Acyloxyacyl Hydrolase is a Host Determinant of Gut Microbiome-Mediated Pelvic Pain

Afrida Rahman-Enyart^{1*}, Wenbin Yang^{2*}, Ryan E. Yaggie¹,
Bryan White^{4,5}, Michael Welge⁵, Loretta Auvil⁵, Matthew Berry⁵, Colleen Bushell⁵, John M.
Rosen^{6,7}, Charles N. Rudick⁸, Anthony J. Schaeffer¹, and David J. Klumpp^{1,3†}

Department of Urology¹

Division of Thoracic Surgery²

Department of Microbiology-Immunology³

Feinberg School of Medicine

Northwestern University, Chicago, IL

Department of Animal Sciences⁴

National Center for Supercomputing Applications⁵

University of Illinois at Urbana-Champaign, Urbana, IL

Department of Gastroenterology⁶

Children's Mercy, Kansas City, MO

Department of Pediatrics⁷

University of Missouri, Kansas City, MO

Clinical Pharmacology and Toxicology⁸

Indiana University School of Medicine

Bloomington, IN

28
29
30
31
32
33
34
35
36
37
38
39

[†]Address all correspondence to
d-klumpp@northwestern.edu
16-719 Tarry Building
303 East Chicago Avenue
Chicago, IL
312.908.1996 P
312.908.7275 F

The authors have declared that no conflicts of interest exist

^{*}These authors contributed equally

ABSTRACT

Dysbiosis of gut microbiota is associated with many pathologies, yet host factors modulating microbiota remain unclear. Interstitial cystitis/bladder pain syndrome (IC/BPS or “IC”) is a debilitating condition of chronic pelvic pain often with co-morbid urinary dysfunction and anxiety/depression, and recent studies find fecal dysbiosis in IC/BPS patients. We previously identified the locus encoding acyloxyacyl hydrolase, *Aoah*, as a modulator of pelvic pain severity in a murine IC/BPS model. AOAH-deficient mice spontaneously develop rodent correlates of pelvic pain, increased responses to induced pelvic pain models, voiding dysfunction, and anxious/depressive behaviors. Here, we report that AOAH-deficient mice exhibit dysbiosis of GI microbiota. AOAH-deficient mice exhibit an enlarged cecum, a phenotype long associated with germ-free rodents, and reduced trans-epithelial electrical resistance consistent with a “leaky gut” phenotype. AOAH-deficient ceca showed altered gene expression consistent with inflammation, Wnt signaling, and urologic disease. 16S rRNA sequencing of stool revealed altered microbiota in AOAH-deficient mice, and GC-MS identified altered metabolomes. Co-housing AOAH-deficient mice with wild type mice resulted in converged microbiota and altered predicted metagenomes. Co-housing also abrogated the pelvic pain phenotype of AOAH-deficient mice, which was corroborated by oral gavage of AOAH-deficient mice with stool slurry of wild type mice. Converged microbiota also alleviated comorbid anxiety-like behavior in AOAH-deficient mice. Oral gavage of AOAH-deficient mice with anaerobes cultured from IC/BPS stool resulted in exacerbation of pelvic allodynia. Together, these data indicate that AOAH is a host determinant of normal gut microbiota, and the dysbiosis associated with AOAH deficiency contributes to pelvic pain. These findings suggest that the gut microbiome is a potential therapeutic target for IC/BPS.

INTRODUCTION

Interstitial cystitis/bladder pain syndrome (IC/BPS or “IC”) is a chronic condition characterized by bladder discomfort and moderate to severe pelvic pain affecting as many as eight million patients in the United States [1–3]. Patients suffering from IC/BPS commonly exhibit co-morbid conditions including voiding dysfunction of increased urinary frequency and urgency and anxiety/depression [4–6]. The etiology of IC/BPS is unknown and pathophysiologic mechanisms remain unclear, so IC/BPS is a clinical challenge with no widely effective therapy. Because of the profound impact of chronic pelvic pain, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) established the Multi-Disciplinary Approaches to Chronic Pelvic Pain (MAPP) Research Network in an effort to study urologic chronic pelvic pain syndromes at the clinical, epidemiologic, and basic levels. In our MAPP studies, we previously conducted a genetic screen for loci modulating pelvic pain severity and identified *Aoah*, the locus encoding acyloxyacyl hydrolase, as a modulator of pelvic pain in a murine model of IC/BPS [7].

AOAH is a host enzyme best known for detoxifying bacterial lipopolysaccharides (LPS) by selectively removing secondary acyl chains from the lipid A moiety, mitigating the host response to bacterial infection and attenuating sustained inflammation [8–12]. Previous studies in our lab demonstrated that mice deficient for AOAH spontaneously develop pelvic allodynia consistent with pelvic pain and exhibit heightened response in induced pelvic pain models [7]. AOAH-deficient mice also have voiding dysfunction [13], and display rodent correlates of anxious/depressive behaviors [14]. Expressed along the bladder-brain axis, AOAH functions as an arachidonyl acyl transferase, and AOAH-deficient mice exhibit defects in central nervous system (CNS) arachidonic acid homeostasis and increased accumulation of PGE₂, contributing to pelvic pain [15]. Moreover, corticotropin-releasing factor (CRF) is the initiator of the hypothalamic-pituitary-adrenal (HPA) axis and a modulator of pain responses, and AOAH-deficient mice have increased *Crf* expression and increased serum corticosterone consistent with

HPA axis dysregulation [14]. IC/BPS patients also have aberrant diurnal cortisol fluctuations indicating HPA axis dysregulation [16], another similarity with AOA^H-deficient mice. Thus, AOA^H-deficient mice recapitulate several key aspects of IC/BPS.

Gut dysbiosis has been increasingly recognized as a key player in visceral pain, and recent studies have indicated altered fecal microbiota in women with IC/BPS as well as in male patients suffering an analogic condition, chronic prostatitis/chronic pelvic pain syndrome [17–20]. Visceral convergence of sensory inputs that drives pelvic organ crosstalk and the gut-brain axis provide mechanisms by which gut dysbiosis may influence IC/BPS urologic and cognitive symptoms [21–25]. Given the strong parallels between phenotypes of AOA^H-deficient mice and IC/BPS, we hypothesized that AOA^H-deficient mice would exhibit gut dysbiosis that mediates pelvic pain and other comorbidities. We observed multiple phenotypes supporting this hypothesis including altered cecal morphology and transcriptomes, fecal and cecal dysbiosis, and a role for gut microbiota in pelvic pain behaviors.

MATERIALS AND METHODS

Animals. Ten to twelve-week-old male and female wild type (WT) C57BL/6 mice were purchased from The Jackson Laboratory. *Aoah*^{-/-} mice (B6.129S6-Aoah^{tm1Rsm}/J) were obtained as a generous gift from Dr. Robert Munford of NIAID and maintained on a 12h:12h light:dark cycle as previously described [14]. All animals were maintained at the Center for Comparative Medicine under Northwestern IACUC approved protocols.

GI characterization. Animals were euthanized and placed on a pan balance to obtain body weight. The cecum and bladder were excised and weighed. The cecum was then dissected, and cecal stool was removed and weighed.

Transepithelial electrical resistance. Cecum and bladder permeability were measured as transepithelial electrical resistance (TEER) as previously described for bladders in Aguiniga et al., 2020 [13]. Excised organs were bisected and immediately placed in Ringer's solution at room temperature. After rinsing, ceca and bladders were mounted onto cassettes with apertures of 0.126cm² and 0.04cm² respectively. Cassettes were inserted between two-halves of an Ussing chamber (Physiologic Instruments EM-CSYS-2). KCl saturated salt-bridge electrodes were inserted. The chambers were filled with Ringer's solution and bubbled with carbogen (95% O₂/5% CO₂) and maintained at 37 °C until preliminary resistance became stabilized, typically at least 1 hr. Voltage was clamped and current passed every 3 s at 30 s intervals using a VCC MC2 multichannel voltage-current clamp amplifier controlled with Acquire and Analyze software v2.3.4 (Physiologic Instruments).

Hematoxylin and eosin staining. All hematoxylin and eosin (H&E) staining in wild type and AOAH-deficient ceca was performed by Northwestern University Mouse Histology & Phenotyping Laboratory (MHPL).

Cecum staining. Animals were euthanized and ceca were dissected. The cecum was flushed first with PBS and then 10% Neutral Buffered Formalin (NBF). Tissue was Swiss-rolled

and placed in 15 ml of 10% NBF prior to embedding in paraffin. Tissue was then sectioned at 4 μ m. Tissue was de-paraffinized with a series of washes as follows: 2 x 3 min with 100% xylene, 3 x 3 min with 100% ethanol, 1 x 3 min with 94% ethanol, 1 x 3 min with 70% ethanol, 1 x 3 min with 50% ethanol, and 1 x 3 min with H₂O. Antigen retrieval was performed in a pressure cooker using retrieval solution (DAKO, Santa Clara, CA) at 100 °C for 10 min, 90 °C for 10 min, and then washed in PBS for 3 min. Tissue was blocked in FC Receptor Block (Innovex Biosciences, Richmond, CA) for 10 min and Background Buster (Innovex) for 30 min. Tissue was incubated with AOA primary antibody (Abcam, Cambridge, United Kingdom) at 1:100 in Antibody Dilution Buffer (DAKO) overnight at 4 °C. Tissue was then washed in 1X PBS 3 x 5 min followed by incubation with Alexa Fluor 488 secondary antibody (Invitrogen, Carlsbad, CA) diluted to 1:500 in Antibody Dilution Buffer (DAKO) for 2 hrs at room temperature. Secondary was washed in 1X PBS 3 x 5 min and nuclei were stained using DAPI followed by imaging.

Gut microbiome analyses. Fecal and cecal gut microbiota were analyzed as previously reported in Braundmeier-Fleming et al., 2016 [19]. Briefly, DNA was extracted from fecal pellets or cecal stool by QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) by homogenizing with a Mini-Beadbeater and 0.1 mm zirconia/silica beads (Biospec, Bartlesville, OK) in 1.4 mL ASL buffer (QIAGEN). Phylotype profiles of microbiota were generated by deep amplicon sequencing of the 16S rRNA V3–V5 hypervariable region. Barcoding samples followed by MiSeq tag sequencing yielded approximately 50,000 reads/sample, detecting dominant and poorly represented taxa. The V3–V5 hypervariable region was selectively amplified through 30 cycles with primers 357F (CCTACGGGAGGCAGCAG) and 926R (CCGTCAATTCMTTTRAGT). Amplicon pools were quantified on a Qubit fluorimeter, and fragment sizes were determined by Agilent bioanalyzer High Sensitivity DNA LabChip (Agilent Technologies, Wilmington, DE) followed by dilution to 10 nM. To accurately calculate matrix, phasing, and prephasing, amplicons were spiked with a PhiX control library to 20%. Mixtures were sequenced on an Illumina MiSeq V2 (250nt from each end). To define OTU abundance profiles and phylogenetic relationships, sequence reads were binned at 97% identity using QIIME and Galaxy.

Predictive functional analysis. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was implemented using 16S rRNA sequence data from fecal samples to predict function as previously described [19, 26].

Fecal metabolomics. Analyses of fecal and cecal gut metabolomes were analyzed as previously reported in Braundmeier-Fleming et al., 2016 [19]. Briefly, duplicate samples were dried, and one was derivatized [27] with minor modifications: 90 minutes at 500 °C with 80 µl of methoxyamine hydrochloride in pyridine (20 mg/ml) followed by incubating 60 min at 500 °C with 80 µl MSTFA. A 5 µl aliquot of a C31 fatty acid internal standard was added to each; in derivatized samples this occurred prior to trimethylsilylation. 1 ml samples were injected with a split ratio of 7:1 into an Agilent GC-MS system (Agilent Inc, Palo Alto, CA) consisting of an 7890A gas chromatograph, a 5975C mass-selective detector, and a 7683B autosampler. GC was performed on 60 m HP-5MS columns (0.25 mm inner diameter, 0.25 mm film thickness; Agilent Inc) at 2500 °C injection and interface temperatures and 2300 °C ion source and helium carrier flow rate of 1.5 ml min⁻¹. A 5-min isothermal heating program at 700 °C was followed by a 50 °C min⁻¹ increase to 3100 °C and finally 20 min at 3100 °C. The mass spectrometer was operated in positive electron impact mode at 69.9 eV ionization energy in a scan range of m/z 30–800. All resulting chromatogram were compared with mass spectrum libraries NIST08 (NIST, Gaithersburg, MD), WILEY08 (Palisade Corporation, Ithaca, NY), and a University of Illinois Metabolomics Center custom library. All data were normalized to internal standards to facilitate direct comparisons, and chromatograms and mass spectra were evaluated using MSD ChemStation (Agilent) and AMDIS (NIST), where retention time and mass spectra were implemented within the AMDIS method formats.

Cecum RNA preparation and microarray. The cecum was dissected from mice immediately following euthanasia by cervical dislocation and stored at –80°C. Cecal epithelium was homogenized in ice-cold TRIzol with a homogenizer, and total RNA was purified according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Gene expression was quantified using Affymetrix Mouse Genome 430 2.0 array that contain 45,101 probes and measure the expression

level of 20,022 unique NCBI Entrez-identified genes. The data sets were preprocessed with normalization, variance stabilization, and $\log_2$ transformation. Student's *t*-tests were used to identify genes significantly differentially expressed ($P < 0.05$ and 2-fold) between WT and AOA-deficient mice. Average difference values were normalized to median over the arrays. The data were filtered so that only those genes that were adequately measured on 75% of the arrays were included. A class comparison protocol was used to identify genes whose degree of expression differed significantly by twofold or more among the three groups. To visualize whole genome expression level by function and pathway, the microarray data were analyzed with MetaCore Analysis (IPA; Ingenuity Systems, Redwood City, CA). IPA analysis identified canonical pathways differentially expressed ($P < 0.05$) between WT and AOA-deficient mice.

Co-housing and fecal microbiota transfer (FMT) experiments. For co-housing experiments, two WT and two AOA-deficient mice were housed in the same cage (4 mice/cage) for at least 1 week prior to experimentation. For FMT experiments, mice from both groups received oral FMT from either WT or AOA-deficient mice. Fecal suspensions were prepared from three FMT donor mice by suspending one fecal pellet in 1 mL sterile phosphate buffered saline using a syringe and 18-gauge needle (Becton Dickinson, Vernon Hills, IL) until the entire volume could pass through the needle. We allowed particulates to settle for 30 s before drawing supernatant into a syringe attached to a 22-gauge gavage needle (Fine Science Tools, Foster City, CA). We pooled together 500 μ L of the supernatants from each of the three 1 mL suspensions and deposited 200 μ L of this pooled supernatant into the stomach of each recipient mouse by gavage. Each FMT recipient was gavaged every other day for 7 days. We cleaned gavage needles with 70% ethanol between mice, using different gavage needles for each treatment group.

FMT of human samples. Human stool samples from healthy patients and patients with IC/BPS were placed in pre-reduced PBS with 0.1% cysteine (PBSc). Diluted (10^{-4}) samples were plated on 10cm plates and grown under anaerobic conditions at 37 °C. Once plates were dense they were scraped into PBSc. 100ul of sample was gavaged into male mice 3 x 3 days. After 7 days mice were tested for tactile allodynia to assess pelvic pain.

Pelvic allodynia. Mice were tested for pelvic allodynia using von Frey filament stimulation to the pelvic region, as previously described in Rudick et al., 2012 [28]. Briefly, prior to co-housing, baseline pelvic allodynia was measured for WT and AOAH-deficient mice. Mice were co-housed for 28 d followed by testing. After co-housing, mice were placed in a test chamber and allowed to acclimate for 5 min. Starting with the lowest force filament, five von Frey filaments were applied 10 times to the pelvic region. A response was considered to be painful if the animal jumped, lifted and shook the hind paws, or excessively licked the pelvic region.

Urinary bladder distention evoked visceromotor response (VMR). Mice were anesthetized with 2% isoflurane. Silver wire electrodes were placed on the superior oblique abdominal muscle and subcutaneously across the abdominal wall (as a ground) to allow differential amplification of the abdominal VMR signals. A lubricated angiocatheter was inserted into the bladder via the urethra for bladder distention. After completion of the surgical preparation, isoflurane anesthesia was reduced to ~0.875% until a flexion reflex response was present (evoked by pinching the paw) but spontaneous escape behavior and righting reflex were absent. The animals were not restrained in any fashion. Body temperature was maintained using an overhead radiant light and monitored throughout the experiment. The conditions were optimized to establish a stable depth of anesthesia and consistent baseline VMR activity. Phasic bladder distention with PBS was then used to evoke bladder nociception. The PBS pressure was controlled by an automated distention control device custom made in the Washington University School of Medicine Electronic Shop. The distention stimulus applied 20–80 mmHg pressure for 20 seconds every 2 min. The VMR signal was relayed in real-time using a Grass CP511 preamplifier to a PC via a WinDaq DI-720 module. Data were exported to Igor Pro 6.05 software (Wavemetrics, Portland, OR). Using a custom script, VMR signals were subtracted from the baseline, rectified and integrated over 20 s to quantify the area under the curve and presented in arbitrary units. The investigator who quantified the VMR was blinded to treatment.

Defensive burying. In order to measure anxiety-like behavior, AOAH-deficient and WT mice were singly placed in a novel holding cage with 7 cm of fresh bedding for 15 min. Anxiety-

like behavior was recorded as latency of first (measured in seconds) and number of burying behaviors. Burying behavior was defined as the mouse aggressively sifting through bedding with both front paws and with their head facing down.

Statistical analysis. Results were analyzed by Student's t-test or one-way/two-way analysis of variance (ANOVA) followed by Tukey's Multiple comparisons test with the use of Prism software, version 6 (GraphPad, Inc). Differences were considered statistically significant at $P < 0.05$.

RESULTS

AOAH-deficient mice have enlarged ceca and increased gut permeability.

Dissection of AOAH-deficient mice suggested a GI phenotype relative to wild type mice where AOAH-deficient mice ceca appeared larger and distended in both females (Fig. 1A) and males (Fig. S1A). This difference in appearance was at least partially borne out in mass because the cecum and cecal stool as a function of body mass were significantly heavier in male AOAH-deficient mice compared to wild type mice (Fig. S1B). In contrast, despite the enlarged morphology of AOAH-deficient ceca, neither cecal tissue nor cecal stool were significantly more massive in AOAH-deficient females relative to wild type mice (Fig. 1B). We further characterized the cecum phenotype by assessing cecum barrier function in female and male wild type and AOAH-deficient mice by quantifying cecum transepithelial electrical resistance (TEER) ex vivo via an $\ddot{U}$ ssing chamber (Fig. 1C and Fig. S1C respectively). Cecum TEER was significantly lower in AOAH-deficient mice compared to wild type in both females ($33.58 \pm 4.77 \Omega \cdot \text{cm}^2$ for wild type and $19.94 \pm 6.66 \Omega \cdot \text{cm}^2$ for *Aoah*^{-/-}; Fig. 1C) and males ($27.66 \pm 6.63 \Omega \cdot \text{cm}^2$ for wild type and $17.11 \pm 6.11 \Omega \cdot \text{cm}^2$ for *Aoah*^{-/-}; Fig. S1C), indicating that AOAH-deficient mice have increased cecum permeability or “leakiness” in both sexes. Since IC/BPS more commonly afflicts women, we focused further characterization on female mice.

AOAH-deficient mice have altered gut signaling pathways.

The compromised cecal TEER of AOAH-deficient mice suggest functionally altered epithelium, yet histologic examination uncovered no obvious differences in cecal epithelia of wild type and AOAH-deficient mice (Fig. 2A, top row). Immunostaining of AOAH protein in wild type mouse cecum revealed sparse expression of AOAH in the epithelium, which was mainly absent in AOAH-deficient cecum (Fig. 2A, bottom row). Transcriptome profiling by microarray analyses revealed significant differences in gene expression consistent with altered signaling pathways and disease risk in the AOAH-deficient ceca (Fig. 2B, C, and S2 respectively). Examination of genes increased/decreased at least 2-fold indicated altered mRNA abundance in

AOAH-deficient mice of genes associated with immune response/inflammation (53%), epithelia (13%), bacteria (7%), lipids (6%), neurons (5%), cell survival (5%), protein transport (4%), thioredoxin (2%), P450 (2%), and erythrocytes (2%) (Fig. 2B). Consistent with this, pathway analyses also revealed increased immune response signaling in AOAH-deficient mice compared to wild type (Fig. 2C). Together, these findings suggest that an inflammatory milieu and alterations in mRNA associated with epithelia contribute to increased cecal epithelial permeability underlying a leaky gut phenotype of AOAH-deficient mice.

AOAH-deficient mice have altered gut microbiota.

Previous studies have linked increased gut permeability to gut dysbiosis, and cecal enlargement is a long-known phenotype of germ-free rodents, both suggest potential gut dysbiosis in AOAH-deficient mice [29–31]. We utilized 16S rRNA amplicon sequencing data to assess large compositional differences in fecal (Fig. 3) and cecal (Fig. S3) microbiota between wild type and *Aoah*^{-/-} mice. As shown by principal component analysis (PCA) and the corresponding heat maps, AOAH-deficient mice demonstrate an altered gut microbiome compared to wild type control in both fecal and cecal stool (Fig. 3A and C and Fig. S3A and C respectively). AOAH-deficient mice show group variance along the PC1 axis in bacterial composition of gut flora compared to wild type mice in both fecal and cecal stool (Fig. 3A and S3A, respectively). In addition, α -diversity calculations revealed a greater number of operational taxonomic units (OTUs) in AOAH-deficient fecal stool (444.4 $\pm$ 30.67 OTUs) compared to WT fecal stool (366.6 $\pm$ 43.93 OTUs, Fig. 3B). We did not observe a significantly different number of observed OTUs between groups in cecal stool (Fig. S3B). However, PC2 axes reveal larger variances amongst individual AOAH-deficient mice compared to wild type, suggesting greater bacterial diversity in mice deficient for AOAH (Fig. 3A and S3A).

To determine the specificity of AOAH modulation of gut microbiota, we examined the relative abundance of bacterial phyla (Fig. 3D). Over 99% of bacteria identified in both mouse groups belonged to Bacteroidetes and Firmicutes phyla, with no significant differences in

abundance of these groups between wild type and AOA-deficient stool. However, there were differences in less common phyla such as Verrucomicrobia (0% expression for WT and 0.2% expression for *Aoa^{-/-}*), Cyanobacteria (0% expression for wild type and 0.3% expression for *Aoa^{-/-}*), and TM7 (0% expression for wild type and 0.1% expression for *Aoa^{-/-}*), where AOA-deficient mice showed more abundant expression of these phyla compared to wild type. A decrease in abundance was observed for Actinobacteria in AOA-deficient mice compared to wild type (0.10% expression for wild type and 0% expression for *Aoa^{-/-}*). These findings suggest a gut microbiome that is richer in diversity in AOA-deficient mice compared to wild type.

To identify changes in the presence and absence of bacterial species between wild type and AOA-deficient mice, the top 500 bacterial OTUs were compared in fecal (Table 1) and cecal (Table S1) samples. We observed several bacterial species that were present in AOA-deficient stool but absent in wild type stool (Table 1). In addition, several bacterial species were absent in AOA-deficient stool but present in wild type stool. Cecal samples exhibited a modest overlap with our findings in fecal samples but were identified to have several more bacterial species unique to AOA-deficient mice compared to wild type (Table S1 for full list). Upon further analyses of bacterial functionality, many of the bacterial species with altered abundance were associated with carbohydrate metabolism (Table 1 and Table S1).

Co-housing alters the functional characterization of bacterial communities.

We utilized in silico metagenome prediction of function with PICRUSt to gain functional insights into the effects of AOA-deficiency and the impacts of microbiome manipulation [26]. PICRUSt revealed that AOA-deficient mice have several significant differences relative to controls including genes that mediate cell growth and death, signaling molecules and interaction, DNA replication and repair, transcription, human diseases, lipid metabolism, the circulatory system, the digestive system, and the immune system (Fig. 4 and Table S2). Upon further analyses, we observed that co-housing AOA-deficient mice with wild type mice, to allow for

coprophagia-mediated microbiome manipulation, altered the predictive metagenomes in both mouse strains (Fig. 4). Wild type and AOA-deficient mice showed differences in genes associated with several different taxa, including the RIG-I-like receptor signaling pathway (Fig. 4A) which plays a role in the innate immune system's ability to recognize pathogens [32]. The differences observed in a subset of functional classes were rescued in co-housed AOA-deficient mice (Fig. 4A-D and H). Interestingly, for other functional classes, co-housing resulted in no changes in AOA-deficient mice but altered the predictive values in co-housed wild type to similar values observed in AOA-deficient mice (Fig. 5E-G). These data suggest that both wild type and AOA-deficient microbiota can influence changes in the metagenome.

AOA-deficient mice have altered gut metabolites.

To identify the biochemical consequences of dysbiosis associated with AOA deficiency, we performed untargeted metabolomics. In both fecal and cecal stool, AOA-deficient mice show altered gut metabolomes compared to controls (Figs. 5 and S3B, respectively). Among the top 500 feature metabolites, we identified numerous metabolites either uniquely present or absent from fecal or cecal AOA-deficient mice (Tables 2, S3 and S4). We observed that 24-Ethyl-delta(22)-coprostenol, cellobiose, and hexadecanol were present in AOA-deficient fecal samples but absent in wild type whereas, methionine was absent in AOA-deficient fecal samples (Table 2). Similarly, cecal samples also expressed the same unique metabolites as fecal samples in AOA-deficient mice (Tables S3 and S4). In addition to overlap with fecal metabolites, we identified 23 newly present metabolites and 8 absent metabolites in AOA-deficient in cecal stool compared to controls (Tables S3 and S4). Similar to our observations of bacterial species, functionality of the metabolites with aberrant presence/absence were primarily associated with carbohydrate metabolism (compare Tables 1 and S1 with Tables 2, S3, and S4). In addition, we observed the presence/absence of gut metabolites associated with lipid and amino acid metabolism (Tables 2, S3, and S4). Taken together, our findings show that AOA-deficient mice have an altered gut microbiome which corresponds with altered accumulation of gut metabolites.

Co-housing converges microbiota and alters gut phenotype in AOA-deficient mice.

To determine if gut dysbiosis could be rescued in AOA-deficient mice, we co-housed AOA-deficient and wild type mice for 7 days to allow for coprophagia-mediated microbiome manipulation. Co-housing resulted in a convergence of cecum size between wild type and AOA-deficient mice, in contrast with the distinct morphologies in like-housed mice (Fig. 6A, compare with Fig. 1A). We next determined whether our phenotypic observations in co-housed mice would match in genotype. Similar to our observations of converged gut morphology (Fig. 6A), 16S rRNA amplicon sequencing revealed that co-housing resulted in a microbiota genotype that was converged in both mouse strains and distinct from both wild type and AOA-deficient mice that were like-housed (Fig. 6B).

We next addressed whether co-housing altered the leaky gut phenotype of AOA-deficient mice (Fig. 6C). Similar to cecum morphology, quantifying TEER showed a convergence of cecum barrier function ($4.85 \Omega \cdot \text{cm}^2$ for co-housed wild type mice, and $21.58 \pm 7.85 \Omega \cdot \text{cm}^2$ for co-housed *Aoa*^{-/-} mice; Fig. 6C). However, co-housed AOA-deficient mice still exhibited significantly increased cecum permeability compared to like-housed wild type mice, suggesting that co-housing does not fully resolve barrier dysfunction in AOA-deficient mice (Fig. 6C).

IC/BPS patients exhibit defects in bladder barrier function [33–35], and similarly we reported that AOA-deficient mice have increased bladder permeability. Because previous studies in pelvic organ crosstalk have shown the gut can modulate bladder inflammation and pelvic pain [36–40], we examined whether microbiome manipulation altered the bladder permeability phenotype of AOA-deficient mice. Co-housed AOA-deficient mice showed bladder TEER similar to co-housed wild type mice ($5062 \pm 1037 \Omega \cdot \text{cm}^2$ for co-housed WT mice and $5416 \pm 1634 \Omega \cdot \text{cm}^2$ for *Aoa*^{-/-} mice, Fig. 6D). In addition, bladder permeability was not significantly different between co-housed mice and like-housed wild type mice (Fig. 6D), suggesting that convergence of microbiota can alleviate the leaky bladder phenotype of AOA-deficient mice.

Microbiota manipulation alters pelvic pain.

AOAH-deficient mice exhibit pelvic pain behaviors that include pelvic allodynia and increased visceromotor response (VMR) [7]. To determine whether altered gut flora affected the pelvic pain phenotype of AOAH-deficient mice, we co-housed wild type and AOAH-deficient mice for 28 d and quantified allodynia in response to von Frey filaments applied to the pelvic region (Fig. 7A). Following co-housing, AOAH-deficient mice showed a 35% reduction in pelvic allodynia from baseline in response to the highest stimulus compared to AOAH-deficient mice prior to being co-housed (Fig. 7A, $83.57 \pm 20.98\%$ response at baseline vs $54.00 \pm 40.06\%$ response after co-housing). These data suggest that changes in the gut microbiome, in part, can decrease pelvic allodynia in AOAH-deficient mice but do not completely alleviate the pelvic pain phenotype.

To confirm the findings of altered allodynia, we quantified the effects of co-housing on VMR measured by superior oblique abdominal muscle EMG activity following urinary bladder distension (Fig. 7B and C). Co-housing significantly reduced bladder distension-induced VMR in AOAH-deficient mice to levels similar to wild type (Fig. 7B), suggesting decreased pelvic pain following microbiome manipulation. These findings were further corroborated through FMT by stool slurry gavage. AOAH-deficient mice that received gavage of wild type stool slurry showed reduced EMG activity upon bladder distension compared to AOAH-deficient mice that received gavage of stool from other AOAH-deficient donors (Fig. 7C).

Previous reports have shown gut dysbiosis in patients with IC/BPS [19, 20], but whether this dysbiosis modulates pelvic pain remains speculative. To address this question AOAH-deficient mice were gavaged with anaerobic cultures of human stool from healthy controls or IC/BPS patients. After one week of microbiota gavage, mice were assessed for pelvic allodynia. AOAH-deficient mice that were exposed to IC/BPS microbiota showed significantly increased pelvic allodynia from baseline compared to AOAH-deficient mice gavaged with control microbiota (Fig. 7D), suggesting that the gut microbiota of IC/BPS patients can influence pelvic

pain. Taken together, our data from manipulating the microbiome of AOA-deficient mice suggest that gut microbiota modulate pelvic pain.

Fecal microbiota transplantation reduces anxiety-like behavior in AOA-deficient mice.

We have previously reported that AOA-deficient mice exhibit comorbid anxious/depressive behaviors, similar to patients with IC/BPS [4, 6, 14]. To assess whether the gut microbiome contributes to the anxious/depressive phenotype of AOA-deficient mice, we measured defensive burying behavior after placing mice into a novel holding cage with deep, clean bedding. Anxious behaviors were quantified as latency to the first burying event and number of burying behaviors. Corroborating our prior findings, AOA-deficient mice exhibited higher levels of anxious behaviors as observed by decreased latency to burying and increased number of burying events (Figs. 8A and S4A). Anxiety-like behavior was rescued in AOA-deficient mice that received gavage of wild type stool, showing longer latency to burying and fewer burying events compared to AOA-deficient mice that received gavage of AOA-deficient stool (Fig. 8B and Fig. S4B). Our findings show that the gut microbiome plays a significant role in the anxiety phenotype of AOA-deficient mice.

DISCUSSION

AOAH deficiency mimics many aspects of IC/BPS, and here we identified *Aoah* as a novel genetic modulator of gut microbiota. Prior studies have implicated host genetic factors in microbiota, including twin studies that showed increased microbiota heritability among monozygotic twins and identified specific host loci in interacting with specific taxa and mediating metabolic processes [41, 42]. Additional human genome-wide association studies (GWAS) and murine quantitative trait loci studies have furthered this understanding of interactions between microbiota and host genetics and provided new mechanistic insights, yet few loci implicated by such studies have been interrogated functionally. For example, 42 human loci were identified that were associated with bacterial diversity, including the vitamin D receptor, and vitamin D receptor-deficient mice had increased *Parabacteroides* abundance [43]. Although loci on chromosome 7, where *AOAH* resides, were significantly associated with altered microbiota, none of these lay nearer than 2MB from *AOAH*, so GWAS studies have not yet directly implicated human *AOAH* in microbiome modulation. However, *PLA2G3* was significantly associated with microbiota diversity, suggesting a role for arachidonic acid metabolism as a modulator of the human microbiome [43]. That finding dovetails with in silico metagenome analyses that identified arachidonic acid metabolism as significantly associated with microbiota of patients with IC/BPS [19]. We recently reported that *AOAH*-deficient mice accumulate elevated levels of arachidonic acid and PGE2 in the CNS and found evidence that *AOAH* is a transacylase mediating arachidonic acid homeostasis via sequestration in phospholipid pools [15]. Thus, together these studies are consistent with an emerging model where loss of *AOAH*-dependent arachidonic acid homeostasis contributes to gut dysbiosis associated with IC/BPS symptoms.

AOAH-deficient mice exhibited enlarged ceca with decreased barrier function, and transcriptome analyses indicated association with Wnt signaling and inflammatory/immune pathways (Figs. 1, 2 and S1). Altered Wnt signaling in *AOAH*-deficiency may be due to elevated PGE2 in these mice and the known role of PGE2 as a modulator of Wnt expression [15]. Moreover,

Wnt expression has been previously implicated in pelvic pain, where a putative urinary marker of IC/BPS was identified as a glycosylated fragment of Frizzled-8 [44]. Alternatively, in the absence of AOA^H that would otherwise detoxify LPS, upregulation of inflammatory and immune responses may be due to excess toll-like receptor (TLR)-mediated signals evoked by LPSs of the gut microbiota. Increased inflammatory responses, in turn, may contribute to loss of barrier function and consequent "leaky gut" [45, 46]. Consistent with this possibility, *in silico* metagenome analyses by PICRUSt revealed changes in AOA^H-deficient mice associated increased RIG-I-like receptor signaling pathway, a regulator of innate immune activation in various cell types [32]. These data were particularly interesting as at least a subset of IC/BPS patients exhibit bladder inflammation associated with activated mast cells [47], and we previously reported that AOA^H-deficient mice exhibit significantly more bladder mast cells [7]. In addition, mast cells in the gastrointestinal (GI) tract show increased activation in patients with GI disorders such as irritable bowel syndrome (IBS) [48, 49]. As dysfunctional bowel-bladder crosstalk may also influence GI mast cells in AOA^H-deficient mice and gut "leakiness", activation of these cells should be analyzed.

Immunostaining did not reveal widespread expression of AOA^H in gut epithelium or underlying tissue layers (Fig. 2), suggesting that AOA^H effects on gut gene expression lie elsewhere. Indeed, we previously reported that AOA^H is a genetic regulator of the locus encoding corticotropin-releasing factor (CRF), in brain sites mediating bladder and stress responses where AOA^H deficiency leads to increased *Cr_f* expression and stress responses [13, 14]. CRF alters gut function at multiple levels (reviewed in [50, 51]). At the enteric epithelium, elevated CRF engages CRFR1 receptors on underlying mast cells, resulting in increased epithelial permeability [52]. In addition, centrally administered CRF suppresses gut motility. This raises the possibility that elevated CRF alters cecal motility, contributing to cecal enlargement in AOA^H-deficient mice while also altering the luminal environment and driving dysbiosis. The resulting combination of dysbiosis and leaky gut may then contribute to AOA^H-deficient phenotypes of pain and anxiety [17].

Although AOA expression is not widespread in enteric mucosa, we observed occasional cells within the epithelium that label brightly (Fig. 2). The appearance and prevalence of these AOA-positive cells in enteric epithelium is reminiscent of neuropods that mediate gut sensory responses [53]. Neuropods are neuroendocrine cells of the enteric epithelium that synapse with vagal neurons, hence providing enteric sensory information to the brainstem through a single synapse and thus poised to convey information about microbiota [54]. Centrally, AOA deficiency leads to arachidonic acid accumulation, a known mediator of neuronal excitability and nociception [15, 55]. If arachidonic acid metabolism is similarly dysregulated in putative neuropods, this may lead to increased excitability and enhanced sensory responses to microbiota in AOA-deficient mice. Alternatively, increased intestinal permeability can lead to altered CNS responses when microbial constituents cross the blood brain barrier [46, 56], and future studies will dissect the relative contributions of neuropods and gut leakage to *Aoa* phenotypes.

We observed increased mass of the cecum and cecal stool in male AOA-deficient mice compared to male WT (Fig. S1), but not in female AOA-deficient mice compared to female WT (Fig. 1). These findings suggest potential sex differences in cecum phenotype and the differential roles that AOA may play in cecum development and maintenance. Several studies in mice and humans have shown sex-specific differences in gut microbiota [57]. Although levels of testosterone and estrogen are directly linked to altered gut flora, other factors such as metabolism, body mass index, and colonic transit time differ among males and females and have been linked to changes in the gut microbiome [57], so future studies will examine sex differences in AOA-mediated microbiota. Nonetheless, we observed significant differences in female microbiota at the levels of phyla and individual taxa, where AOA-deficient mice showed more bacterial diversity in both fecal and cecal stool and increased verrucomicrobia, cyanobacteria, and TM7 in AOA-deficient feces (Fig. 3). Cyanobacteria have previously been linked to gastrointestinal symptoms in humans, including abdominal pain, and mucosa of Crohn's disease and ulcerative colitis patients show significant differences in TM7 bacteria composition [58, 59]. Together these studies suggest a role for cyanobacteria and TM7 phyla in microbiota-associated pelvic pain.

We also observed several OTUs that were present/absent in AOA-deficient fecal and cecal stool compared to wild type (Table 1 and Table S1 respectively). Based on the known functions of these OTUs, our findings suggest that AOA-deficient mice may have altered carbohydrate metabolism. This possibility is corroborated by the increased abundance of metabolites that play a role in carbohydrate metabolism (Table 2 and Tables S3 and S4). For example, cecal stool show an absence of *B. acidifaciens* and *P. distasonis* in AOA-deficient mice (Table S1), bacterial species that regulate glucose metabolism and homeostasis as well as play a role in obesity [60–62]. Previous studies have shown that *B. acidifaciens* and *P. distasonis* prevent obesity or weight gain in mice [61, 62]. Here, we also observed that female AOA-deficient mice have increased body mass compared to controls, consistent with previous studies that associated reduced *Aoa* expression with increased poultry size [63]. Thus, it is possible that AOA modulation of microbiota, resulting in altered prevalence of species including *B. acidifaciens* and *P. distasonis* and consequent altered carbohydrate metabolism may explain the increase in body mass observed in diverse species.

Manipulating microbiota demonstrated that gut flora underlie phenotypes of AOA-deficient mice (Figs. 6-8). Co-housing AOA-deficient and wild type mice to manipulate microbiota by coprophagia resulted in a merged gut flora composition that was distinct from like-housed mice and corresponded to converged cecum morphology and epithelial barrier function (Fig. 6). In addition, we observed that co-housing or direct FMT by stool slurry gavage resulted in reduced pelvic pain in AOA-deficient mice (Fig. 7). These findings are similar to the beneficial effects of co-housing on symptomatology and epithelial barrier function of the colon in ulcerative colitis models [64, 65]. However, co-housing colitis mice with controls also increased histologic colitis scores in control mice, suggesting that microbiota transmission may result in a diseased phenotype in previously healthy mice [65]. Although we observed improvement in the pelvic pain phenotype of AOA-deficient mice after co-housing without adverse pain effects on co-housed control mice, we nonetheless found that co-housing impacted several PICRUSt functional classes in wild type mice, including steroid hormone biosynthesis, mineral absorption,

and carotenoid biosynthesis (Figs. 4 and 7). Thus, microbiota alterations associated with AOA-H-deficiency are likely sufficient to impact biologic processes and phenotypes.

Our previous studies show that AOA-H-deficient mice mimic symptoms and comorbidities of IC/BPS [7, 13, 14]. Here we show that the pelvic pain phenotype and anxiety / depressive-like behavior can be regulated by microbiota (Figs 7 and 8). We identified 2-Quinolincarboxylic acid, 4,8—dihydroxy (a.k.a., xanthurenic acid) as a gut metabolite of AOA-H-deficient cecal stool of mice (Table S3). Xanthurenic acid is a tryptophan metabolite and mGluR_{II} agonist that is elevated in urine of depressed patients, drawing another clinical parallel between patients and AOA-H-deficient mice and suggesting this metabolite in AOA-H-deficient mice ceca may contribute to the depressive-like phenotype [66–68]. Finally, we found IC/BPS microbiota exacerbate the pelvic pain phenotype of AOA-H-deficient mice (Fig. 7). In light of our prior report of altered microbiota among IC/BPS patients [19], this suggests dysbiosis in IC/BPS is functionally significant and sufficient to impact the defining clinical parameter of IC/BPS, pelvic pain. Thus, addressing gut dysbiosis is a therapeutic target for IC/BPS, and novel IC/BPS therapies should be developed that modify the gut microbiome. Such therapies may include prebiotic diets, probiotic supplements to complement deficiencies in specific OTUs or the functions thereof, and even transplant of stool or complex communities derived from stool.

In summary, the data presented here show that AOA-H-deficient mice exhibit gut dysbiosis and an altered gut phenotype which mediates pelvic pain and anxiety-like behavior. These findings demonstrate that the gut microbiome is a promising therapeutic target for treating IC/BPS.

ACKNOWLEDGEMENTS

This work was supported by NIH/NIDDK award R01 DK103769 (B.A.W., A.J.S., and D.J.K) and by NIH/NIDDK T32 DK062716 postdoctoral fellowship to Dr. Rahman-Enyart. Histology services were provided by the Northwestern University Mouse Histology and Phenotyping Laboratory which is supported by NCI P30-CA060553 awarded to the Robert H Lurie Comprehensive Cancer Center. We thank Dr. Robert Munford for generously providing AOA- deficient mice and for many helpful discussions.

REFERENCES

1. Akiyama Y, Homma Y, Maeda D. Pathology and terminology of interstitial cystitis/bladder pain syndrome: A review. *Histol Histopathol*. 2019;34:25–32. doi:10.14670/HH-18-028.
2. Kim H-J. Update on the pathology and diagnosis of interstitial cystitis/bladder pain syndrome: A review. *Int Neurourol J*. 2016;20:13–7. doi:10.5213/inj.1632522.261.
3. Mullins C, Bavendam T, Kirkali Z, Kusek JW. Novel research approaches for interstitial cystitis/bladder pain syndrome: thinking beyond the bladder. *Transl Androl Urol*. 2015;4:524–33. doi:10.3978/j.issn.2223-4683.2015.08.01.
4. Chuang Y-C, Weng S-F, Hsu Y-W, Huang CL-C, Wu M-P. Increased risks of healthcare-seeking behaviors of anxiety, depression and insomnia among patients with bladder pain syndrome/interstitial cystitis: a nationwide population-based study. *Int Urol Nephrol*. 2015;47:275–81. doi:10.1007/s11255-014-0908-6.
5. Hanno PM, Erickson D, Moldwin R, Faraday MM, American Urological Association. Diagnosis and treatment of interstitial cystitis/bladder pain syndrome: AUA guideline amendment. *J Urol*. 2015;193:1545–53. doi:10.1016/j.juro.2015.01.086.
6. McKernan LC, Walsh CG, Reynolds WS, Crofford LJ, Dmochowski RR, Williams DA. Psychosocial co-morbidities in Interstitial Cystitis/Bladder Pain syndrome (IC/BPS): A systematic review. *Neurourol Urodyn*. 2018;37:926–41. doi:10.1002/nau.23421.
7. Yang W, Yaggie RE, Jiang MC, Rudick CN, Done J, Heckman CJ, et al. Acyloxyacyl hydrolase modulates pelvic pain severity. *Am J Physiol Regul Integr Comp Physiol*. 2018;314:R353–65. doi:10.1152/ajpregu.00239.2017.
8. Erwin AL, Munford RS. Deacylation of structurally diverse lipopolysaccharides by human acyloxyacyl hydrolase. *J Biol Chem*. 1990;265:16444–9.
9. Hagen FS, Grant FJ, Kuijper JL, Slaughter CA, Moomaw CR, Orth K, et al. Expression and characterization of recombinant human acyloxyacyl hydrolase, a leukocyte enzyme that deacylates bacterial lipopolysaccharides. *Biochemistry*. 1991;30:8415–23.

doi:10.1021/bi00098a020.

10. Lu M, Varley AW, Ohta S, Hardwick J, Munford RS. Host inactivation of bacterial lipopolysaccharide prevents prolonged tolerance following gram-negative bacterial infection. *Cell Host Microbe*. 2008;4:293–302. doi:10.1016/j.chom.2008.06.009.
11. Munford RS, Hunter JP. Acyloxyacyl hydrolase, a leukocyte enzyme that deacylates bacterial lipopolysaccharides, has phospholipase, lysophospholipase, diacylglycerolipase, and acyltransferase activities in vitro. *J Biol Chem*. 1992;267:10116–21.
12. Staab JF, Ginkel DL, Rosenberg GB, Munford RS. A saposin-like domain influences the intracellular localization, stability, and catalytic activity of human acyloxyacyl hydrolase. *J Biol Chem*. 1994;269:23736–42.
13. Aguiniga LM, Searl TJ, Rahman-Enyart A, Yaggie RE, Yang W, Schaeffer AJ, et al. Acyloxyacyl hydrolase regulates voiding activity. *Am J Physiol Renal Physiol*. 2020;318:F1006–16. doi:10.1152/ajprenal.00442.2019.
14. Aguiniga LM, Yang W, Yaggie RE, Schaeffer AJ, Klumpp DJ, MAPP Research Network Study Group. Acyloxyacyl hydrolase modulates depressive-like behaviors through aryl hydrocarbon receptor. *Am J Physiol Regul Integr Comp Physiol*. 2019;317:R289–300. doi:10.1152/ajpregu.00029.2019.
15. Yang W, Yaggie RE, Schaeffer AJ, Klumpp DJ. AOA remodelling arachidonic acid-containing phospholipid pools in a model of interstitial cystitis pain: A MAPP Network study. *PLoS One*. 2020;15:e0235384. doi:10.1371/journal.pone.0235384.
16. Lutgendorf SK, Kreder KJ, Rothrock NE, Hoffman A, Kirschbaum C, Sternberg EM, et al. Diurnal cortisol variations and symptoms in patients with interstitial cystitis. *J Urol*. 2002;167:1338–43.
17. Pusceddu MM, Gareau MG. Visceral pain: gut microbiota, a new hope? *J Biomed Sci*. 2018;25:73. doi:10.1186/s12929-018-0476-7.
18. Defaye M, Gervason S, Altier C, Berthon J-Y, Ardid D, Filaire E, et al. Microbiota: a novel regulator of pain. *J Neural Transm*. 2020;127:445–65. doi:10.1007/s00702-019-02083-z.

19. Braundmeier-Fleming A, Russell NT, Yang W, Nas MY, Yaggie RE, Berry M, et al. Stool-based biomarkers of interstitial cystitis/bladder pain syndrome. *Sci Rep*. 2016;6:26083. doi:10.1038/srep26083.
20. Lee KW, Song HY, Kim YH. The microbiome in urological diseases. *Investig Clin Urol*. 2020;61:338–48. doi:10.4111/icu.2020.61.4.338.
21. Du H-X, Liu Y, Zhang L-G, Zhan C-S, Chen J, Zhang M, et al. Abnormal gut microbiota composition is associated with experimental autoimmune prostatitis-induced depressive-like behaviors in mice. *Prostate*. 2020;80:663–73. doi:10.1002/pros.23978.
22. Codagnone MG, Spichak S, O'Mahony SM, O'Leary OF, Clarke G, Stanton C, et al. Programming bugs: microbiota and the developmental origins of brain health and disease. *Biol Psychiatry*. 2019;85:150–63. doi:10.1016/j.biopsych.2018.06.014.
23. Moloney RD, Johnson AC, O'Mahony SM, Dinan TG, Greenwood-Van Meerveld B, Cryan JF. Stress and the Microbiota-Gut-Brain Axis in Visceral Pain: Relevance to Irritable Bowel Syndrome. *CNS Neurosci Ther*. 2016;22:102–17. doi:10.1111/cns.12490.
24. Felice VD, Moloney RD, Cryan JF, Dinan TG, O'Mahony SM. Visceral pain and psychiatric disorders. *Mod Trends Pharmacopsychiatri*. 2015;30:103–19. doi:10.1159/000435936.
25. Malykhina AP, Wyndaele J-J, Andersson K-E, De Wachter S, Dmochowski RR. Do the urinary bladder and large bowel interact, in sickness or in health? ICI-RS 2011. *Neurourol Urodyn*. 2012;31:352–8. doi:10.1002/nau.21228.
26. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol*. 2013;31:814–21. doi:10.1038/nbt.2676.
27. Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L. Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. *Plant J*. 2000;23:131–42. doi:10.1046/j.1365-313x.2000.00774.x.
28. Rudick CN, Jiang M, Yaggie RE, Pavlov VI, Done J, Heckman CJ, et al. O-antigen modulates infection-induced pain states. *PLoS One*. 2012;7:e41273. doi:10.1371/journal.pone.0041273.

29. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. *N Engl J Med*. 2016;375:2369–79. doi:10.1056/NEJMra1600266.
30. Dupont HL, Jiang Z-D, Dupont AW, Utay NS. The intestinal microbiome in human health and disease. *Trans Am Clin Climatol Assoc*. 2020;131:178–97.
31. Viganò D, Zara F, Usai P. Irritable bowel syndrome and endometriosis: New insights for old diseases. *Dig Liver Dis*. 2018;50:213–9. doi:10.1016/j.dld.2017.12.017.
32. Loo Y-M, Gale M. Immune signaling by RIG-I-like receptors. *Immunity*. 2011;34:680–92. doi:10.1016/j.immuni.2011.05.003.
33. Tomaszewski JE, Landis JR, Russack V, Williams TM, Wang LP, Hardy C, et al. Biopsy features are associated with primary symptoms in interstitial cystitis: results from the interstitial cystitis database study. *Urology*. 2001;57 6 Suppl 1:67–81. doi:10.1016/s0090-4295(01)01166-9.
34. Ratliff TL, Klutke CG, McDougall EM. The etiology of interstitial cystitis. *Urol Clin North Am*. 1994;21:21–30.
35. Johansson SL, Fall M. Clinical features and spectrum of light microscopic changes in interstitial cystitis. *J Urol*. 1990;143:1118–24. doi:10.1016/s0022-5347(17)40201-1.
36. Kaplan SA, Dmochowski R, Cash BD, Kopp ZS, Berriman SJ, Khullar V. Systematic review of the relationship between bladder and bowel function: implications for patient management. *Int J Clin Pract*. 2013;67:205–16. doi:10.1111/ijcp.12028.
37. Rudick CN, Chen MC, Mongiu AK, Klumpp DJ. Organ cross talk modulates pelvic pain. *Am J Physiol Regul Integr Comp Physiol*. 2007;293:R1191-8. doi:10.1152/ajpregu.00411.2007.
38. Malykhina AP. Neural mechanisms of pelvic organ cross-sensitization. *Neuroscience*. 2007;149:660–72. doi:10.1016/j.neuroscience.2007.07.053.
39. Pezzone MA, Liang R, Fraser MO. A model of neural cross-talk and irritation in the pelvis: implications for the overlap of chronic pelvic pain disorders. *Gastroenterology*. 2005;128:1953–64. doi:10.1053/j.gastro.2005.03.008.
40. Qin C, Malykhina AP, Akbarali HI, Foreman RD. Cross-organ sensitization of lumbosacral spinal neurons receiving urinary bladder input in rats with inflamed colon. *Gastroenterology*.

2005;129:1967–78. doi:10.1053/j.gastro.2005.09.013.

41. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. *Cell*. 2014;159:789–99. doi:10.1016/j.cell.2014.09.053.

42. Goodrich JK, Davenport ER, Beaumont M, Jackson MA, Knight R, Ober C, et al. Genetic determinants of the gut microbiome in UK twins. *Cell Host Microbe*. 2016;19:731–43. doi:10.1016/j.chom.2016.04.017.

43. Wang J, Thingholm LB, Skiecevičienė J, Rausch P, Kummén M, Hov JR, et al. Genome-wide association analysis identifies variation in vitamin D receptor and other host factors influencing the gut microbiota. *Nat Genet*. 2016;48:1396–406. doi:10.1038/ng.3695.

44. Keay SK, Szekely Z, Conrads TP, Veenstra TD, Barchi JJ, Zhang C-O, et al. An antiproliferative factor from interstitial cystitis patients is a frizzled 8 protein-related sialoglycopeptide. *Proc Natl Acad Sci USA*. 2004;101:11803–8. doi:10.1073/pnas.0404509101.

45. Camilleri M. Leaky gut: mechanisms, measurement and clinical implications in humans. *Gut*. 2019;68:1516–26. doi:10.1136/gutjnl-2019-318427.

46. Obrenovich MEM. Leaky gut, leaky brain? *Microorganisms*. 2018;6. doi:10.3390/microorganisms6040107.

47. Grover S, Srivastava A, Lee R, Tewari AK, Te AE. Role of inflammation in bladder function and interstitial cystitis. *Ther Adv Urol*. 2011;3:19–33. doi:10.1177/1756287211398255.

48. Wouters MM, Vicario M, Santos J. The role of mast cells in functional GI disorders. *Gut*. 2016;65:155–68. doi:10.1136/gutjnl-2015-309151.

49. Ramsay DB, Stephen S, Borum M, Voltaggio L, Doman DB. Mast cells in gastrointestinal disease. *Gastroenterol Hepatol (NY)*. 2010;6:772–7.

50. Taché Y, Perdue MH. Role of peripheral CRF signalling pathways in stress-related alterations of gut motility and mucosal function. *Neurogastroenterol Motil*. 2004;16 Suppl 1:137–42. doi:10.1111/j.1743-3150.2004.00490.x.

51. Tache Y, Larauche M, Yuan P-Q, Million M. Brain and gut CRF signaling: biological actions and role in the gastrointestinal tract. *Curr Mol Pharmacol*. 2018;11:51–71.

doi:10.2174/1874467210666170224095741.

52. Rodiño-Janeiro BK, Alonso-Cotoner C, Pigrau M, Lobo B, Vicario M, Santos J. Role of Corticotropin-releasing Factor in Gastrointestinal Permeability. *J Neurogastroenterol Motil.* 2015;21:33–50. doi:10.5056/jnm14084.

53. Kaelberer MM, Buchanan KL, Klein ME, Barth BB, Montoya MM, Shen X, et al. A gut-brain neural circuit for nutrient sensory transduction. *Science.* 2018;361. doi:10.1126/science.aat5236.

54. Liddle RA. Neuropods. *Cell Mol Gastroenterol Hepatol.* 2019;7:739–47. doi:10.1016/j.jcmgh.2019.01.006.

55. Jang Y, Kim M, Hwang SW. Molecular mechanisms underlying the actions of arachidonic acid-derived prostaglandins on peripheral nociception. *J Neuroinflammation.* 2020;17:30. doi:10.1186/s12974-020-1703-1.

56. Logsdon AF, Erickson MA, Rhea EM, Salameh TS, Banks WA. Gut reactions: How the blood-brain barrier connects the microbiome and the brain. *Exp Biol Med.* 2018;243:159–65. doi:10.1177/1535370217743766.

57. Kim YS, Unno T, Kim BY, Park MS. Sex differences in gut microbiota. *World J Mens Health.* 2020;38:48–60. doi:10.5534/wjmh.190009.

58. Lévesque B, Gervais M-C, Chevalier P, Gauvin D, Anassour-Laouan-Sidi E, Gingras S, et al. Prospective study of acute health effects in relation to exposure to cyanobacteria. *Sci Total Environ.* 2014;466–467:397–403. doi:10.1016/j.scitotenv.2013.07.045.

59. Kuehbach T, Rehman A, Lepage P, Hellmig S, Fölsch UR, Schreiber S, et al. Intestinal TM7 bacterial phylogenies in active inflammatory bowel disease. *J Med Microbiol.* 2008;57 Pt 12:1569–76. doi:10.1099/jmm.0.47719-0.

60. Miyamoto Y, Itoh K. *Bacteroides acidifaciens* sp. nov., isolated from the caecum of mice. *Int J Syst Evol Microbiol.* 2000;50 Pt 1:145–8. doi:10.1099/00207713-50-1-145.

61. Wang K, Liao M, Zhou N, Bao L, Ma K, Zheng Z, et al. Parabacteroides distasonis Alleviates Obesity and Metabolic Dysfunctions via Production of Succinate and Secondary Bile Acids. *Cell Rep.* 2019;26:222–235.e5. doi:10.1016/j.celrep.2018.12.028.

730 62. Yang JY, Lee YS, Kim Y, Lee SH, Ryu S, Fukuda S, et al. Gut commensal *Bacteroides*
731 *acidifaciens* prevents obesity and improves insulin sensitivity in mice. *Mucosal Immunol.*
732 2017;10:104–16. doi:10.1038/mi.2016.42.

733 63. Claire D'Andre H, Paul W, Shen X, Jia X, Zhang R, Sun L, et al. Identification and
734 characterization of genes that control fat deposition in chickens. *J Anim Sci Biotechnol.*
735 2013;4:43. doi:10.1186/2049-1891-4-43.

736 64. Wu M, Li P, An Y, Ren J, Yan D, Cui J, et al. Phloretin ameliorates dextran sulfate sodium-
737 induced ulcerative colitis in mice by regulating the gut microbiota. *Pharmacol Res.*
738 2019;150:104489. doi:10.1016/j.phrs.2019.104489.

739 65. Garrett WS, Lord GM, Punit S, Lugo-Villarino G, Mazmanian SK, Ito S, et al. Communicable
740 ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell.* 2007;131:33–45.
741 doi:10.1016/j.cell.2007.08.017.

742 66. Maes M, De Ruyter M, Suy E. Xanthurenic acid flow in 24-hour urine following L-
743 tryptophan loading in depressive patients. *Acta Psychiatr Belg.* 1986;86:120–30.

744 67. Hoes MJ. The clinical significance of an elevated excretion of xanthurenic acid in psychiatric
745 patients. *Acta Psychiatr Belg.* 1979;79:638–46.

746 68. Hoes MJAJ., Sijben N. Xanthurenic Acid in Depression. *J Orthomol Med.* 1987.

747 69. Song Y, Könönen E, Rautio M, Liu C, Bryk A, Eerola E, et al. *Alistipes onderdonkii* sp. nov.
748 and *Alistipes shahii* sp. nov., of human origin. *Int J Syst Evol Microbiol.* 2006;56 Pt 8:1985–90.
749 doi:10.1099/ijss.0.64318-0.

750 70. Renson A, Mullan Harris K, Dowd JB, Gaydos L, McQueen MB, Krauter KS, et al. Early
751 signs of gut microbiome aging: biomarkers of inflammation, metabolism, and macromolecular
752 damage in young adulthood. *J Gerontol A, Biol Sci Med Sci.* 2020;75:1258–66.
753 doi:10.1093/gerona/glaa122.

754 71. Cotta M, Forster R. The family *Lachnospiraceae*, including the genera *Butyrivibrio*,
755 *Lachnospira* and *Roseburia*. In: *The Prokaryotes*. Dworkin M, Falkow S, Rosenberg E, Schleifer
756 K-H, Stackebrandt E, editors. New York, NY: Springer US; 2006. p. 1002–21. doi:10.1007/0-387-

- 30744-3_35.
72. Watthanalamloet A, Tachaapaikoon C, Lee YS, Kosugi A, Mori Y, Tanasupawat S, et al. *Cellulosibacter alkalithermophilus* gen. nov., sp. nov., an anaerobic alkalithermophilic, cellulolytic-xylanolytic bacterium isolated from soil of a coconut garden. *Int J Syst Evol Microbiol.* 2012;62 Pt 10:2330–5. doi:10.1099/ijs.0.027854-0.
73. Kondo F, Tottori J, Soki K. Ulcerative enteritis in broiler chickens caused by *Clostridium colinum* and in vitro activity of 19 antimicrobial agents in tests on isolates. *Poult Sci.* 1988;67:1424–30. doi:10.3382/ps.0671424.
74. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, et al. Gut microbiota functions: metabolism of nutrients and other food components. *Eur J Nutr.* 2018;57:1–24. doi:10.1007/s00394-017-1445-8.
75. Hedberg ME, Moore ERB, Svensson-Stadler L, Hörstedt P, Baranov V, Hernell O, et al. *Lachnoanaerobaculum* gen. nov., a new genus in the Lachnospiraceae: characterization of *Lachnoanaerobaculum umeaense* gen. nov., sp. nov., isolated from the human small intestine, and *Lachnoanaerobaculum orale* sp. nov., isolated from saliva, and reclassification of *Eubacterium saburreum* (Prevot 1966) Holdeman and Moore 1970 as *Lachnoanaerobaculum saburreum* comb. nov. *Int J Syst Evol Microbiol.* 2012;62 Pt 11:2685–90. doi:10.1099/ijs.0.033613-0.
76. Ntougias S, Lapidus A, Han J, Mavromatis K, Pati A, Chen A, et al. High quality draft genome sequence of *Olivibacter sitiensis* type strain (AW-6(T)), a diphenol degrader with genes involved in the catechol pathway. *Stand Genomic Sci.* 2014;9:783–93. doi:10.4056/sigs.5088950.
77. Velasco SE, Yebra MJ, Monedero V, Ibarburu I, Dueñas MT, Irastorza A. Influence of the carbohydrate source on beta-glucan production and enzyme activities involved in sugar metabolism in *Pediococcus parvulus* 2.6. *Int J Food Microbiol.* 2007;115:325–34. doi:10.1016/j.ijfoodmicro.2006.12.023.
78. Táncsics A, Kéki Z, Márialigeti K, Schumann P, Tóth EM. *Siphonobacter aquaeclarae* gen. nov., sp. nov., a novel member of the family 'Flexibacteraceae', phylum Bacteroidetes. *Int J Syst*

784 Evol Microbiol. 2010;60 Pt 11:2567–71. doi:10.1099/ijs.0.019398-0.

785 79. Shah HN. *Anaerorhabdus*. In: Bergey's manual of systematics of archaea and bacteria.

786 Whitman WB, Rainey F, Kämpfer P, Trujillo M, Chun J, DeVos P, et al., editors. Wiley; 2015. p.

787 1–2. doi:10.1002/9781118960608.gbm00237.

788 80. Saman S, Sarman S, Slattery P. Isolation of a potential new member of the *Bacillus cereus*

789 group from snow covered soil. Life Sciences and Medicine Research. 2010.

790 81. Himelbloom BH, Canale-Parola E. *Clostridium methylpentosum* sp. nov.: a ring-shaped

791 intestinal bacterium that ferments only methylpentoses and pentoses. Arch Microbiol.

792 1989;151:287–93. doi:10.1007/BF00406553.

793 82. Cornick NA, Jensen NS, Stahl DA, Hartman PA, Allison MJ. *Lachnospira pectinoschiza* sp.

794 nov., an anaerobic pectinophile from the pig intestine. Int J Syst Bacteriol. 1994;44:87–93.

795 doi:10.1099/00207713-44-1-87.

796 83. Arai R, Tominaga K, Wu M, Okura M, Ito K, Okamura N, et al. Diversity of *Melissococcus*

797 *plutonius* from honeybee larvae in Japan and experimental reproduction of European foulbrood

798 with cultured atypical isolates. PLoS One. 2012;7:e33708. doi:10.1371/journal.pone.0033708.

799 84. Sakamoto M, Suzuki N, Matsunaga N, Koshihara K, Seki M, Komiya H, et al.

800 *Parabacteroides gordonii* sp. nov., isolated from human blood cultures. Int J Syst Evol

801 Microbiol. 2009;59 Pt 11:2843–7. doi:10.1099/ijs.0.010611-0.

802 85. Gérard P. Metabolism of cholesterol and bile acids by the gut microbiota. Pathogens.

803 2013;3:14–24. doi:10.3390/pathogens3010014.

804 86. Andrić P, Meyer AS, Jensen PA, Dam-Johansen K. Reactor design for minimizing product

805 inhibition during enzymatic lignocellulose hydrolysis: I. Significance and mechanism of

806 cellobiose and glucose inhibition on cellulolytic enzymes. Biotechnol Adv. 2010;28:308–24.

807 doi:10.1016/j.biotechadv.2010.01.003.

808 87. Peng B, Zhao C, Kasakov S, Foraita S, Lercher JA. Manipulating catalytic pathways:

809 deoxygenation of palmitic acid on multifunctional catalysts. Chem Eur J. 2013;19:4732–41.

810 doi:10.1002/chem.201203110.

811 88. Seyyedini S, Nazem MN. Histomorphometric study of the effect of methionine on small
812 intestine parameters in rat: an applied histologic study. *Folia Morphol (Warsz)*. 2017.
813 doi:10.5603/FM.a2017.0044.

814 89. Brosnan JT, Brosnan ME, Bertolo RFP, Brunton JA. Methionine: A metabolically unique
815 amino acid. *Livest Sci*. 2007;112:2–7. doi:10.1016/j.livsci.2007.07.005.

816 90. Sanderson SM, Gao X, Dai Z, Locasale JW. Methionine metabolism in health and cancer: a
817 nexus of diet and precision medicine. *Nat Rev Cancer*. 2019;19:625–37. doi:10.1038/s41568-019-
818 0187-8.

819 91. Al-Jasser AM, Enani MA, Al-Fagih MR. Endocarditis caused by *Abiotrophia defectiva*.
820 *Libyan J Med*. 2007;2:43–5. doi:10.4176/061223.

821 92. Rautio M, Eerola E, Väisänen-Tunkelrott M-L, Molitoris D, Lawson P, Collins MD, et al.
822 Reclassification of *Bacteroides putredinis* (Weinberg et al., 1937) in a new genus *Alistipes* gen.
823 nov., as *Alistipes putredinis* comb. nov., and description of *Alistipes finegoldii* sp. nov., from
824 human sources. *Syst Appl Microbiol*. 2003;26:182–8. doi:10.1078/072320203322346029.

825 93. Parker BJ, Wearsch PA, Veloo ACM, Rodriguez-Palacios A. The genus *alisticipes*: gut bacteria
826 with emerging implications to inflammation, cancer, and mental health. *Front Immunol*.
827 2020;11:906. doi:10.3389/fimmu.2020.00906.

828 94. Murray WD, Sowden LC, Colvin JR. *Bacteroides cellulosolvens* sp. nov., a Cellulolytic
829 Species from Sewage Sludge. *Int J Syst Bacteriol*. 1984;34:185–7. doi:10.1099/00207713-34-2-185.

830 95. Benítez-Páez A, Gómez Del Pulgar EM, Sanz Y. The Glycolytic Versatility of *Bacteroides*
831 *uniformis* CECT 7771 and Its Genome Response to Oligo and Polysaccharides. *Front Cell Infect*
832 *Microbiol*. 2017;7:383. doi:10.3389/fcimb.2017.00383.

833 96. Desvaux M, Guedon E, Petitdemange H. Cellulose catabolism by *Clostridium cellulolyticum*
834 growing in batch culture on defined medium. *Appl Environ Microbiol*. 2000;66:2461–70.
835 doi:10.1128/aem.66.6.2461-2470.2000.

836 97. Koendjibiharie JG, Wiersma K, van Kranenburg R. Investigating the Central Metabolism of
837 *Clostridium thermosuccinogenes*. *Appl Environ Microbiol*. 2018;84. doi:10.1128/AEM.00363-18.

98. Surkov AV, Dubinina GA, Lysenko AM, Glöckner FO, Kuever J. *Dethiosulfovibrio russensis* sp. nov., *Dethiosulfovibrio marinus* sp. nov. and *Dethiosulfovibrio acidaminovorans* sp. nov., novel anaerobic, thiosulfate- and sulfur-reducing bacteria isolated from “Thiodendron” sulfur mats in different saline environments. *Int J Syst Evol Microbiol.* 2001;51 Pt 2:327–37. doi:10.1099/00207713-51-2-327.
99. Collins MD, Hutson RA, Foster G, Falsen E, Weiss N. *Isobaculum melis* gen. nov., sp. nov., a *Carnobacterium*-like organism isolated from the intestine of a badger. *Int J Syst Evol Microbiol.* 2002;52 Pt 1:207–10. doi:10.1099/00207713-52-1-207.
100. Weiss N, Schillinger U, Laternser M, Kandler O. *Lactobacillus sharpeae* sp. nov. and *Lactobacillus agilis* sp. nov., two new species of homofermentative, meso-diaminopimelic acid-containing lactobacilli isolated from sewage. *Zentralblatt für Bakteriologie Mikrobiologie und Hygiene: I Abt Originale C: Allgemeine, angewandte und ökologische Mikrobiologie.* 1981;2:242–53. doi:10.1016/S0721-9571(81)80005-1.
101. Nakajima M, Yoshida R, Miyanaga A, Abe K, Takahashi Y, Sugimoto N, et al. Functional and Structural Analysis of a β -Glucosidase Involved in β -1,2-Glucan Metabolism in *Listeria innocua*. *PLoS One.* 2016;11:e0148870. doi:10.1371/journal.pone.0148870.
102. Khan H, Chung EJ, Kang DY, Jeon CO, Chung YR. *Mucilaginibacter jinjuensis* sp. nov., with xylan-degrading activity. *Int J Syst Evol Microbiol.* 2013;63 Pt 4:1267–72. doi:10.1099/ijms.0.043828-0.
103. Zhang B, Tong H, Dong X. *Pediococcus cellicola* sp. nov., a novel lactic acid coccus isolated from a distilled-spirit-fermenting cellar. *Int J Syst Evol Microbiol.* 2005;55 Pt 5:2167–70. doi:10.1099/ijms.0.63778-0.
104. Carlier J-P, K’ouas G, Han XY. *Moryella indoligenes* gen. nov., sp. nov., an anaerobic bacterium isolated from clinical specimens. *Int J Syst Evol Microbiol.* 2007;57 Pt 4:725–9. doi:10.1099/ijms.0.64705-0.
105. Orelli LR, Garcia MB, Niemevz F, Perillo IA. Selective Monoformylation Of 1,3-Diaminopropane Derivatives. *Synth Commun.* 1999;29:1819–33.

doi:10.1080/00397919908086172.

106. Venkatesan K, Srinivasan KV. A novel stereoselective synthesis of pachastrissamine (jaspine B) starting from 1-pentadecanol. *Tetrahedron: Asymmetry*. 2008;19:209–15. doi:10.1016/j.tetasy.2007.12.001.

107. Hu X, Shen J, Pu X, Zheng N, Deng Z, Zhang Z, et al. Urinary Time- or Dose-Dependent Metabolic Biomarkers of Aristolochic Acid-Induced Nephrotoxicity in Rats. *Toxicol Sci*. 2017;156:123–32. doi:10.1093/toxsci/kfw244.

108. Hansen KB, Rosenkilde MM, Knop FK, Wellner N, Diep TA, Rehfeld JF, et al. 2-Oleoyl glycerol is a GPR119 agonist and signals GLP-1 release in humans. *J Clin Endocrinol Metab*. 2011;96:E1409-17. doi:10.1210/jc.2011-0647.

109. Chang YF. Lysine metabolism in the human and the monkey: demonstration of pipecolic acid formation in the brain and other organs. *Neurochem Res*. 1982;7:577–88. doi:10.1007/BF00965124.

110. Lepkovsky S, Roboz E, Haagen-Smit AJ. Nutrition classics from *The Journal of Biological Chemistry* 149:195-201, 1943. Xanthurenic acid and its rôle in the tryptophane metabolism of pyridoxine-deficient rats. *Nutr Rev*. 1974;32:338–9. doi:10.1111/j.1753-4887.1974.tb07326.x.

111. Gomez A, Petrzalkova KJ, Burns MB, Yeoman CJ, Amato KR, Vlckova K, et al. Gut microbiome of coexisting baaka pygmies and bantu reflects gradients of traditional subsistence patterns. *Cell Rep*. 2016;14:2142–53. doi:10.1016/j.celrep.2016.02.013.

112. Appeldoorn MM, Vincken J-P, Aura A-M, Hollman PCH, Gruppen H. Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-gamma-valerolactone as the major metabolites. *J Agric Food Chem*. 2009;57:1084–92. doi:10.1021/jf803059z.

113. Xiong X, Liu D, Wang Y, Zeng T, Peng Y. Urinary 3-(3-Hydroxyphenyl)-3-hydroxypropionic Acid, 3-Hydroxyphenylacetic Acid, and 3-Hydroxyhippuric Acid Are Elevated in Children with Autism Spectrum Disorders. *Biomed Res Int*. 2016;2016:9485412. doi:10.1155/2016/9485412.

892 114. Ichihara A, Greenberg DM. Pathway of serine formation from carbohydrate in rat liver.
893 Proc Natl Acad Sci USA. 1955;41:605–9. doi:10.1073/pnas.41.9.605.

894 115. Howell RK, Lee M. Influence of alpha-ketoacids on the respiration of brain in vitro. Proc
895 Soc Exp Biol Med. 1963;113:660–3. doi:10.3181/00379727-113-28456.

896 116. Hidese R, Mihara H, Esaki N. Bacterial cysteine desulfurases: versatile key players in
897 biosynthetic pathways of sulfur-containing biofactors. Appl Microbiol Biotechnol. 2011;91:47–
898 61. doi:10.1007/s00253-011-3336-x.

899 117. Zerenturk EJ, Sharpe LJ, Ikonen E, Brown AJ. Desmosterol and DHCR24: unexpected new
900 directions for a terminal step in cholesterol synthesis. Prog Lipid Res. 2013;52:666–80.
901 doi:10.1016/j.plipres.2013.09.002.

902 118. Maiuolo J, Oppedisano F, Gratteri S, Muscoli C, Mollace V. Regulation of uric acid
903 metabolism and excretion. Int J Cardiol. 2016;213:8–14. doi:10.1016/j.ijcard.2015.08.109.

904 119. Martínez E, Hamberg M, Busquets M, Díaz P, Manresa A, Oliw EH. Biochemical
905 characterization of the oxygenation of unsaturated fatty acids by the dioxygenase and
906 hydroperoxide isomerase of *Pseudomonas aeruginosa* 42A2. J Biol Chem. 2010;285:9339–45.
907 doi:10.1074/jbc.M109.078147.

908 120. Zhou X, Liu L, Lan X, Cohen D, Zhang Y, Ravindran AV, et al. Polyunsaturated fatty acids
909 metabolism, purine metabolism and inosine as potential independent diagnostic biomarkers for
910 major depressive disorder in children and adolescents. Mol Psychiatry. 2019;24:1478–88.
911 doi:10.1038/s41380-018-0047-z.

912 121. Hodgson E, Casida JE. Insecticide Metabolism in Mammals, Mammalian Enzymes
913 Involved in the Degradation of 2,2-Dichlorovinyl Dimethyl Phosphate. J Agric Food Chem.
914 1962;10:208–14. doi:10.1021/jf60121a012.

915 122. Ellingboe J, Karnovsky ML. Origin of glycerol ethers. J Bio Chem. 1967;242:5693–9.
916 doi:10.1016/S0021-9258(18)99356-7.

917 123. Nowak A, Libudzisz Z. Influence of phenol, p-cresol and indole on growth and survival of
918 intestinal lactic acid bacteria. Anaerobe. 2006;12:80–4. doi:10.1016/j.anaerobe.2005.10.003.

- 919 124. Costantini A, Pietroniro R, Doria F, Pessione E, Garcia-Moruno E. Putrescine production
920 from different amino acid precursors by lactic acid bacteria from wine and cider. *Int J Food*
921 *Microbiol.* 2013;165:11–7. doi:10.1016/j.ijfoodmicro.2013.04.011.
- 922 125. Mahdi JG. Biosynthesis and metabolism of β -d-salicin: A novel molecule that exerts
923 biological function in humans and plants. *Biotechnol Rep (Amst).* 2014;4:73–9.
924 doi:10.1016/j.btre.2014.08.005.
- 925 126. Kolenbrander HM, Berg CP. Role of urocanic acid in the metabolism of l-histidine. *Arch*
926 *Biochem Biophys.* 1967;119:110–8. doi:10.1016/0003-9861(67)90435-3.
- 927 127. Kwok KM, Choong CKS, Ong DSW, Ng JCQ, Gwie CG, Chen L, et al. Hydrogen-Free Gas-
928 Phase Deoxydehydration of 2,3-Butanediol to Butene on Silica-Supported Vanadium Catalysts.
929 *ChemCatChem.* 2017;9:2443–7. doi:10.1002/cctc.201700301.
- 930 128. Nikitina MA, Ivanova II. Conversion of 2,3-Butanediol over Phosphate Catalysts.
931 *ChemCatChem.* 2016;8:1346–53. doi:10.1002/cctc.201501399.
- 932 129. Nishizawa A, Yabuta Y, Shigeoka S. Galactinol and raffinose constitute a novel function to
933 protect plants from oxidative damage. *Plant Physiol.* 2008;147:1251–63.
934 doi:10.1104/pp.108.122465.
- 935 130. Hochstein LI, Dalton BP, Pollock G. The metabolism of carbohydrates by extremely
936 halophilic bacteria: identification of galactonic acid as a product of galactose metabolism. *Can J*
937 *Microbiol.* 1976;22:1191–6. doi:10.1139/m76-174.
- 938 131. Snyder R, Hedli CC. An overview of benzene metabolism. *Environ Health Perspect.*
939 1996;104 Suppl 6:1165–71. doi:10.1289/ehp.961041165.
- 940 132. Kim SK, Lee DH, Hong J-I, Yoon J. Chemosensors for pyrophosphate. *Acc Chem Res.*
941 2009;42:23–31. doi:10.1021/ar800003f.
- 942 133. Nićiforović N, Abramović H. Sinapic acid and its derivatives: natural sources and
943 bioactivity. *Comp Rev Food Sci Food Safety.* 2014;13:34–51. doi:10.1111/1541-4337.12041.
- 944 134. Keppler K, Humpf H-U. Metabolism of anthocyanins and their phenolic degradation
945 products by the intestinal microflora. *Bioorg Med Chem.* 2005;13:5195–205.

946 doi:10.1016/j.bmc.2005.05.003.

947 135. Hidalgo M, Oruna-Concha MJ, Kolida S, Walton GE, Kallithraka S, Spencer JPE, et al.

948 Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial

949 growth. J Agric Food Chem. 2012;60:3882–90. doi:10.1021/jf3002153.

950 136. Elbein AD, Pan YT, Pastuszak I, Carroll D. New insights on trehalose: a multifunctional

951 molecule. Glycobiology. 2003;13:17R–27R. doi:10.1093/glycob/cwg047.

952

953

954

Table 1. Characterization of Fecal Bacteria in *Aoah*^{-/-} Stool Compared to WT

Fecal Bacterial Species	Absent or Present	Functionality
<i>A. onderdonkii</i>	P	Carbohydrate fermentation; end-product: succinic acid [69]
<i>B. crossotus</i>	P	Th1/Th2 inflammation balance; fermentation of maltose, starch, glycogen, and dextrin [70, 71]
<i>C. alkalithermophilus</i>	P	Cellulose and xylan hydrolysis [72]
<i>C. colinum</i>	P	Fatty acid metabolism; associated with enteric disease [73]
<i>L. longoviformis</i>	P	Dehydrogenation of enterodiol to enterolactone [74]
<i>L. orale</i>	P	No active metabolism [75]
<i>O. sitiensis</i>	P	Diphenol metabolism [76]
<i>P. parvulus</i>	P	Carbohydrate metabolism [77]
<i>S. aquaeclarae</i>	P	Production of catalase and gelatinase [78]
<i>A. furcosa</i>	A	Esculin hydrolysis; end-products: acetic and lactic acids [79]
<i>B. samanii</i>	A	Glucose, lactose, maltose, sucrose, and trehalose fermentation [80]
<i>C. methylpentosum</i>	A	Fermentation of methylpentoses and pentoses [81]
<i>L. pectinoschiza</i>	A	Carbohydrate metabolism [82]
<i>M. plutonius</i>	A	Glucose, fructose, and mannose metabolism [83]
<i>P. gordonii</i>	A	L-arabinose production [84]

955

956

Table 2. Characterization of Fecal Metabolites in *Aoah*^{-/-} Stool Compared to WT

Fecal Metabolites	Absent or Present	Functionality
24-Ethyl-delta(22)-coprostenol	P	Derived from cholesterol metabolism [85]
Cellobiose	P	Reducing sugar; Glucose hydrolysis [86]
Hexadecanol	P	Palmitic acid reduction [87]
Methionine	A	Polyamine and creatine synthesis; conversion to S-adenosylmethionine; goblet cell development in small intestine [88–90]

957

958

FIGURE LEGENDS

Figure 1. Female acyloxyacyl hydrolase-deficient (*Aoah*^{-/-}) mice exhibit enlarged ceca and compromised gut permeability. **A.** Comparison of representative cecum sizes (arrows) of female wild-type (WT) C57BL/6 (bottom) and *Aoah*^{-/-} (top) mice. **B.** Female *Aoah*^{-/-} mice showed increased body weight versus WT mice (WT mice: n=4 and *Aoah*^{-/-} mice n=7, P<0.0001, Student's t test, two tailed). No differences were observed in the mass of the cecum and cecal stool (WT mice: n=4 and *Aoah*^{-/-} mice n=6, P > 0.05, Student's t test, two tailed). Bars represented as average ± SEM **C.** Ceca of female *Aoah*^{-/-} mice showed a decrease in transepithelial electrical resistance (TEER) compared to WT (WT mice: n=6 and *Aoah*^{-/-} mice n=5, P<0.01, Student's t test, two tailed). Data represented as individual values and average (represented by horizontal line) ± SEM.

Figure 2. AOAHD-deficient mice have normal gut morphology but altered gut signaling pathways. **A.** Comparison of representative cecum H&E staining of female WT (top left) and *Aoah*^{-/-} (top right) mice (scale bar: 50 µm). Immunostaining of AOAHD (green) in WT (bottom left) and *Aoah*^{-/-} (bottom right) cecum. DAPI staining nuclei shown in blue (scale bar: 17 µm). **B.** Pie chart of transcriptome profiling by microarray analysis showing levels of mRNA with 2-fold difference in AOAHD-deficient cecum compared to wild-type. **C.** Pathway analysis indicating top 10 signaling pathways altered in AOAHD-deficient cecum compared to wild-type.

Figure 3. *Aoah*^{-/-} mice exhibit gut dysbiosis. **A.** PCA plot of 16S rRNA analyses of fecal stool. Dots represent individual mice; AOAHD-deficient mice shown in blue (n=5) and wild-type mice shown in red (n=5). **B.** α-diversity as measured by observed operational taxonomic units (OTUs), n=5 for both groups, P<0.05. **C.** Heat map of 16S rRNA analyses of AOAHD-deficient (blue) and wild-type (red) fecal stool (n=5). **D.** Quantification of bacterial phyla using 16S rRNA sequencing in wild-type and AOAHD-deficient fecal stool (n=5).

Figure 4. Microbiome-dependent changes in the functional characterization of bacterial communities. Biological functions were predicted by implementing Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analyses using 16S rRNA sequence data from wild-type and AOA-deficient fecal samples. Changes in the gut microbiome by genetic manipulation (*AoaH*^{-/-}) or by co-housing (*AoaH*^{-/-} co-h and B6 co-h) resulted in altered predictions including functions related to the RIG-I-like receptor signaling pathway (A), basal transcription factors (B), African trypanosomiasis (C), alpha-linolenic acid metabolism (D), meiosis (yeast, E), steroid hormone biosynthesis (F), mineral absorption (G), and carotenoid biosynthesis (H). n=5, *P<0.05, **P<0.01, ***P<0.001, One-Way ANOVA followed by post-hoc Tukey HSD. Data represented as average ± SEM.

Figure 5. *AoaH*^{-/-} mice exhibit altered fecal metabolomics. A. PCA plot of metabolomics of fecal stool. Dots represent individual mice; AOA-deficient mice shown in blue (n=5) and wild-type mice shown in red (n=5). B. Heat map of metabolomics of AOA-deficient (blue) and wild-type (red) fecal stool (n=5).

Figure 6. Converging microbiota by co-housing results in altered gut morphology and TEER. A. Comparison of representative cecum sizes of female WT (top left), *AoaH*^{-/-} (bottom left), co-housed WT (top right), and co-housed *AoaH*^{-/-} (bottom right) mice. B. PCA plot of 16S rRNA analyses of fecal stool in like-housed and co-housed animals. Dots represent individual mice; AOA-deficient mice shown in red and wild-type mice shown in blue. n=5 for like-housed conditions and n=10 for co-housed condition (circled in green). C. *AoaH*^{-/-} mice showed an improvement in cecum TEER after co-housing (like-housed WT mice: n=6, like-housed *AoaH*^{-/-} mice: n=5, co-housed WT mice: n=8, co-housed *AoaH*^{-/-} mice: n=6; *P<0.05, **P<0.01, One-Way ANOVA followed by post-hoc Tukey HSD). Data represented as individual values and average (represented by horizontal line) ± SEM. D. *AoaH*^{-/-} mice showed an improvement in bladder

TEER after co-housing (like-housed WT mice: n=6, like-housed *Aoah*^{-/-} mice: n=6, co-housed WT mice: n=9, co-housed *Aoah*^{-/-} mice: n=6; *P<0.05, One-Way ANOVA followed by post-hoc Tukey HSD). Data represented as individual values and average (represented by horizontal line) ± SEM.

Figure 7. Pelvic pain phenotype is microbiome-dependent. **A.** AOA-deficient mice exhibited increased pelvic allodynia compared to WT, which was partially alleviated by co-housing for 28 d as shown through response to von Frey filaments stimulating the pelvic region (n=10; *P<0.05, Student's t test, two tailed). Data represented as average response (%) ± SEM. **B.** Visceromotor response (VMR) was quantified in mice as electromyography (EMG) activity in response to bladder distension under anesthesia. EMG was elevated in AOA-deficient mice, which was alleviated by co-housing for 7 d (WT mice: n=15, *Aoah*^{-/-} mice: n=9, co-housed (co-h) *Aoah*^{-/-} mice: n=9; *P<0.05, One-Way ANOVA followed by post-hoc Tukey HSD). Data represented as average EMG activity (%) ± SEM. **C.** Measurement of VMR following stool slurry gavage (SSG) from WT and *Aoah*^{-/-} donors. EMG was elevated in AOA-deficient mice that received SSG from other AOA-deficient mice compared to WT mice that received SSG from other WT mice. SSG in AOA-deficient mice from WT donors resulted in reduced EMG activity (WT mice: n=5 and *Aoah*^{-/-} mice conditions: n=4; *P<0.05, One-Way ANOVA followed by post-hoc Tukey HSD). Data represented as average EMG activity (%) ± SEM. **D.** Male AOA-deficient mice exhibited increased pelvic allodynia following SSG of anaerobic cultures from IC/BPS patients compared to AOA-deficient mice that received SSG from healthy patients, as shown through response to von Frey filaments stimulating the pelvic region (n=10 for healthy flora, n=12 for IC/BPS flora; *P<0.05, Student's t test, two tailed). Data represented as average response (%) ± SEM.

Figure 8. Anxiety-like behavior in AOA-deficient mice is microbiome-dependent. **A.** Analyses of defensive burying behavior revealed AOA-deficient mice exhibited increased anxiety-like behavior as measured through number of burying attempts compared to WT mice (WT mice: n=5

1047 and *Aoah*^{-/-} mice conditions: n=4; *P<0.05, One-Way ANOVA followed by post-hoc Tukey
 1048 HSD). Data represented as average number of burying attempts (for 15 min) ± SEM. **B.** AOA-
 1049 deficient mice showed reduced burying attempts 7 d following SSG from WT donors compared
 1050 to AOA-deficient mice that received SSG from AOA-deficient donors (WT mice: n=5 and
 1051 *Aoah*^{-/-} mice conditions: n=4; *P<0.05, One-Way ANOVA followed by post-hoc Tukey HSD).
 1052 Data represented as average number of burying attempts (for 15 min) ± SEM.
 1053
 1054

Figure 1 Rahman-Enyart et al.

Figure 2 Rahman-Enyart et al.

Figure 3 Rahman-Enyart et al.

Figure 4 Rahman-Enyart et al.

Figure 5 Rahman-Enyart et al.

A

B

Figure 6 Rahman-Enyart et al.

Figure 7 Rahman-Enyart et al.

Figure 8 Rahman-Enyart et al.