

Complex system modelling reveals oxalate homeostasis is driven by diverse oxalate-degrading bacteria

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In this **valuable** contribution, the authors present an approach based on a complex systems theoretical framework to characterize diet-host-microbe interactions and to develop targeted bacteriotherapies using a three-phase workflow. Overall, the **solid** results provide a reference for microbial community research and insights to guide future studies. However, the theoretical systems approach would benefit from further description, and some claims regarding oxalate bacterial metabolism in complex microbial communities could be strengthened. This study will interest researchers working on gut microbiomes specifically those seeking to modulate host-microbial interactions.

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Abstract

Decades of research have made clear that host-associated microbiomes touch all facets of health. However, effective therapies that target the microbiome have been elusive given its inherent complexity. Here, we experimentally examined diet-microbe-host interactions through a complex systems framework, centered on dietary oxalate. Using multiple, independent molecular, animal, and *in vitro* experimental models, we found that microbiome composition influenced multiple oxalate-microbe-host interfaces. Importantly, administration of the oxalate-degrading specialist, *Oxalobacter formigenes*, was only effective against a poor oxalate-degrading microbiota background and gives critical new insights into why clinical intervention trials with this species exhibit variable outcomes. Data suggest that, while heterogeneity in the microbiome impacts multiple diet-host-microbe interfaces, metabolic redundancy among diverse microorganisms in specific diet-microbe axes is a critical variable that may impact the efficacy of bacteriotherapies, which can help guide patient and probiotic selection criteria in probiotic clinical trials.

Introduction

Research into the host-associated microbiome is at an inflection point. Decades of research have revealed that host-associated microbiomes are intimately linked to host health and touch all aspects of host physiology^{1,2}. The first phase of microbiome research was focused on descriptive studies that characterized differences in the microbiome by host species, body site, and disease phenotypes^{3–5}. Clinical case:control metagenome wide association studies that found differences in microbiome composition between healthy and disease cohorts gave rise to the ambiguous term “dysbiosis” and much of the literature is focused on “balancing” the gut microbiota⁶. In the second, and current, phase of research, studies have moved towards gaining more mechanistic insight into specific microbe-host interactions that influence host physiology and disease^{6–9}. However, it is increasingly being recognized that, given the wide variability in microbiome composition, host genetics, and lifestyles, understanding mechanisms and causal relationships between gut bacteria and host physiological responses is not enough for the development of effective bacteriotherapies that target the gut microbiota^{10,11}. In fact, many probiotic clinical trials, with known mechanistic links between the probiotics and host physiology, have exhibited wide variability in results^{12–15}. It is currently unclear what sources of variability may drive the response to targeted bacteriotherapies. Some variables that have been proposed include heterogeneity in microbiome composition, alcohol consumption, and bowel movement quality¹⁰.

Complex system theory is one means to potentially constrain hypotheses and bridge the gap between mechanistic studies and effective bacteriotherapies^{16,17}. Studies of the host-associated microbiome often casually acknowledge that these systems are complex and, while, there has been progress made in developing mathematical approaches to complex modelling of host-microbiome relationships^{16–19}, few studies have bridged the gap between experimental biology and complex system theory.

Mammals and their gut microbiome, are often considered as a holobiont²⁰, defined as a discrete unit that exhibits collective action and evolves as a unit. Using evolutionary theory approaches, it is hypothesized that the form and function of the microbial part of the holobiont is driven primarily by host-microbe influences, but which require microbe- microbe interactions to help manage the enormous burden of microbiome constraint, which is termed an “ecosystem on a leash”²¹. Under this hypothesis, microbe-host interactions, in which host-associated microbes evolve to produce metabolic by- products for the sole benefit of the host, are necessarily rare. Essential features of complex systems include functional redundancy^{21,22} and cooperation through chains of direct interactions^{21–23}, distinct functional nodes that process and transfer resources^{22,23}, fractality^{22,23}, and adaptability through specific homeostatic feedback mechanisms to maintain relatively consistent internal conditions given a dynamic external environment^{21,24}. While considerable progress has been made in understanding the nature of complex systems, there is a lack of consensus on basic terminology and broadly applicable analytical or experimental models²⁵. This limitation is due in part because complex systems necessarily transect many divergent entities in nature and is thus studied in diverse scientific disciplines²⁵. Thus, while some essential features of complexity have been elucidated within specific real-world systems, such as the conversion of polysaccharides to butyrate by multiple species in the gut²⁶, or feedback loops in blood pressure regulation²⁷, experimental evidence of multiple features of complexity within a single system is sparse, especially in microbe-host systems.

To overcome the limitations of applying complex systems theory to the microbe-host holobiont, new models and experimental frameworks are needed that balance complexity with tractability. Antinutrients, produced in plants to deter herbivory, disrupt homeostasis by targeting the function

of the microbiome, host, or both²⁸, and provide an effective focal point to study complexity. Oxalate is an antinutrient present in many plant-based foods, which typically provides the majority of oxalate in circulation²⁹, but is also produced as a terminal metabolite in the liver³⁰. While some host genetic mutations increase endogenous production of oxalate³¹, mammals do not produce enzymes capable of degrading oxalate³². However, multiple oxalate-degrading bacteria exist in the gut, which degrade oxalate through one of a handful of simple metabolic pathways involving one or two genes^{33–35}, which isolates this function to the gut microbiota. As such, oxalate degradation as a function, exhibits a moderate amount of complexity compared to other gut microbiota functions, such as the production of trimethylamine N-oxide (TMAO), that requires host input³⁶. Beyond the moderate complexity, it is known that oxalate-degrading bacteria are susceptible to antibiotics and that antibiotic use decreases oxalate degradation^{37–41}. Elevated levels of oxalate induce oxidative stress, activates the inflammasome, and disrupts epithelial barrier function through tight junction proteins^{42–44}. This molecule has been linked to diseases including kidney stones and chronic kidney disease^{45–47}, breast cancer⁴⁸, and cardiometabolic disorders such as atherosclerosis⁴⁹, obesity⁵⁰, and diabetes⁵⁰. *Oxalobacter formigenes*, which uses oxalate as a sole carbon and energy source³³, is a well-studied oxalate-degrading species of bacteria. The negative, at times lethal, effects of oxalate, along with the identification and mechanisms of oxalate-degrading bacteria in the gut, have been worked out for several decades^{33,51–54}, pre-dating the current microbiome era.

Despite this knowledge, clinical intervention trials involving *Oxalobacter formigenes*, which is perhaps the most effective oxalate-degrading species known, have successfully resulted in a significant reduction in urine oxalate levels in only 43% of studies^{15,55}. Oxalate-degrading lactic acid bacteria have been successful in 37.5% of studies¹⁵. Both treatments led to a wide variability in patient responses. Given these data and the well worked out mechanisms of oxalate metabolism, it is clear that having an understanding of the mechanistic links between the gut microbiota and host physiology, alone, is demonstrably not sufficient to develop effective bacteriotherapies. Therefore, even though the history of oxalate-microbe-host interactions is much greater than most other microbe-host systems, oxalate degradation represents an accurate reflection of the challenges faced by microbiome research and is a prime candidate for complex system modelling to understand the critical variables that determine responsiveness to bacteriotherapies.

The objectives of the current study were to evaluate oxalate-microbe-host interactions, within the framework of complex systems. We used multi-omic approaches utilizing multiple independent *in vivo* and *in vitro* models to understand the critical variables that influence the gut microbiota's maintenance of oxalate homeostasis and its impact on the host. We targeted several potential oxalate-microbe-host interfaces that include the gut microbiota itself, which is one of the first lines of defense against antinutrients^{56,57}, intestinal epithelium, which is an important barrier between microbe and host²¹, the liver, which is important for the biotransformation of dietary or microbial metabolites⁵⁸, along with the kidneys and vasculature, where calcium oxalate can potentially form calcified deposits or induce inflammation^{49,59–61}. Collectively, data indicate that multiple oxalate-microbe-host interfaces are influenced significantly by gut microbiota composition and that harboring diverse microorganisms capable of degrading oxalate can limit the impact of *O. formigenes* as a probiotic. Based on results from this study, we propose a phased approach for the development of bacteriotherapies whereby clinical case:control studies determine whether or not a clinical phenotype is associated with the microbiome and the microbial taxa/functions associated with specific phenotypes (Phase I).

Hypothesized taxa and/or microbial functions should be mechanistically resolved through *in vitro* and germ-free animal studies (Phase II). Finally, mechanistic insights should be applied to a complex systems theoretical framework to identify those variables that most impact the potential

success of bacteriotherapies (Phase III). Such a phased approach would have broad implications for patient and probiotic selection in the development of targeted bacteriotherapies.

Results

Defining the oxalate-microbe-host as a complex system

To use complex system modelling as a means to identify the most pertinent variables that impact oxalate-microbe-host interactions, we first had to define the system as a series of nodes, whereby oxalate is processed, connections, where the end-product of one node is transferred to another node, and fractals, which are autonomous subunits or layers that exhibit analogous, but unique, functions to other layers. For antinutrients, such as oxalate, we can define four unique nodes where these molecules can either be transformed or where they can transform the host. The first node is the stomach where the low pH can biotransform some molecules, such as glucosinolates⁶². Some proportion of the molecules can be absorbed into the circulation in the stomach.

Stomach contents are then transferred to the intestines, the second node, where they are first exposed to high densities of gut bacteria, particularly in the colon. Within the gut microbiota, there are two fractal layers that include microbial species and the genes within each species. In each of these fractal layers, there are three hypotheses that can be generated about how microbes can degrade oxalate or other antinutrients. These include a) one species/gene, one function; b) multiple species/genes, one function; or c) multiple species, multiple functions. Canonically, the hypothesis is that one single species, *O. formigenes*, is primarily responsible for oxalate degradation⁶³, which is achieved through a two gene metabolic pathway (oxalyl-CoA decarboxylase and formyl-CoA transferase)^{53,54}. However, more recent studies show that many species of gut bacteria degrade oxalate and multiple metabolic pathways can be used to degrade oxalate that comprise of one or two genes^{34,35,64,65}. Furthermore, studies have shown that there are broad interactions between the gut microbiota and oxalate degradation^{66–72}. Finally, two of the most common by-products of oxalate degradation are CO₂ and formate. Importantly, these by-products can be used in downstream metabolic pathways such as acetogenesis, methanogenesis, and sulfate-reduction^{73–76}. Given the inconsistent results in *O. formigenes* trials and the recognition that there are broad gut microbiota-oxalate interactions that maintain oxalate homeostasis, we can hypothesize that, instead of relying on a single species, oxalate homeostasis is maintained either by diverse oxalate-degrading bacteria or by cooperative networks whereby a small number of oxalate-degrading bacteria produce substrates to be used in downstream metabolic pathways (i.e. multiple species, one function or multiple species, multiple functions).

Again, some proportion of oxalate in the intestines gets absorbed into circulation or is excreted in the stool. However, oxalate can also be secreted back into the gut through transporters such as SLC26a6^{77,78}. Another proportion of the molecule can be transported through the portal vein to the liver for hepatic biotransformation⁷⁹.

Importantly, for oxalate, the liver is a source, rather than a sink³². However, for other molecules, such as trimethylamine that is produced by gut bacteria, the liver is critical for converting the molecule to the more pathogenic TMAO form³⁶. Some complex antinutrients, such as creosote, require both the gut microbiota and the liver⁸⁰. The liver can also be divided into fractal layers based on cell type and genes. Oxalate or other antinutrients that are neither excreted in the stool, nor degraded by the gut microbiota, nor degraded by the liver, can impact one or more organs and interact with the host through localized immune responses, with the remaining being excreted in the urine.

As such, this model sets up multiple testable hypotheses, which are explored in depth, below.

Constitutive and oxalate-dependent effects of the microbiome impact host hepatic gene expression

Both the gut microbiota and the liver are important organs for the neutralization of antinutrients^{58,81}, indicative of functional redundancy, cooperation, and fractality. While it is known that the liver does not degrade oxalate itself, it could still be impacted by oxalate, in gut microbiota dependent ways. To determine the effects of oxalate exposure and microbiome on host hepatic gene expression, we used a fecal transplant model to examine different host microbiomes with the same host genetics. Specifically, a five-day course of neomycin was used to suppress the native gut microbiota of Swiss- Webster mice (SWM)³⁸, followed by fecal transplants either from SWM (allograft; SW- SW) or *N. albigulia* (NALB), which has a highly effective and transferable oxalate- degrading gut microbiota^{68,69,72}, (xenograft; SW-NALB). Subsequently, animals were fed either a 0% or 1.5% oxalate diet (Evigo, **Fig. 1A**, Table S1). Liver tissue was obtained after three weeks and processed for bulk RNAseq. The RNAseq data analyses revealed constitutive microbiome and microbiome-dependent oxalate effects (**Fig. 1B,C**), whereby SW-SW mice exhibited an oxalate-dependent alteration of 219 hepatic genes, with a net increase in activity, while the SW-NALB mice exhibited an oxalate- dependent alteration of 21 genes with a net decrease in activity (**Fig. 1C**, Table S2). In the SW-NALB mice, the primary response was a decrease in sulfation activity with oxalate exposure, which is involved in the deactivation, detoxication, and excretion of xenobiotics (**Fig. 1C**), and suggests oxalate may be beneficial for this host-microbe system⁸². The primary response in the SW-SW mice was an increase in mitochondrial activity, translation, protein regulation, and ribosome biogenesis, indicative of oxalate- induced hepatic stress^{49,83}. Since hosts only differed by gut microbiota composition, these data demonstrate causative interactions between the gut microbiota and liver activity through oxalate-dependent and independent pathways. Within the framework of complex systems, results show microbe-host cooperation whereby oxalate effectively processed within the SW-NALB gut microbiota reduced overall liver activity, indicative of a beneficial impact. Data also suggest that both the gut microbiota and the immune system are involved in oxalate remediation (redundancy), such that if oxalate cannot be neutralized in the gut microbiota or liver, then the molecule will be processed through host immune response mechanisms (fractality), in this case indicated through an overall increase in hepatic activity and specifically in mitochondrial activity.

Constitutive and oxalate-dependent effects of the microbiome impact microbial metabolic activity in the gut

Microbe-microbe interactions are important features of the microbe-host holobiont within the context of complex systems, both in terms of constraining the microbiome and in processing dietary components^{21,22,26,56}. To assess changes to microbial metabolic output with oxalate exposure, we used the same animals as above. Following the diet trial, colon stool was collected post-necropsy and processed for untargeted metabolomics, which is a measure of total microbial metabolic output. Collectively, results are indicative of constitutive microbiome and microbiome-dependent oxalate effects (**Fig. 2A,B**). The SW-SW mice exhibited an alteration of 162 microbial metabolites upon oxalate exposure, with a net decrease in activity compared to the no oxalate group, whereas the SW-NALB mice exhibited an alteration of 83 microbial metabolites, with a net increase in activity compared to the no oxalate group (**Fig. 2B**, Table S3). In SW-NALB mice, the primary response was an increase in lipid metabolism and a shift (increase/decrease) in fatty acid, secondary metabolite, and alkaloid profiles. In the SW-SW mice, the primary response was an increase in fatty acid synthesis and phenylalanine metabolism, and a decrease in synthesis of secondary metabolites, cholesterol, and alkaloids (**Fig. 2B**). Therefore, while oxalate had a much greater impact on gut microbial metabolism in SW-SW mice overall, the metabolic pathways impacted were similar. Net changes in microbial metabolites produced are indicative of a negative

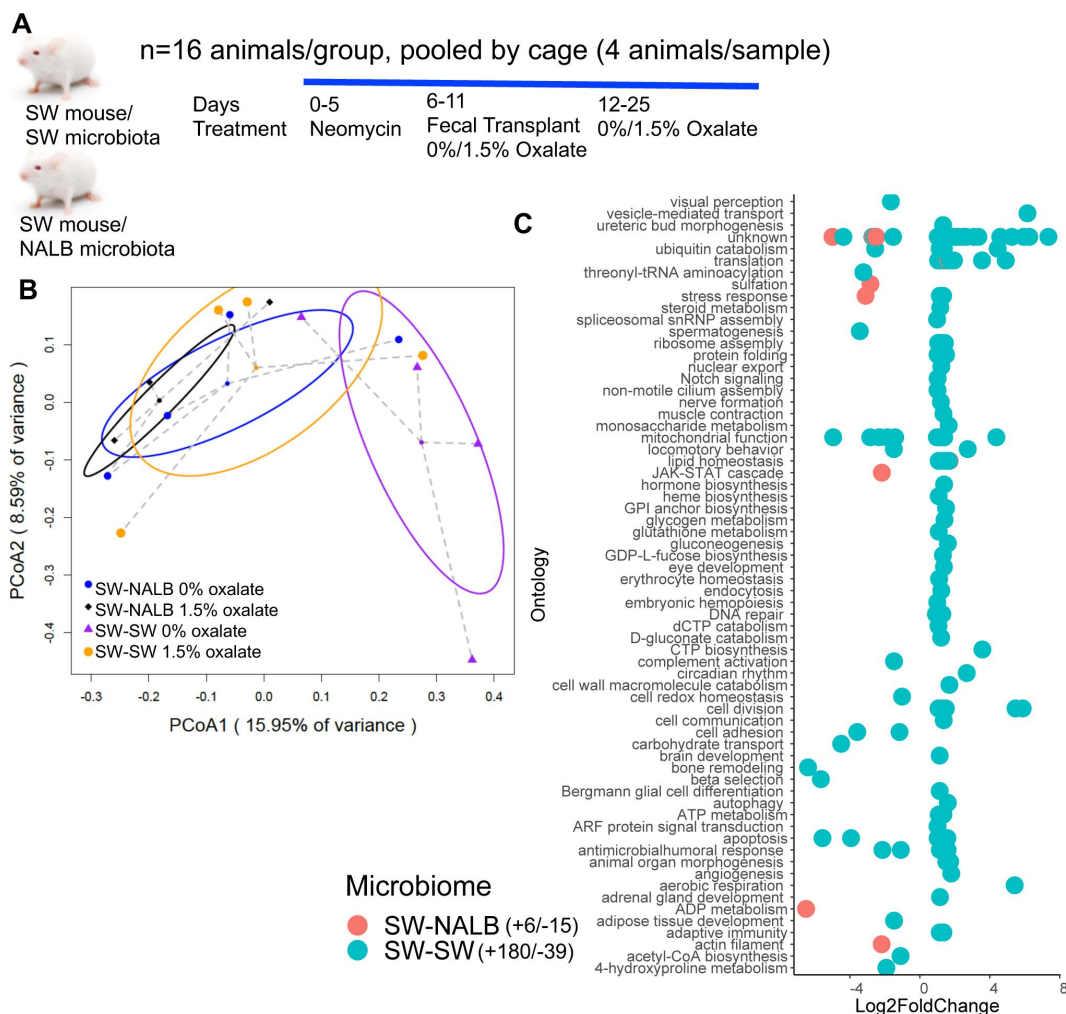


Fig. 1.

Oxalate exposure impacted host hepatic activity in a microbiota dependent fashion.

A) Swiss Webster mice were given neomycin, followed by an allograft (SWM) or xenograft (NALB) fecal transplant, then maintained on a 0% or 1.5% oxalate diet prior to necropsy for fecal metabolomics ([Fig. 2](#)) and hepatic transcriptomics. B) PCoA of normalized, whole-transcriptome data. $p=0.02$ for microbiome composition, $p=0.07$ for dietary oxalate content, $p=0.3$ in 2-way analysis; 2-way PERMANOVA. C) Total number of hepatic genes significantly stimulated or inhibited by dietary oxalate. Significant genes are plotted by Log2FoldChange. Positive values reflect genes increased with oxalate exposure and negative values are genes decreased with exposure. FDR < 0.05, Wald test. Hepatic genes are annotated to pathway (Kegg, Uniprot, PubChem, Metacyc) and the total number of genes that exhibit a positive or negative shift with oxalate exposure are listed in the legend. Complete gene list is in Table S2.

impact of oxalate on microbial activity in SW-SW and positive impact in SW- NALB mice. Integration of host hepatic gene expression and gut metabolomic data shows that oxalate induces a small decrease in hepatic activity overall for SW-NALB mice and a large increase in hepatic activity for SW-SW mice (**Fig. 2C** [↗](#), Table S4). From a complex systems perspective, data reflect a causative effect of oxalate for the shift in microbial metabolic output. Specifically, the SW-NALB mice exhibit hallmarks of homeostatic feedback with oxalate exposure to maintain a consistent metabolic output, defined by the relatively small, net negative, microbial metabolite-hepatic gene network compared to the large, net positive, network of SW-SW mice. Additionally, data further support the cooperation, redundancy, and fractality of the gut-liver axis. While SW- NALB exhibit a small increase in microbial metabolic activity and decrease in liver activity, the SW-SW mice saw a large decrease in microbial metabolic activity, coupled with a large increase in hepatic activity, which is reflected in the multi-omic network profiles (**Fig. 2C** [↗](#)).

Oxalate stimulates the growth of microbial populations involved in oxalate metabolism, formate metabolism, and their precursors

To gain a deeper understanding of microbe-microbe interactions associated with oxalate exposure and to identify specific microorganisms that positively respond to oxalate exposure, NALB with their native microbiota were pair-fed increasing amounts of oxalate, from 0.2% to 6% (**Fig. 3A** [↗](#)). Stool was sampled after 5 days on the 0.2% and 6% diets and processed for shotgun metagenomics. Analysis of metagenomic data revealed that oxalate had a significant impact on metagenome composition (**Fig. 3B** [↗](#)), with a significant increase of 1073 gene populations and decrease of 382 gene populations (**Fig. 3C** [↗](#); Table S5). Changes in gene abundance included four oxalate metabolism gene populations, a shift in 48 gene populations involved in formate metabolism (32 +/16 -), and a shift in genes related to glycine and glyoxylate/dicarboxylate metabolism (6+/4-), which are precursors to oxalate (**Fig. 3D** [↗](#)). A total of 128 differentially abundant genes were involved in sugar metabolism. Altered gene populations primarily belonged to *O. formigenes* and *Alistipes senegalensis*, with many genes belonging to the *Muribaculum* genus (**Fig. 3E** [↗](#)). *Muribaculum* spp harbor oxalate-degrading genes, which suggests oxalate metabolic redundancy in the gut microbiota⁸⁴ [↗](#). The oxalate-dependent metagenomic divergence of the NALB gut microbiota (**Fig. 3** [↗](#)), combined with the lack of change in the microbial metabolomic profile with oxalate exposure (**Fig. 2** [↗](#)), suggest that oxalate stimulates taxonomically diverse, but metabolically redundant microorganisms, in support of maintaining homeostasis. Given that data came from the same hosts sampled longitudinally, these data also reflect a microbiota that is adaptive to oxalate exposure, which is another important characteristic of complex systems.

Oxalate and formate metabolism are highly redundant functions in the NALB gut To investigate the hypothesis that oxalate stimulates a taxonomically diverse, metabolically redundant community, 248 full length genomes were extracted from shotgun metagenomic data (Fig. S1). Genes for oxalate metabolism, formate metabolism, or the formate metabolic pathways of acetogenesis, methanogenesis, and sulfate reduction, were derived from the KEGG pathway database⁸⁵ [↗](#) and mapped to full length genomes. This analysis provides a targeted assessment for metabolic redundancy aimed at oxalate metabolism and pathways associated with the by-products of oxalate metabolism (potential cooperation). A total of 59.3% of genomes contained at least one gene associated with oxalate metabolism or handling (**Fig. 4A** [↗](#)), most represented by oxalyl-CoA decarboxylase, glycerate dehydrogenase, and formyl- CoA:oxalate CoA transferase (**Fig. 4B** [↗](#)). However, only 27.8% of genomes harbored a complete metabolic pathway for oxalate degradation (**Fig. 4C** [↗](#)). Taxa with oxalate genes were dominated by *Bacteroides*, *Muribaculaceae*, *Clostridium*, *Ruminococcus*, and *Lachnospiraceae* (**Fig. 4D** [↗](#)). Formate metabolism genes were found in 97.18% of genomes, which was dominated by serine hydroxymethyltransferase, and formate-tetrahydrofolate ligase (Fig. S2A-C). Acetogenic genes were also present in 97.18% of genomes, dominated by acetate kinase and formate-tetrahydrofolate ligase (Fig. S3A- C). Methanogenic genes were present in 100% of genomes, dominated by phosphoserine phosphatase, atp-dependent 6-

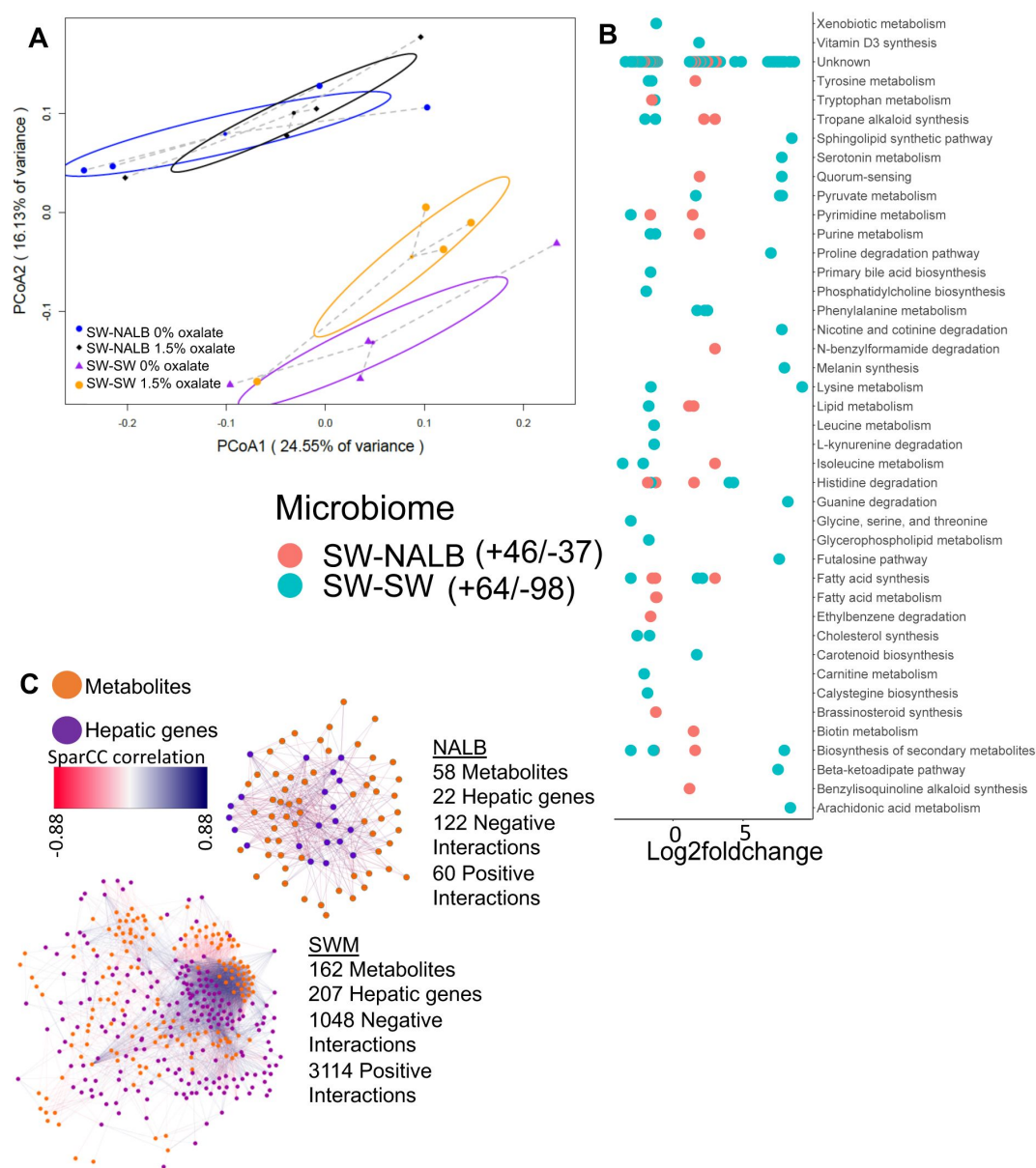


Fig. 2.

Oxalate exposure impacted microbial metabolic activity in a microbiota dependent fashion.

A) PCoA of protein normalized, log-transformed metabolomic data. $p=0.001$ for microbiome composition, $p=0.1$ for dietary oxalate content, $p=0.3$ in 2-way analysis; 2-way PERMANOVA. B) Total number of fecal metabolites significantly stimulated or inhibited by dietary oxalate. Significant metabolites are plotted by Log2FoldChange. Positive values reflect metabolites increased with oxalate exposure and negative values are metabolites decreased with exposure. FDR < 0.05, Mann-Whitney U or Fisher's exact test. Metabolites are annotated to pathway (Kegg, Uniprot, PubChem, Metacyc) the total number of metabolites that exhibit a positive or negative shift with oxalate exposure are listed in the legend. Complete list is in Table S3. C) Change in the host-microbe interaction network upon exposure to oxalate, quantified as hepatic gene-microbe metabolite correlations $R > \pm 0.3$ and FDR < 0.05 with SparCC, visualized in Cytoscape. All host-microbe interactions are listed in Table S4.

phosphofructokinase, and phosphate acetyltransferase (Fig. S4A-C). Sulfate-reducing genes were present in 31.05% of genomes, dominated by bifunctional oligoribonuclease and PAP phosphatase, FMN reductase, and cysteine synthase (Fig. S5A-C). Data show highly redundant oxalate-associated metabolic pathways and thus provide evidence for very robust homeostatic feedback mechanisms to handle oxalate and metabolic by-products within the NALB gut microbiota. Additionally, the broad diversity of species that contain oxalate-related genes suggest that the distribution of metabolic genes is somewhat independent of the distribution of microbial species, which suggests that microbial genes exist in an autonomous fractal layer, to some degree. This hypothesis is supported by studies which show a high degree of horizontal gene transfer within the gut microbiota as a means of adaptation⁸⁶.

Oxalate metabolism is driven both by substrate availability and microbiota composition Adaptability and homeostatic feedback within complex systems is driven by the convergence of system components and resource availability²⁴. To examine the confluence of resource availability and oxalate metabolism, a custom medium based on previously published gut microbiota media⁸⁷ was modified by adding substrates associated with metabolic pathways enriched by oxalate (Fig. 3C, Table S6). The oxalate-degrading species *Enterococcus gallinarum*, previously isolated from NALB⁷⁰ and the whole NALB community were assessed. Chosen substrates impacted oxalate metabolism and the impact of oxalate on growth, particularly at the community level (Fig. 5A,B). A minimal media with the same substrates added as sole carbon and energy sources (Table S7) allowed for quantification of the proportion of the NALB microbiota that could use each substrate as sole carbon and energy sources.

Culturomic data recapitulates molecular data to show a considerable amount of redundancy surrounding oxalate metabolism (Fig. 5C). Isolates generated from this assay were used for subsequent study (metabolic cohort; Figure 5D). Additionally, a second cohort was defined and commercially purchased based both on known metabolic functions and the proportion of studies that saw an increase in their taxonomic population with oxalate consumption (Fig. 5D; taxonomic cohort). Where possible, isolates from human sources were obtained. Cohorts, defined in the STAR methods, were used to delineate hypotheses that either carbon and energy substrates are sufficient to explain known effects of the oxalate-degrading microbial network or that additional aspects of taxa commonly stimulated by dietary oxalate are required to explain past results (taxa defined through previous meta-analysis of studies)¹⁵. Oxalate metabolism with the metabolic and taxonomic cohorts was assessed *in vitro* in minimal media with oxalate as a sole carbon and energy source (Fig. 5E,F). There were considerable differences in oxalate metabolism in both cohorts, dependent on the microbes present. However, significant oxalate metabolism occurred even in the absence of *O. formigenes*, indicative of metabolic redundancy. Collectively, data show that both resource availability and community composition impacts oxalate metabolism, which helps to define the adaptive nature of the NALB gut microbiota. Additionally, results further bolster evidence for redundancy surrounding oxalate metabolism.

Severity of oxalate-induced microbe-host effects is dependent on microbial oxalate metabolism, independent of taxonomy

To delineate hypotheses of metabolic redundancy or cooperation for mitigating the negative effects of oxalate on the gut microbiota and host, two independent diet trials were conducted with analogous microbial communities derived from the metabolic and taxonomic cohorts. Following antibiotic suppression of the gut microbiota, SWM were given microbial transplants from either the metabolic or taxonomic cohorts in a longitudinal, crossover experimental design with either a 0% or 3% oxalate diet (Envigo; Fig. 6A, S8A; Table S1). The 0% oxalate diet was designed to test the stability of oxalate-degraders since *O. formigenes* is often lost when oxalate is removed from the diet¹⁵. Transplanted microbial communities included the same as those for *in vitro* studies (Fig. 5D-F). Animal metrics and microbial were tracked over the course of the trial as was urinary/fecal oxalate, urinary formate, inflammatory cytokines, and creatinine. Renal calcium

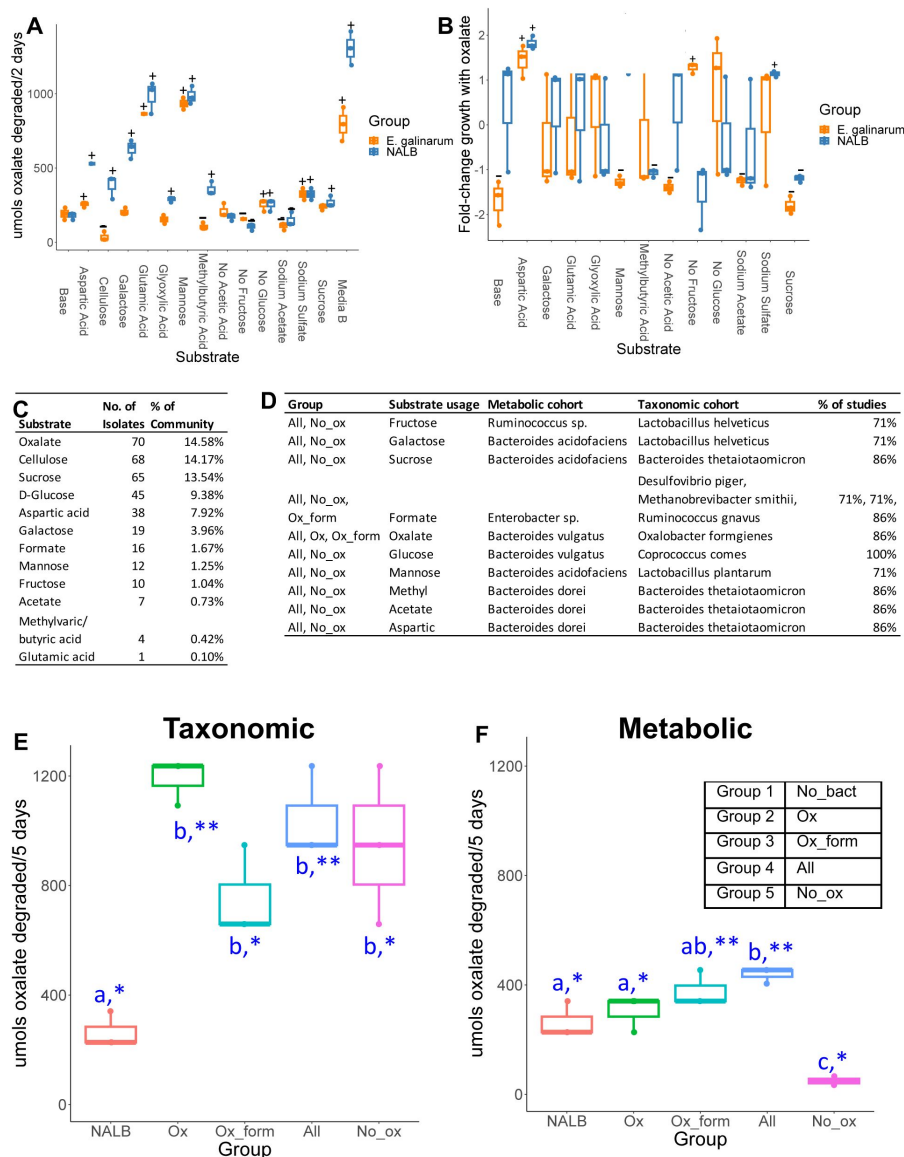


Fig. 5.

Microbial community composition and available substrates impact oxalate metabolism and the impact of oxalate on growth.

A) Substrates associated with metabolic pathways enriched by exposure to dietary oxalate *in vivo* differentially impact oxalate metabolism. $p < 0.001$ in one-way and two-way ANOVA against bacterial group and substrate; +/- reflects $p < 0.05$, Holm's-corrected, pairwise t-test compared to base media for an increase (+) or decrease (-) in oxalate degradation. B) Substrates differentially impact the influence of oxalate on microbial growth; $p < 0.001$ in two-way ANOVA against substrate and bacterial group, and one-way analysis against substrate; +/- reflects $p < 0.05$, Holm's-corrected, one-sample t-test compared 0 (no impact of oxalate) for an increase (+) or decrease (-) in growth due to oxalate exposure. The impact of oxalate on growth was not calculated for cellulose or Media B. C) Culture-based means to quantify proportion of the NALB community that can use substrates identified through shotgun metagenomics as sole carbon and energy sources. D) Defined microbial communities to assess oxalate metabolism *in vitro* and *in vivo*. Listed are the microbial consortia, which substrate the microbes utilize that corresponds to the shotgun metagenomic data, taxonomic classification of microorganisms used in the two cohorts, and the proportion of studies in which microorganisms in the taxonomic cohort were stimulated by oxalate exposure. E,F) Oxalate metabolism in minimal media with 20mM oxalate from the microbial communities listed in 5D, in comparison to the NALB community. $p < 0.001$, ANOVA comparing microbial groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Holm's corrected, one sample t-tests against 0 (no oxalate metabolism). Blue letters reflect statistical groups between microbial groups for oxalate metabolism.

oxalate (CaOx) deposition, cardiac fibrosis, and colitis was assessed through histopathology. CaOx deposition and cardiac fibrosis was quantified through a semi-automated process, based on stain color that differentiates calcium deposits (Von Kossa) or collagen (Mason's Trichrome). Colitis severity was assessed by two independent reviewers in hematoxylin and eosin stained tissues through a standardized, multi-factorial assessment⁸⁸.

Using microorganisms from the taxonomic cohort, while the change in urinary and fecal oxalate levels were greatest in mice given *O. formigenes*, the change in oxalate levels were significantly greater than the no bacteria controls (Group 1) even in the absence of *O. formigenes*, consistent with *in vitro* results (Fig. 6B,C). Interestingly, the change in urinary formate levels was not different in any microbial group for the taxonomic cohort (Fig. 6D). While IL1 β was below detection levels, IL6 exhibited levels consistent with oxalate induction, which decreased over the course of the trial (Fig. 6B, S6A). Differences in IL18 were only seen as an increase over time in the No_ox (Group 5) (Fig. S6B). The only changes in urinary creatinine seen was an increase and decrease in the NALB and All (Group 4) (Fig. S6C), respectively, which indicates that while the NALB bacteria may induce some inflammation, the minimal community present in the All (Group 4) group may limit these effects and actually improve kidney health under conditions of oxalate exposure. While some differences were seen in water or food metrics, these can largely be explained by batch effects of the two trials conducted (Fig. S6D-G). We did see a greater increase in body mass in animals receiving the No_ox (Group 5) microorganisms than when *O. formigenes* was administered alone (Group 2), which can not be explained by batch effects. Renal CaOx deposition, cardiac fibrosis, and colitis severity all closely tracked oxalate levels (Fig. 6E-J, S6H-I) and did not depend on *O. formigenes* if the other probiotic microbes were present. Collectively, data suggest that oxalate homeostasis and oxalate-induced pathologies are mitigated by the presence of diverse oxalate-degrading bacteria and that adding *O. formigenes* on an already effective oxalate-degrading microbiota will not improve oxalate homeostasis.

The microbiota composition of colon contents from this diet trial was significantly different from excreted feces (Fig. S7A). The microbiome composition in stool from mice was not different based on probiotic administration (Fig. S7B), but within group differences for the All (Group 4) group was significantly lower than other groups (Fig. S7C), indicative of a protective effect against oxalate exposure. This conclusion is corroborated by the change in alpha diversity in which the microbiota of animals given the All (Group 4) group exhibited the greatest post-antibiotic recovery and is the only group that saw recovery beyond a marginally significant increase in diversity, post-antibiotics (Fig. S7D). In general, there was not much loss of the inoculated bacteria throughout the study, in any group (Fig. S7E).

Overall, metabolic cohort transplants were less effective than the taxonomic cohort in terms of inducing oxalate metabolism, particularly in the group without bacteria (Group 1) isolated against oxalate (Figs. 6B,C, S8B,C). However, there was a much greater change in formate metabolism, dependent on the microbes present (Fig. S8D). Despite the more moderate change in oxalate levels, there were similar oxalate-associated effects on renal calcium oxalate deposition (Fig. S8F,G), which was not dependent on formate levels (Fig. S8H). With the metabolic cohorts, the presence of known oxalate degraders appeared to be more important for oxalate homeostasis than the taxonomic cohort, since absence of known oxalate-degraders led to oxalate levels similar to the No_bact (Group 1) controls (Figs. 6B,C; S8B,C). Collectively, data show that while the taxa chosen for the taxonomic cohort enabled efficient oxalate even in the absence of *O. formigenes*, the bacteria present in the metabolic cohort had a greater influence on formate levels. In both diet trials, urinary oxalate, but not formate correlated with CaOx deposition (Fig. S8G,H – metabolic, data not shown - taxonomic). The effects of oxalate and transplant group differed between the two cohort studies with mice in the metabolic cohort exhibiting higher levels of IL-6 of microbial transplant groups compared to the negative controls. However, similar to the taxonomic cohort, there were no differences in IL18. Urinary creatinine increased significantly over time for all groups receiving a microbial transplant, in contrast with the taxonomic cohort (Figs. S6A-C, S9A-C).

Taxonomic cohort

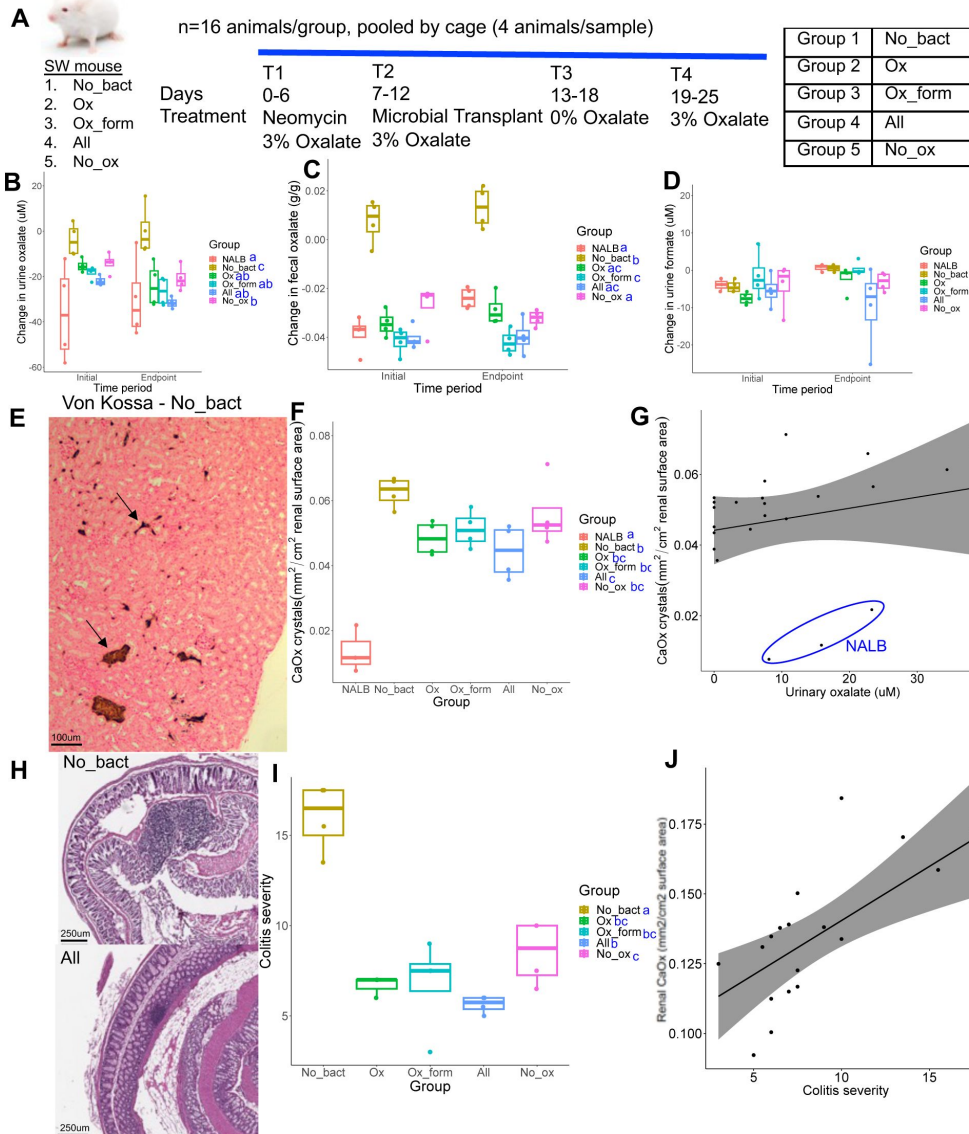


Fig. 6.

Microbial community composition (taxonomic cohort) impacted the effect of exogenous oxalate on host health.

A) Swiss Webster mice were given neomycin, followed by inoculation of microbial consortia that included either no bacteria or the taxonomic cohort listed in [Figure 5C](#). B,C) The effect of microbial transplants on urinary (B) or fecal (C) oxalate levels over the course of the diet trial, compared to baseline. ANOVA $p < 0.001$ for microbial group, but was not significant by timeperiod or 2-way analyses for both B & C. D) The effect of microbial transplants on urinary formate levels over the course of the diet trial, compared to baseline. ANOVA was not significant in one-way and two-way analyses. E) Renal calcium oxalate deposition. Arrows show stained calcium deposits, which were quantified through an automated algorithm in QuPath. F) Quantification of renal calcium oxalate deposition by group. $p < 0.001$, ANOVA. Blue letters reflect statistical groups between microbial groups for renal calcification by Holm's corrected paired t-tests. G) Pearson correlation between urinary oxalate and renal calcium oxalate deposition. $R = 0.22$, $p = 0.32$ with NALB samples included (blue circle); $R = 0.7$, $p = 0.001$ excluding the NALB group. H) Representative colon tissues from the No_bact (Group 1) and All (Group 4) groups, exhibiting high and low colitis severity scores, respectively. Tissues were stained with hematoxylin and eosin and scored based on standardized, multifactorial metrics. I) Quantification of colitis severity by group. $p < 0.001$, ANOVA. Blue letters reflect statistical groups between microbial groups for renal calcification by Holm's corrected paired t-tests. J) Pearson correlation between colitis severity and renal calcium oxalate deposition. $R = 0.7$, $p = 0.002$. Colitis severity was not quantified for the NALB group.

For the metabolic cohort, there similar group-based differences observed in water based metrics. However, animals in the taxonomic cohort exhibited greater positive changes in food intake and body mass than the metabolic cohort (Fig. S9D-G).

In the metabolic cohort, we did not observe the same trends in the microbiota composition, antibiotic recovery, or persistence as we did in the taxonomic trial indicative of less effective microbial communities at protecting the community as a whole (Fig. S10A-D), though probiotic bacteria were generally persistent (Fig. S10E).

Overall, data from the taxonomic and metabolic cohorts indicate that oxalate metabolism results from a defined community of microorganisms that includes redundancy in oxalate metabolism as the primary driver. Importantly, the efficacy of *O. formigenes* was apparent when administered alone, but the effect was diminished when co-inoculated with other oxalate-degrading microbes.

Discussion

While research in the last two decades have made clear that the gut microbiota is intimately tied to all facets of host health, translating those insights into actionable biotherapies has been difficult due to the inherent complexity and heterogeneity present in the microbe-host system. The study of complex systems holds enormous potential to constrain hypotheses and offer insight into effective bacteriotherapy development²³.

However, the field is limited by inconsistent terminologies, concepts, and tractable experimental frameworks²⁵. The objective of the current study was to examine oxalate- microbe-host interactions within a complex systems framework. Collectively, results of the study offer quantifiable and generalizable metrics of diet-microbe-host systems that can be used to guide more effective patient and probiotic selection criteria for clinical trials involving bacteriotherapies that target the gut microbiota.

Microbial oxalate metabolism, which has been researched for several decades, pre- dating the current microbiome era, is an ideal focal point to understand pertinent variables that impact probiotic success. Here, we found that heterogeneity in microbiome composition, independent of host genetics, significantly impacted multiple diet-microbe-host interfaces that included hepatic activity (Figs. 1, 2), gut microbiota metabolism (Figs. 2, 3), renal mineralization (Fig. 6E,F; S8E,F), intestinal inflammation (Fig. 6H,I), and cardiac fibrosis (Fig. S6H,I). Importantly, we found that oxalate degradation was highly redundant among diverse species in the gut of *N. albigula*, which consumes a high oxalate diet in the wild (Fig. 4). Consistent with past studies, administering *O. formigenes* led to a significant reduction in both stool and urine oxalate (Fig. 6B,C). However, when co-administered with other oxalate-degrading microorganisms, there was no additive effect. Oxalate-based results were similar, but to a lower degree, in the metabolic cohort (Fig. S8B,C). In taxonomic cohort studies, we did not see significant differences in the change in urine formate, based on microbial transplant group (Fig. 6D), in contrast to metabolic cohort studies (Fig. S8D).

Collectively, data offer strong support for the hypothesis that metabolic redundancy among diverse taxa, is the primary driver of oxalate homeostasis, rather than metabolic cooperation in which the by-products of oxalate degradation are used in downstream pathways such as acetogenesis, methanogenesis, and sulfate reduction. However, data on the metabolic cooperation hypothesis were inconclusive and there are multiple known microbe and host sources of formate, which may confound results in these studies^{74,89-91}.

Modelling the microbe-host system through an evolutionary perspective, it has previously been suggested that while host control over the microbiome is the primary driver of the form and function of the gut microbiota, given the enormous biomass and diversity of the gut microbiota, microbe-microbe competition is also required to constrain the microbiota in mammalian microbe-host systems²¹. In the current (Figs. 2B, 5B) and previous studies^{33,34,64,65}, we and others have found that oxalate can differentially exhibit positive or negative effects on microbial growth and metabolism dependent on the species and environment present. These data provide two alternative ecological pressures to degrade oxalate. The first is to use oxalate as a carbon and energy source for growth, as is the case with *O. formigenes*³³. The second is to degrade oxalate to remove it as a toxin, as is the case with some *Lactobacilli* and *Bifidobacteria*^{64,65}. The data showing oxalate degradation as a metabolically redundant function among multiple diverse microorganisms that maintains oxalate homeostasis (Figs. 4, 6, S8), suggest that the amount of oxalate consumed or produced by the liver is too great for a single, slow-growing oxalate-degrading specialist can handle alone, in contrast to prevailing hypotheses⁶³, and in support of the ecosystem on a leash hypothesis. Importantly, the near universal presence of formate metabolism genes suggest that formate may be an even greater source of ecological pressure (Figs. S2-S5). Collectively, data from the current and previous studies on the effect of oxalate exposure on the gut microbiota^{68,71} support the hypothesis that the gut microbiota serves as an adaptive organ⁹²⁻⁹⁴ in which specific, metabolically redundant microbes respond to and eliminate dietary components, for the benefit of themselves, but which can residually protect or harm host health depending on the dietary molecules and gut microbiota composition^{6,92-94}.

Oxalate degradation, as a focal point in diet-microbe-host interactions, is a special case in which the effects of oxalate on host health and the mechanisms of microbial oxalate degradation have largely been worked out for decades^{33,51-54}. Furthermore, oxalate degradation exhibits moderate complexity, both in that it is isolated to the gut microbiota and is performed through a handful of simple metabolic pathways³¹⁻³⁵. Despite this knowledge, clinical trials designed to reduce systemic oxalate using either *O. formigenes* or other oxalate-degrading bacteria have exhibited a wide variety of patient and trial responses¹⁵. However, knowledge about other diet-microbe-host links are largely in their infancy. Many of the other known features exhibit much greater complexity than oxalate degradation. For instance, while the production of short chain fatty acids are widely viewed as beneficial for both the gut microbiota and host health⁹⁵⁻¹⁰⁰, many short chain fatty acids are known and are produced by a wide variety of species^{101,102}. Other important diet-microbe-host links, such as the production of secondary bile acids or TMAO^{6,36,103-105}, also involve host hepatic activity, increasing the complexity further. Given the complexity of diet-microbe-host interactions and the fact that understanding the mechanisms through which the gut microbiota modifies the diet to influence host health is demonstrably insufficient for the development of effective bacteriotherapies¹²⁻¹⁵, we propose a three-phase, pre-clinical experimental workflow for the development of targeted bacteriotherapies. In the first phase of research, case:control metagenome-wide studies and research into other factors that modify the microbiome, such as prior antibiotic exposure, can identify disease phenotypes that are influenced by the microbiome, along with the microbial taxa and genes responsible for this association. In the second phase of research, hypothesized taxa and/or metabolic functions can be explored through *in vitro* and germ-free animal studies, using appropriate models to determine the mechanistic links that drive disease phenotypes through diet-microbe-host interactions. Finally, in phase three, these mechanistic links can be applied to a complex system theoretical framework, as done in the current study, to identify those variables most pertinent to successfully influence specific phenotypes through targeted changes to the gut microbiota. Such a phased research structure will provide for much more effective probiotic and patient selection criteria prior to clinical trials.

There were some important limitations to the current study. First, while we did conduct one study with *N. albigula*, most animal studies here sought to eliminate host genetics from the equation, as a means of simplification. Host genetics are another layer of complexity that were not examined

here and may have an impact on oxalate homeostasis beyond the gut microbiota. Second, in animal studies with the refined communities of microorganisms, the taxonomic and metabolic cohorts studies were conducted separately and some batch effects are apparent, such as with the change in creatinine values or urine output. Finally, it is apparent from our data and the literature that there are multiple host and microbial sources of formate. As such, data pertaining to formate utilization by our transplant communities are inconclusive.

In conclusion, using a complex systems theoretical framework, we examined the oxalate-microbe-host interactions of multiple oxalate-microbe-host interfaces and found multiple microbiome-dependent effects of oxalate. The negative effects of oxalate were mitigated by metabolically redundant oxalate-degrading bacteria, more so than by metabolic cooperation or a single oxalate-degrading species. Critically, we found that while *O. formigenes* can lower urine oxalate when placed on a background of a poor oxalate-degrading community, as current theory predicts, this effect is lost when co-administered with other oxalate-degrading bacteria. Collectively, data help to resolve why the gut microbiota of the white throated woodrat, *Neotoma albigula*, effectively responds to and degrades even very high dietary oxalate levels^{68,69,72} and offer a clear pathway for more effective patient and probiotic selection for future clinical trials to reduce urine oxalate. More importantly, the conceptual and experimental framework developed in this study, based on complex systems theory, paves the way for a phased approach to microbiome research in which clinical microbiome insights (Phase I) drive mechanistic insights between the gut microbiota and host physiology (Phase II), that can be applied to a complex system model to constrain hypotheses and identify the most pertinent variables that drive microbe-host interactions that influence host physiology and health (Phase III). Such an approach will allow for much more efficacious probiotic and patient selection criteria in clinical intervention trials.

STAR Methods

Animal studies

Tracking animal health

Throughout all diet trials, animals were monitored for evidence of trauma, dehydration, pain, or other forms of suffering. Additionally, water and food intake, urine output, and body mass was monitored daily (i.e., Figs. S6, S9). If animals lost >10% of their baseline body mass, they were removed from the diet trial and placed back onto standard mouse chow.

Effect of oxalate on the native gut microbiota of *Neotoma albigula*

To determine the impact of dietary oxalate on *N. albigula* with their native microbiota, 14 adult *N. albigula* woodrats were collected from Castle Valley, UT (38.63°N, 109.41°W) in September 2014, using Sherman live traps. Animals were transported to the University of Utah and housed in individual cages (48 by 27 by 20 cm) with a 12-h/12-h light/dark cycle, at 28°C and 20% humidity. Animals were initially maintained on a 0.2% oxalate, high-fiber rabbit chow (Harlan Teklad formula 2031; Envigo) for 7 months prior to experimentation. This diet reduces the overall detectable diversity of the microbiota of these animals but maintains the members of the native microbiota overall¹. All methods were approved by the IACUC under protocol no. 12-12010.

Subsequently, *N. albigula* woodrats were placed in a diet trial in which the oxalate concentration of the food was gradually increased over time along a gradient from 0.2% (for days 1 to 5; herein referred to as 0%), 3% (days 6 to 10), and 6% (days 11 to 15; **Fig. 3A**). The oxalate concentration of the diet was adjusted by adding the appropriate amount of sodium oxalate (Fisher Scientific,

Pittsburgh, PA) into the powdered rabbit chow on a dry weight basis. The schedule for each concentration of dietary oxalate was chosen to ensure that the gut microbiota had time to respond to the specific diet^{2,3}.

During diet trials, oxalate did not have a negative impact on *N. albigula* health and neither fecal nor urinary oxalate levels increased significantly⁴.

During the diet trial, animals were placed in metabolic cages to separate urine and feces into sterile 50-ml conical tubes. A subsample of feces was collected from each animal every 4 to 5 days on the 0% and 6% oxalate diets for shotgun metagenomic sequencing. Samples were frozen at -80°C until DNA extraction.

Stool collection for bacterial isolations and fecal transplant studies

Feces for fecal transplants were collected from two sources. For the Swiss-Webster feces, 20 individual animals (Taconic Farms, female, six weeks old) were placed on a 0% oxalate diet in cages with a custom-designed insert to separate urine and feces at the Cleveland Clinic (IACUC #2016-1653)⁵. For the *N. albigula* feces, 14 individual animals (mixed sex and age) were placed on a 3% oxalate diet in a metabolic chamber at the University of Utah (IACUC #12-12010) to acclimate the microbiome to oxalate metabolism⁵. After 3 days of acclimation on the respective diets, feces were collected from animals within 2 h of defecation, submerged in sterile 15% glycerol and flushed with CO₂ prior to freezing at -80 °C until use in animal studies approximately 12 months after receipt (IACUC #2016-1653) or for bacterial isolations.

Microbiota-dependent effects of oxalate on liver and gut microbiota

All animal studies were approved through the Cleveland Clinic's IACUC (IACUC #'s 2016-1653, 2020-2312). To examine the host-independent effects of oxalate on gut microbiota with either a low or high efficiency at degrading oxalate, two distinct host- microbe model systems were developed. For both models, equal numbers of male and female Swiss-Webster mice were used as the host. For the low oxalate-degrading gut microbiota, Swiss-Webster mice were given fecal transplants using feces from other Swiss-Webster mice (allografts), as discussed below. A 1.5% oxalate diet is sufficient to induce hyperoxaluria in Swiss-Webster mice, defined as a 50% increase in urinary oxalate excretion⁶⁻⁸. For the high oxalate-degrading gut microbiota, Swiss-Webster mice were given fecal transplants from *N. albigula* (xenografts). We have previously shown that fecal transplants from *N. albigula* are sufficient to induce significant and persistent oxalate metabolism^{7,8}. During and after fecal transplants, animals were grouped four to a cage, with four cages assigned to each treatment group, for a total of 16 animals per experimental group. To minimize individual variability and eliminate any cage effect in subsequent metrics, samples and data collected from all animals in a cage were pooled together into an individual sample. To develop the two different mouse models, the conversion of the gut microbiota from the native microbiota to the grafted microbiota (allografts or xenografts) was completed in two stages. First, the native microbiota was depleted with a five-day course of neomycin (0.5g/L water, combined with 2g/L sucralose), while on a 0% oxalate diet. Mice were given *ad libitum* access to water during this time and throughout the experiment. Neomycin is a broad spectrum antibiotic that is poorly absorbed across the gut, and effectively reduces gut microbiota density by up to 90%⁹. After antibiotic depletion, mice were given the respective fecal transplants by first thawing feces, then aseptically mixing 32 g feces per kg body weight directly into powdered mouse chow. Fecal transplants were performed daily for 6 days (Fig. 1A), as previously described⁵, during which animals were switched to either a 0% or 1.5% oxalate diet. All animals were maintained on their respective diets for two weeks following fecal transplants (Table S1). A total of four treatment groups were defined based on allografts/xenografts and oxalate diet.

Following diet trials, animals were sacrificed through CO₂ asphyxiation and cervical dislocation. Upon necropsies, liver tissue from each animal was dissected out and placed into RNAlater on ice, prior to freezing at -80°C, within two hours of necropsies.

Additionally, colon feces were collected and placed into screw-cap tubes with o-rings, on ice, prior to freezing at -80°C.

Microbial cohort-dependent effects on mitigating the systemic effects of oxalate on host and microbiome

To determine the effect of specific microbial cohorts at mitigating the systemic host- microbiome effects of oxalate, different combinations of two microbial cohorts were established. First, based on the metabolic pathways enriched by oxalate in the *N. albigula* gut microbiota (Fig. 3D [↗](#), 5C [↗](#), Table S5), assessed through comparative shotgun metagenomics (described below), we hypothesized that microorganisms that engage in those metabolic pathways help to mitigate the systemic effects of oxalate. To test this hypothesis, we isolated bacteria from *N. albigula* stool (described below) and grouped microorganisms into four cohorts (Fig. 5D [↗](#)). These included oxalate-degrading bacteria alone (Ox; Group 2), microorganisms that can utilize formate or oxalate (Ox_form; Group 3), oxalate and formate users, in addition to the metabolism of selected sugars (All; Group 4), or the complete cohort except for oxalate degraders (No_ox; Group 5).

Microorganisms isolated from *N. albigula* stool and used in microbial transplants were termed the “metabolic cohort”. In addition to the metabolic cohort, a “taxonomic cohort” was defined by cross-referenced metabolic pathways enriched by oxalate with microbial taxa previously reported to be stimulated by oxalate (Fig. 5D [↗](#))¹⁰ [↗](#). Microorganisms from this cohort were purchased commercially from the ATCC or DSMZ (Table S8). In addition to these cohorts, we also examined a negative control in which animals were inoculated with sterile media (No_bact (Group 1); Group 1), and a positive control in which animals were inoculated with the whole gut microbiota of *N. albigula*, extracted from stool by first vortexing in a 1:1 mixture of stool and sterile PBS, followed by centrifuging at 8000RPM for 2 minutes. The supernatant was inoculated into gut microbiota media with 20mM sodium oxalate (Table S6) overnight prior to microbial transplant.

To prepare each isolate for transplants, an overnight culture of each species was grown and mixed in equal proportions by volume for each cohort. The community was then centrifuged and supernatant was decanted. After antibiotic depletion of the native gut microbiota in Swiss-Webster mice, 38mg of pelleted bacteria of each preparation was added directly to the food and administered over the course of six days (Figs. 6 [↗](#), S8), as done previously^{6,7} [↗](#). Preparations corresponded to approximately 5×10^8 [↗](#) microorganisms for each isolate in the appropriate transplant groups. Quantification of bacterial numbers was performed through absorbance of the microbial preparations on a spectrophotometer at 600 nm, calibrated to direct microscopic counts.

As above, animals were grouped four to a cage, with samples from each cage pooled together prior to collection and analyses. Similarly, animals were maintained on custom inserts that mimic metabolic cage for the effective separation of urine and stool prior to collection. During the microbial transplant animal studies, stool and urine was collected daily and inserts disinfected. For oxalate and formate quantification, samples were collected at the end of the antibiotic depletion, microbial transplants, a 0% oxalate washout period, and after return to a 3% oxalate diet (Figs. 6B-D [↗](#), S8B-D). Prior to biochemical assays, urine was frozen (-20°C) and feces were dried at 45°C overnight. Prior to freezing, aliquots of urine were equally divided into six fractions for each of the biochemical assays performed (discussed below). Fractions for oxalate quantification were collected into 4N HCl prior to freezing to prevent the non-enzymatic conversion of ascorbic acid into oxalate² [↗](#). For microbial inventories, a portion of feces and colon feces (discussed below) were frozen at -80°C. All animals were maintained on a 3% oxalate diet, except during the washout period (Table S1).

Following diet trials, animals were sacrificed through CO₂ asphyxiation and cervical dislocation. Necropsies were performed to aseptically remove colon feces, colon, kidneys, and heart tissue. Colon tissue was longitudinally bisected, swiss-rolled, and placed into 4% paraformaldehyde overnight for fixation at 4°C for histopathology ¹¹ [↗](#).

Kidney and heart tissues were placed into 4% paraformaldehyde overnight for fixation at 4°C prior to histopathology. Hearts were first perfused with paraformaldehyde solution prior to immersion.

After fixation, colon, kidney, and heart tissue were placed in 70% ethanol at 4°C prior to paraffin embedding, serial sectioning, and staining. To quantify colitis, colon tissue was stained with hematoxylin and eosin and imaged with light microscopy (**Fig. 6H** [↗](#)). Whole colons were scored for colitis by two blinded, independent reviewers using a semi-quantitative protocol that considers inflammatory infiltrates, goblet cell loss, crypt density, crypt hyperplasia, muscle thickening, submucosal inflammation, crypt abscesses, and ulcerations ¹² [↗](#). To quantify calcium oxalate deposition, kidney tissue was stained with Von Kossa staining ¹³ [↗](#), which turns calcium deposits black and the remaining tissue pink (**Figs. 6E** [↗](#), S8E). Kidneys were imaged under light microscopy and calcium oxalate deposition was quantified using an automated process in QuPath, based on stain color. The area of the black calcium oxalate deposits was normalized to total kidney area. To quantify cardiac fibrosis, heart tissue was stained with Masson's trichrome stain, which stains collagen fibrosis blue and the remaining tissue red (Fig. S6H). Hearts were imaged under light microscopy and fibrosis was quantified with an automated process in QuPath, quantifying the blue and red stained areas ¹⁴ [↗](#). Fibrosis was normalized to total heart surface area.

Biochemical assays

Urinary creatinine (Fisher Scientific), formate (VWR), IL-6 (R&D Systems), IL-18 (Fisher Scientific), and IL-1 β (R&D Systems) were quantified with ELISA-based assays, following manufacturer's instructions. Positive controls that included a known amount of substrate along with no substrate negative controls were included in all batches of ELISA assays. Samples, standards and controls were all run in duplicate and values averaged. Urinary and fecal oxalate were quantified with an ELISA-based assay (Sigma-Aldrich), with a modified protocol, as follows. Upon initial testing of oxalate and formate assays using solutions of sodium oxalate and sodium formate in water, it was discovered that while formate assays only had affinity for formate, the oxalate assays had equal affinity for both oxalate and formate (Figs. S11A). Specifically, when oxalate alone was added to solution, the quantified concentration of oxalate matched the amount added. However, when oxalate and formate was added at equal concentrations, the quantified oxalate was approximately twice as high as what was added (Fig. S11B). Furthermore, when adding 300uM of formate or oxalate to human urine specimens, the amount of oxalate quantified was approximately 300uM higher than urine without any modifications (Fig. S11C). To eliminate the formate contamination on oxalate measurements, oxalate was extracted by first acidifying to pH 3 with 3M HNO₃. Acidified urine was centrifuged, and supernatant collected. The pH was then brought up to 7 with NaOH. Subsequently, 5M CaCl₂ was added to solution to precipitate calcium oxalate. The precipitates were extracted by centrifugation and decanting. Finally, calcium oxalate solution was acidified in 1:4 parts of 4N HCl prior to enzymatic assay, as previously described ² [↗](#). When oxalate was extracted from urine specimens in this way, prior to quantification, we saw a significant decrease in quantified oxalate (Fig. S11C), as expected given the assay affinity for formate. When solutions containing formate alone went through the extraction process, no oxalate was quantified. Given these results, urinary and fecal oxalate was extracted prior to ELISA-based assays using the manufacturer's recommendations, post-extraction. For fecal oxalate, samples were acidified with 6N H₂SO₄ to solubilize oxalate prior to following the extraction protocol above. For creatinine, cytokines, and oxalate, values generated on a 0% oxalate diet were subtracted from all other values, matched by cage, with the assumption that this would isolate oxalate-induced molecule generation. For urinary formate, this subtraction was not done since formate can come from multiple sources other than oxalate degradation and values on a 0%

oxalate diet were not clearly lower than on a 3% oxalate diet. For all values, data are presented as the change from baseline. Data were statistically compared with two-way analyses and post-hoc, Holm's-corrected, paired t-tests.

Culturomic assays

All assays were conducted under strict anaerobic conditions (90% N₂, 5% H₂, 5% CO₂) in an anaerobic chamber (Bactron 300).

The stool from *N. albigula* were used to determine the impact of environmental factors on oxalate metabolism *in vitro* and to isolate bacteria for the metabolic cohort.

Enterococcus gallinarum, an oxalate-degrading species previously isolated from *N. albigula*, was also used in culturomic assays to determine the impact of environmental factors on oxalate metabolism¹⁵. To test environmental factors on oxalate metabolism, gut microbiota media, previously designed to support a broad array of gut bacteria¹⁶, was used as a base media. From there, compounds were added to the media based on metabolic pathways enriched by dietary oxalate in the *N. albigula* gut microbiota (Fig. 3D, Table S6). Concentrations of added chemicals were based on previously published media recipes where necessary. To assess oxalate metabolism in different culture conditions, oxalate was quantified by extracting oxalate and quantification through titration with KMnO₄, as we have done previously¹⁵.

To isolate bacteria for the metabolic cohort and to validate the metabolic redundancy of oxalate metabolism and other pathways stimulated by oxalate, a minimal media with the same substrates from above were added as sole carbon and energy sources (Table S7). In this medium, oxalate was added at 50mM to specifically select for bacteria that can use oxalate as a carbon and energy source. Subsequently, the NALB microbiota from the stool was first diluted to ~1 bacterium/100ul and then inoculated into 5 or 10 96-well plates, depending on the rarity of positive hits, and quantifying growth through spectrophotometry. Wells in which the absorbance values at 600nm for greater than one standard deviation above the average for all well were considered positive for growth. Wells with positive growth had bacteria isolated by streaking on rich media, transferred to broth cultures, and had stocks made in 15% glycerol that were frozen at -80°C until needed. Those isolates grown in oxalate or formate containing media were subsequently enriched in the minimal media and oxalate or formate degradation was validated through broth cultures and ELISA-based assays.

Metabolomics

For untargeted metabolomic assays, fecal samples were re-suspended in Optima LC/MS grade water (50mg stool to 150ul water). The samples were then placed briefly in a water bath at 37°C and placed on dry ice. An aliquot of each sample was taken out for protein concentration measurement. Then, 150 µL of chilled acetonitrile containing internal standards (Betaine-d₉, Carnitine-d₉, Orinithine-d₆, Valine-13C₃, Tyrosine-13C, 15N, Estrone-13C₃, and Cholesterol-13C₃) was added to the remaining samples followed by centrifugation at 14,000g for 10 minutes to precipitate out the protein pellet. The supernatants were recovered and transferred to fresh tubes. The samples were dried briefly and re-suspended in 2% acetonitrile and 0.1% formic acid for subsequent LCMS analysis. One-microliter aliquots taken from each sample were pooled and this QC standard was analyzed every 10 injections. The untargeted metabolomics was performed by injecting 0.5uL of each sample onto a 10 cm C18 column (Thermo Fisher CA) coupled to a Vanquish UHPLC running at 0.35mL/min using water and 0.1% formic acid as solvent A and acetonitrile and 0.1% formic acid as solvent B. The 15-min gradient used is given below. The Orbitrap Q Exactive HF was operated in positive and negative electrospray ionization modes in different LC-MS runs over a mass range of 50-750 Da using full MS at 120,000 resolution. The data dependent acquisitions were obtained on the pooled QC sample. The DDA acquisition (DDA) include MS full

scans at a resolution of 120,000 and HCD MS/MS scans taken on the top 10 most abundant ions at a resolution of 30,000 with dynamic exclusion of 4.0 seconds and the apex trigger set at 2.0 to 4.0 seconds. The resolution of the MS2 scans were taken at a stepped NCE energy of 20.0, 30.0 and 45.0.

XCMS was used to deconvolute the data using 5 ppm consecutive scan error, 7 to 75 seconds as minimum and maximum peak width, S/N threshold of 10, and span of 0.2 in positive and negative electrospray ionization modes for retention time correction. The resulting peak table was normalized to total protein concentration and further analyzed via MetaboLyzer¹⁷. Briefly, the ion presence threshold was set at 0.7 in each study group. Data were then log-transformed and analyzed for statistical significance via non-parametric Mann-Whitney U test. Ions present in just a subset of samples were to be analyzed as categorical variables for presence status via Fisher's exact test. All p-values were set to be corrected via the Benjamini-Hochberg step-up procedure for false discovery rate (FDR < 0.05). The data could then be utilized for PCA, putative identification assignment, and pathway enrichment analysis via KEGG¹⁸, HMDB¹⁹,

LIPIDMAPS²⁰, and BioCyc²¹ databases. In addition to differential abundance analysis provided by MetaboLyzer, whole metabolome comparisons were made as a Binomial dissimilarity matrix, which is optimal for metabolomic datasets²², with statistical analysis provided by PERMANOVA with 999 permutations and a principal component visualization in Vegan²³.

Transcriptomics

For transcriptomic analysis, RNA was extracted from liver tissue with a TRIzol RNA purification kit (Invitrogen), following manufacturer's recommendations. Extracted RNA was converted to cDNA using a cDNA synthesis kit (Fisher Scientific) and stored at -20°C. The cDNA quality was checked on a bioanalyzer and libraries were prepared with the TruSeq stranded mRNA library prep kit. Sequencing occurred on an Illumina HiSeq 2500 at the Genomics Core (Cleveland Clinic) for paired 100bp sequencing.

The raw sequencing files, with 33.5 million +/- 950,000 reads per sample, were pre-processed using an established protocol²⁴. Specifically, FastQC and SortMeRNA was used to remove low quality reads, remove rRNA reads, and merge paired reads^{25,26}.

Subsequently, trimmomatic was used to remove adapter sequences²⁷ and the remaining high quality reads were mapped to the mm10 mouse reference genome for annotation, using HISAT^{28,29}. The annotated gene counts were normalized to fragments per kilobase of transcript per million mapped reads. Comparative whole transcriptome analyses were conducted as a Bray-Curtis dissimilarity index and principal components visualization with statistical analysis provided by PERMANOVA with 999 permutations, in the Vegan package of R statistical software^{23,30}. Differential abundance analysis was conducted in Cuffdiff2, with significance defined at a false discovery rate <0.05³¹.

Sequences mapping to mouse genes were annotated to metabolic pathway with the gene ontology database³².

To integrate transcriptomic and metabolomic data, the normalized metabolite concentrations and normalized transcript counts of metabolites and genes that were significantly different between the SWM-SWM and SWM-NALB groups were correlated, within each group, using SPARCC correlations in R³³. Significant positive and negative correlations were calculated at an R greater or less than 0.4 or -0.4, respectively and an FDR <0.05. Significant correlations were visualized in Cytoscape version 3.3.0³⁴.

Shotgun metagenomics

For shotgun metagenomic analysis, DNA from *N. albigula* stool was extracted with the QIAmp DNA stool minikit (Qiagen). The extracted DNA was submitted to Argonne National Laboratory (Chicago, IL) for 150bp paired-end shotgun metagenomic sequencing on an Illumina HiSeq 2500. The low quality reads were trimmed from raw sequencing data and paired ends were merged using default parameters in BBMerge³⁵. Reads were first mapped to the *Neotoma* genome³⁶ with BWA mem with default settings³⁷, and removed. Paired, non-rodent sequences were assembled in MetaSpades with default parameters³⁸.

Full-length microbial genes were extracted and annotated from the assembled contigs using PROKKA software³⁹. Gene annotation was achieved by mapping to the UniProt and Hamap protein databases^{40,41}. To dereplicate genes, ensure common nomenclature across samples, and build a reference gene catalog for the quantification of gene counts, full-length genes were clustered at 90% homology in CD-hit⁴². After the creation of a non-redundant gene catalogue, high quality reads for each sample were mapped to the annotated gene catalog to generate gene-level count tables with BWA mem.

Gene count tables were normalized and differential abundance analysis was conducted with a negative binomial Wald test in DESeq2⁴³. Significantly different genes were considered at an FDR <0.05. The normalized count tables were also used to generate a weighted Bray–Curtis dissimilarity matrix in the Vegan R package, with statistical comparisons with a PERMANOVA at 999 permutations²³. To annotate differentially abundant genes to taxonomy, genes were mapped to full length genomes extracted directly from the shotgun metagenomic data, as described below.

de novo genome construction and calculation of metabolic redundancy

For the *de novo* construction of genomes, the metaspades assembled contigs were binned to genomes with Autometa software⁴⁴, as previously described⁴⁵. The Autometa algorithm takes assembled contigs as input, bins the data to genomes using contig coverage values, GC content, and the Barnes-Hut t-Distributed Stochastic Neighbor Embedding (BH t-SNE) distribution of the contigs. Taxonomy is assigned based on the consensus classification of all contigs in a genomic bin. Completeness and purity calculations are based on the presence of known, unique universal single copy genes in Autometa. After genomic binning, the contigs were oriented and scaffolded in CSAR⁴⁶, which compares the genomic bins to reference genomes of close relatives. Gaps within the genomes were filled with Abyss-Sealer⁴⁷ using the original raw sequencing reads as input. The completeness and purity of genomes were recalculated and taxonomic assignment was validated through phylogenetic analysis of the *de novo* constructed genomes in comparison with all complete NCBI genomes (Fig S1), achieved through PhyloPhlan, using the “—accurate” parameter which considers more phylogenetic positions at the cost of computational speed⁴⁸. PhyloPhlan allows for the phylogenetic analysis of bacteria and archaea using complete genomes rather than gene amplicons, which provides greater resolution on phylogenetic analysis. Collectively, we extracted 248 high quality, full length genomes, defined as being >80% complete and >90% pure, based on current standards⁴⁹.

To quantify the proportion of the *N. albigula* microbiota that contained genes related to oxalate metabolism, formate metabolism, acetogenesis, methanogenesis, or sulfate reduction, all genes that contribute to those metabolic pathways were extracted from the KEGG database⁵⁰. Subsequently, PROKKA³⁹ was run on the 248 full length genomes to extract and annotate full length genes. The PROKKA extracted genes were then cross-referenced with the gene databases from KEGG to determine the proportion of genomes that contained at least one gene in the target pathways and determine which taxa had those genes.

16S rRNA metagenomics

Fecal DNA from mice in the SWM-NALB and SWM-SWM studies, underwent DNA extraction through a semi-automated protocol on a KingFisher Duo Prime System (Thermo Scientific) following the manufacturer's protocol for stool. The protocol includes mechanical lysis with bead-beating, piston driven lysis, and proteinase K chemical lysis. The extracted DNA, which attaches to magnetic beads in a proprietary salt solution, is removed from solution and eluted in buffer. Duplicate samples of a commercial DNA positive of known composition (ZymoBIOMICS, USA) were included as positive controls, as well as the DNA reagents that went through the entire workflow, sterile water, and a PCR negative as negative controls.

The fecal DNA was submitted to the Microbial Sequencing and Analytics Core at Cleveland Clinic for high throughput sequencing of the 16S rRNA gene on an Illumina MiSeq. The DNA was first PCR amplified with the 515F and 806R primers that target the V4 hypervariable region of the 16S gene. The DNA concentrations were quantified before and after PCR amplification on a Qubit and normalized prior to library prep with the Illumina Nextera XT library prep kit. The sequence run was conducted to generate 150bp, paired-end sequences.

The raw sequencing data were processed in the R statistical package (4.2.0)³⁰. Quality control, removal of bimeras, and assignment of amplicon sequence variants (ASV) were completed in dada2⁵¹. For ASV taxonomic assignment, a combined, non-redundant database of the Silva 138 SSURF and NCBI 16S rRNA databases were used⁵². Taxa assigned to mitochondria or chloroplasts were removed from subsequent analysis. The resulting ASV's were aligned in MSA⁵³ and arranged into a maximum likelihood phylogeny in phangorn⁵⁴. The resulting phylogenetic tree and ASV table were merged with sample data for loading into PhyloSeq⁵⁵. The sequencing depth threshold required to adequately capture microbial diversity was calculated with a rarefaction analysis in Vegan²³. Non-control samples below the depth threshold were removed from further analysis.

The raw count table of 16S rRNA sequences was normalized with the DESeq2 algorithm and α -diversity was calculated as phylogenetic diversity in PhyloSeq, along with beta-diversity as a weighted UniFrac distance⁵⁶. Alpha diversity was analyzed with a paired t-test with a Holm's correction where applicable, while beta-diversity analyses were conducted with a PERMANOVA after 999 permutations.

Data availability

Sequence reads from the animal study are available at the Sequence Read Archive under Accession numbers for gut microbiota genomes: PRJNA833303, Shotgun metagenomics: PRJNA839366, Liver transcriptomics: PRJNA1018952 Gut microbiota data: 16SPRJNA1018559

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Additional information

Author contributions

SM, DD, MM and AWM conceived and designed the study. SM, CAB, AA, AN, and AWM designed and performed most *in vitro* and animal-based biological experiments. JA, MS, AZ, TO performed additional animal and *in vitro* experiments and contributed to data analysis. SM and AWM wrote the manuscript with input from all authors. AWM supervised the study. All authors reviewed the manuscript.

Supplementary figures and tables

Supplementary figures

A: Bacteroidetes
 B: Firmicutes
 C: Proteobacteria
 D: Synergistetes
 E: Tenericutes
 F: Actinobacteria
 G: Actinobacteria
 H: Thermotogae
 I: Thermotogae
 J: Spirochaetes
 K: Spirochaetes
 L: Chrysiogenetes
 M: Chrysiogenetes
 N: Thermi
 O: Thermi
 P: Thermodesulfobacteria
 Q: Thermodesulfobacteria
 R: Deferribacteres
 S: Deferribacteres
 T: Cyanobacteria
 U: Caldithrix
 V: Caldithrix
 W: Chloroflexi
 X: Chloroflexi
 Y: Fusobacteria
 Z: Fusobacteria
 a: Aquificae
 b: Aquificae
 c: Verrucomicrobia
 d: Planctomycetes
 e: Acidobacteria
 f: Acidobacteria
 g: Chlamydiae
 h: Chlamydiae
 i: Deinococcus-Thermus
 j: Chlorobi
 k: Lentisphaerae
 l: Lentisphaerae
 m: WWE1
 n: Elusimicrobia
 o: Elusimicrobia
 p: TM7
 q: Dictyoglomi
 r: Nitrospirae
 s: Gemmatimonadetes
 t: Gemmatimonadetes
 u: Fibrobacteres
 v: Fibrobacteres
 w: Euryarchaeota
 x: Crenarchaeota
 y: Nanoarchaeota

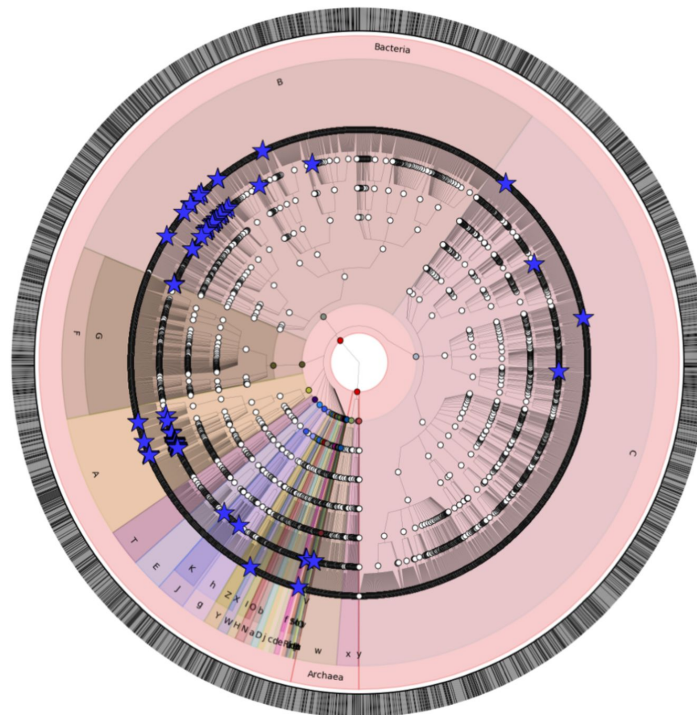


Fig. S1.

Phylogeny of genomes extracted from the *N. albigula* metagenome, in comparison to >3000 full length microbial genomes.

Stars indicate taxonomic placement of extracted genomes. All other points indicate reference genomes.

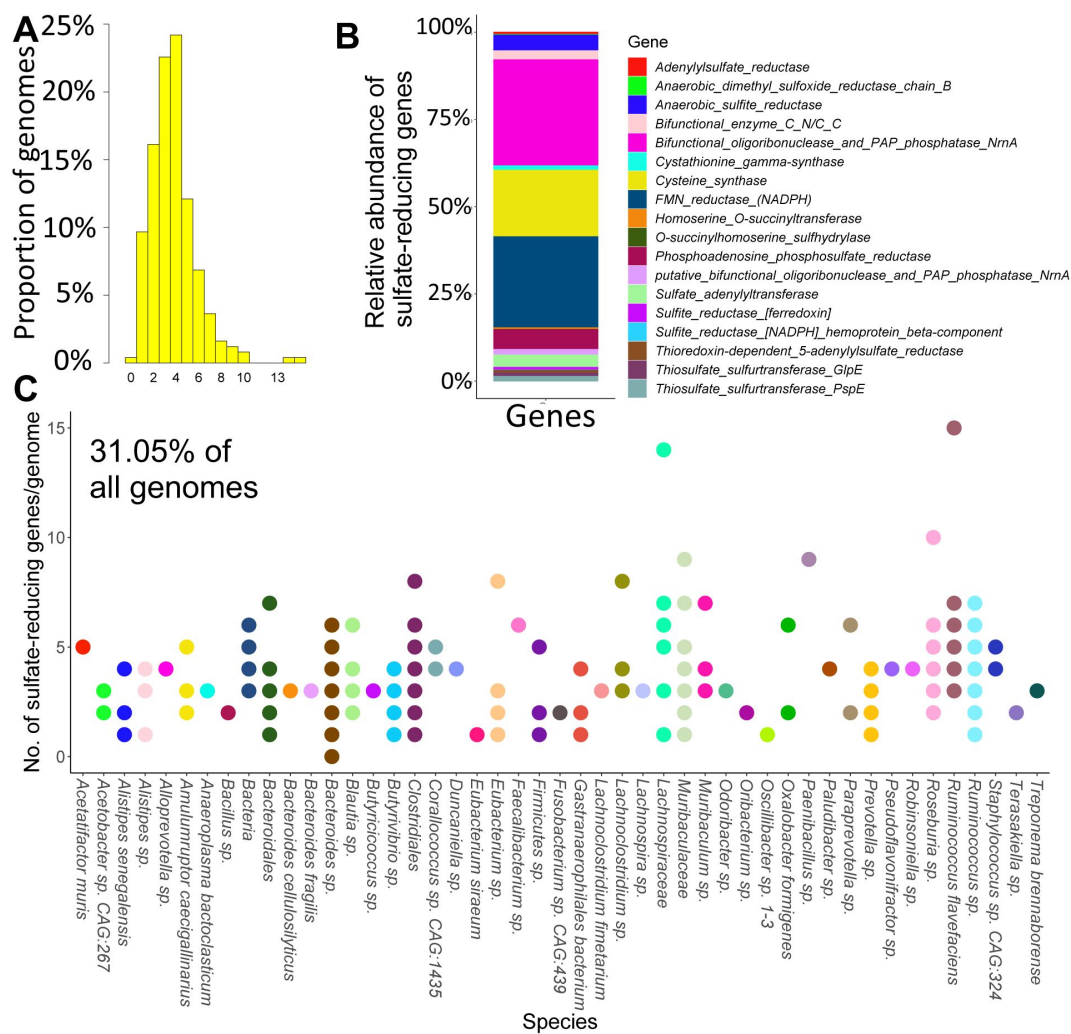
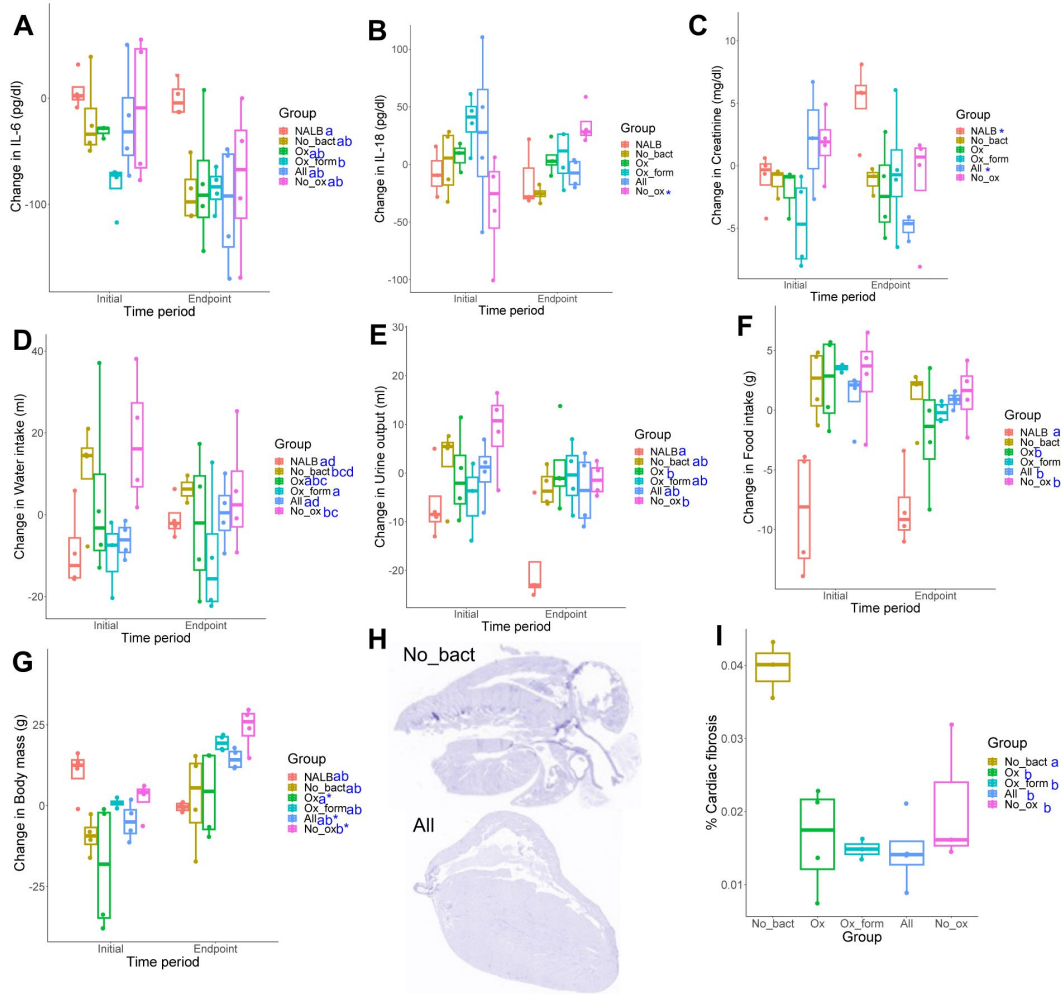


Fig. S5.

Representation of sulfate-reducing genes in the NALB metagenome derived from 248 full length genomes.

A) Proportion of the genomes extracted from *N. albigula* that had at least one sulfate-reducing gene. B) Representation of sulfate-reducing genes among the metagenome, broken down by gene function and proportion of total sulfate-reducing genes. C) Representation of sulfate-reducing genes broken down by taxon.

Taxonomic cohort



Group 1	No_bact
Group 2	Ox
Group 3	Ox_form
Group 4	All
Group 5	No_ox

Fig. S6.

Microbial transplant composition impact on urinary inflammation, renal health, and overall mouse health for the taxonomic cohort.

A-G) The effect of microbial transplants on urinary IL-6 (A), IL-18 (B), and creatinine (C) levels, along with water intake (D), urine output (E), food intake (F) and body mass (G). H) Masson's Trichrome staining of heart tissue reveals fibrosis. Shown are representative images from the No_bact (Group 1) and All (Group 4) groups. I) Quantification of cardiac fibrosis from different microbial transplant groups. Statistical significance - 2-way ANOVA (shown on charts) and post-hoc, Holm's corrected, paired t-tests. Blue letters reflect statistical groups between microbial transplants and * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ for longitudinal comparisons within each microbial transplant group.

Taxonomic cohort

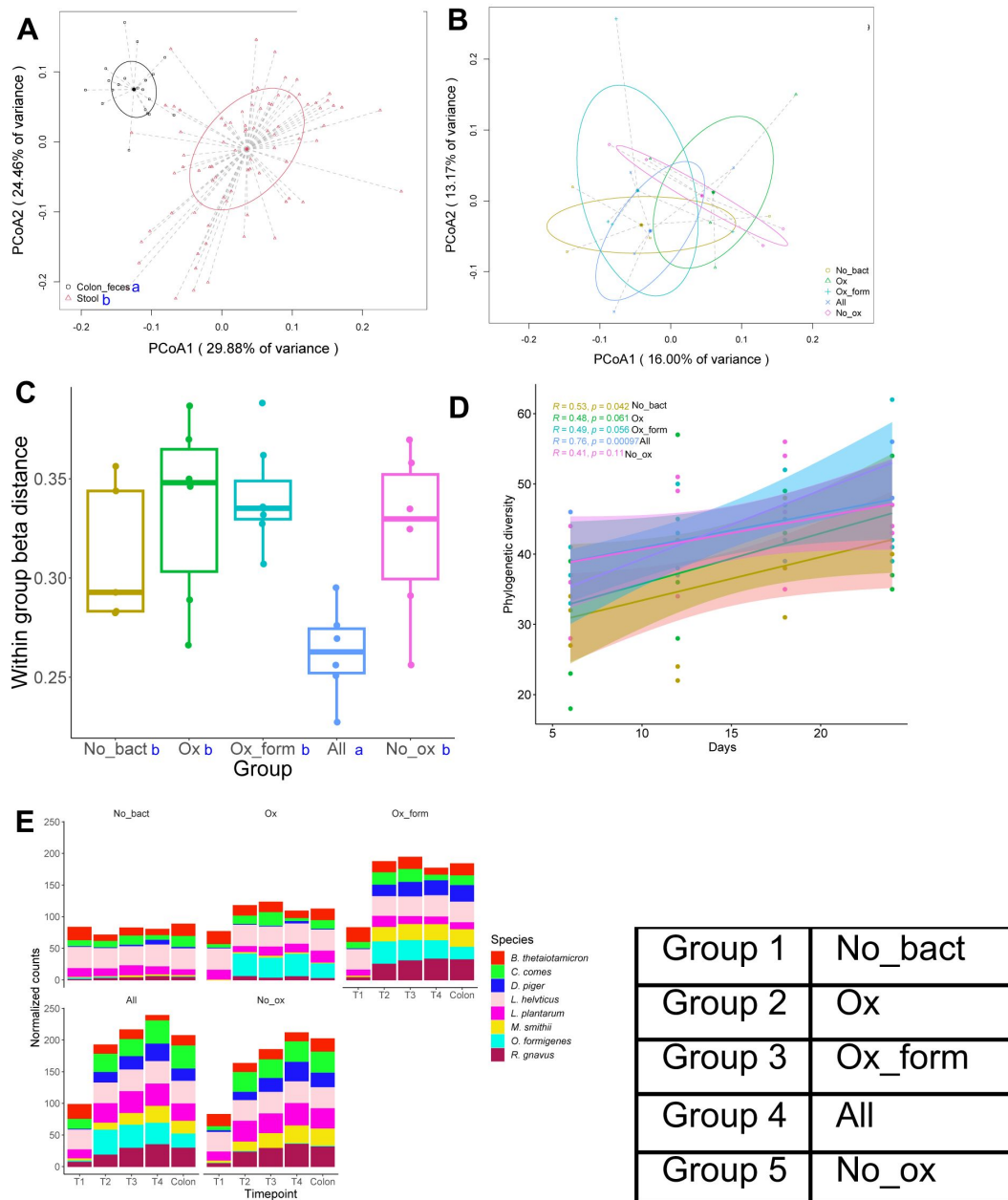


Fig. S7.

Effect of microbial transplants on microbial community composition for the taxonomic cohort.

A,B) Beta-diversity analysis based on a weighted UniFrac dissimilarity matrix of colon feces collected at the end of the study period vs. stool (A; $p = 0.006$, PERMANOVA) or in the endpoint stool samples by group (B; no significant differences). Similar results for (B) were obtained with colon feces. Statistical differences shown by blue letters in legend. C) Within group beta-diversity distance of colon feces. $p = 0.006$, one-way ANOVA. Blue letters represent statistical groups, based on post-hoc, Holm's corrected, paired t-tests. Similar results were found with endpoint stool samples. D) Change in phylogenetic diversity of stool for each microbial transplant group. Stats, shown on graph, are based on Pearson correlations. E) Normalized counts of transplanted microorganisms in the stool across timepoints and in the colon feces at the end of the diet trial.

Metabolic cohort

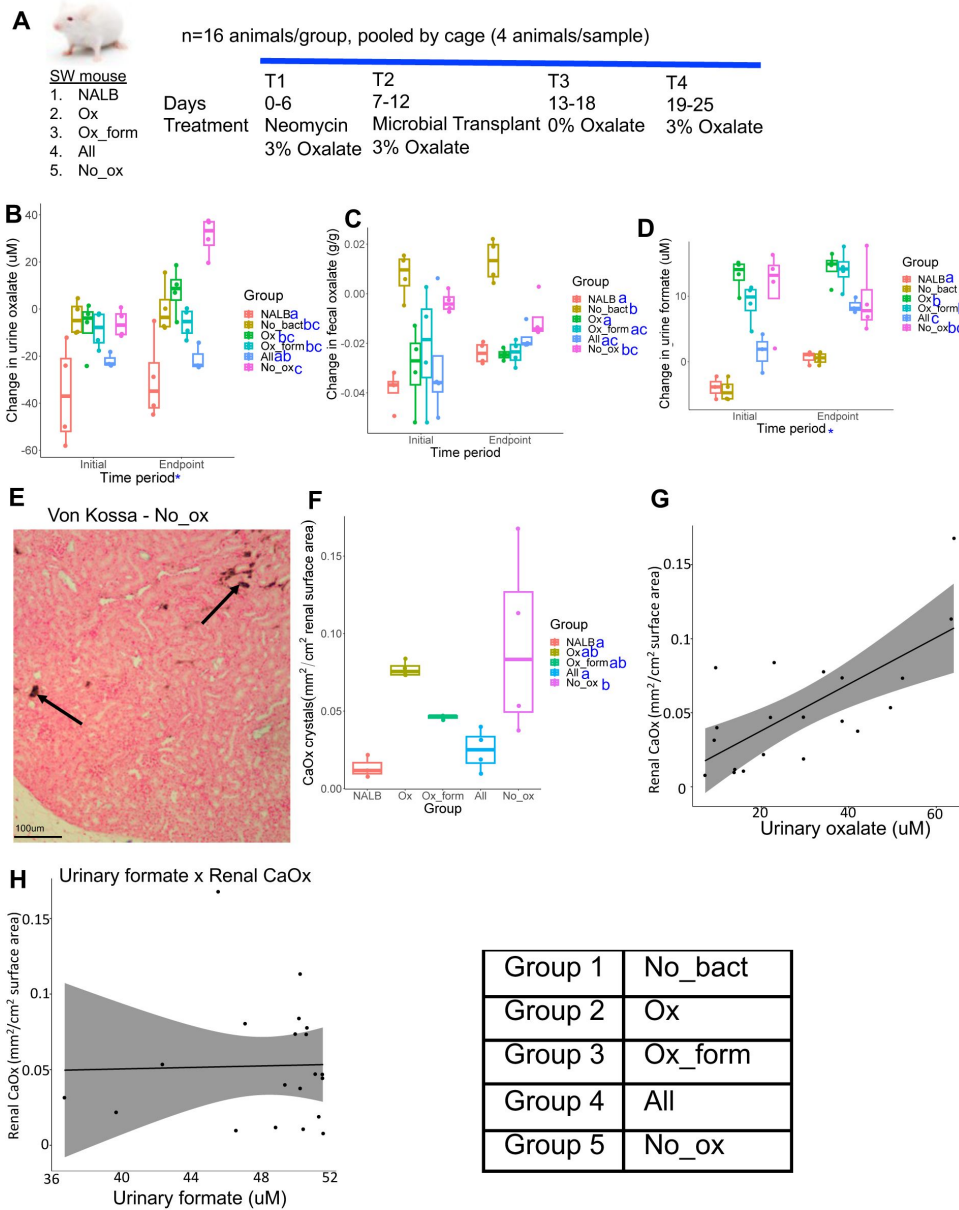


Fig. S8.

Microbial community composition (metabolic cohort) impacted the effect of exogenous oxalate on kidney health.

A) Experimental design. Swiss Webster mice were given neomycin, followed by inoculation of one of five microbial consortia that included either no bacteria, the NALB community, or the metabolic cohort listed in [Figure 5C](#). Animals were maintained on a 3% oxalate diet throughout the trial except for a 0% oxalate washout period after the microbial transplant period. B,C) The effect of microbial transplants on urinary (B) or fecal (C) oxalate levels over the course of the diet trial, compared to baseline. ANOVA $p < 0.001$ for microbial group (B&C), and $p < 0.01$ for both timeperiod and in 2-way analysis for (B only). D) The effect of microbial transplants on urinary formate levels over the course of the diet trial. ANOVA $p < 0.001$ for microbial group and timeperiod. E) Renal calcium oxalate deposition based on Von Kossa staining of renal tissue sections. Arrows show calcium deposits stained black, which were quantified through an automated algorithm in QuPath. F) Quantification of renal calcium oxalate deposition by group. $p = 0.014$; ANOVA. G,H) Pearson correlation between urinary oxalate ($R = 0.7$, $p < 0.001$) (G) or formate ($R = 0.026$, $p = 0.9$) (H) and renal calcium oxalate deposition. Where applicable, statistical significance was assessed through an ANOVA and post-hoc, Holm's corrected, paired t-tests. Statistical groups shown by blue letters.

Metabolic cohort

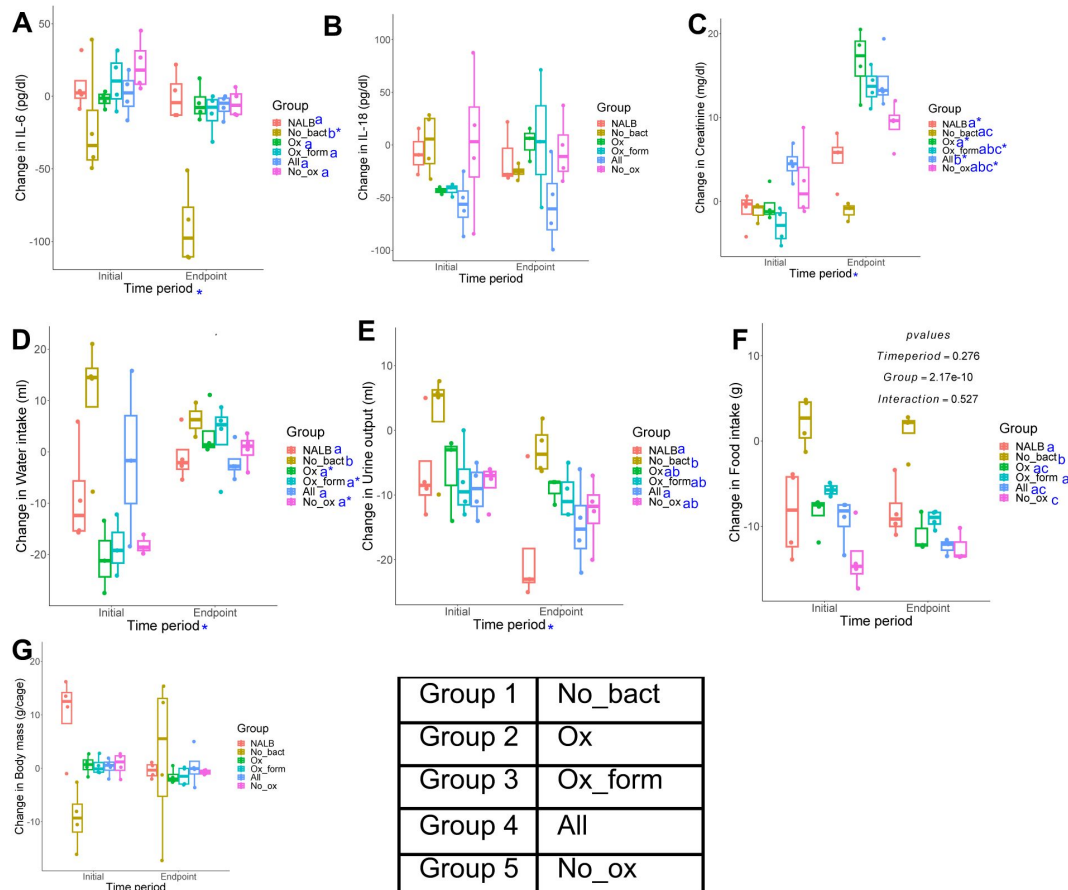


Fig. S9.

Microbial transplant composition impact on urinary inflammation, renal health, and overall mouse health for the metabolic cohort.

A-G) The effect of microbial transplants on urinary IL-6 (A), IL-18 (B), and creatinine (C) levels, along with water intake (D), urine output (E), food intake (F) and body mass (G). Statistical significance - 2-way ANOVA (shown on charts) and post-hoc, Holm's corrected, paired t-tests. Blue letters reflect statistical groups between microbial transplants and * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ for longitudinal comparisons within each microbial transplant group.

Metabolic cohort

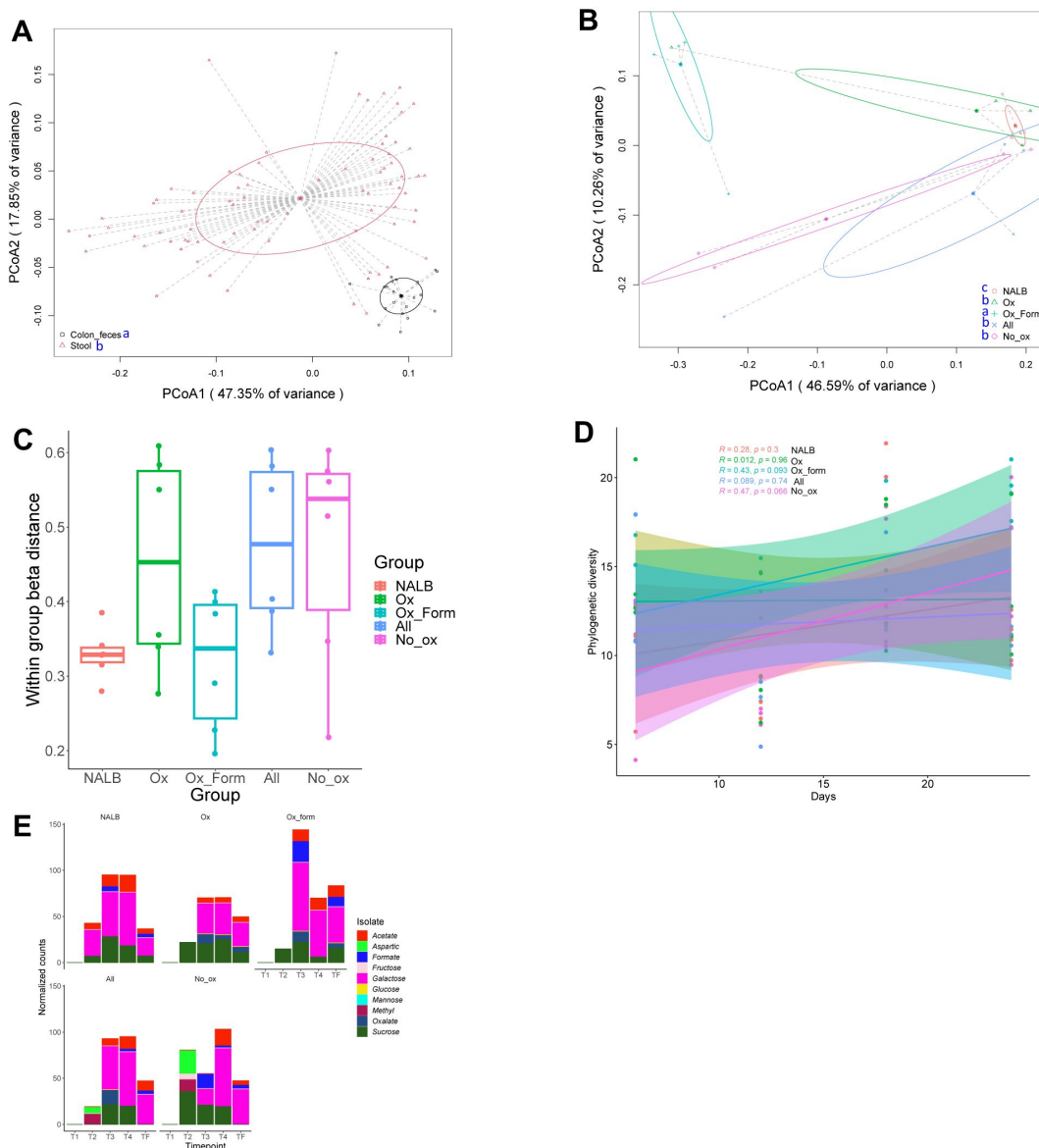


Fig. S10.

Effect of microbial transplants on microbial community composition for the metabolic cohort.

A,B) Beta-diversity analysis based on a weighted UniFrac dissimilarity matrix of colon feces collected at the end of the study period vs. stool (A; $p=0.006$, PERMANOVA) or in the endpoint stool samples by group (B; no significant differences). Similar results for (B) were obtained with colon feces. Statistical differences shown by blue letters in legend. C) Within group beta-diversity distance of colon feces. $p=0.006$, one-way ANOVA. Blue letters represent statistical groups, based on post-hoc, Holm's corrected, paired t-tests. Similar results were found with endpoint stool samples. D) Change in phylogenetic diversity of stool for each microbial transplant group. Stats, shown on graph, are based on Pearson correlations. E) Normalized counts of transplanted microorganisms in the stool across timepoints and in the colon feces at the end of the diet trial.

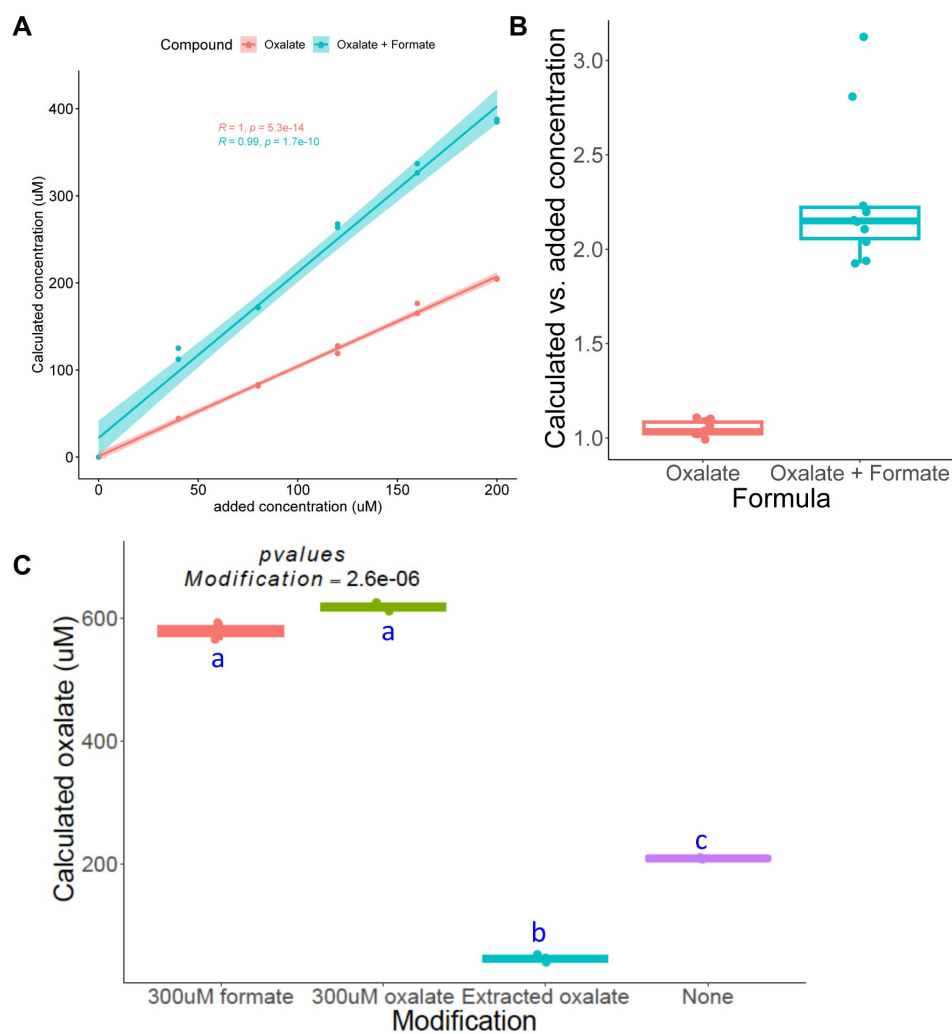


Fig. S11.

Effect of oxalate and formate on oxalate quantification using an enzymatic, ELISA-based assay.

A) Correlation between oxalate or oxalate + formate, added to water, on oxalate concentration quantified through the enzymatic kit. B) Ratio of the calculated oxalate vs. the added concentration of oxalate and formate. C) The effect of adding oxalate or formate to urine, or of extracting oxalate from urine. R values and p-values (A) or p-values from one-way ANOVA (B,C) shown on chart. Blue letters represent statistical groups, based on pairwise t-tests.

Supplementary tables

Ingredients	0% oxalate (g/Kg)	1.5% oxalate (g/Kg)	3% oxalate (g/Kg)
Casein	200	200	200
L-Cysteine	3	3	3
Corn Starch	285.5	NA	NA
Maltodextrin	120	120	120
Sucrose	100	185.4	185.4
Anhydrous Milkfat	NA	200	200
Soybean Oil	70	70	70
Cellulose	150	150	150
Mineral Mix	42	42	42
Vitamin Mix	12	12	12
Choline Bitartrate	2.5	2.5	2.5
TBHQ, antioxidant	0.014	0.054	0.054
Sodium Oxalate	0	15	30

Table S1.

Composition of the diets used in the diet trials.

Component	Amount/L	Concentration	
KH ₂ PO ₄	100 mL	100 mM	
NaHCO ₃	0.4 g	4.8 mM	
NaCl ₂	0.08 g	1.37 mM	
CaCl ₂	1 mL	0.80%	0.8g/100mL stock; autoclave
Vitamin K (menadione)	1 mL	5.8 mM	1 mg/mL stock solution; protect from light; autoclave
FeSO ₄	1 mL	1.44 mM	0.4 mg FeSO ₄ /mL stock solution; filter sterilize
Histidine Hematin Solution	1 mL	0.1%	1.2 mg hematin/mL in 0.2M histidine; filter sterilize after preparation of stock; protect from light; store at 4C
NH ₄ Cl	1 g		
Tween 80	2 mL	0.05%	
MgSO ₄ ·7H ₂ O***	1 mL	0.008 mM	
ATCC Vitamin Mix***	10 mL	1%	
ATCC Trace Mineral Mix***	10 mL	1%	
For sole C&E media, add one of the following to Media A			
Aspartic acid	0.02g		
Mannose	0.9g		
Galactose	1.8g		
Sodium acetate	6g	0.60%	
Sodium formate	4g	0.40%	Requires sodium sulfate
D-glucose	0.4 g	2.2 mM	Add to 10ml DI H ₂ O and filter sterilize
Fructose	1 g	2.2 mM	Add to 10ml DI H ₂ O and filter sterilize
Sucrose	1.71g		Add to 10ml DI H ₂ O and filter sterilize
Methylvaleric acid	561ul		Add both methylvaeric and methylbutyric
Methylbutyric acid	517ul		
Oxalate	6.75g	50mM	
pH to 7			
Filter sterilize and bring into anaerobic chamber			
Cysteine***	0.5g		Add to 10ml DI H ₂ O and filter sterilize in anaerobic chamber
sodium sulfate	2g	0.20%	Only add if adding sodium formate
***Let media sit in anaerobic chamber for at least 24hrs to be fully reduced before inoculation			

Table S7.

Media recipe used to test the proportion of the NALB community that can utilize substrates as sole carbon and energy sources.

Species	Commercial source	Isolation ¹⁶¹⁸ source
<i>Oxalobacter formigines</i>	ATCC 35274	human
<i>Desulfovibrio piger</i>	DSMZ749	Sheep rumen
<i>Methanobrevibacter smithii</i>	DSMZ11975	animal feces
<i>Ruminococcus gnavus</i>	ATCC 29149	human
<i>Coprococcus comes</i>	ATCC 27758	human
<i>Bacteroides thetaiotaomicron</i>	ATCC 700349	human
<i>Lactobacillus helveticus</i>	ATCC 55796	Unknown
<i>Lactobacillus plantarum</i>	ATCC 700210	Unknown

Table S8.

Microorganisms used in the taxonomic cohort.

Additional files

Table S2. The number of host hepatic genes in each metabolic pathway that exhibited a significant increase (positive numbers) or decrease (negative numbers) in expression upon consumption of a 1.5% oxalate diet. [🔗](#)

Table S3. The number of microbial metabolites in each metabolic pathway that exhibited a significant increase (positive numbers) or decrease (negative numbers) in expression upon consumption of a 1.5% oxalate diet by the host. [🔗](#)

Table S4. The significant positive and negative correlations between microbial metabolites and host hepatic gene expression for those metabolites/genes significantly altered by 1.5% oxalate consumption. Data are listed with the host microbiome, metabolite and gene ID, long with correlation values and false discovery rate corrected p-values. [🔗](#)

Table S5. The number of microbial genes in each metabolic pathway that exhibited a significant increase (positive numbers) or decrease (negative numbers) in abundance upon consumption of a 6% oxalate diet by the host. [🔗](#)

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Reviewer #1 (Public review):

Summary:

This study experimentally examined diet-microbe-host interactions through a complex systems framework, centered on dietary oxalate. Multiple, independent molecular, animal, and in vitro experimental models were introduced into this research. The authors found that microbiome composition influenced multiple oxalate-microbe-host interfaces. *Oxalobacter formigenes* were only effective against a poor oxalate-degrading microbiota background and give critical new insights into why clinical intervention trials with this species exhibit variable outcomes. Data suggest that, while heterogeneity in the microbiome impacts multiple diet-host-microbe interfaces, metabolic redundancy among diverse microorganisms in specific diet-microbe axes is a critical variable that may impact the efficacy of

bacteriotherapies, which can help guide patient and probiotic selection criteria in probiotic clinical trials.

Strengths:

The paper has made significant progress in both the depth and breadth of scientific research by systematically comparing multiple experimental methods across multiple dimensions. Particularly through in-depth analysis from the enzymatic perspective, it has not only successfully identified several key strains and redundant genes, which is of great significance for understanding the functions of enzymes, the characteristics of strains, and the mechanisms of genes in microbial communities, but also provided a valuable reference for subsequent experimental design and theoretical research.

More importantly, the establishment of a novel research approach to probiotics and gut microbiota in this paper represents a major contribution to the current research field. The proposal of this new approach not only breaks through the limitations of traditional research but also offers new perspectives and strategies for the screening, optimization of probiotics, and the regulation of gut microbiota balance. This holds potential significant value for improving human health and the prevention and treatment of related diseases.

Weaknesses:

While the study has excellently examined the overall changes in microbial community structure and the functions of individual bacteria, it lacks a focused investigation on the metabolic cross-feeding relationships between oxalate-degrading bacteria and related microorganisms, failing to provide a foundational microbial community or model for future research. Although this paper conducts a detailed study on oxalate metabolism, it would be beneficial to visually present the enrichment of different microbial community structures in metabolic pathways using graphical models.

Furthermore, the authors have done a commendable job in studying the roles of key bacteria. If the interactions and effects of upstream and downstream metabolically related bacteria could be integrated, it would provide readers with even more meaningful information. By illustrating how these bacteria interact within the metabolic network, readers can gain a deeper understanding of the complex ecological and functional relationships within microbial communities. Such an integrated approach would not only enhance the scientific value of the study but also facilitate future research in this area.

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Reviewer #2 (Public review):

Summary:

Using the well-studied oxalate-microbiome-host system, the authors propose a novel conceptual and experimental framework for developing targeted bacteriotherapies using a three-phase pre-clinical workflow. The third phase is based on a 'complex system theoretical approach' in which multi-omics technologies are combined in independent in vivo and in vitro models to successfully identify the most pertinent variables that influence specific phenotypes in diet-host-microbe systems. The innovation relies on the third phase since phase I and phase II are the dominant approaches everyone in the microbiome field uses.

Strengths:

The authors used a multidisciplinary approach which included:

- (1) fecal transplant of two distinct microbial communities into Swiss-Webster mice (SWM) to characterize the host response (hepatic response-transcriptomics) and microbial activity (untargeted metabolomics of the stool samples) to different oxalate concentrations;
- (2) longitudinal analysis of the *N. albigulia* gut microbiome composition in response to varying concentrations of oxalate by shotgun metagenomics, with deep bioinformatic analyses of the genomes assembled; and
- (3) development of synthetic microbial communities around oxalate metabolisms and evaluation of these communities' activity in oxalate degradation in vivo.

Weaknesses:

However, I have concerns about the frame the authors tried to provide for a 'complex system theoretical approach' and how the data are interpreted within this frame. Several of the conclusions the authors provide do not seem to have sufficient data to support them.

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