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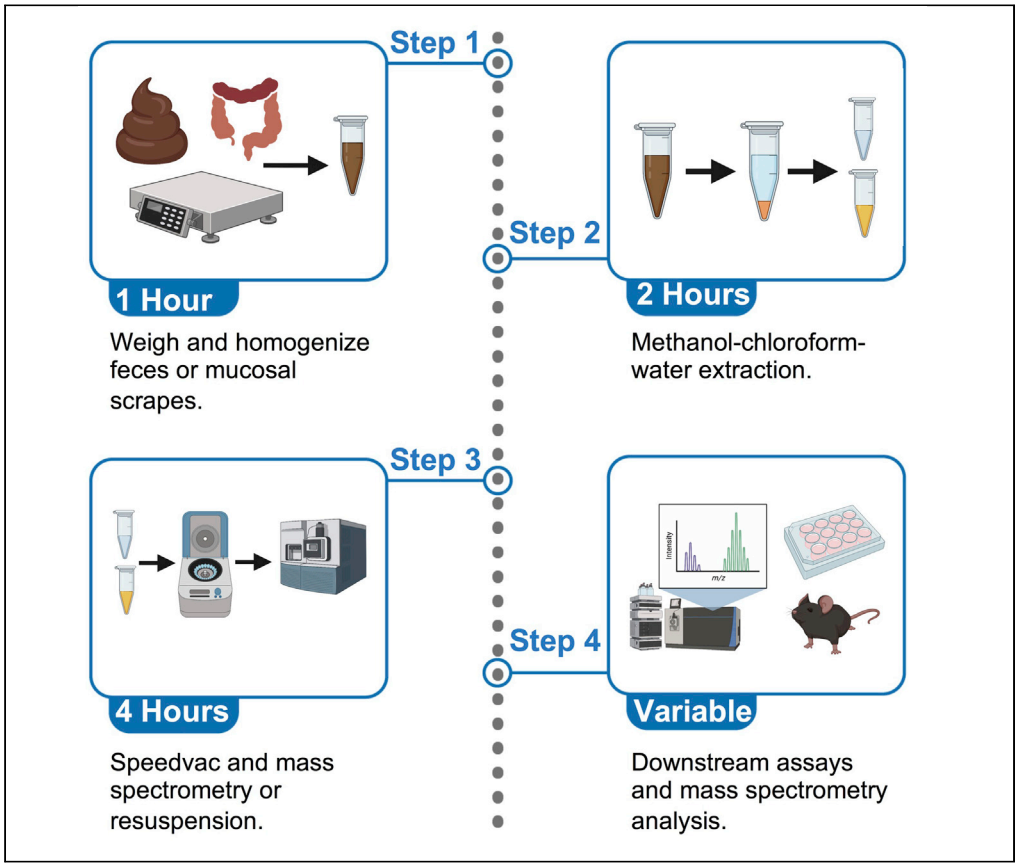
Protocol for isolation and analysis of small volatile microbiome metabolites from human or mouse samples



Metabolites are crucial for bidirectional communication between host and microbiome. We describe a protocol for the isolation of organic and aqueous metabolites from mucosal scrapes and feces from mouse and human samples. Although some of the most reactive organic compounds may be lost, this approach generates a functionally reproducible metabolic extract containing both host and microbial compounds appropriate for quantitative mass spectrometry and functional characterization. Our mass spectrometry approach identifies low-abundant and difficult to identify microbially derived metabolites.

Hannah N. Bell, Nupur K. Das, Yatrik M. Shah

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Highlights

Quickly isolates microbial metabolites from human or mouse feces or tissue

Metabolites can be quantified and used for *in vitro* or *in vivo* applications

Organic or aqueous metabolites can be isolated separately

Identifies low-abundant and difficult to identify microbially derived metabolites

Bell et al., STAR Protocols 3, 101311
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101311

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Protocol for isolation and analysis of small volatile microbiome metabolites from human or mouse samples

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https://doi.org/10.1016/j.xpro.2022.101311

SUMMARY

Metabolites are crucial for bidirectional communication between host and microbiome. We describe a protocol for the isolation of organic and aqueous metabolites from mucosal scrapes and feces from mouse and human samples. Although some of the most reactive organic compounds may be lost, this approach generates a functionally reproducible metabolic extract containing both host and microbial compounds appropriate for quantitative mass spectrometry and functional characterization. Our mass spectrometry approach identifies low-abundant and difficult to identify microbially derived metabolites.

For complete details on the use and execution of this protocol, please refer to Bell et al. (2021) and Das et al. (2020).

BEFORE YOU BEGIN

Obtain institutional permission for animal or human study

Timing: Variable

1. Obtain proper institutional permission to perform animal studies, and collect human tissues under an approved IRB protocol. Our protocol was approved by Michigan's Institutional Animal Care and Use Committee and human subject use was approved by an Institutional Review Board.

Pre-weigh microcentrifuge tubes

Timing: 20 min

2. Pre-weigh three micro-centrifuge tubes for each sample.

Prepare working reagents

Timing: 20 min

3. Prepare 3 mL of methanol-chloroform mixture per sample. Methanol to chloroform ratio should be 2:1 by volume. Chill on dry ice and handle all samples on dry ice whenever possible.
4. Chill 1.2 mL of deionized distilled water per sample on ice.

⚠ CRITICAL: Maintain samples and reagents on dry ice at all times except when cold centrifuging and homogenizing.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Methanol	Fisher Scientific	CAT# 67-56-1
Chloroform	Fisher Scientific	CAT# 67-66-3
Corn Oil	MilliporeSigma	CAT# 8001-30-7
Dimethyl Sulfoxide (DMSO)	MilliporeSigma	CAT# 67-68-5
Trichloroacetic Acid	MilliporeSigma	CAT# T6399
Sodium Hydroxide	MilliporeSigma	CAT# 06203
1-Propanol	MilliporeSigma	CAT# 34871
Pyridine	MilliporeSigma	CAT# 270407
Propylchloroformate	MilliporeSigma	CAT# 249467
Hexane, suitable for HPLC	MilliporeSigma	CAT# 34859
Glass Beads, 1.0 mm	MilliporeSigma	CAT# Z250473
DMEM	Thermo Fisher Scientific	CAT# 11995065
1× PBS, pH 7.4	Gibco	CAT# 10010023
[2,3-$^{13}C_2$]-Alanine	Sigma-Aldrich	CAT# 604682
[2,2,4,4-D_4]-Citric Acid	Sigma-Aldrich	CAT# 485438
N-Methyl-N-(Trimethylsilyl)Trifluoroacetamide (MSTFA)	MilliporeSigma	CAT# 69479
Biological samples		
Mouse Fecal Sample, 1–2 Pellets	N/A	N/A
Human Fecal Sample	N/A	N/A
Experimental models: Cell lines		
HCT116: Human, colorectal cancer, male	ATCC	N/A
Experimental models: Organisms/strains		
Mouse: C57Bl/6J age 6–12 Weeks, Male or Female	The Jackson Laboratory	N/A
Other		
Eppendorf Centrifuge	Thermo Scientific	N/A
Eppendorf Vacufuge SpeedVac	Thermo Scientific	N/A
70 Micron Strainer	Fisher Scientific	CAT# 07-201-432
Cytation 5 Cell Imaging Multi-Mode Reader	BioTek	N/A
HP5 ms (5% phenylmethylpolysiloxane) Capillary Column	Agilent	CAT# DB-624
7890A-5975C GC-MS System	Agilent	N/A
7697A Headspace Sampler	Agilent	N/A
Tissue Culture Incubator	Thermo Scientific	N/A
TissueLyser	QIAGEN	N/A
Q55 Sonicator	Thomas Scientific	CAT# 1204P05

MATERIALS AND EQUIPMENT

Methanol/Chloroform (2:1, v:v) Solution	
Reagent	Amount
Methanol	2 mL
Chloroform	1 mL
Total	**To 3 mL per sample**

Note: Prepare solution immediately before use and do not store between experiments.

Trichloroacetic Acid Solution (6% by volume)		
Reagent	Final concentration	Amount
Trichloroacetic Acid	6%	60 μL
Water		940 μL
Total	**n/a**	**1 mL**

Note: Prepare solution immediately before use and do not store between experiments.

[2,3-$^{13}C_2$]-Alanine Internal Standard (10 μg/mL) Spiking Solution		
Reagent	Final concentration	Amount
[2,3-$^{13}C_2$]-Alanine IS stock solution	10 μg/mL	1 mg
Trichloroacetic Acid Solution (6% by volume)		To 100 mL
Total	**n/a**	**100 mL**

Note: Can be aliquoted and stored at −20 degrees Celsius for 7 days.

Sodium Hydroxide Solution (50 mM)		
Reagent	Final concentration	Amount
Sodium Hydroxide	50 mM	2 mg
Water	Solvent	Bring to 1 mL
Total	**n/a**	**1 mL**

Note: Can store indefinitely at 4 degrees Celsius.

Propanol/Pyridine (3:2, v:v) Solution	
Reagent	Amount
Propanol	600 μL
Pyridine	400 μL
Total	**1 mL**

Note: Can store indefinitely at 4 degrees Celsius.

[2,2,4,4-D4]-Citric Acid IS Solution (1 mM)		
Reagent	Final concentration	Amount
[2,2,4,4-D_4]-Citric Acid	1 mM	196.15 mg
Deionized distilled water	Solvent	
Total	**n/a**	**To 10 mL**

Note: Make using deionized, distilled water and pure compound. Aliquot and store at −20 degrees Celsius indefinitely.

Methanol:Water (80:20, v:v) Solution	
Reagent	Amount
Methanol	800 μL
Deionized distilled water	200 μL
Total	1 mL

Note: Prepare solution immediately before use and do not store between experiments.

Pyridine:N-Methyl-N-(Trimethylsilyl)Trifluoroacetamide (MSTFA) Derivatization Solution		
Reagent	Final concentration	Amount
N-Methyl-N-(Trimethylsilyl) Trifluoroacetamide	10 mg/mL	10 mg
Pyridine	Solvent	To 10 mL
Total	n/a	10 mL

Note: Prepare this reagent immediately before use, store at 4 degrees Celsius and do not reuse.

Reagent safety statements

⚠ CRITICAL: Methanol and hexane solvents are flammable and toxic if inhaled, ingested, or applied to skin. Proper PPE (i.e., gloves, googles) and engineering controls (i.e., chemical fume hood) should be used when handling these solvents – consult the SDS.

⚠ CRITICAL: Propanol and pyridine solvents are flammable, corrosive, irritants, and are harmful upon all exposure routes. Proper PPE (i.e., gloves, googles) and engineering controls (i.e., chemical fume hood) should be used when handling these solvents – consult the SDS.

⚠ CRITICAL: Chloroform is harmful if ingested and is acutely toxic if inhaled, is a suspected carcinogen, and may be a fetal toxin. Proper PPE (i.e., gloves, googles) and engineering controls (i.e., chemical fume hood) should be used when handling this solvent – consult the SDS.

⚠ CRITICAL: Dimethyl sulfoxide (DMSO) is a carrier solvent, and substances dissolved in DMSO can immediately breakthrough incompatible gloves and be absorbed through the skin upon skin exposure. Proper PPE (i.e., gloves, googles) should be used when handling DMSO-based solutions – consult the SDS and the institutional Environmental Health and Safety Officer for advice.

⚠ CRITICAL: Trichloroacetic acid (TCA) is a volatile and extremely corrosive substance that can cause severe burns by all exposure routes, and is a suspected carcinogen. Proper PPE (i.e., gloves, googles) and engineering controls (i.e., chemical fume hood) should be used when handling crystalline TCA solids and dilute aqueous solutions – consult the SDS.

⚠ CRITICAL: Propyl chloroformate is a flammable, corrosive, and acutely toxic substance upon all routes of entry. Proper PPE (i.e., gloves, googles) and engineering controls (i.e., chemical fume hood) should be used when handling this material – consult the SDS.

⚠ CRITICAL: Sodium hydroxide (NaOH) is an extremely caustic substance that can cause severe burns by all exposure routes. Proper PPE (i.e., gloves, googles) should be used when handling NaOH pellets and solutions – consult the SDS.

⚠ CRITICAL: N-Methyl-N-(Trimethylsilyl)Trifluoroacetamide (MSTFA) is a flammable liquid that can cause skin, eye, and respiratory tract irritation. Proper PPE (i.e., gloves, googles) and engineering controls (i.e., chemical fume hood) should be used when handling this material – consult the SDS.

STEP-BY-STEP METHOD DETAILS

Metabolite extraction

Timing: 2 h

This step involves using a methanol-chloroform-water extraction to isolate the organic and aqueous metabolites from mucosal scrapes and feces (Figure 1).

1. Sample preparation for metabolite analysis.
   a. Obtain mouse fecal sample.
      i. Restrain the mouse by holding the skin on the dorsal side of the mouse between the shoulders.
      ii. Rotate your wrist so the mouse is belly up. Gently stroke the mouse's belly until it defecates then collect feces using sterile forceps.
   b. Alternatively, a 100 milligram of flash frozen human feces can be used. We used human feces from male and female participants between the ages of 18 and 80.
      i. Place frozen feces on dry ice and use a sterile fresh razor blade to cut off an approximately 100 milligram portion.
      ii. Use a sterile fresh razor blade to remove this section on dry ice. Flash freezing throughout the paper indicates rapid freezing in liquid nitrogen.
   c. Obtain mouse mucosal colon scrapes:
      i. Mouse mucosal scrapes from the colon can also be used at this step. Euthanize a mouse by carbon dioxide asphyxiation or other institutionally approved method of euthanasia.
      ii. Use scissors to cut through the skin and the peritoneum of the mouse. Using forceps, isolate the colon and lift out of the abdominal cavity. Identify the large pouch of the cecum at the proximal end of the colon, and the connection of the colon to the rectum.
      iii. Transect the colon at the anus and the cecum and remove from the body cavity. Using sharp surgical scissors, slit the colon horizontally to form a long tube.
      iv. Wash thoroughly in 1× PBS and lay flat on a glass plate. Using a sterile microscope slide, gently but firmly scrape the luminal portion of the colon. Mucus and epithelial layer should be easily removed and deposited into a pre-weighed Eppendorf tube.
   d. Alternatively, metabolites can be isolated from the cecum of a mouse.
      i. The benefits of cecal metabolites are the large abundance of material, the lack of contamination upon collection, and a snapshot of the metabolites in the more proximal colon.
      ii. Disadvantages are the necessity of sacrificing the mouse to obtain fecal contents.
      iii. The cecum can be identified by the large pouch at the proximal end of the colon, which can be slit open using surgical scissors, and the contents can be directly taken and weighed.

Pause point: Samples can be stored at minus 80 degrees Celsius for up to one month.

2. Preparation of organic-aqueous extraction.
   a. Deposit two fecal pellets, or approximately 100 milligram of tissue or feces into pre-weighed Eppendorf tube. As most tissue is frozen at this point, dry weight is used. A fecal pellet is one individual mouse poop. Reweigh tube and record weight of the sample.
   b. Add 3 mL of dry ice-chilled methanol/chloroform (2:1, v:v) solution to each sample tube. Add one 1.0 mm glass homogenization bead to tube. Homogenize for 2 min using a Tissue Lyser at a rate of 20–30 Hertz.

c. Incubate sample on dry ice for 10 min.

Pause point: Samples can be safely stored at minus 80 degrees Celsius for up to one month.

d. Centrifuge at 16,000 g for 10 min at 4°C.
e. Pass the supernatant through a 70-μm cell strainer and centrifuge at 16,000 g for 10 min.
f. Add 0.6 mL of ice-cold water per 1.5 mL of supernatant and then vortex thoroughly for at least 15 s. Centrifuge the sample at 16,000 g for 10 min to obtain phase separation in a microcentrifuge tube.
g. Separately collect the upper and lower phases into pre-weighed Eppendorf sample tubes without disturbing the interface.

Note: Amounts of upper and lower phases are variable depending on fecal pellet quantity and extraction, but anticipate approximately 0.6 mL of upper aqueous layer and 1.4 mL of bottom organic layer.

h. Place the samples into a vacuum centrifuge and evaporate the solvent over the course of 8 h cooled to 4°C.
   i. Confirm that all liquid is removed.
   ii. Ensure that there is a pellet.

Note: Organic pellet will be a yellowish-brown color, and the aqueous pellet will be clear.

i. Resuspend samples in dimethyl sulfoxide (DMSO) for *in vitro* applications and corn oil *in vivo* at a concentration of 5 mg per 100 microliters as described below. Keep pellet lyophilized and store at −80°C until mass spectrometry analysis.

⚠ CRITICAL: Try to keep all samples on dry ice throughout the procedure. Many metabolites are volatile and are prone to evaporation if sample is warmed. Keeping reagents and samples chilled helps to prevent degradation of unstable compounds as well.

Extraction and derivatization

Timing: 4 and 16 h incubation

This step describes the extraction and derivatization methods used for volatile metabolites such as diaminopropane and reuterin.

3. An example of extraction and derivatization method for Diaminopropane (DAP).
   a. Mix 10 mg of lyophilized sample pellet with a 150 μL volume of [2,3-$^{13}C_2$]-Alanine Internal Standard Solution. Homogenize at 30,000 RCF for 25 s with one 1.0 mm glass homogenization bead per sample.
   b. Centrifuge at 20,000 g for 10 min at 4°C.
   c. Collect the supernatant in a glass vial and mix with 300 μL volume of a 50 mM sodium hydroxide solution and a 300 μL volume of a 1-propanol/pyridine (3:2, v:v) solution.
   d. Add a 100 μL volume of propylchloroformate on ice and vortex for 1 min.
   e. The samples were derivatized in an incubator at 35°C for 30 min, then extracted with a 75 μL volume of hexane twice.
   f. The 150 μL of extract volume were stored at 4°C for headspace analysis.
4. An example of extraction and derivatization method for reuterin.
   a. Mix 10 mg of sample with a 5 μL volume of 1 mM [2,2,4,4-D_4]-]citric acid and a 500 μL volume of ice-cold methanol:water (80:20, v:v). Homogenize at 20–30 Hertz for 25 s with one 1.0 mm glass homogenization bead.

b. Add a 200 μL volume of ice-cold water and a 200 μL volume of chloroform and homogenize again.
c. Centrifuge at 20,000 g for 10 min at 4°C.
d. Collect the supernatant liquor in a glass vial and dry with a speed vac.
e. Mix the dried sample with a 50 μL volume of a Pyridine:MSTFA (X:X, v:v) Derivatization Solution (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA)), and vortex-mix for 5 min.
f. Derivatize the samples in a minus 20°C freezer for at least 16 h.
g. Sonicate the glass vial containing samples for 20 min using a probe sonicator at 20% power, then centrifuge at 20,000 g for 10 min at 4°C.
h. Store the extracts at 4°C for gas chromatography mass spectrometry (GC-MS) analysis. Samples should be used within 24 h of extraction.

Mass spectrometry analysis

Timing: 2 h

This step describes the mass spectrometry protocol that we used to analyze bacterial metabolites from extracted fecal pellets.

5. GC-MS Method.
   a. Use a headspace sampler (details of the one used in our protocol are in key resources table). Employ a HP5 ms (5% phenylmethylpolysiloxane) capillary GC column (30 m by 250 μm, with 0.25 μm film thickness, Agilent Technologies) with helium as the carrier gas at a constant flow rate of 1 mL/min.
   b. Use an initial column oven temperature of 70°C for 2 min, then increase to 230°C at a rate of 20°C/min.
   c. Set the temperature of the front inlet to 250°C, the transfer line to 280°C and the mass spec ionization source to 230°C.
   d. Use a total run time of 20 min and collect mass spectral data in full scan mode with a mass range *m/z* 30–400. Use an injection volume of 1 μL.
   e. Run calibration standards of diaminopropane and reuterin first.

   Note: Appropriate concentrations will vary depending on experiment, and will need to be individually determined. We used 0.1, 1, and 10 micromolar.

   f. Quantify diaminopropane and reuterin by peak areas of extracted *m/z* at 276 and 219 (Ghassempour et al., 2004; Talarico and Dobrogosz, 1989).

In vitro and *in vivo* applications of metabolic extract

Timing: Variable

This step describes some potential applications of the metabolic extract in cell culture and *in vivo* mouse applications.

6. In vitro cell culture treatment:
   a. Resuspend samples in DMSO for *in vitro* applications. Recommended DMSO concentration is 50 μL per 5 mg of initial dry tissue weight.

   Note: Resuspension will require significant vortex-mixing and pipetting. At this point, subaliquot to smaller volumes of metabolic extract in order to reduce freeze-thaw cycles.

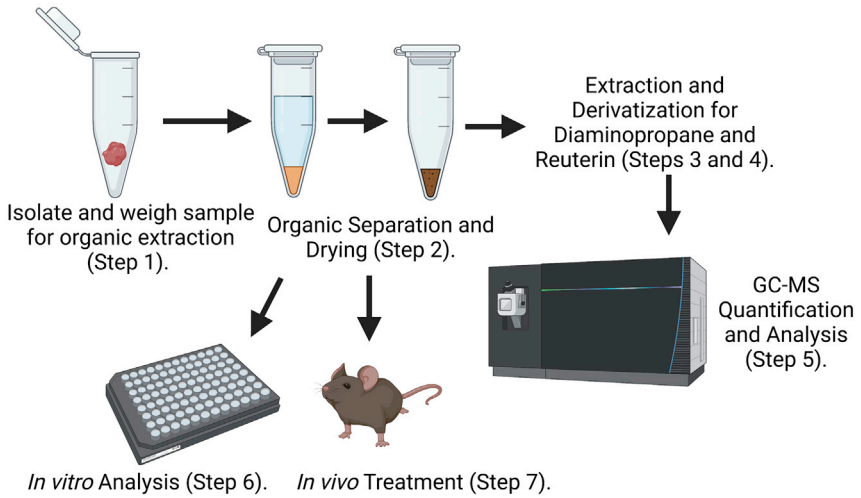

Figure 1. A step by step schematic of the preparation, extraction, and cell culture use of fecal metabolic extracts

b. Concentration needed for functional cell assays must be titrated upon each extraction due to variable conditions and initial starting concentrations.

Note: We found that 1 μL volumes of the suggested resuspension quantity of above extracts from wildtype mouse feces was sufficient to reduce growth of HCT116 cells by 80% on Day 3.

Note: Use low passage HCT116, ideally under 15 passages.

Note: We recommend performing a dose curve. We used Cytation 5 to quantify cell growth after treatment with the extract.

7. *In vivo* Applications:
   a. Resuspend samples in corn oil for *in vivo* applications.

Note: Recommended corn oil concentration is 100 μL of corn oil per 5 mg of initial dry tissue weight.

   b. Inject a 200 μL volume of previously resuspended metabolites intraperitoneally into subject mice daily.

Note: We have used the concentration of 5 mg per 100 μL for our *in vivo* applications, but titration may be necessary for any other application.

EXPECTED OUTCOMES

Step 1 of this protocol should result in two separate pellets, which can be resuspended to obtain the aqueous and organic metabolites. The organic pellet should be yellow-brown, and larger than the aqueous pellet. For example, we have quantified diaminopropane and reuterin in human normal and colorectal cancer tissue as described above (Figure 2). We have resuspended this pellet for *in vitro* applications, and have seen consistent trends, although the necessary concentration varies based on the starting sample. An excellent positive control is extracting wild type mouse feces and assessing growth effects in the human colorectal cancer cell line HCT116. We have reliably seen that wild type fecal extracts repress growth in a dose dependent manner (Figure 3). We have shown that different mouse model fecal metabolite extracts have reproducibly different metabolite profiles and that wild type mouse fecal metabolites

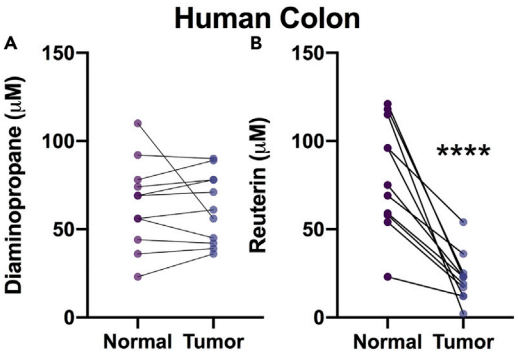

Figure 2. Quantification of reuterin and diaminopropane from human colorectal cancer tumors.

(A) Mass spectrometry quantification of diaminopropane in human colon tumors.

(B) Mass spectrometry quantification of reuterin in human colon tumors.

reliably repress the growth of a variety of colorectal cancer cell lines in a dose dependent manner. We have treated mice with cecal extracts resuspended in corn oil from wild type mice and seen reproducible effects on colon ferritin levels (Figure 4). Using mass spectrometry, this protocol will allow the quantification of metabolites in extracts prepared from mouse or human feces, mucosal scrapes, or tumor tissues.

QUANTIFICATION AND STATISTICAL ANALYSIS

Due to inherent variability between mouse and human metabolite profiles, we recommend using a separate sample for each biological replicate when comparing metabolites between conditions or genotypes. We also recommend performing technical triplicates for end-point assays such as growth, for each individual sample. For large-scale treatment with metabolites from one group, samples from multiple mice can be pooled after extraction in order to obtain a homogenous, higher volume sample.

LIMITATIONS

This method does not separate host and microbial linked metabolites. Further analysis using germ-free mice or metabolic tracing are necessary. In addition, extremely volatile or gaseous metabolites may be lost during speed vacuuming for drying.

TROUBLESHOOTING

Problem 1

No pellet after evaporating the aqueous and organic samples (step 2h).

Potential solution

Begin with a larger starting quantity. Feces tend to provide much larger metabolite pellets than tissue samples. Ensure the use of a sample at least 100 mg if pellet is not seen with smaller sample.

Problem 2

Difficulty re-dissolving the pellet in DMSO or corn oil (step 2i).

Potential solution

The pellet is very sticky and difficult to fully dissolve, or to combine pellets. We recommend adding the DMSO, letting the pellet incubate in the liquid for 15 min, vortex-mixing vigorously, then pipetting up and down several times to remove the pellet from the bottom of the tube. If the pellet still remains viscous or crystallized, repeat. It is necessary for the pellet to be fully dissolved with no crystals for reproducible activity.

Problem 3

Variability between samples extracted on different days for *in vitro* or *in vivo* treatment (step 6).

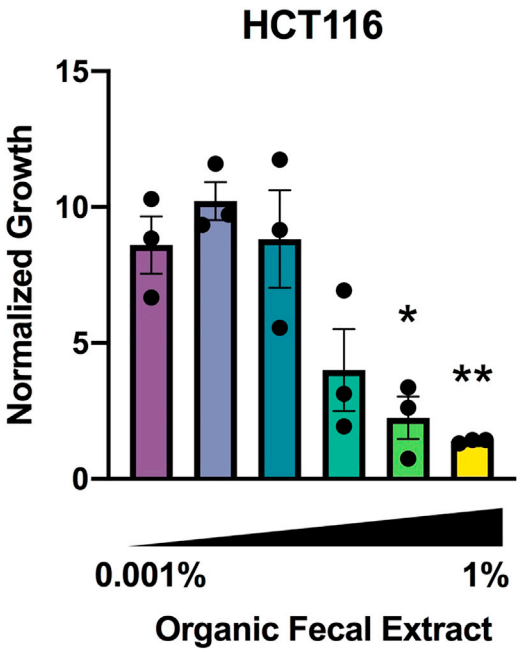

Figure 3. Quantification of growth suppression of HCT116 colorectal cancer cells in a dose-dependent manner by the organic fecal extracts of wildtype mice.

Potential solution

Variability in active concentration between samples has been seen in our hands. To counteract this, we performed a dose curve with a known readout (wild type mouse fecal metabolites on HCT116 colorectal cancer cells), with each extraction, and standardized downstream working concentrations to the same growth inhibition.

Problem 4

No separation between aqueous and organic layers (step 2g).

Potential solution

We have seen cases where there is no defined phase separation. In this case, it is typically a problem of either too much initial starting sample or an incomplete vortexing. It is important to keep the starting sample between 10 and 100 milligrams for ease of use and complete separation and to vortex

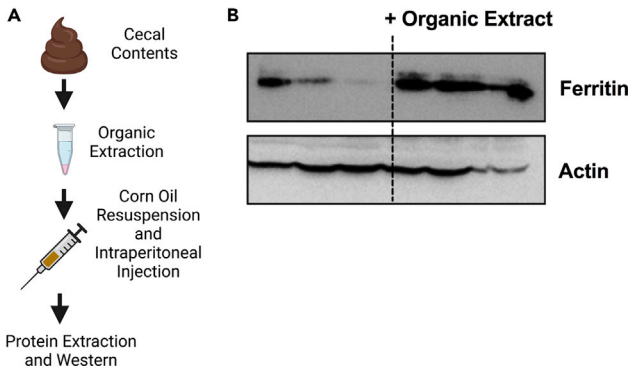

Figure 4. Schematic of animal treatment scheme with metabolic extracts and example of extract effect on Ferritin expression in the duodenum.

(A) Treatment schematic.

(B) Mice on 350 ppm iron diet, treated with antibiotics, and either treated or not with organic metabolic extracts three days in a row before sacrifice. Lysates are prepared from the duodenum.

the sample after adding the water for 15–45 s. If phase separation is a problem, we would recommend reducing the starting sample size or increasing the vortex time. If phase separation cannot be achieved after this troubleshooting tip, the sample should be discarded.

Problem 5

No activity of metabolites *in vivo* or *in vitro* (step 6).

Potential solution

Metabolites have differential effects on both *in vivo* and *in vitro* applications depending on many factors such as the mouse line, if the metabolite preparation is warmed, a frequent freeze thaw cycles. If the metabolites are not effective, we recommend confirming repressiveness in colon cancer cell line HCT116 of wildtype mouse fecal metabolites as a positive control to determine proper metabolite preparation as described in Bell et al.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be responded to by Yatrik Shah, shahy@umich.edu.

Materials availability

This study did not generate any new unique reagents.

Data and code availability

This study did not generate or analyze any new datasets or codes.

ACKNOWLEDGMENTS

This work was supported by NIH grants; R01CA148828, R01CA245546, and R01DK095201 to Y.M.S. The work was also supported by the University of Michigan Comprehensive Cancer Center Core Grant P30CA046592; the GI SPORE Molecular Pathology and Biosample Core (P50CA130810); the Center for Gastrointestinal Research (DK034933); and the Department of Defense (CA171086 to Y.M.S.). H.N.B. was supported by a T32 training grant (T32- of Michigan Systems and Integrative Biology grant).

AUTHOR CONTRIBUTIONS

H.N.B., N.K.D., and Y.M.S. designed the method. H.N.B. and N.K.D. acquired the data and analyzed the data. H.N.B. and Y.M.S wrote the manuscript. Y.M.S. supervised the study.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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