fluid in the luminal chamber, (C) Ussing chamber serosal chamber sample with SCFA solution in the luminal chamber, and (D) Ussing chamber serosal chamber sample with Parson's solution in the two chambers to serve as a blank, in which case serosal glucose is the only carbon source. Each spectrum is normalized to the resonance of the internal standard DSS allowing direct comparison of resonance intensities.

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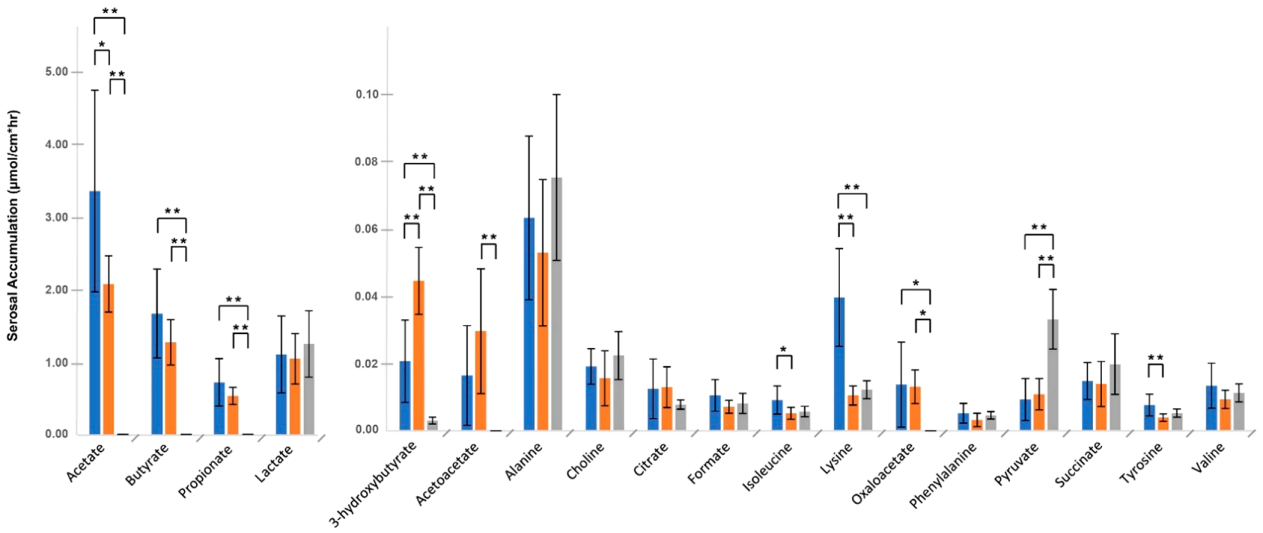

Figure 3. Rates of serosal accumulation (μmol/cm·h) of acetate, butyrate, propionate, lactate, 3-hydroxybutyrate, acetoacetate, alanine, choline, citrate, formate, isoleucine, lysine, oxaloacetate, phenylalanine, pyruvate, succinate, tyrosine, and valine, reflecting the rates of absorptive transport and diffusion across the cecal epithelium. Blue (n = 10), orange (n = 10), and gray (n = 6) represent samples removed from the serosal chamber in which cecal stool fluid, SCFA solution, and Parson's solution respectively, were loaded in the corresponding luminal chamber. Statistical significance is indicated by ** ($p \leq 0.01$) and * ($p \leq 0.05$), determined by one-way ANOVA (analysis of variance) post hoc Tukey test.

To assess the effect of luminal solutes on absorptive transport, experiments were carried out with the luminal chamber loaded with stool fluid (the water-soluble metabolome), SCFA solution (the SCFA component of the metabolome), or Parson's solution (metabolome-free). The ^{1}H NMR spectra obtained (Figure 2) allow for direct comparison of metabolite profiles from each chamber. The spectrum of stool fluid (Figure 2A) contained numerous resonances, confirming the known chemical diversity of the metabolome. With the stool fluid in the luminal chamber, the spectrum of the corresponding serosal solution (Figure 2B) included a subset of the same resonances, including the prominent signatures of the SCFAs, reflecting the selective transport or diffusion of various luminal metabolites across the epithelium over the 45 min intervals used in these experiments. For example, several aromatic compounds are detected in luminal stool fluid around 6.6−7.6 ppm but not in the corresponding serosal solution, indicating that these are excluded by the epithelium or transported at rates that yield serosal concentrations below our limits of detection. To assess the transport of only the SCFA component of the metabolome, experiments were repeated with a luminal solution containing SCFAs at concentrations resembling those in native cecal stool fluid (59.5 mM acetate, 43 mM butyrate, 18 mM propionate, pH 6.50). With this SCFA solution in the luminal chamber, the ^{1}H NMR spectrum obtained for the serosal solution (Figure 2C) was less complex, but included the prominent resonance signatures of the major SCFAs, which are known to be rapidly transported by the cecal epithelium.[52] The spectrum obtained for the serosal sample with luminal Parson's solution, acting as a blank, is shown in Figure 2D; because no absorptive transport can occur in the absence of luminal metabolites, the resonances detected in Figure 2D are presumed to reflect the generation of metabolites by the epithelial cells and the supportive cells of the cecal mucosa and submucosa.

Table 1 lists 18 compounds that were identified and quantified in the serosal solution directly by ^{1}H NMR. Among these 18 molecules, 14 were detected in stool fluid. The others (3-hydroxybutyrate, citrate, oxaloacetate, and pyruvate) were observed only in serosal solutions, and therefore are presumed to be generated by the metabolic processes of the tissue. The ability to detect both transported solutes and cellular metabolic products highlights an advantage of NMR as a platform for the nontargeted analysis of organic compounds. The serosal samples in Table 1 are segregated into 3 sample types based on the solution in the luminal chamber (stool fluid, SCFA solution, and Parson's solution). Additional resonances were observed in our experiments including those of nicotinic acid (niacin), hydrocinnamate, and tyramine (Table 1). These molecules could be reliably detected but not consistently quantified due to insufficient signal-to-noise ratios. This analytical limitation could be overcome in future experiments through the use of a higher field spectrometer (e.g., operating at 700 or 800 MHz) equipped with a cryogenically cooled probe or by the acquisition of additional transients with a corresponding increase in experiment times.[35]

For the three experimental treatments studied (stool fluid, SCFA or Parson's solution in the mucosal chamber), serosal accumulation rates (μmol/cm^2·h) were calculated for each metabolite by averaging the extent of serosal accumulation over the two sequential 45 min intervals (Figure 3). In the case of the ketone body 3-hydroxybutyrate, when SCFA solution is placed in the luminal chamber, the rate of serosal accumulation increased significantly compared to the analogous experiment with stool fluid (Figure 3). Although we did not detect 3-hydroxybutyrate in luminal stool fluid, it may have been present below our detection limits. The SCFA solution, however, is prepared to be free of 3-hydroxybutyrate, providing evidence that it is generated by tissue metabolism. With luminal Parson's solution (no luminal metabolites), the rate of serosal 3-hydroxybutyrate accumulation decreased significantly, reinforcing our hypothesis that this ketone body is produced by the colonic epithelium largely through transformation of SCFAs. Similarly, two other ketone bodies, acetoacetate and the

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oxaloacetate, were detected in serosal samples when either stool fluid or SCFA solution were present in the luminal chamber; however, the rates at which these metabolites accumulated in the serosal solution decreased markedly when SCFAs were removed from the luminal solution (Parson's solution) (Figure 3). Given this context, it is interesting to note that serosal pyruvate accumulation was significantly lower when the luminal chamber contained native concentrations of SCFA (either stool fluid or SCFA Solution). Because glucose was always included in the serosal chamber to maintain tissue viability, glycolysis could be a source of this serosal pyruvate. However, the high rate of serosal pyruvate accumulation and low rate of oxaloacetate accumulation with luminal Parson's solution could indicate that the epithelial tissue lacks sufficient ATP to convert pyruvate to oxaloacetate via pyruvate carboxylase. With butyrate the preferred energy source of colonocytes,[53] the presence of stool fluid or SCFA solution in the luminal chamber appears to overcome this energy limitation, as lower rates of pyruvate and higher rates of oxaloacetate accumulation are observed. These results also support the hypothesis that the serosal accumulation of the ketone bodies acetoacetate and 3-hydroxybutryate primarily results from SCFA metabolism.

Although serosal acetate accumulation decreased significantly when luminal stool fluid was replaced by SCFA solution, the rates of butyrate and propionate absorption were comparable to those observed with stool fluid (Figure 3), indicating that their absorption is largely unaffected by other components of the metabolome. Moreover, while stool fluid contains 1.63 mM lactate (Table 1), the rate of serosal lactate accumulation was similar when the luminal chamber contained lactate free SCFA or Parson's solutions, indicating that the main source of serosal lactate is tissue metabolism rather than absorptive transport, and that cellular lactate production is unaffected by the luminal presence or transcellular transport of the metabolome (Figure 3). This same trend is observed for alanine, choline, citrate, formate, succinate, and valine, suggesting that they are generated by the cecal tissue at rates that are not impacted by the composition of the luminal solution.

We observed several essential amino acids in cecal stool, including lysine, isoleucine, phenylalanine, and valine, in addition to tyrosine, which is conditionally essential (Table 1).[54] The rate at which these amino acids accumulated in the serosal solution was found to decrease, in some cases significantly, when luminal stool fluid was replaced by the amino acid-free SCFA or Parson's solutions (Figure 3), indicating that a component of this accumulation represents absorptive transport or diffusion across the cecal epithelium. The appearance of all 5 essential amino acids in the serosal solution at low yet detectable rates with luminal SCFA and Parson's solutions suggests that these amino acids are released from existing intracellular pools by the tissue or produced by contaminating bacteria. To evaluate the latter possibility, we preincubated freshly excised and thoroughly rinsed cecal mucosa for 1 h at 37 °C in oxygenated Parson's solution containing two antibiotics with broad coverage of the gut microbiota: metronidazole (50 μg/mL) and streptomycin (100 μg/mL). Antibiotic pretreatment had no measurable effect on tissue viability (TER, response to secretory stimuli) or serosal accumulation of the 18 quantified metabolites. The only detectable change in the ^{1}H NMR spectra of serosal samples using the treated tissue was 2 singlets (indicated by *) at 2.50 and 8.05 ppm matching the resonances of metronidazole (Figure S2). The concentration of metronidazole in the serosal samples was ~1.0 μg/mL suggesting that a small amount of metronidazole remained after rinsing the tissue. Thus, the serosal appearance of metabolites not initially present in luminal solutions reflects their synthesis, transformation, or in the case of amino acids, possible cellular protein catabolism rather than production by contaminating bacteria.

CONCLUSIONS

To the best of our knowledge, this is the first experimental report using the Ussing chamber in conjunction with metabolite detection by ^{1}H NMR. This approach enabled the simultaneous identification and quantification of 18 metabolites that were transported or generated by the colonic epithelium of the rat cecum. The quantitative ability of ^{1}H NMR analysis allowed the direct comparison of samples distinguishing rates of those metabolites being both generated by the tissue and transported from stool fluid. For example, significant differences in serosal accumulation were observed for the ketone bodies 3-hydroxybutyrate and acetoacetate depending on whether SCFAs were present at native concentrations in the luminal chamber. Similarly, in the absence of the luminal metabolome or SCFAs (Parson's solution), the rate of serosal pyruvate accumulation increased while the rate of oxaloacetate generation decreased. This may indicate that without SCFA's, the tissue has insufficient ATP stores to complete the enzymatic conversion of pyruvate to oxaloacetate. These results are especially significant when one considers that 3-hydroxybutyrate, pyruvate, and oxaloacetate were not detected in stool fluid and would have likely been missed by a more targeted analytical approach focused on the transport of identified stool fluid metabolites. The analytical approach employed herein provides promise that future studies will expand our knowledge of colonic epithelial tissue metabolism and provide a better understanding of how the composition of the luminal environment affects transport rates and vice versa. The use of higher field magnets and more sensitive NMR probes in future experiments will likely expand the number of serosal metabolites identified and quantified with this approach. In addition, the ability to use stable isotope labeled precursors could clarify the extent to which critical metabolites such as butyrate are transformed by the epithelial tissue during absorptive transport.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b00393.

Method of metabolite quantification, T_1 calculations, table of T_1 correction values, and ^{1}H NMR spectra of antibiotic treatment (PDF)

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

^{1}H NMR based identification of intestinally-absorbed metabolites by Ussing chamber analysis of the rat cecum

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S-1. T_1 determinations and concentration calculations for Ussing chamber analyses

Metabolite concentrations were measured relative to the integrated intensity of the resonance of the internal standard 3-(trimethylsilyl)propane-1-sulfonic acid (DSS-d_6, Cambridge Isotope Laboratories, Tewksbury, MA). To determine the DSS concentration in the deuterated NMR buffer added to each sample, the primary standard KHP (Sigma Aldrich, St. Louis, MO) was dried in an oven for 24 h at approximately 100°C. A known quantity of 513.01 mg KHP was weighed and diluted with H_2O to 25.00 mL in a volumetric flask yielding a stock solution with a concentration of 100.5 mM. A 15 µL aliquot of the KHP stock solution was pipetted into a 600 µL aliquot of deuterated NMR buffer containing DSS and the resultant solution mixed well.

A fully relaxed ^{1}H NMR spectrum was acquired with 32 transients coadded into 32768 points for the solution containing KHP and DSS using a relaxation delay of 90 s. Free induction decays (FIDs) were apodized by multiplication by an exponential decay equivalent to 1.0 Hz line broadening, zero-filled to 131072 points, manually phased and 2.31 Hz (Whittaker smoothing) baseline correction applied. Eq. S1 was used to calculate the DSS concentration relative to the known concentration of KHP from the integrals measured for resonances of KHP and DSS with their respective normalization factors (NF). The normalization factor corresponds to the reciprocal of the number of protons that give rise to the resonance.[1]

$$[DSS] = [KHP] \times \frac{(integral_{DSS} \times NF_{DSS})}{(integral_{KHP} \times NF_{KHP})} \quad \text{(S1)}$$

Having determined the concentration of the DSS in the deuterated buffer solution, it can be used in a similar equation (Eq. S2) to calculate the metabolite concentration [M] in a sample, being careful to consider dilution factors.

$$[M] = [DSS] \times \frac{(integral_{M} \times NF_{M})}{(integral_{DSS} \times NF_{DSS})} \quad \text{(S2)}$$

Many quantitative NMR measurements use repetition times that are at least 5 times the longest T_1 relaxation time to ensure complete relaxation.[2] For the dilute Ussing chamber samples analyzed in this work, it was necessary to coadd a large number of transients to achieve a signal-to-noise ratio sufficient for quantitative measurements (S/N ≥ 10:1), thus a short relaxation delay of 0.05 s was used to minimize the total experiment time. In these experiments, the short repetition time (2.38 s) required that a correction factor be applied to compensate for incomplete T_1 relaxation during the recovery period between pulses. To determine the correction factors necessary for quantitative analysis, T_1 values were measured for each compound of interest using the inversion-recovery experiment.[3]

Standard solutions of the identified metabolites were prepared to match the matrix of the Ussing chamber NMR samples with the same composition of Parson's solution and deuterated phosphate buffer. A total of 3 separate standard solutions included the following metabolites at concentrations of ~50 mm: sodium acetate (Sigma Aldrich, St. Louis, MO), sodium butyrate (Alfa Aesar, Ward Hill, MA), sodium propionate (Sigma Aldrich, St. Louis, MO), lithium acetoacetate (Sigma Aldrich, St. Louis, MO), sodium citrate (Fisher, Pittsburg, PA), choline chloride (Sigma Aldrich, St. Louis, MO), phenylalanine (Fisher, Pittsburg, PA), tyrosine (Fisher, Pittsburg, PA), DL-β-hydroxybutyric acid sodium salt (3-hydroxybutyric acid, Sigma Aldrich, St. Louis, MO), sodium formate (Fisher, Pittsburg, PA), succinic acid (Spectrum chemical, Gardena, CA), sodium pyruvate (Sigma Aldrich, St. Louis, MO), oxaloacetic acid (Alfa Aesar, Ward Hill, MA), lactic acid (Sigma Aldrich, St. Louis, MO), L-alanine (Sigma Aldrich, St. Louis, MO), valine (Sigma Aldrich, St. Louis, MO), lysine (Sigma Aldrich, St. Louis, MO), and isoleucine (Fisher, Pittsburg, PA). All standard solutions also included the internal standard DSS.

Inversion recovery NMR spectra of the standard metabolite solution were recorded by coaddition of 96 transients using recovery delays between 0.01 and 60.0 s, and repetition times equal to the largest recovery delay plus the acquisition time of 2.38 s. In individual experiments, the relaxation delay varied from 50 to 60 s, however, in all experiments the total relaxation time was more than 10 times the largest T_1 relaxation time for the resonances of interest.

The inversion recovery spectra were processed using Mnova 10.0 software (Mestrelab Research, Bajo, Spain) with zero filling to 131072 points, 1.0 Hz apodization, manual phasing, and 2.31 Hz Whittaker smoothing baseline correction applied. One resonance was selected for each compound for purposes of quantitation based on the spectral resolution (avoiding nearby peaks as possible) and the signal-to-noise ratio of the resonance. The same metabolite resonances were used for the T_1 calculations as well as for quantification relative to DSS in the individual Ussing chamber samples. The selected resonances were deconvoluted and peak-fit in Mnova using a generalized Lorentzian model to obtain peak areas.

To extract the T_1 relaxation times from the inversion recovery spectra, peak areas were plotted versus recovery delay time in Mathematica (Wolfram Research, Champaign, IL) as illustrated in Figure S-1. T_1 relaxation times were calculated for each resonance used for quantification by fitting Eq. S3 to the inversion recovery data.

$$M(t) = M\left(1 - 2e^{\frac{-t}{T_1}}\right) \qquad \text{(S3)}$$

In Eq. S3, M(t) is the observed peak area for a particular resonance in a spectrum, and M is the peak area of the fully relaxed spectrum. The recovery time is denoted by t and the relaxation time constant is T_1. The T_1 relaxation times summarized in Table S-1 were used to correct the integrals measured for each compound as illustrated by Eq. S4, where t is the recovery time used in the acquisition of the one dimensional ^{1}H NMR spectrum.

$$M = \left[\frac{M(t)}{\left(1-2e^{\frac{-t}{T_1}}\right)}\right] \times \frac{1}{P} \quad \text{(S4)}$$

$$[MET] = \left(\frac{[DSS]\times M_{MET}}{M_{DSS}}\right) \times \mathrm{D} \quad \text{(S5)}$$

Eq. S4 uses the T_1 relaxation times determined with Eq. S3, as well as the number of protons (P) for the corresponding resonance to solve for M which can be converted to concentration using Eq S5. Here we use the ratio of the predetermined DSS concentration and its corresponding T_1-corrected and normalized peak area (M_{DSS}) to determine the concentration of the metabolite of interest ([MET]), taking into consideration the dilution factor (D) applied when deuterated buffer is added to the sample prior to NMR analysis.

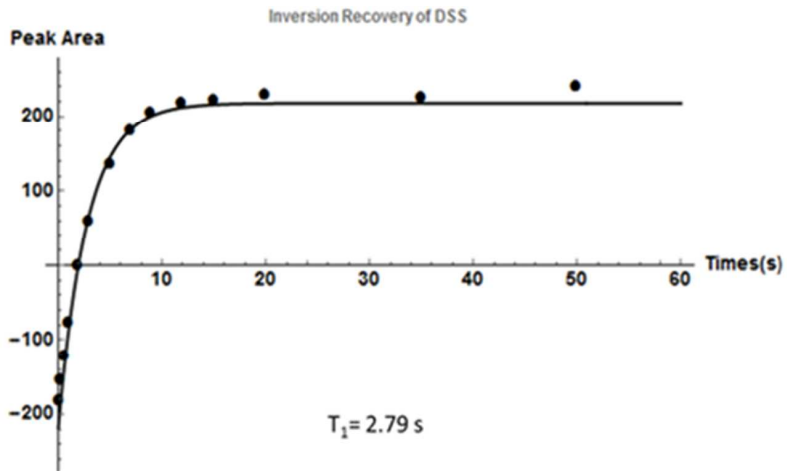

Figure S-1. Integrated peak areas (Y-axis) obtained from the inversion recovery spectra plotted against the recovery times (X-axis) for the representative ^{1}H-NMR resonance of DSS. The T_1 relaxation time is determined from the fit of the line in Mathematica.

Table S-1. Values for ^{1}H NMR T_1 relaxation times measured for metabolite standards using the inversion recovery experiment. The relaxation times were used to calculate correction factors to compensate for incomplete T_1 relaxation in the Ussing chamber NMR experiments.

	Metabolite	**T_1 Value (s)**
1	3-hydroxybutyric acid	1.51
2	Acetate	5.30
3	Acetoacetate	5.85
4	Alanine	1.93
5	Butyrate	2.84
6	Choline	2.42
7	Citrate	0.84
8	DSS	3.06
9	Formate	8.17
10	Isoleucine	1.47
11	Lactic acid	1.78
12	Lysine	1.31
13	Oxaloacetic acid	5.43
14	Phenylalanine	2.55
15	Propionate	4.89
16	Pyruvate	4.76
17	Succinate	2.47
18	Tyrosine	2.12
19	Valine	2.48

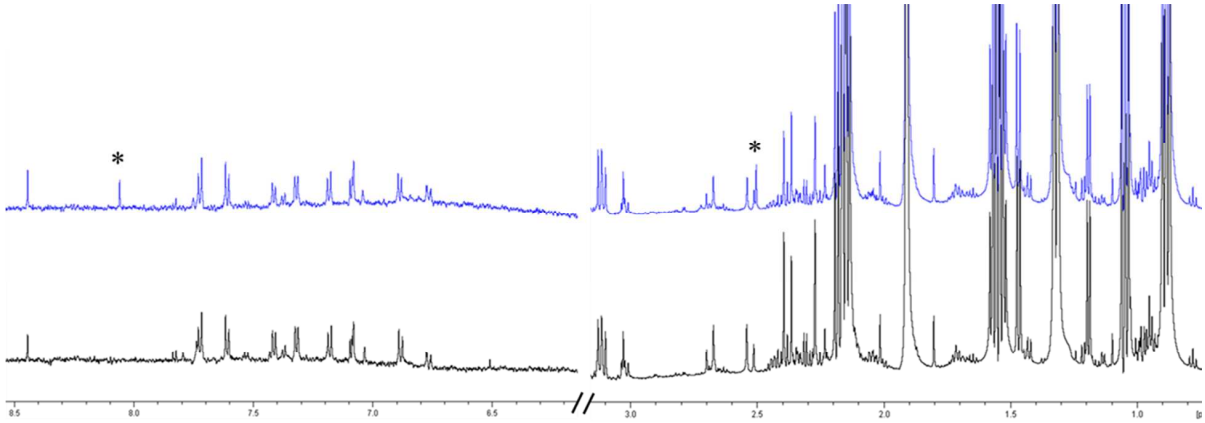

Figure S-2: ^{1}H-NMR spectra of samples removed from the serosal chamber with stool fluid in the luminal chamber. The top (blue) spectrum represents experiments in which the epithelial tissue was pretreated with the antibiotics metronidazole (50 µg/mL) and streptomycin (100 µg/mL) for 45 min prior to loading bacteria-free stool fluid into the luminal chamber. For the bottom (black) spectrum, the epithelial tissue was not pretreated with antibacterial compounds prior to the Ussing chamber experiments.

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