Gut dysbiosis in experimental kidney disease: a meta-analysis of repository data

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Significance statement

Alterations in gut microbiota contribute to the pathophysiology of a diverse range of diseases, leading to suggestions that that chronic uremia may cause intestinal dysbiosis which contributes to the pathophysiology of chronic kidney disease. Various small, single-cohort rodent studies have supported this hypothesis. This meta-analysis of publicly-available repository data demonstrates that cohort variation far outweighs any effect of experimental kidney disease on the gut microbiome. No dysbiotic changes were seen in kidney disease animals across all cohorts, although a few trends shared between the majority of experiments may be attributable to kidney disease. We conclude that rodent studies do not provide evidence for the existence of 'uremic dysbiosis', and that single-cohort studies are unsuitable for produce generalizable results in microbiome research.

Abstract

Background: Single-cohort rodent studies have yielded insights into host:microbiome relationships in various disease processes, but their relevance is limited by cohort and cage effects. In nephrology, rodent studies have popularized the notion that uremia may induce pathological changes in the gut microbiota which contribute to progression of kidney disease.

Methods: All data describing the molecular characterization of the gut microbiota in rodents with and without experimental kidney disease were downloaded from two online repositories and re-analyzed using the *DADA2* and *Phyloseq* packages in *R*. Data were analyzed both in a combined dataset of all samples, and at the level of individual experimental cohorts.

Results: Cohort effects accounted for 69% of total sample variance ($p<0.001$), substantially outweighing the effect of kidney disease (1.9% of variance, $p=0.026$). No microbial trends were seen universally in kidney disease animals, but some (increased alpha diversity, relative decreases in *Lachnospiraceae* and *Lactobacillus*, increases in other *Clostridia* and opportunistic taxa) were seen in many cohorts, and may represent the effects of kidney disease on the gut microbiome.

Discussion: We argue that there is inadequate evidence that kidney disease causes reproducible patterns of dysbiosis. We advocate meta-analysis of repository data as a way of identifying broad themes that transcend experimental variation.

Introduction

It has been widely suggested that alterations in the composition and function of the gut microbiome may play an important role in the development of chronic kidney disease (CKD). [1] One potential mechanism involves systemic uremia altering the gut microenvironment and exerting selective pressures on bacterial populations, causing structural or functional alterations to the resident microbiota which have been termed 'uremic dysbiosis' [2-4]. It has been suggested that abnormal gut microbiota may drive progression of CKD and its complications through increased generation of uremic toxins, reduced production of beneficial metabolites such as short-chain fatty acids, or disruption of the mucosal barrier of the gut resulting in chronic inflammation.

There is little consensus, however, as to exactly which microbial changes are caused by uremia. In humans, case:control studies have identified changes in host microbiota associated with CKD, [8-13] but confounding factors including dietary restrictions and use of gut-active or anti-microbial medications make the effects of kidney disease itself hard to define.

Several animal studies have described relative changes in the abundances of various microbial taxa in experimental uremia. Most notably, *Vaziri et al* demonstrated that 175 operational taxonomic units were differentially abundant in the gut microbiota of rats following either subtotal nephrectomy or sham surgery, concluding that uremia profoundly affects the gut microbiota. [14] However, other animal studies have yielded contrasting results, [15-17] and since each of these animal studies employed a single, small cohort of animals, the generalizability of the findings they describe is limited. We have previously demonstrated the extent of variability in the intestinal microbiota between two batches of animals from the same supplier, and the ability of such batch differences to influence the metabolomic phenotype of host rats. [18]

In this meta-analysis, we re-analyze publicly available datasets describing the gut microbiota of animals with or without experimental kidney disease from two online repositories, comprising a total of 127 rodents across ten experimental cohorts, to attempt to find common microbial signatures that transcend batch variability and may be confidently attributed to the effect of kidney disease.

Methods

Selection of studies

We searched the Sequencing Reads Archive (SRA) operated by the National Centre for Biotechnology Information (NCBI) for relevant studies, using the search term (uremia OR uraemia OR kidney OR renal) AND (microbiome OR microbiota) AND (rodent OR rat OR mouse OR mice), on the 24th May, 2021. This search returned gut microbiome data from 412 experimental subjects across fourteen studies, which were assessed for suitability for inclusion using the Run Selector facility. Eligibility criteria were: use of rodent subjects, use of experimental techniques to induce chronic (>2 weeks) uraemia, and the use of non-culture dependent, DNA-based tools to assess the gut microbiota. Exclusion criteria included use of other experimental interventions, other than the induction of kidney disease; however, in some studies employing a four-group design (e.g, control, control plus intervention, uremic, uremic plus intervention), data from animals in the non-intervention control and kidney disease groups were included.

Eight studies were excluded: three because there was no induction of kidney disease (NCBI BioProject IDs PRJNA576633, PRJNA596575 and PRJNA325943); two which used RNA rather than DNA sequencing (PRJNA631843 and PRJNA492322); one which employed an acute kidney injury rather than chronic kidney disease model (PRJDB6225); one which studied kidney tissue rather than gut microbiome samples (PRJEB27588), and one which included only human samples (PRJEB11419). Seven of these excluded studies have subsequently been published. [19-25]

The remaining six studies were included, [15-18, 26, 27] including our own study (Randall 2019) which comprised data from two cohorts of animals. Each of these studies have been published in a peer-reviewed journal.

One cohort (Al-Asmakh2020) included sequencing samples from the ileum, cecum and colon for each animal; we elected to include only cecal samples in this analysis to match the majority of the samples from other rat cohorts.

We also included data from two further cohorts of our own (Randall 2021a and Randall 2021b) which are also publicly available via the SRA with the publication of this article. These cohorts have not previously been published in journals; the experimental conditions of these animals and subsequent sample analysis and DNA sequencing are described below.

Finally, phylochip microbiota data from the older, Greengenes repository was obtained for a final study, Vaziri2013, [14] which was the first major study to claim an effect of kidney disease on the gut microbiome. All other phylochip datasets also in the Greengenes repository were manually screened for eligibility using the criteria above but none were suitable.

Data processing

Datasets downloaded from the SRA were converted into fastq format using the fastq-dump software from the SRA toolkit. Raw sequences were analysed in R version 3.6.1, using the DADA2 pipeline (version 1.4), [28] with each dataset pre-processed separately because of differences in primer pairs and sequencing quality, with filtering and trim parameters being optimised for each dataset. One dataset (Al-Asmakh2020) used widely separated primer pairs (337F/805R) which meant that after adjusting for quality, only a very small proportion of reads could be successfully merged, and so for this dataset the decision was made to include only forward reads in order to avoid bias. Two datasets (Mishima2015 and

Kikuchi2017) used 454 pyrosequencing instead of Illumina paired-end sequencing, and so only longer, forward reads were available for these datasets.

Amplicon sequencing variants (ASVs) were aligned against Silva v138 [29] in order to assign taxonomy. Raw abundance data of ASVs were used with taxonomic assignments and sample metadata to create phyloseq objects for each cohort. [30] These phyloseq objects were retained for analysis within each dataset at the level of individual ASVs, but then agglomerated at family level and merged to allow analysis of the whole dataset as described below.

Phylochip data for the Vaziri2013 dataset was substantially different in nature from the sequencing data of all the other datasets; partly because of the nature of the data acquisition (consisting of fluoroscopic intensity scores for each of several thousand probes on the chip, rather than simply those sequences present in the sample), and partly because the taxonomic identities attributed to the different 25-mer probes on the phylochip are incommensurable with the modern Silva taxonomy. Thus, otu table, taxonomy and meta-data were combined for this dataset to allow it to be individually analysed in *phyloseq* in parallel with the other datasets, but this dataset was not agglomerated and merged into the whole-dataset object for combined analysis.

Quantification and statistical analysis

Combined analysis of whole dataset

A combined dataset was constructed to permit comparison between microbial communities from all samples (excluding the Vaziri2013 dataset), irrespective of the sequencing methodologies and primer pairs used.

To allow this, taxa from the individual cohort *phyloseq* objects were agglomerated to family level (the lowest taxonomic level at which all sequencing variants received a confident taxonomic identity), using the tax_glom function in *phyloseq* (version 1.36.0). Taxa were manually renamed across datasets to allow comparison of like with like between cohorts; then a combined taxonomy, meta-data and ASV table were used to construct a *phyloseq* object incorporating all samples.

These data were rendered compositional using centered log-ratio transformation via the transform function in the R package *microbiome* (version 1.14.0), [31] and redundancy analysis (RDA) was carried out using the ordinate function in *phyloseq* which was plotted using the plot_ordination function. Scores and loadings were extracted from the RDA model and used to calculate spatial means and the vector between control and uremic samples within each cohort on the combined RDA axes. The ADONIS function in R package *vegan* (version 2.5.7) [32] was used for permutational analysis of variance (PerMANOVA) calculations.

Separate analysis of individual datasets

Each cohort was then analysed independently at the level of individual ASVs, without agglomeration at higher taxonomic levels. Redundancy analysis and PerMANOVA were carried out using the same methods as for the combined dataset. Additionally, alpha diversity analyses were carried out on log-ratio transformed datasets using the estimate_richness function in *phyloseq*, and beta dispersion was calculated for control and uremic groups using the betadisper function in *vegan*. Abundance data from the combined *phyloseq* object were aggregated to phylum level and rendered compositional before being used to generate the bar charts demonstrating compositional community abundance.

To reflect the composition nature of microbiota datasets, and to allow for multiple hypothesis correction, testing for ASVs displaying differential abundances according to kidney disease was carried out for all cohorts using the analysis of the composition of microbiomes *(ANCOM2)* statistical framework. [33] Code for ANCOM2 (version 1) was obtained from GitHub (https://github.com/FrederickHuangLin/ANCOM, accessed 26th August 2019) and used according to default parameters. ANCOM analysis was carried using data agglomerated at family, order, class and phylum levels to pick out differences between control and uremic samples at each of these levels. For the data presented in supplementary data table 4, only taxa detected at a cut-off of 0.7 were treated as significant, and at each level the differentially-abundant taxa were listed in descending order according to their W score. Also on this table, to allow a crude comparison of the significance of association, is the 2-sample t-test; in some cases this is higher than the set alpha of 0.05, but these all actually had an adjusted significance of <0.05 after multiple hypothesis correction. A simple ratio between mean abundance in uremics and mean abundance in controls is presented to show whether uremic animals had increased or decreased abundance relative to controls.

Experimental method for the two previously unpublished datasets

This paper includes data from two experimental cohorts (Randall2021a and Randall2021b) that were not previously published in a peer-reviewed format. Details of these animal experiments are provided here.

All animal experiments were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986, with local ethical committee approval. All animal work was carried out in the Biological Services Units of Queen Mary University of London at

Charterhouse Square; and complied fully with all relevant animal welfare guidance and legislation (UK Home Office Project License number PPL 70/8350 and P73DE7999).

Randall2021a cohort: This cohort consisted of two experimental groups from a larger study which had been designed to investigate the effects of lactulose on the gut microbiota, using a 2x2 experimental design (control with lactulose, control without lactulose, uremic with lactulose, uremic without lactulose). Only samples from the non-lactulose groups were included in this analysis. In the whole experiment, twenty-seven male wild-type outbred Wister IGS rats were obtained at seven weeks of age from Charles Rivers (Kent, UK). During a week of acclimatization, rats were swapped between cages each day for a week in order to homogenize resident microbiota. Seventeen underwent subtotal nephrectomy (SNx) and ten underwent sham procedures Subtotal nephrectomy involved exteriorisation of the left kidney with decapsulation and removal of the upper and lower poles and subsequent replacement of the middle pole only, followed by total right nephrectomy 2 weeks later. Sham procedures involved exteriorisation, decapsulation and replacement of the left kidney, followed by the same procedure on the right kidney 2 weeks later.

Four weeks after the completion of surgery, lactulose was administered mixed into drinking water to eight SNx animals and six controls, with the remaining animals in each group (nine SNx, six sham) continuing to receive tap water. Only samples from these latter groups were included in the meta-analysis. All animals received free access to RM1 standard rodent diet (SDS dietary services, Essex, UK), and water, and were housed under standard 12 hour light-dark cycles in individually ventilated cages.. There were up to four rats per cage, and the animals housed according to surgical procedure, with no co-housing between batches.

In weeks 5-8 post surgery, rats underwent individual housing in metabolism cages weekly to allow the collection of a 24-h urinary specimen which was frozen at − 80 °C until the time of analysis. Rats were killed by lethal injection of sodium thiopentone (LINK Pharmaceuticals, Horsham, UK), and caecal fluid was expressed, stored in foil and snap-frozen in liquid nitrogen and then at − 80 °C until the time of analysis. Blood samples were taken by cardiac puncture, and after centrifugation, the serum was frozen at − 80 °C until the time of analysis. Data describing the housing, weights and serum biochemistry is available in supplementary data file 5.

Randall2021b cohort: 20 male wild-type C57/BL6 mice were obtained from Charles Rivers at 7 weeks of age. After a week-long period of acclimatization, ten animals were placed on an intervention diet (RM1 with 0.15% adenine as published previously by our group [34]), whilst ten remained on standard RM1 diet (both diets from SDS dietary services, Essex, UK). All animals received free access to food and water, and were housed under standard 12 hour light-dark cycles, with five animals in each individually ventilated cages. Mice were weighed weekly, and housed individually in metabolism cages every four weeks to allow the collection of a 24-h urinary and fecal specimen. All mice were sacrificed 18 weeks after the start of the experimental protocol (at 26 weeks of age), by lethal injection of sodium thiopentone (LINK Pharmaceuticals, Horsham, UK). Cecal fluid was stored at -80^{o} pending DNA extraction. Data describing the housing, weights and urine volumes is available in supplementary data file 6.

DNA extraction and next-generation sequencing: DNA from cecal fluid samples from both cohorts was extracted using the PowerSoil© kit from Qiagen, according to manufacturer's

instructions. Polymerase chain reaction (PCR) was carried out in-house using barcoded 27F/338R primer pairs, targeting the V1/V2 hypervariable region of the 16S rRNA gene. PCR was carried out in a sterile 96-well plate using Phusion Green Hot Start II High Fidelity PCR Master Mix (ThermoFisher Scientific), using an initial denaturation step for 5 mins at 98°C followed by 25 cycles of 98°C for 10s, 53°C for 30s, 72°C for 45s and a final extension of 72°C for 10 min. Normalization of DNA concentrations was carried out using SequalPrep™ Normalization Plates (ThermoFisher) and DNA was quantified using a Qubit® 4 Fluorometer (also ThermoFisher). Pooled samples were then sent for next generation sequencing at the DNA Sequencing Facility, Department of Biochemistry, University of Cambridge. Samples with <4,500 reads were excluded from further analysis, and all remaining fastq files were uploaded to the NCBI SRA database.

Results

Published datasets exhibit significant variability in experimental technique

A search of the NCBI Sequence Read Archive [35] and the Greengenes repository [36] was conducted to discover publicly-available data describing the bacterial composition of the gut microbiota in experimental kidney disease (figure 1). Data were included from seven published studies, including one specifically designed to investigate the effect of kidney disease on the gut microbiome, [14] three designed to investigate bacterial production of uremic toxins, [17, 18, 26] and three investigating gut-acting medications for the improvement of the uremic syndrome (only samples from non-intervention animals were included). [15, 16, 27] In addition, two unpublished but publicly available datasets were included from our own group; a description of the experimental treatment of cohorts is included in the methods section below.

In total, we included data from 127 animals; 73 rats across six cohorts and 54 mice across four cohorts. There were significant differences between these datasets in the animal strains used, the methods used to induce kidney disease, the age of animals at the time of sacrifice, and the sample types used; as well as in the methods of DNA amplification and sequencing employed (table 1).

Raw sequencing data were re-analysed using the DADA2 pipeline and the Silva (v138) taxonomy database. [29] There was a broad but non-significant positive correlation between sequencing depth (mean reads per sample) and observed species richness (amplicon sequencing variants, ASVs, per sample); Spearman rank coefficient 0.55, p=0.133.

Cohort is the key driver of variation across all datasets

Sequencing data from different cohorts were agglomerated at family level (the lowest taxonomic level at which all ASVs were assigned a clear taxonomic identity) and combined to allow broad trends in variation to be visualized across all datasets (figure 2). Redundancy analysis revealed clustering to be most significantly influenced by cohort, with the Al-Asmakh2020 and Kikuchi2017 cohorts completely separate from other samples and only the Randall2019a and Randall2019b cohorts, which comprised animals obtained a few weeks apart from the same supplier, displaying broadly overlapping ordination. We have previously shown, however, significantly differential ordination between these two cohorts when analysed in a reduced dataset comprising only these samples. [18]

PerMANOVA of the log-ratio transformed datasets was used to establish how much variation could be attributed to different experimental variables; this revealed that cohort accounted for the largest amount of variation (69% of variance, $p<0.001$), with host species (rat vs mouse) accounting for 13.3% ($p<0.001$).

Other significant associations were found between clustering and primer type (V1/V2 vs V3 vs V3/V4, 23.9% of variation, $p<0.001$), method of inducing kidney disease (surgery vs adenine feed, 13.2% of variation, $p<0.001$), sequencing methodology (454 pyrosequencing vs Illumina, 9.7% of variance, $p<0.001$) and sample type (feces vs cecal fluid, 6.7% of variance, $p<0.001$); although these variables were closely associated with cohort.

Treatment effect (control vs uremic) did influence sample clustering, but to a much lesser extent (1.9% of variance, $p=0.026$). Scores and loadings from the redundancy analysis model were interrogated to understand the basis for this small observed effect of kidney disease, and it became clear that whilst control and uremic samples were not significantly separated in axes 1 and 2, when plotted on axes 3 and 4 of the redundancy analysis model, there was a significant shift between control and uremic samples in both dimensions (supplementary data figure 1). Loadings for these axes revealed that a ‘uremic’ deflection along both was notably associated with increased abundances of certain families from class

Clostridia (including *Peptostreptococcaceae*, *Peptococcaceae*, *Clostridiaceae* and *Christenellaceae*), supplementary data table 1.

Significant compositional differences exist between experimental cohorts

Each experimental dataset was agglomerated at phylum level, and community composition was plotted for each sample to allow comparison at a high taxonomic level between cohorts (figure 3).

Dominant phyla in all samples were *Firmicutes*, accounting for 76% of reads in rat samples vs 40% in mouse samples ($p<0.001$); and *Bacteroidetes* (58.7% of sequencing reads in mouse samples but only 9.9% in rat samples; $p<0.001$). Mouse samples were simpler than those from rats, with the contribution of these major taxa accounting for an average of 98.4% of reads in mice, but only 84% in rats ($p<0.001$).

The Al-Asmakh2020 and Kikuchi2017 datasets appeared to be outliers compared to other rat cohorts. The Al-Asmakh2020 cohort demonstrated clear differences between control and uremic samples at phylum level, with a substantial increases in *Proteobacteria* and *Actinobacteria* in uremic animals, accounting for an average of 39.9% and 9.4% of reads, respectively, in this group. Conversely, samples in the Kikuchi2017 cohort were very simple, with reads from the phylum *Firmicutes* accounting for 98.5% of reads across all samples and kidney disease having no discernible effect. In other datasets, there were no obvious high-level community differences between control and uremic samples.

Kidney disease increases alpha diversity in rats but not in mice

There were no significant differences in alpha diversity between control and uremic animals across the whole dataset (supplementary data table 2, supplementary data figure 2). However, samples from rats were found to have higher alpha diversity than samples from mice (significantly so for observed ASVs per sample, 338 in rats vs 232 in mice, p=0.006, and the related Chao1 and ACE indices).

Among rat samples, those from uremic animals showed higher alpha diversity than those from controls across all measures; significantly so for the Shannon (4.135 in control vs 4.656 in uremic, p=0.011), Simpson (9.952 vs 9.975, p=0.01) and Inverse Simpson (40.74 vs 62.59, p=0.012) indices. Although this was chiefly driven by the highly diverse uremic samples in the Al-Asmakh2020 cohort, a trend towards increased alpha diversity was seen universally across all measures of diversity in every rat cohort. No measures of alpha diversity showed significant differences between control and uremic samples in mice.

Beta dispersion was assessed to test the hypothesis that kidney disease increases the heterogeneity of gut communities, but no reproducible differences were seen between groups, supplementary data table 3.

Samples from control and uremic animals cluster apart in most cohorts

Plots of redundancy analysis ordination for log-ratio transformed datasets at the level of individual ASVs were constructed for all cohorts (figure 4). Samples from uremic animals clustered separately from those from control animals in most cohorts, and this was confirmed using PerMANOVA which quantified significant between-group differences associated with kidney disease in seven of the ten cohorts. Nevertheless, significantly divergent clustering between similarly treated animals (seemingly attributable to cage effects) was seen in a number of cohorts; especially the Mishima2015, Nanto-Hara2020, Randall2019a and Randall2021b cohorts.

Kidney disease is associated with reduced abundances of health-associated taxa and an increased abundance of opportunistic species in some cohorts

The ANCOM2 methodology was used to assess differential abundances of bacterial taxa between control and uremic samples in all cohorts, at each taxonomic level between individual ASVs and phyla (supplementary data table 4).

In two cohorts (Al-Asmakh2020 and Vaziri2013), a classically dysbiotic picture emerged with reductions in health-associated taxa (prominently genus *Lactobacillus*, also *Bacteroides* and *Akkermansia*), an increase in Gram-negative bacteria (including families from the class *Gammaproteobacteria*, such as *Enterobacteriaceae* and *Pseudomonadaceae)*; and an increase in families from the high-GC content, Gram-positive phylum *Actinobacteria* (including *Corynebacteriaceae* and *Bifidobacteriaceae)*.

However, these changes were not seen universally, and in some cohorts – including the Randall2019a and Kikuchi2017 cohorts which did not show significantly differential clustering in ordination plots and PerMANOVA – there were no differentially abundant taxa between control and uremic groups at any taxonomic level. In other cohorts, conflicting results were seen, such as in the two mouse cohorts Mishima2015 and Nanto-Hara2020, where some *Lactobacillus* species were seen to increase in abundance in samples from uremic animals, an opposite trend from that seen in the Al-Asmakh2020 and Vaziri2013 cohorts.

To assess whether similar trends were seen across multiple groups, the mean relative abundance of all families was compared between control and uremic animals within each sequencing cohort (figure 5). There were no families or organisms for which kidney disease caused uniform changes in relative abundance across all cohorts. However, two highly prevalent taxa showed a trend to reduced abundances

in uremic animals (*Lachnospiraceae*, the most proportionally abundant family overall, present at lower relative abundances in uremic animals in 7/9 cohorts, and *Lactobacillaceae*, the third most proportionally abundant family overall, present at lower relative abundances in 7/8 cohorts). Other highly abundant families (including *Oscillospiraceae* and *Ruminococcaceae*) did not show anything approaching a uniform association with kidney disease. A number of lower-abundance taxa showed relatively uniform increases in uremic animals, including *Clostridiaceae*, increased in 8/9 cohorts; *Erysipeltrichaceae*, increased in 7/9 cohorts; *Peptostreptococcaceae*, increased in 6/7 cohorts; *Tannerellaceae*, increased in 6/7 cohorts; and *Eggerthellaceae*, increased in 7/9 cohorts). In most cohorts the difference in mean relative abundances between control and uremic samples was small, and there were no families in which the mean relative abundances were significantly different between control and uremic animals.

Discussion

This meta-analysis demonstrates that between-cohort differences eclipse the effect of experimental kidney disease in explaining compositional variation between samples of rodent gut microbiota. The limitations of animal models of uremia means that it is impossible to say whether the greater degree of 'uremia' seen in human subjects on dialysis may exert more selective pressure than is seen in the animal models described in the animals studies re-analyzed here. Likewise, limitations of 16S amplicon sequencing (which describes only the composition, and not the activity of the microbiota), mean it is impossible to determine on the basis of the data described her whether a similar population of gut microbes may be exhibiting a different metabolic phenotype in the gut environment of a host organism with kidney disease than they would in the gut environment of a healthy host.Nevertheless, these findings do fundamentally question the idea that kidney disease itself, in the absence of drug, dietary or behavioural interventions commonly used in humans with CKD, causes distinct and reproducible changes in the composition of the gut microbiome.

Although it is possible that differences in experimental technique and sequencing methodologies may account for some of the cohort differences described in this meta-analysis, we suggest that the majority is likely to be accounted for by baseline differences in the gut microbiota of animals used in different studies. These results are consistent with previous reports showing batch variation to be a major confounder in microbiome research; [37] microbial variation being demonstrated based on animal vendor, [18, 38] differences in husbandry, [39-44] animal strain, sex and even diurnal rhythm. [45-47]

Significant heterogeneity between experimental cohorts makes it difficult to describe with confidence any reproducible pattern of 'uremic dysbiosis'. For example, whereas in the Al-Asmakh2020 and Vaziri2013 cohorts, large and classically 'dysbiotic' effects were associated with induction of kidney disease, in the Kikuchi2017 and Randall2019a datasets (which employed the same intervention – five sixths

nephrectomy, in the same host species, rats), there was no discernable difference at all between the microbiota of experimental groups. These data suggest that microbial community changes may vary widely between groups of experimental animals in response to a similar biological insult, and pose significant questions about the generalizability of any single-cohort rodent experiments where the gut microbiota might play a significant physiological or pathological role. Publication bias risks obscuring genuine heterogeneity of response by highlighting experiments reporting more striking results.

A drawback of most of the studies included in this analysis is that caging was defined by treatment class (i.e. control animals were housed with other controls, and uremic animals with other uremics), presumably because of the practicalities of administering a modified feed, or allowing for different recovery times after sham surgery or subtotal nephrectomy. The consequence of separate housing is that it becomes impossible to distinguish the effects of kidney disease from the diverging effects that would be seen in microbiota between any two groups of animals housed in separate cages. Profound cage effects were seen between different groups of similarly-treated animals in a number of the cohorts analyzed (Mishima2015, Nanto-Hara2020, Randall2019a and Randall2021b), and in several of the cohorts there were ASVs present in high abundance in one experimental group but totally absent in the other group; we feel this situation is highly likely to reflect cage effects rather than the biological effect of kidney disease. Interestingly, the fewest differences between control and uremic groups were seen in cohorts where attempts had been made to reduce caging effects, either by moving animals between cages prior to the initiation of surgery to homogenize microbial populations (Randall2021a), by housing control and uremic animals together after post-operative recovery (Randall2019a and Randall2019b), or by housing all animals in individual cages (Kikuchi2017).

We conclude that single-cohort studies comparing control and intervention animals are an unsuitable tool for investigating the role of the gut microbiota in health and disease. Future microbiome experiments

should assess baseline gut microbiota in all animals before experimental interventions are undertaken, allowing comparison of longitudinal changes in bacterial taxa and allowing every animal to act as its own control. Studies should ideally employ several batches of animals, take measures to reduce caging effects, and if describing 'dysbiosis', should demonstrate trends that transcend batch and cage variability, as has been advocated elsewhere. [48, 49] We furthermore suggest that meta-analysis of published data from different studies, such as this one, can be used to discriminate batch effects from genuine biological trends.

In the specific context of experimental kidney disease, no bacterial trends could be demonstrated that were present universally in uremic animals across all animal cohorts. However, the following features were each seen in many cohorts, implying that the common factor of kidney disease may be causative: increased alpha diversity (in samples from rats); an increase in lower-abundance taxa including non-*Lachnospiraceae Clostridia*, *Gammaproteobacteria* and *Actinobacteria*; and a decrease in core, health-associated taxa, particularly *Lactobacillus* and *Lachnospiraceae*. It is possible that the effect of kidney disease is to disrupt dominant members of the gut microbiota and create an environment where less-prevalent, opportunist organisms, varying at species level between cohorts, can expand in number. However, it must be noted that some animal cohorts (eg Kikuchi2017 and Randall2019a) did not display even these broad trends. Reassuringly, the effects we describe in our re-analysis of others' data are broadly the same as those reported by the authors in the original descriptions of their research.

These data also present a broadly similar picture to results in published human studies. We are aware of twenty studies describing the molecular characterization of the gut microbiota in kidney disease; findings from these studies are summarized in Table 2. [8, 10-14, 50-63] There are many differences between these studies in the patient populations included, sequencing techniques used and statistical analysis employed, and many of these studies not meet quite basic requirements for modern microbiological work,

as discussed in a recent review article. [64] Whilst most of these studies compare patients with end-stage renal disease on dialysis with healthy controls, several consider those with much milder renal impairment [8, 13, 56, 58]. Similar to the rodent data reviewed in this paper, there is a significant degree of heterogeneity in the results described by authors, alongside some broader themes that may reflect the effect of kidney disease, and in some case match trends in the animal data presented here. Measures of alpha diversity were reported in 11/20 studies; in six diversity was reduced in kidney disease [8, 11, 13, 50, 59, 61], in three it was not significantly different [9, 12, 14] and in two it was increased [56, 65]. Eleven studies reported the results of ordination between samples; in five there was clear separation between control and kidney disease samples [11-13, 50, 52]; in two samples there was partial separation [53, 61] and in five there was no separate clustering; [8, 14, 56, 58, 66] although two of these studies noted that samples from kidney disease subjects showed a greater degree of beta dispersion than those from controls. [14, 58] At the level of individual microbial taxa, changes in abundance between subjects with kidney disease and controls were especially notable for *Enterobacteriaceae* (where the whole family, or subtaxa within it, were increased in subjects with kidney disease in 13 studies, [8, 10, 11, 14, 50, 53, 56, 57, 60-62, 65] but decreased in five [11, 12, 50, 54, 60]), *Clostridia* (increased in subjects with kidney disease in four studies [10, 11, 13, 53] but decreased in one [55]), *Bifidobacteria* (decreased in subjects with kidney disease in three studies [8, 10, 60] but increased in one [12]), and a group of well-recognized, health-associated producers of short chain fatty acids (genera *Roseburia, Faecalibacteria, Romboustia, Blautia* and *Eubacteria*; decreased in subjects with kidney disease in nine studies, [11-13, 50-53, 55, 61] but increased in two [8, 50]).

Many questions remain in seeking to define the relationship between kidney disease and the gut microbiome. Firstly, what microbiological factors underlie the heterogeneity of changes seen in response to kidney disease in the host organisms? Are there features of organisms, or consortia of organisms, that make them more or less able to survive in a uremic environment? Longitudinal studies showing how

different taxa fare over time as a host organism becomes uremic may be helpful in this regard, as may *in vitro* testing of urea tolerance by batch culture.

Secondly, might the functions of the microbiota change in kidney disease, even if structural shifts vary? For example, in the setting of non-alcoholic fatty liver disease it has been shown that functional changes in the microbiota precede compositional changes, but nevertheless affect host phenotype. [68] The family *Lachnospiraceae* (which tended to reduce in relative abundance in uremic animals) includes numerous species which are highly metabolically active and associated with production of short-chain fatty acids, which have a variety of beneficial health outcomes. [69, 70] *Gammaproteobacteria*, [71, 72] which we have shown to be variably increased in the gut microbiota of uremic animals, possess the metabolic potential for production of toxic molecules from dietary protein [73, 74] and production of forms of lipopolysaccharide which have been associated with an exaggerated inflammatory response to systemic endotoxemia. [75, 76] Studies using different -omics techniques (metagenomics, metatranscriptomics, metabolomics, metaproteomics) may answer these key mechanistic questions. For example, recent metabolomic data has suggested that fears of increased toxin generation by bacteria in the context of CKD may be wide of the mark. [77]

Finally, is the microbiome of uremic animals amenable to the kinds of modifications that have been demonstrated in other contexts, for instance using prebiotic or probiotic interventions? Studies looking at manipulation of the microbiota for therapeutic purposes may lead us to view the microbiome in CKD not primarily as a factor in pathology, but rather as a potential therapeutic resource.

We conclude that single-cohort, intervention/control rodent studies are not fit for purpose in describing the effect of experimental conditions on gut microbiota, or on the wider host phenotype where the host:microbiome interactions might be a key pathophysiological factor in the disease process studied. We conclude further that there is no definite and reproducible effect of experimental kidney disease on the

rodent gut microbiome, but that trends seen in several different experiments may be caused by the effects of kidney disease. Finally, we advocate meta-analysis of repository data as a way of addressing experimental variation and identifying trends that transcend batch effect.

Authors' contributions

DR was the main investigator and downloaded and analyzed the raw data and compiled much of the information presented in this paper. JK was responsible for animal husbandry of each of the Randall2019 and Randall2021 datasets included here. LH completed sequencing analysis for the Randall2019a&b datasets, and provided bioinformatic support. KM, MC and MMY were responsible for overall conception and supervision of the project and analysis and presentation of the data.

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Competing interests

The authors declare that they have no competing interests.

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Availability of data and material

All data is available via the online SRA and Greengenes repositories using accession numbers described in the text.

Supplementary material: table of contents

Figure and table legends

Figure 1: Consort diagram of the meta-analysis. Searches were conducted on 24th May 2021. The NCBI BioProject IDs of excluded studies were PRJDB6225, PRJEB27588, PRJEB11419, PRJNA576633, PRJNA596575, PRJNA631843, PRJNA325943 and PRJNA492322; seven of these studies are published. [19-25] BioProject IDs of included studies are shown in table 1. All datasets in the Greengenes repository were manually screened but none met inclusion criteria. NCBI, National Center for Biotechnology Information; SRA, Sequencing Reads Archive.

Table 1: Protocols for animal cohorts and techniques used for molecular characterization of gut microbiota in the datasets included in this study. ≠The publicly available phylochip data from the Vaziri2013 dataset consists of mean fluouroscopic intensity data from 4,522 probes each consisting of a 25 bp DNA strand against a portion of the 16S rRNA gene unique to one bacterial taxon. Each of these probes was treated as a separate ASV for the purposes of *phyloseq* analysis. ǂThe Al-Asmakh2020 dataset included paired samples from the ileum, cecum and distal colon of each animal; we included only the cecal fluid samples to match the majority of other rat datasets. ASV, amplicon sequencing variant; NCBI, National Center for Biotechnology Information.

Figure 2: Ordination plot of redundancy analysis of combined, log-ratio transformed data from all sequencing samples, agglomerated at family level. Each point represents an individual sample; circles represent samples from control animals and triangles samples from uremic animals; colors represent samples from different cohorts.

Figure 3: Proportional abundances of bacterial phyla in all experimental cohorts. Rat cohorts are on the top row and mouse cohorts below. Each vertical bar represents a sample from a single animal, grouped within cohorts with control samples on the left and uremic samples on the right. Because of the nature of phylochip analysis, the Vaziri2013 cohort included data for 43 phyla, including many making negligible contributions to the overall population, thus for the Vaziri2013 cohort only the 12 most abundant phyla are shown and a different legend is provided to reflect the different taxonomy used in phylochip analysis compared to the other datasets. The relatively high abundances of minor phyla in the Vaziri2013 samples may reflect increased prominence of these probes in the design of the phylochip.

Figure 4: Ordination plots of compositionally-transformed data for all cohorts at the level of individual ASVs. Each point represents a sample from an individual animal, colored according to treatment (control vs uremic). R^2 and p-values from PerMANOVA analysis of the same data are superimposed on each plot. Divergent clusters between similarly-treated animals seen in the Mishima2015, Nanto-Hara2020, Randall2019a and Randall2021b are attributed to caging effects.

Figure 5: Relative abundances of the fifteen most abundant families of bacteria in the overall dataset in uremic vs control animals within different experimental cohorts. Graphs are presented in order of decreasing overall abundance, with *Lachnospiraceae* having the highest overall relative abundance, *Muribaculaceae* the second highest, and *Lactobacillaceae* the third highest, and so on. Each point represents the mean proportional contribution of a particular family of bacteria within control or uremic animals in a given cohort; when a family is represented by fewer dots than the number of studies, this is because that family was not detected in all studies; bars link control and uremic animals within a given cohort so that upwards slopes suggest that the family forms a higher proportion of the microbiota in uremic animals within that cohort, and downwards slopes indicate that the family forms a higher proportion of the microbiota in control animals in that cohort. None of the differences between the average proportional abundances between control and uremic animals were significant by two-sample unequal variances t-test, and the average of all slopes on a given graph was never significantly different from zero by the one-sample t-test.

Supplementary figure and table legends

Supplementary figure 1: Ordination plot showing axes 3 and 4 from the redundancy analysis model of all samples. Axis 3 represented 14.3% of total variance and axis 4 represented 6.7%. Each point represents an individual sample, colored according to treatment class. Ellipses represent different cohorts. Within most cohorts, uremic samples were clustered below and to the left of control samples, and overall there was a vector of [-0.035, -0.046] between the spatial mean of all uremic samples compared to the spatial mean of all control samples (p=0.045 and p=0.009, respectively). Furthermore there was a uniform negative vector of movement in both axes between the spatial mean of uremic and control samples within each individual cohort, implying the same microbial shifts are occurring in all datasets.

Supplementary data table 1: Loadings for axes 3 and 4 in the RDA model, the basis for the scores plot in Supplementary data figure 1. These axes showed significant associations with the shift in spatial means between control and uremic samples (p=0.045 and p=0.009, respectively). The five families most positively and negatively associated with each axis are listed along with their respective contributions to the model.

Supplementary data table 2: Alpha diversity. The mean alpha diversity for groups of samples is calculated using measures including observed amplicon sequencing variants per sample, and the Chao1 index, the abundance-based coverage estimators (ACE) index and the Shannon, Simpson, Inverse Simpson and Fisher indices. Comparisons are made using all samples in the dataset between control and uremic samples and between rat and mouse samples; and between control and uremic samples in all and all mouse samples, and then within each individual cohort. Significance is assessed using the unequal variances t-test. Calculation of these measures for each individual sample was carried out in R using the

estimate_richness function in *phyloseq*. The Vaziri2013 cohort was not included in this analysis because the nature of the phylochip analysis makes the results directly incomparable with sequencing methodologies.

Supplementary data table 3: beta dispersion. The mean beta dispersion, calculated as mean distance between an individual point and the group median, is calculated for all groups using the method developed by Anderson. [78] Comparisons are made using all samples in the dataset between control and uremic samples and between rat and mouse samples; and between control and uremic samples in all and all mouse samples, and then within each individual cohort. Significance is assessed using the unequal variances t-test. Calculation of these measures for each individual sample was carried out in R using the betadisp function in *vegan*. Rat samples were significantly more heterogeneous than mouse communities overall (average distances of individual points to group median 0.399 vs 0.312, $p<0.001$); however, uremic animals did not demonstrate increased beta dispersion compared with control animals either in the whole dataset (0.352 in controls vs 0.364 in uremics, $p=0.53$). The Vaziri2013 cohort was not included in this analysis because the nature of the phylochip analysis makes the results directly incomparable with sequencing methodologies.

Supplementary data table 4: Bacterial taxa showing significant differences in abundance between control and uraemic samples at each taxonomic level within each cohort. Analysis was carried out in R using the ANCOM2 methodology. All significantly differentially abundant taxa at an ANCOM cut-off of 0.7 are included; at species level these are aggregated at genus level.

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