Understanding artificial mouse-microbiome heterogeneity and six actionable themes to increase study power

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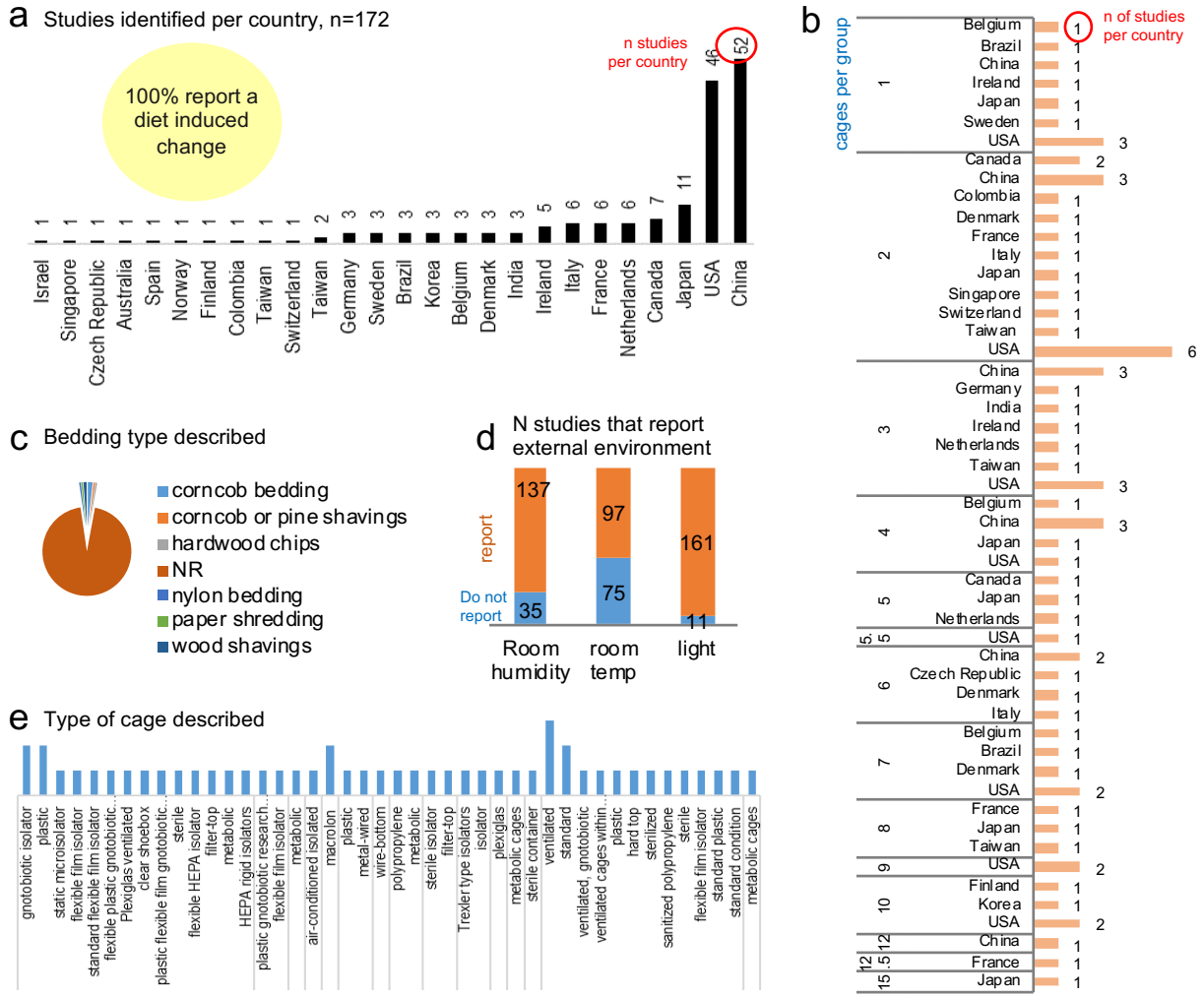
1. Supplementary Methods

Historic and quantitative verification of husbandry variability in published literature. As a test topic, we chose to use 'dietary studies in mice' as a theme to screen for reporting animal husbandry practices in the published literature. Original peer-review research publications were identified using the search terms 'diet and gut microbiome and mice' in PubMed. Abstract screening, and then Article review and data extraction were conducted by two authors (AL, GL) under the supervision of AB and iterative testing/training and problem-solving sessions with ARP. Studies were included if they met the following criteria/definition: 'original article examining the gut microbial community in fecal or cecum samples in mice administered a diet-based intervention'. Studies published in letter or abstract format were excluded as they did not include enough information on animal husbandry for our review. Extracted study information was conducted using a standardized extraction form and included the following variables**; *i)*** study location (country), ***ii)*** whether the diet intervention altered the microbiome, ***iii)*** number of mice per cage, ***iv)*** number of mice per experimental group, as well as reporting of; ***v)*** sterile diets (autoclave, irradiated), ***vi)*** water source and decontamination process, ***vii)*** frequency of bedding change, ***vii)*** light/dark cycle, ***ix)*** room temperature, ***x)*** room humidity, ***xi)*** season/time of year, ***xii)*** mouse strain and GF status of mice. Where appropriate, binary interpretation of data was used for quantitative assessment.

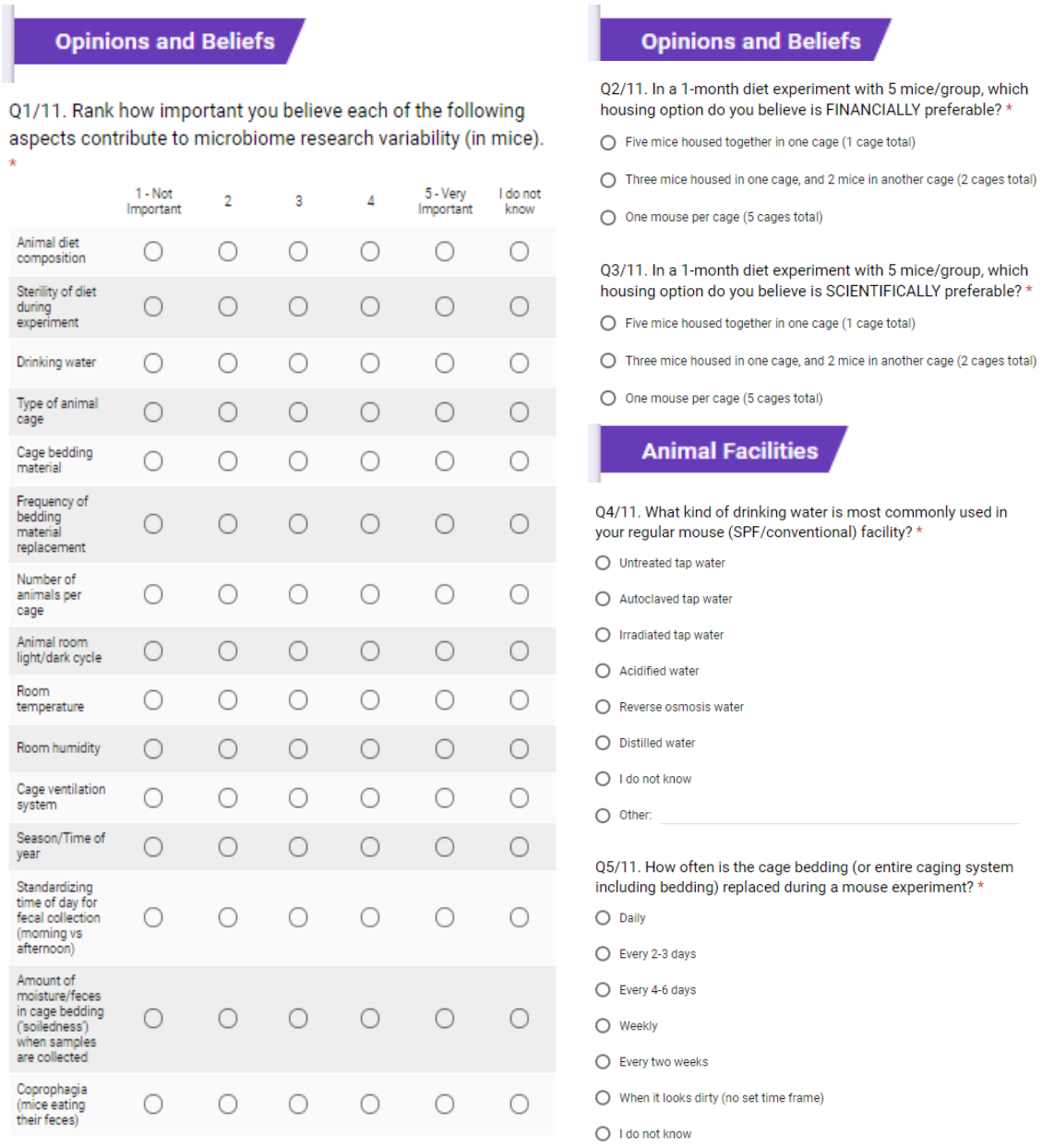
Verification of current husbandry variability among academicians via electronic survey. A one-time online survey with multiple-choice questions pertaining to opinions on animal husbandry practices that influence microbiome data variability was used. Survey questions are described within the results section (and in Supplementary Materials). All research was approved by the Case Western Reserve University Institutional Review Board (STUDY20180138). The survey was administered via secure email (Google Survey) links to eligible participants between August and November in 2018. Informed electronic consent preceded the voluntary, anonymous, and pretested (with 4 individuals) survey. All subjects had the option to withdraw at any time; and no compensation was provided. Eligible participants were >18 years old and recruited via email using the following list servers were used: *i)* membership lists of faculty affiliated to the 17 NIH National Institute Diabetes and Digestive and Kidney Diseases (NIDDK) Silvio O'Conte Digestive Diseases Research Core Centers (DDRCC), which provide research support to investigators in local and national institutions ***ii)*** registrants and attendees of the 2018 Cleveland International Digestive Education and Science (IDEAS) Symposium hosted by the Cleveland DDRCC, CWRU (September 16th- 18th 2018)***, iii)*** registrants of the Taconic Biosciences Webinar (August, Thursday 16th, 2018) titled 'Cyclical Bias and Variability in Microbiome Research', ***iv)*** members and affiliates of the American Association of Laboratory Animal Science (AALAS), and ***v)*** members of the 'Gnotobiotics ListServ', an open professional forum affiliated with the National Gnotobiotics Association. Incomplete surveys were discarded. All multiple-choice questions had an "I do not know" option. A total of 3 follow up reminder emails were sent.

Literature to support evidence-based expert-graded recommendations. To provide evidence-based recommendations and support the development of a consensus report that can be implemented throughout microbiome research practices, we prioritized topics using the ranking of husbandry practices derived from the survey analysis. Using the keywords contained in the individual survey question or topic (*e.g.,* mouse, water), as well as secondary screening of relevant papers using google search and grey literature performed by three individuals, we create a list of relevant supporting or contrasting evidence and potential solutions for consideration to be used as an information tool by the key experts to propose the number and the description/wording of overarching recommendations. Finally, a table containing the list of the core recommendations were submitted to external experts for comments and grading using a simplified objective Delphi protocol based on the agreement between experts. The presented recommendations are listed based on the average grading (ranked from 1 – 10, each Recommendation is graded separately for sentence structure, content, and experimental relevance as integrated solutions; 10 is the highest quality). Secondary experts were provided with the abstract of this manuscript.

2. Supplementary Figures



Supplementary Figure 1. Literature review of studies in 'diet gut microbiome and mice' illustrates heterogeneity in animal husbandry in experiments (complement to data presented in Figure 2). a) Distribution of studies identified per country. **b)** Average number of cages per experimental group used illustrated by country. **c)** Type of bedding described by studies that report bedding type. **d)** Number of studies that do report vs. do not report aspects of external environment. Pie chart shows that most studies (58%) do not report how many animals were housed per cage. **d)** Ranking shows number of studies per country based on estimation of number of cages per experimental group (#cages per group = #mice per cage / #mice per group).



Opinions and Beliefs

Q1/11. Rank how important you believe each of the following aspects contribute to microbiome research variability (in mice). *

	1 - Not Important	2	3	4	5 - Very Important	I do not know
Animal diet composition	○	○	○	○	○	○
Sterility of diet during experiment	○	○	○	○	○	○
Drinking water	○	○	○	○	○	○
Type of animal cage	○	○	○	○	○	○
Cage bedding material	○	○	○	○	○	○
Frequency of bedding material replacement	○	○	○	○	○	○
Number of animals per cage	○	○	○	○	○	○
Animal room light/dark cycle	○	○	○	○	○	○
Room temperature	○	○	○	○	○	○
Room humidity	○	○	○	○	○	○
Cage ventilation system	○	○	○	○	○	○
Season/Time of year	○	○	○	○	○	○
Standardizing time of day for fecal collection (morning vs afternoon)	○	○	○	○	○	○
Amount of moisture/feces in cage bedding ('soiledness') when samples are collected	○	○	○	○	○	○
Coprophagia (mice eating their feces)	○	○	○	○	○	○

Opinions and Beliefs

Q2/11. In a 1-month diet experiment with 5 mice/group, which housing option do you believe is FINANCIALLY preferable? *

- ○ Five mice housed together in one cage (1 cage total)
- ○ Three mice housed in one cage, and 2 mice in another cage (2 cages total)
- ○ One mouse per cage (5 cages total)

Q3/11. In a 1-month diet experiment with 5 mice/group, which housing option do you believe is SCIENTIFICALLY preferable? *

- ○ Five mice housed together in one cage (1 cage total)
- ○ Three mice housed in one cage, and 2 mice in another cage (2 cages total)
- ○ One mouse per cage (5 cages total)

Animal Facilities

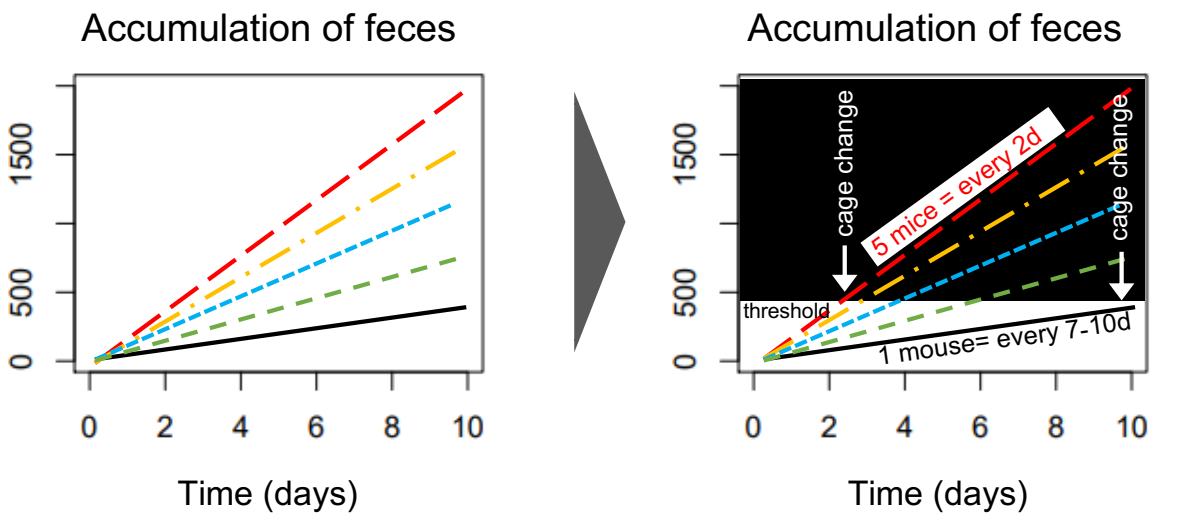
Q4/11. What kind of drinking water is most commonly used in your regular mouse (SPF/conventional) facility? *

- ○ Untreated tap water
- ○ Autoclaved tap water
- ○ Irradiated tap water
- ○ Acidified water
- ○ Reverse osmosis water
- ○ Distilled water
- ○ I do not know
- ○ Other: _____

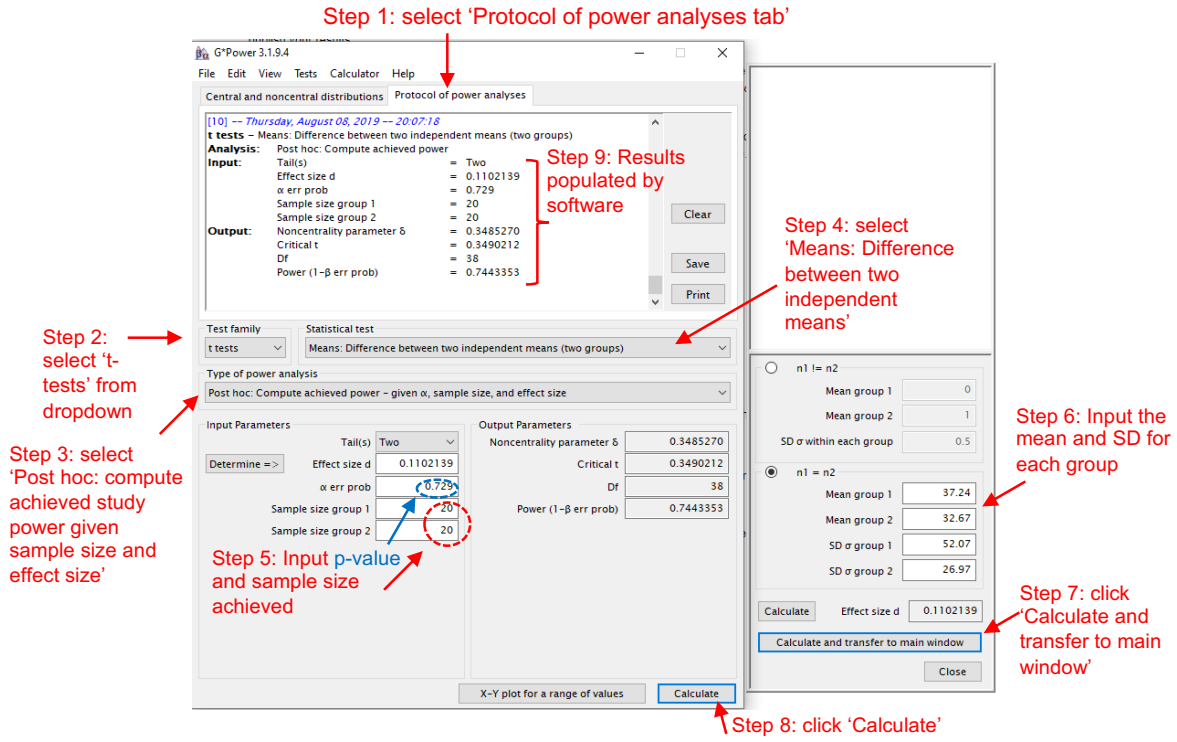
Q5/11. How often is the cage bedding (or entire caging system including bedding) replaced during a mouse experiment? *

- ○ Daily
- ○ Every 2-3 days
- ○ Every 4-6 days
- ○ Weekly
- ○ Every two weeks
- ○ When it looks dirty (no set time frame)
- ○ I do not know

Supplementary Figure 2. Physical appearance of survey pertaining to opinions/beliefs and animal facilities. a) Ranking question on the importance of animal husbandry-related factors in variability of microbiome research**. b)** Opinion question on scientific vs. financial of different animal housing options. **c)** 'Animal facilities' question to determine facility-dependent practices on type of water used and cage change frequency. To understand academia perceptions, we formulated four questions, using 'diet composition' as a positive control because intuitively, we know the quality of responses would be reflected appropriately. The first was a ranking based question (from 1-to-5 with 1 being not important and 5 being very important) comprised of 11 variables pertaining to animal husbandry practices and each in context to the primary question; "Rank how important you believe each of the following aspects contribute to microbiome research variability (in mice)?**"**. **c)** Facility specific husbandry practices.



Supplementary Figure 3. Accumulation of organic matter (feces) in cage bedding. The plot illustrates the linear accumulation of fecal material which increases linearly based on the number of mice housed in one cage (assuming the production of feces by each mouse is consistent). Note that frequency of cage change increases with higher animal density based on the faster accumulation of fecal content in bedding. Cage changes represent a 'clean break' in bacterial overgrowth. From left to right, each line represents 5, 4, 3, 2 and 1 mouse projections. Adapted illustration.[1]



Supplementary Figure 4. Screen shot of open-source power calculator G*Power (complement to data presented in Figure 8). Screenshot of G*Power window for Figure 8A following completion of steps 1-8 (described above) and results populated by software. Open-source software G*Power and detailed manual can be downloaded from https://www.gpower.hhu.de.

3. Supplementary Tables

Supplementary Table 1. Five leading causes of cage-cage variability ('cage effect') in mouse research relevant to experiments and simple solutions*

Animal husbandry factor & Comment on how element introduces variability and solutions
Microbiota drifts and cage type Ammonia levels vary across cages based on cage differences in ventilation, animal density, bedding material, temperature, humidity (moisture level), fecal accumulation, diet, and microbiota. **Variable consequences:** Variable high levels in ammonia have shown to disrupt the 'normal' mouse environment, causing stress, inhibition of small intestine motility, as well as affect upper respiratory functions.[2,3] Cyclical 'dirty cage' effect. **Solution**: Fecal homogenization. Here, it is important to consider the presence of immunomodulatory organisms (*e.g.*, SFB)[4], as well as richness and diversity of inoculum with differences in outcome transfer reported in mice colonized with simpler microbiota.[5]
Cage position on cage rack Cages located in high shelves are exposed to *brighter light* and *warmer air*, (chimney effect, warm air floats), more *vibration*. The opposite applies to cages on bottom shelves. Cages next to corridor are exposed to *human traffic*; cages facing the wall are quieter. Not all *ventilation ports* in pressurized ventilated cages are expected to function equally. **Variable consequences:** animal distraction such as sleep deprivation, visual & auditory stimulation, anxiety, and abnormal feeding/grooming. Closer cages to ventilator will have faster sir flow (less resistance to airflow. **Solution:** 'The more cages, the better' in any experiment to increase heterogeneity and account for statistical errors (*i.e.*, statistical residuals in y=bo + b1x1 + E).
Animal density/cage soiledness N of mice/cage affect Behavioral responses; hierarchy, aggression, and barbering. More mice in a cage will consume food faster, therefore more food replacements mean fresher diet. **Variable consequences: High levels of ammonia (see above).** Coprophagia or coprophagy is considered a normal behavior in rodents and mice and refers to the consumption of expelled feces that are present in the animal cage. While the behavior has been shown to contribute to the animal's nutrition (B-vitamins),[6,7] it also holds strong potential to introduce microbial variability in microbiome research studies, especially as the degree of cage bedding 'soiledness' (dirtiness) increases.[1] In addition, inter-individual variations in mouse gut microbiota are common in animals housed within the same facility room. To address coprophagy as a potential confounding factor, previous studies have proposed housing mice with floors of larger mesh size (to allow feces to fall through)[8] as well as to increase cage-change frequency.[1] **Solution**: Individual housing of mice.
Expected less controllable Cage-variable Batch effect Diet is an expected, but less controlled and reported variable that influences cage effect. **Variable consequences:** Differences in nutritional composition, specifically fatty acids of commercial laboratory rodent diets have been shown to alter exocrine pancreatic function in rodents.[9] Equally, potential for batch-to-batch variability could exist with closed-formula commercial diets (ingredients not publicly available).[10] In this context, studies performed over extended periods may include data generated from diets comprised of different ingredients even if sourced from a single vendor. Diet quality (*i.e.,* ingredients, mold, micro toxins, dust, expiration) and storage of experimental diets (*i.e.,* were diets refrigerated or frozen upon receipt to ensure freshness throughout experiment) should also be considered whether using open- or closed-formula diets.[10,11] ***Diet sterility.*** Intuitively, diet sterility is also an essential factor to minimize external microbial exposure to animals, with non-irradiated diets acting as a variable source of bacteria and fungi. Autoclaving and irradiation are used to improve diet sterility, although in-house autoclaving methods are not standardized and likely lead to variations in nutritional composition of the autoclaved diet.[12] **Solution**: Every effort should be made to ensure diet sterility (autoclaving, irradiation).
Unexpected sporadic/ accidental/non-anticipated sources of technical error Flooding (*e.g.*, accidental water bottle leak). Provision of incorrect bedding or diet treatment.

*Cages can experience different levels of noise, lighting, vibration, and humidity based on rack location. Differences in individual cage humidity levels are a factor of bedding moisture level, type of cage ventilation (static vs mechanically ventilated), number of mice per cage.

Supplementary Table 2. Microbiome variability: sources of cage-cage differences prior/during experimentation

Gut microbiota modulators	Comment
Vendor, Shipping and Institution	Vendor, shipping, the individual institution and facility have been shown to influence and alter gut microbiota with transfer of mice from one facility to another, resulting in microbial shifts.[13,14] One of the most recognized differences is Segmented filamentous bacteria (SFB) presence (commercial vendors) or absence (Jackson Laboratory).[15] Facility-dependent differences in animal husbandry (*e.g.* caging, bedding, cage change freq.; refer to **Supplementary Table 1**), diet[16,17] and water contamination methods.[18,19]
Breeding	Established colonies harbor a stable microbiota over generations.[20] However, the introduction of new dams or a sire harboring difference microbiota can cause downstream variation in gut microbiota of pups.
Methods to eliminate unwanted bacteria	Bacterial elimination methods with downstream effects on gut bacteria include; ***i)*** Rederivation[21-23] and mode of pup delivery (cesarean vs. vaginal),[24] ***ii)*** transfer of mice between facilities or from conventional to barrier settings,[12] ***iii)*** resuscitation of mice from cryopreserved germplasm or *iv)* therapeutic antibiotic use to eliminate unwanted pathogens[25,26] all hold potential to introduce microbial variation. Antibiotic efficacy is highly dependent on regime and baseline richness and diversity of microbiota before initiation.[12,27,28] More targeted measures include reconstituting germ-free mice with specific microbial communities and rederivation via surgical embryo transfer, although these are not without their disadvantages.[12]
Intestinal habitants	The presence or absence of certain members of the gut microbiota can dramatically modulate model immune response and phenotype.[29-31] and thus reproducibility These include; ***i)*** rodent **helicobacters** (*e.g., Helicobacter hepaticus),* [32-41] ***ii)*** **segmented filamentous bacteria** (see detailed review), [15] ***iii)*** certain **protozoans** (*e.g.*, *Tritrichomonas* spp.),[42,43] ***iv)*** the **human isolates** (*Bifidobacterium adolescentis),* [44] and ***v)*** mouse parvoviurs (MPV).[45] It is also highly likely that multiple agents collectively contribute to model phenotype.[46,47]

Co-housing and cross-fostering (transfer of pups to a timed-mating foster dam) are non-targeted solutions that cannot rule out the gut microbiota contribution to phenotype and should be accompanied by next-generation sequencing. See further rational and discussion on how the water type and sterility, which vary by vendor,[18,19,48-52] and the role of coprophagia influence the gut microbiota in **Supplementary discussion**.

Supplementary Table 3. Table for expert grading of proposed recommendation themes for 'sentence clarity', 'potential benefit to increase study power' and 'reproducibility' in mouse research

For each of the five recommendations listed below, please grade from 1-to-10 the sentence for clarity, the potential benefit to improve study power & research reproducibility in mouse research, and the 'recommendability'. Mark with an 'X' your grade for each row.	1 (lowest grade, minimal clarity/ minimal expected benefit to improve study reproducibility, I would not recommend it) 10 (highest grade, complete clarity/ outstanding benefit and potential, I would absolutely recommend it)

Five Recommendations	Rows to grade	1	2	3	4	5	6	7	8	9	10
1. On reporting of diet and animal husbandry factors: Use a *paragraph-style template* (here proposed, as non-plagiarism amenable for future meta-analysis) to facilitate the consistent reporting of detailed diet description and husbandry parameters as publishable accompanying 'Supplementary Materials' in future publications, instead of only following reporting checklists (e.g., ARRIVE guidelines). **Suggestions:**	Grade for Clarity										
	Grade for Potential Benefit										
	Would you recommend it										
2. On cage-cage microbiome variability BEFORE experiments: Use a *fecal matter-based microbiome normalization protocol* (e.g., by orally administering a homogenous pool of feces from a group of mice intended for experimentation, to all the mice, prior the beginning of the study) to homogenize the microbial exposure risk across all mice intended for an experiment, and thus reduce cage-cage microbiome variability that naturally occurs in intensive research mouse production/farming. **Suggestions:**	Grade on Clarity										
	Grade on Potential benefit										
	Would you recommend it										
3. On cage dirtiness and time of sampling DURING experiments: *Prevent the uncontrolled accumulation of animal excrements in the cage* by housing a homogeneous number of animals per cage (ideally low density, 1 mouse/cage), or increase frequency of sanitation of cages, *and collect samples 1-2 days after mice have been in clean bedding/cages* (because coprophagia and 'dirty cages' affect the mouse physiology and microbiota). **Suggestions:**	Grade on Clarity										
	Grade on Potential benefit										
	Would you recommend it										
4. On animal density, clusters, and study power: *House one mouse per cage* (unless more mice per cage is scientifically justifiable) and *increase the number of cages per group* (instead of few cages with many mice, because cohousing results in lower study power due to cage clustered correlated data which requires more mice to compensate for study power loss) to maximize the experimental and statistical value of each animal as a test subject during experimentation. **Suggestions:**	Grade on Clarity										
	Grade on Potential benefit										
	Would you recommend it										
5. On Repeating experiments: *Plan and execute statistically powerful designs* to determine the effect of season and reproducibility of results and *do not repeat underpowered* (cage clustered, low sample size) experiments in different seasons (because several unforeseen factors affecting animal husbandry are very difficult to detect and control in diet and personnel). **Suggestions:**	Grade on Clarity										
	Grade on Potential benefit										
	Would you recommend it										

OTHER RECOMMENDATION/S you may think of? Please describe here: _____

Supplementary Table 4. Compilation of comments made by individuals that graded the initially drafted five recommendations

1. General comments.

Assistant Professor: The way you are putting this paper is interesting. Good luck with the review.

2. Specific comments for each recommendation.

Notice that no comments were excluded from this compilation. In parenthesis are the scores provided for the Recommendation, in the following order, sentence clarity, Potential beneficial impact on study power and reproducibility, and 'recommendability' to other scientists. All graduate research associates were native English speakers.

RECOMMENDATION 1. On reporting of diet and animal husbandry factors:

Use a *paragraph-style template* (here proposed, as non-plagiarism amenable for future meta-analysis) to facilitate the consistent reporting of detailed diet description and husbandry parameters as publishable accompanying 'Supplementary Materials' in future publications, instead of only following reporting checklists (e.g., ARRIVE guidelines).

Comments:

- **Professor**: I cannot understand this paragraph, clearly. (Scores provided 5,6,-).
- **Associate Professor**: Clarity - The portion in parentheses regarding (here proposed, as non-plagiarism amenable) …. is not clear to me. (Scores provided 7, 10, 10)
- **Graduate Research Associate**: Diet description might be determined to be an explanation of macronutrient composition, which many studies include rather than diet sterility and storage details. Scores Provided (9,10.10).
- **Assistant Professor**: not clear what (here proposed, as non-plagiarism amenable for future meta-analysis) refers to. (Scores provided, 5, 10, 10)
- **Graduate Research Associate**: Unclear as written – "Report detailed diet and husbandry descriptions within the supplementary materials section of all submitted work. Information should be presented in a paragraph-style template, instead of only following reporting checklists." (Scores provided, 2,8,8).
- **Associate Professor:** Use a paragraph-style template to facilitate consistent reporting of in vivo study detail such as diet description, husbandry parameters and method of material(s) sterilization. (Suggested example of sentence modification) (Scores provided, 7,10,10).

RECOMMENDATION 2. On cage-cage microbiome variability BEFORE experiments:

Use a *fecal matter-based microbiome normalization protocol* (e.g., by orally administering a homogenous pool of feces from a group of mice intended for experimentation, to all the mice, prior the beginning of the study) to homogenize the microbial exposure risk across all mice intended for an experiment, and thus reduce cage-cage microbiome variability that naturally occurs in intensive research mouse production/farming.

Comments:

- **Associate Professor**: Clarity – are you suggesting that feces from all mice in the experiment be collected at baseline, homogenized, and then a sample be fed orally to all mice in the experiment? How would you ensure that all mice ate the feces sample – an oral gavage? (Scores provided 6,6,6).
- **Associate Professor**: How many samples should be taken for baseline? (Scores provided, 7,10,10).
- **Graduate Research Associate**: Unclear as written and overly verbose. "Prior to study initiation, fecal samples from experimental mice should be collected and processed for oral gavage. Homogenization of the microbiome in this manner reduces cage-to-cage variability." (Scores provided, 2,8,9).
- **Assistant Professor**: Replace mouse production/farming with 'settings'. (Scores provided, 9,10,10).
- **Associate Professor:** Not sure "exposure risk" is optimal terming to homogenize microbial exposure across all mice . . . my understanding is that this is a phenomenon not limited to intensive research mouse production/farming but rather occurs with standard mouse housing practices. (Scores provided, 10,7,7).

RECOMMENDATION 3. On cage dirtiness and time of sampling DURING experiments:

Prevent the uncontrolled accumulation of animal excrements in the cage by housing a homogeneous number of animals per cage (ideally low density, 1 mouse/cage), or increase frequency of sanitation of cages, *and collect samples 1-2 days after mice have been in clean bedding/cages* (because coprophagia and 'dirty cages' affect the mouse physiology and microbiota).

Comments:

- **Assistant Professor**: I agree to this recommendation. The word "dirtiness" in the title could be [added] considered, e.g. environment, because this recommendation has a potential to normalize not only dirtiness but also other cofounding factors as you mentioned. (Scores provided, 9, 10,10)
- **Professor**: can you collect two or three times a day with a group of mice? (Scores provided 8,8,9).
- **Assistant Professor**: Delete low density because it gets confusing and conflicting with the one below. Not clear if this is one or two recommendations. Needs rewording. (Scores provided, 3, 10, 10).

Supplementary Table 5. Compilation of comments made by individuals that graded the initially drafted five recommendations *(cont.)*

- **Graduate Research Associate**: I think that the last part of the suggestion (collecting samples 1-2 days after cleaning) is a little unclear. Perhaps, try saying "collect samples within a few days of cage changes to avoid the effect of coprophagia on the mouse physiology and microbiota. (Scores provided 7,9,10).
- **Graduate Research Associate:** Ok as written, but I would use less parenthesis. (Scores provided 7,6,5).
- **Assistant Professor:** One mouse per cage may not be economically feasible. (Scores provided 10,5,5).
- **Associate Professor:** Frequency of sanitation requires multifactorial considerations; for some phenotypes, "soiled caging" environments are desirable. The emphasis of timing and cognition of sample collection timing relative to cage change is most important. (Scores provided, 10,7,7).

RECOMMENDATION 4. On animal density, clusters, and study power:

House one mouse per cage (unless more mice per cage is scientifically justifiable) and *increase the number of cages per group* (instead of few cages with many mice, because cohousing results in lower study power due to cage clustered correlated data which requires more mice to compensate for study power loss) to maximize the experimental and statistical value of each animal as a test subject during experimentation.

Comments:

- **Assistant Professor**: I think this point might be difficult to understand for some researchers because people are familiar with the "traditional" way (multiple mice per cage). If there is more space, the more detailed explanation on the rationale of this recommendation would be helpful. (Scores Provided, 9, 10, 10).
- **Associate Professor**: My recommendation to follow this suggestion would be based on calculating cost of individual housing versus the savings in lower number of mice needed for the experiment. (Scores provided. 10, 9, 9).
- **Professor**: put one mouse per cage, IACUC may not approve it. (Scores provided 8, 7, 8).
- **Assistant Professor**: The recommendation is partially same as above, so they both need to be refined. (Scores provided 5,10,10).
- **Associate Professor**: It could make mice more depressive and apatic, that will influence microbiome as well. (Scores provided, 7,7,7).
- **Graduate Research Associate**: I'm not entirely convinced on this point. (Scores provided, 8,3,3)
- **Assistant Professor**: Separate your thoughts here. Sentence seems run on. (Scores provided, 3,5,5).
- **Associate Professor:** Lack of social interactions between animals with one mouse per cage may contribute to the altered hormone levels and increase stress, thus affecting the interaction between the host (e.g., immune system) and the microbiome. (Scores provided, 10,8,7).

RECOMMENDATION 5. On Repeating experiments:

Plan and execute statistically powerful designs to determine the effect of season and reproducibility of results and *do not repeat underpowered* (cage clustered, low sample size) experiments in different seasons (because several unforeseen factors affecting animal husbandry are very difficult to detect and control in diet and personnel).

Comments:

- **Professor**: You need to calculate how many mice are really needed for this experiment. (Scores provided 8,9,8).
- **Graduate Research Associate**: This seems wordy to me…try making it more concise (Scores provided 8,6,7).
- **Graduate Research Associate:** This is all well and good in theory, but in practice it seems unrealistic to wait an entire year between experiments. The first part of the suggestion is a given for most experiments. (Scores provided, 8, 3, 1).
- **Assistant Professor:** Not sure what the point is here. (Scores provided, 2, 1,1).
- **Associate Professor:** Presumably, this is to control for variation in natural product variability (diet, bedding substrate). Alternative if this is truly critical for the particular study design, study interpretation may be use of standardized synthetic or purified materials. This may not be needed and maybe cost prohibitive, an area for further assessment I believe. In table 3, no references are provided for seasonal variation and only variation reported in rats is mentioned. I am concerned about the foundation for the strong recommendation made for this category. (Scores provided, 10,3,1).

Supplementary Table 5. Evidence-based husbandry suggestions to improve study power

Animal Husbandry	Peer-Reviewed Scientific Evidence	Considerations to support relevance of the actionable Recommendations
Diet composition	Short- & long-term changes in diet (type, source; e.g., maintenance vs. breeder chow) induce microbial shifts. Such shifts alter their ability to harvest energy and in turn can affect host systemic immune response.[10,53]	• Accurate/thorough description of diet composition. • Reporting should include; *i)* diet type, *ii)* diet source, *iii)* batch, *iv)* storage method (frozen, refrigerated, room temperature).
Diet sterility	Autoclaving and irradiation influence external microbial exposure to animals, with non-irradiated diets (*e.g.,* commercially available rodent chow) providing a variable source of bacteria and fungi (see impact of SFB in Table 2).[10,12] Autoclaving of diet can lead to variations in nutritional composition.	• Every effort should be made to ensure diet sterility. • Thorough description diet sterility (autoclaving, irradiation, none) is required and should include the protocol (*i.e.,,* autoclave settings, irradiation dosage used.
Drinking water	Water source (tap, filtered) and decontamination process (UV light, acidification, autoclaving) alter gut microbiota, specifically via differences in pH, chlorine content, and ion presence. Geographical differences also influence water composition.[49,52] These factors are animal facility-dependent and almost impossible (and not scientifically correct) to standardize.	• Description of water source/decontamination. • Reporting should include; *i)* method of water treatment (*e.g.,* acidification with HCl, autoclave,), *ii)* the water given by the commercial vendor, *iii)* the source of water, and, *iv)* the final pH. • To minimize bias water bottles should be changed at the same time for all cages.
Coprophagia	Coprophagia is a normal rodent behavior and refers to the consumption of expelled feces present in the cage. Although unavoidable, coprophagia holds strong potential to introduce microbial variability, especially as the degree of cage bedding dirtiness increases (bacterial overgrowth).[1]	• Reducing animal density and increasing cage-changing frequency is advisable. • Prior to each study, a **fecal homogenization protocol** that uses fresh fecal pellets from all mice (rather than soiled cage bedding or mixing of bedding protocols) should be used; cages should be replaced after each inoculation.[54-56]
Fecal collection time	Sampling of feces and intestinal gut microbiota is the most popular method to examine different microbial profiles, but is subject to diurnally host-microbiome changes that bear relevance on experimental outcomes of disease.[57,58]	• Systematic fecal collection in all mice under consistent conditions (*e.g.,* morning or afternoon) performed 1-2 days after cage changes.[1] • At sacrifice, fecal/other samples should be collected from one mouse per experimental group until all mice have been sampled.
Bedding material	Many varieties of cage bedding are available such as paper products, wood chips and corn cob, each with its advantages and disadvantages in terms of cost, urine absorbency and ammonia build-up. Collectively, evidence indicates that corncob and hardwood bedding hold higher potential of microbial overgrowth to that of paper bedding, although bedding-dependent microbial overgrowth is a factor of cage ventilation (see below), housing density and most importantly, cage change frequency. [12,59,60]	• Type of animal bedding should be adequately described and details of related animal husbandry factors (number of mice per cage. • Description of animal husbandry factors that influence microbial overgrowth must accompany. • Diet-microbiome studies should use a non-edible bedding material to avoid bias derived from animals consuming cage bedding (*e.g.,* corn bedding).[61]
Housing density (animals per cage)	Under laboratory conditions, it is common practice to house mice by gender, which contrasts the social organization of wild mice. Within-cage differences (e.g. hormonal stress) may be observed among socially housed mice.[62,63] Ultimately, the decision to single or group house animals should be based on the type of experiment and assessments (*e.g.,* single caging is advisable in drug safety/efficacy. studies).	• Animal density should be limited to 1-2 mice/cage with animal feces being sampled on day 2 post-cage replacement ('2 × 2 cage sampling rule').[1] • **Single housing** of mice allows for each cage to be treated as a unit for statistical purposes and thus single-cage studies may be more cost-effective since the number of animals can be reduced.[64]
Light/Dark cycle	Maintaining mice in standard light/dark conditions is a generally accepted practice. Disruptions in the circadian rhythm of light/dark cycle have been shown to affect the gut microbiome significantly.	• Description of light/dark conditions, including any disruptions, should be reported. •
Environmental Temperature	Temperature (and humidity, see below) within a cage affects the rate of microbial overgrowth in bedding material and can vary by as much as 5°F to that of the external room temperature. Factors influencing cage temperature include; ***i)*** cage design/material (filter top), ***ii)*** presence/type of bedding, ***iii)*** frequency of bedding replacement, ***iv)*** animal density (including age/sex) and, ***v)*** the presence of forced ventilation.	• Continuous monitoring of cage temperature levels in individual cages should be performed and reported.

Husbandry variables presented according to the ranking of importance by respondents (Figure 4).

Supplementary Table 5. Evidence-based husbandry suggestions to improve study power *(cont.)*.

Animal Husbandry	Evidence	Suggestion
Cage ventilation	Cage ventilation is a factor of air changes per hour [ACPH=(flowmeter reading (in L/min)/cage volume in Litres) x 60min/hr] that largely determines individual cage measurements of ammonia levels, humidity and cage temperature, thus playing an integral role in bedding moisture levels and rate of microbial overgrowth. The interaction between cage ventilation type and bedding material has strong potential to alter intestinal microbial profiles,[1,59,65] thereby affecting study reproducibility.	• Description of type of cage ventilation should be reported. Ideally, this should be combined with monitoring cage ammonia, temperature/humidity, which will vary with cage density. • Static isolation caging (without forced ventilation) restricts ventilation, and it is important to compensate by adjusting animal husbandry practices such as cage-change frequency, bedding selection, animal density, placement of cages in a secondary enclosure and macroenvironment adjustments to temperature and humidity.
Bedding soiledness	The degree of bedding soiledness ('dirtiness') is directly related to cage change frequency and animal housing density (see above), and exerts a formidable effect on microbiome bias[1,66] primarily due to microbial overgrowth in cage bedding and the coprophagic behavior of mice.	See cage change frequency above
Environmental Humidity	Humidity levels can vary from up to 11% higher within an individual cage.[60] Higher humidity levels within a cage (particularly in combination with higher temperature; see above) increase bedding moisture levels, & promote bedding microbial growth.[1,60,66]	• Continuous monitoring of individual cage humidity levels could be performed and reported, but it may not be realistic. Instead, reporting should include all factors that contribute to individual cage variability in humidity levels (*e.g.,* number of mice, cage ventilation, cage change frequency, etc.).
Cage type	Bacterial community divergence has been reported concerning different cage types such as barrier access vs open cages.	• Description of type of cage used should be reported
Seasonal variation	Changes to gut microbiome with seasonal variation have been reported in rats.	• Details of seasonal variation both within and across different experiments, should be reported.

Husbandry variables presented according to the ranking of importance by respondents (Figure 4). See further details and discussion in Supplementary Discussion.

Supplementary Table 6. Rationale for six actionable recommendation themes

Rationale for six actionable Recommendation Themes
Theme 1 Rationale. Our scoping review of literature and the high number of citations for the ARRIVE guidelines (over 3000 times),[67] show that 'checklists' could be a successful approach to improve reporting quality in mouse research. However, with over half a million papers in mouse research, checklists do not ensure that sufficient details are reported, or that methods are reported in a clear, non-misleading fashion and not open to interpretation. We created, as a template, a methods section from three journals deemed by the authors who conducted the literature search to be of good reporting quality (including the J of the AALAS) and the ARRIVE guidelines. The availability of such paragraph would enforce a more uniform transparency, enhance reproducibility, and enable the rapid data mining of future more comprehensive meta-analyses, widely used to help guide the practice of medicine, but scarcely use in basic science. As described in publishing policies, use of the paragraph proposed here should be allowed if authors cite their previous use in their work, and the rationale that lead to their implementation (*i.e.*, this paper) as described under the 'text recycling' section of well-established publisher[68-71] (see **Supplementary discussion**). Reproducibility will occur only if critical study details are provided in published literature.
Theme 2 Rationale. Fecal bacterial profiles can differ widely between cages within a single mouse strain housed under identical conditions. Fecal homogenization, wherein all mice are administered, via oral gavage, a composite of freshly collected feces for 3 days, before the experiment, has been shown as an effective method to minimize inter-cage heterogeneity in gut microbiota.[54-56] Fecal homogenization inoculum should consist only of fresh fecal pellets collected from all experimental mice (rather than soiled cage bedding or mixing of bedding protocols), and all cages should be replaced following each inoculation.[54] The use of cohousing (discussed elsewhere [12]) is a simple approach that that relies on passive transfer of gut microbiota between co-housed animals. Co-housing and is frequently used to assess the influence of complex gut microbiota, treatment effects and has potential to minimize variation between mice within a cage, but the method does not ensure equal dissemination of low-abundance bacteria,[72,73] or facilitate experiments where males tend to be more aggressive. Although useful in genetic studies, co-housing requires to control for animal density variability and unbalanced clustered designs.
Theme 3 Rationale. Recent culture and microbiome studies of feces and bedding material indicate that increased bedding soiledness ('dirtiness') contributes to period variations in gut microbiome, although the extent to which all potential animal density factor combinations such as body weight, drinking, grinding behavior could ultimately influence gut microbial structure has not yet been examined. Microbiome experiments would benefit if conducted with cages having comparably reduced animal density (*e.g.*, 1-2 mice/cage), with animals being sampled for analysis on day 2 post-cage replacement (*e.g.,* '2×2 cage sampling rule').[1] Mouse biological samples (feces) should be systematically collected at the same time of day (*e.g.*, morning) to avoid diurnal variation.[74]. See further details in **Supplementary Figure 3** and **Supplementary discussion** under 'housing density and cage effect', 'cage frequency and bedding', 'bedding material', and 'time of fecal collection'.
Theme 4 Rationale. Little is known about the impact of season, climate, and latitude in laboratory animal biology, including mouse research. Some studies have shown that the mouse microbiome varies over time in the same facility, but the precise causes of variation are unknown.[13,75,76] Seasonal changes to the community composition of gut microbiota [77] could reflect differences in geographic availability to foodstuff suppliers of food ingredients/crops.[78,79] It is also possible that batch/season variability in diets introduces microbes from distinct locations that are not destroyed by standard irradiation. While it is almost impossible to control for seasonal variation within long-term experiments or multiple experiments spanning over several years, it is crucial to report measures taken to reasonably control for seasonal variation (*e.g.*, food batch storage, inter-experiment fecal homogenization). In certain husbandry settings, the influence of human health and seasonal variation on animal handler microbiome (*e.g.,* respiratory/intestinal infections) could be temporally relevant, but this remains speculative.[80,81]
Theme 5 Rationale: The Guide requires the social housing of social species, mice, except for where scientific justification for variance is requested. One report describing literature trends and reporting solutions[82] determined that investigators fall into four categories with respect to how they handle clustered data: some ignore clustering, while others either reduce clusters (*e.g.,* cages) to independent observations; use fixed effects regression or ANOVA methods; or explicitly account for clustering in design and methods. Before controlling for cluster effects, investigators must thus determine the extent by which data clustering occurs in their design. We provide the statistical rationale[82] to justify the housing of mice at low or individual densities to promote the most efficient use of animals since grouping of mice in cage clusters have also been shown to increase data variability equally in both males and female mice during experimentation.[83] When rearing mice colonies intended for experimental purposes at large scale, we acknowledge that for technological reasons and space limitations it is cost-effective to use the highest possible densities allowed by the Guide or approved by the local institutional/IACUC boards.
Theme 6 Rationale. Conventional statistical analysis of experimental mouse data considers each mouse as an individual data point irrespective of caging allocation. In the case of experiments where multiple mice are housed in one cage (*e.g.,* 5mice/cage and 10 mice per group), this approach leads to a statistical dependence on clustered data. Data of this kind is referred to as cluster-correlated data. For example, when clustering is a result of cage effect, measurement on units within a single cage (with multiple mice) are more similar than measurements on a cage within the same treatment group. Inappropriate analysis of clustered data can give substantially different results that are not reproducible, particularly when the analysis includes between-mouse treatment. As a general statistical approach, mixed models can be used to account for ICC, which can be retrofitted to compute and monitor the achieved study power.

Supplementary Table 7. Methodology reporting template

C1. Recommendation 1: Reporting of diet and husbandry factors**:** *Use of a paragraph-style template (herein provided) to consistently report detailed diet and husbandry factors (e.g., macronutrient, diet sterility) as reproducible publishable accompanying 'Supplementary Materials'.*

Complete the blanks [example text in red italics provided] **as appropriate. Specify details for each experimental group. Refer to the ARRIVE guidelines[67] for a complete description of each reporting recommendation.**

This experiment tested groups [*number of experimental mice per group*] of [*age of mice*]-week *old* [*include terms 'age- and/or sex-matched' as applicable*] [*specify gnotobiotic status of mice*] [*mouse strain/species*] mice, housed using [*type of cage(s)*] as [*number of mice per cage*] to provide a study power of [*specify study power calculation and software used*]. Experimental mice were treated with [*specify treatment; drug formulation,* at a dose of [*dose*] administered via [*administration site and route*] and provided with [*specify analgesia/anesthesia and/or specialist equipment (including vendor) used*] for [*treatment duration*] weeks/days. Mice [*were/were not*] randomly allocated to treatment groups.[*specify controls used for each experiment and experimental group*]. Mice were maintained on [*bedding type*] [*specify type of enrichment added to cage*] and fed an ['*autoclaved', 'irradiated', 'non-sterilized'*] [(*specify irradiation dose and/or autoclave settings*)] diet [*diet name and vendor*] [(*kcal, %CHO, %PRO, %FAT/5g*)] and ['*autoclaved', 'irradiated', 'non-sterilized'*], [*tap, filtered, reverse osmosis, etc*] water [*specify water pH*] [*ad libitum*]. All mouse [*cages*], [*food*], [*water*] was replaced every [*X*] days, and a [*fecal homogenization, mixed bedding, co-housing, none*] protocol was used [*time protocol employed*]. Fresh murine feces were collected routinely in the [*morning, afternoon*] from [*specify whether all cages or select cages*] cages [*number of days*] [*before, after, at time of*] cage replacement and stored for [*#days*] at [*temperature, C*] until analysis.

In studies where multiple experiments span over two or more seasons:

Experiments were conducted in [*specify season and year*]. The batch [*specify if single or multiple*] of food/experimental materials [*were/ were not*] used for all experiments. Homogenous colonization of mice spanning across all experiments [*was/was not*] performed **(see Recommendation 4)**. Data analyses were performed in a [*blinded, non-blinded*] manner with [*single animal, group, cage of animals*] considered as an experimental unit.

Example paragraph:

This experiment tested groups (*12 mice/group'*) of *8*-week *old* '*age- and/or sex-matched' germ-free Black 6* mice housed using *ventilated microisolator* cages with an equal number of *2* mice/cage to provide *80% study power*. Age- and sex-matched untreated GF mice were used as negative controls. Experimental mice were administered *2mg/kg dexamethasone treatment via I.P injection each morning for 7 consecutive days, followed by 14-day recovery. No specialist equipment was used.* Mice *were* randomly allocated to treatment groups. All mice were maintained on *Aspen* bedding (*with nestlet*) and fed *autoclaved (60 minute wet cycle) 40–50 kGy irradiated (6/5 irradiated*) pellet food (*PMI Nutrition Int'l., LLC. Labdiet® Charles River. Vac-Pac Rodent*) diet (*5% kcal% fat*) and *autoclaved, irradiated tap* water (*ph 5*) '*ad libitum'*. All mouse *cages/food/water* was replaced every *7* days. A *fecal homogenization* protocol was used before the start of all experiments. *Fresh* murine feces were collected *routinely, in the morning,* from *all* mice, *2* days *after* cage replacement, and stored for *90* days (*-80C*) until *batch* analysis. Experiments were conducted in *Spring 2019* and *Fall 2020. The same batch of food was used for all experiments.* Data analyses were performed in a *blinded* manner with a *single animal* considered as an experimental unit.

Supplementary Table 8. Summary of actionable items for each Recommendation theme.

Recommendation 1 on 'Reporting of diet and husbandry factors'	• Determine the aspects required by ARRIVE that apply to your institution, laboratory and experiment. • Use the template provided (**Supplementary Table 7**) and create a standard animal methodology section for your laboratory. • Save customized template on a secure folder. Updates/amendments can be saved as 'version xx'. • Upload your customized template to journals with your manuscripts as supplementary materials.
Recommendation 2 on 'Cage-cage microbiome variability BEFORE mouse experiments'	• Determine the number of animals needed and cage density per group, the cage type (e.g., microisolator, static) and bedding material type (*e.g.,* non-edible bedding in diet experiments). • Implement a fecal homogenization; collect fresh feces from all experimental mice and create inoculum to gavage all mice. Repeat for 3 days. Start experiment four days after the final gavage. Detailed protocol previously described. • Collect samples before and after fecal homogenization to confirm microbiota normalization; include in publication.
Recommendation 3 on ''Dirty cages' and time of sampling DURING experiments'	• House an equal number of mice in each cage and maintain a low animal density (see Recommendation 5). • Adjust frequency of cage sanitation (cage changes) to the number of mice per cage (see Figure below) • Systematically collect biological samples (feces) at the same time of day (*e.g.,* morning). • Fresh feces should be collected ideally 2 days after mice placed in clean cages.
Recommendation 4 on 'Repeating experiments in different seasons'	• *In studies where multiple experiments will span over two or more seasons;* • Obtain sufficient food (from a single batch) to sustain all experimental groups. Store food at -20C or as per manufacturer instructions. • Collect and store fresh frozen feces (-80C with cryo-preserving buffer such as glycerol) from the original, 'first round' of experimental mice for use as a normalizing colonization inoculum in mice of future experimental within study. *Note*: this should be performed in addition to Recommendation 2, point 2.
Recommendation 5 on 'Animal density, clusters, and study power'.	• House experimental mice individually housed otherwise scientifically justifiable. • Maintain study power by increasing the total number of mice if implementing higher animal densities (>1 mice/cage) to increase the number of cages per group. • Verify statistical power using open-source study power calculators/software G*power or R.
Recommendation 6 on 'Implementing statistical models to consider ICC in clustered data	• *In studies where multiple mice are housed per cage';* • Analyze data as cluster-correlated data (and not using methods intended for independent data sets). • Mixed models can be used to account and compute the inter-class correlation coefficient (ICC) (the complete annotated code for the statistical methods is available in the Github code repository) • The computed ICC should be retrofitted in customizable power tables to determine whether more cages per group or more mice per cage are needed to achieve a study power of at least 0.7, ideally 0.8. • As a general statistical approach, mixed models can be used to account for ICC, which can be retrofitted to compute and monitor the achieved study power.

4. Supplementary discussion

To date, there are no perception survey based-studies on the relevance of animal husbandry and variability of microbiome research. Animal models, either SPF or human-associated transplantation models arguably represent a useful platform in microbiome research. However, written and verbal commentaries on the subject might suggest inadvertently that animal husbandry practices introduce sources of confounding,

Perception: The discord between perception in academia and experimental practices highlights the need for appropriate reporting guidelines in this regard in peer-reviewed publications. Unless we implement corrective reporting measures, the lack of information, or misinformation will not self-correct over time. Having an accurate reporting is crucial toward promoting study reproducibility and in understanding diet microbiome interactions and improve current models of disease to benefit patients, but also is critical to prevent the systematic spreading of miscommunication and regain public distrust.
The following sections below provide a supplementary evidence-based discussion to support the six actionable Recommendations proposed in this manuscript.

Our collective examination of recent literature in 'mouse microbiome and diet' as a theme illustrates considerable heterogeneity in animal husbandry reporting practices that influence reproducibility in microbiome research, which resembles what happens in the medical literature. [84,85] From a social perspective, a critical finding derived from the survey was the recognition that academicians from three professional organizations ranked 15 husbandry factors in a reproducible manner, providing for the first time a widely applicable framework to prioritize areas for intervention. Efforts to decrease sources of technical variability could improve the quality of published data with enforcement of reporting guidelines to promote reproducibility, which has been deemed to be strongly confounded.[86] To promote the implementability and adoption of practices to improve research quality, *social incentives* (response to opinion of others as an incentive to lead to change [87]), *immediate reward* (significant findings based on highly powered experiment), and *progress monitoring* (controlling for bias could result in better results to support grants) should be considered by respective institutions.

Suppl on *Recommendation 1: Reporting of diet and husbandry factors*:

Our data support discussions that journals should continue to prevent at all costs plagiarism in results, but they should be aware that text recycling in methods sections as here proposed has the tremendous potential of increasing study reproducibility in basic and translational research.

Implementation and availability of a template paragraph for rapid and complete husbandry reporting in manuscripts will enable the rapid data mining of future more comprehensive meta-analyses, widely used to help guide the practice of medicine, but scarcely use in basic science. A paragraph and photographs (an image is worth 1000 words) will facilitate the extraction of data in future studies by scientists interested in meta-analysis of basic research. Extracting data for systematic, scoping, or meta-analysis studies is tedious, slow, and prone to errors due to the lack of consistency in reporting strategies. Such inconsistency in reporting, be it in order, natural variation of methods, or lack of information, could also be the result of limited editorial space to publish, and the pressure that exists to avoid plagiarism.

To alleviate concerns with respect to 'text recycling' for 'Method Sections' in publications, here we present the exact excerpt from the BioMed Central publisher available as; http://media.biomedcentral.com/content/editorial/BMC-text-recycling-editorial_guidelines.pdf: "Use of similar or identical phrases in methods sections where there are limited ways to describe a method is not unusual; in fact text recycling may be unavoidable when using a technique that the author has described before, and it may actually be of value when a technique that is common to a number of papers is described. Editors should use their discretion and knowledge of the field when deciding how much text overlap is acceptable in the methods section. An important factor to consider is whether the authors have been transparent, stating that the methods have already been described elsewhere and providing a citation." This statement and our proposition are in line with the interest that exists in the field across

multiple disciplines. For advanced discussions on the topic (July 26th, 2019), please refer to the summary of a meeting hosted by the International Society of Managing & Technical Editors (https://www.ismte.org/) on March 1st, 2019 on the findings of an NSF-sponsored study on text recycling and self-plagiarism.[71,88]

We propose that to promote reproducibility, studies should follow a basic template where methods sections could be used to support the study and facilitate others to continue and integrate the findings from multiple studies. Of concern, publications in high-tier journals often have limited descriptions of animal husbandry despite growing evidence that multiple husbandry factors serve as sources for confounding. Paraphrasing husbandry baseline practices to avoid infringing on plagiarism is of little value when it results in the omission of crucial details required for study reproducibility. Important methodology components can be described using a paragraph/customizable checklist template. See the rational and discussion below on how the water type and sterility, which vary by vendor[18,19,48-52] influence the gut microbiota.

1.1 Type of water and decontamination

The source and decontamination process (UV light, acidification, autoclaving) of the water is often overlooked in mouse studies even though phenotypic gut microbiome-based changes have been reported as a result of the pH level, chlorine content and ion/mineral content of water. [48,49] Acidification of water (lower pH) is most commonly used across facilities because of its effectiveness in killing gram-negative bacteria (*e.g.,* Pseudomonas spp.) but may also influence phenotypic outcomes of disease. For example, low pH of drinking water has been shown to affect incidence of DM1 and associated gut microbiome differences in non-obese diabetic mice compared to neutral pH.[18,50] This change was associated with a decrease in *Bacteroides* and *Prevotella* and an increase in *Parabacteroides* when mice switched from acidified drinking water to neutral pH. Acidified water also holds potential to increase heavy metal leakage from water bottles into the drinking water.[51] Chlorination of water has been shown to alter gut microbiome by decreasing *Clostridium perfringens*, *C. difficile*, *Enterobacteriaceae* and *Staphylococcus*, which increased incidence of colon cancer in Apc mice,[19] with other reports showing chlorine content can enhance bacterial antibiotic resistance.[19]

Alternative water sterilization methods, autoclaving and UV sterilization retains much of the original ion content of water which can influence the gut microbiome and murine phenotype including inflammation severity and immune response.[52] It is important to note that mouse suppliers like the Jackson Lab provide acidified drinking water (pH 2.5-3.0 with HCl) for their mice, while other distributors, including Charles River and Taconic, filter and hyper chlorinated water, all of which will influence the microbiome.[52]

Suppl. on Recommendation 2: Cage-cage microbiome variability BEFORE mouse experiments

In 2014, our laboratory proposed a fecal homogenization protocol (IsPreFeH) that included use of both fresh mouse feces and bedding material to create the homogenization inoculum.[54] Since then, however, we have discovered a novel source of microbiome bias variability based on microbial overgrowth in bedding material that is depending on degree of cage 'dirtiness'. In light of this discovery, we have amended our original protocol to include only fresh fecal pellets of all mice for the inoculum.

Suppl. on Recommendation 3: 'Dirty cages' and time of sampling DURING experiments

3.1 Cage change frequency and bedding 'soiledness' (dirtiness). Sufficient cage change frequency is necessary to maintain animal wellbeing and microenvironmental conditions and should be based on the number and size of animals, cage size, bedding dirtiness and moisture levels (due to organic matter output; urine, feces) and experimental conditions. Institutional protocols for cage changing range from 3 to 7 days, to that of weekly bedding changes with complete sanitation of cages monthly, to once every 14 days (ventilation rates of 60-90 ACH). [66,89] Although frequent cage changing can have adverse effects on rodent behavior,[90,91] consistent and timely cage changing is a prerequisite for sound

scientific methodology, especially in microbiome research. Inadequacies in prolonged cage-changing presents a formidable confounding effect on intra-cage ammonia (mucosal membrane irritant),[92] as well as gut microbiome composition and heterogeneity due to microbial proliferation of some (e.g. *Enterobacteriaceae*), but not all bacterial species in cage bedding.[1] Systematic, simultaneous changing of cages based on animal density is advisable (**Supplementary Figure 3**).

3.2 Timing of Fecal Collection. Fecal sampling is the most popular method due to the ease of sampling and non-invasiveness. Samples can also be taken post mortem from along the GI tract (like the cecum), which will yield different densities and compositions of microbiota.[59,93] However, recent studies now demonstrate that microbiome profiles can fluctuate within as little as 24hrs and that this microbiota diurnal rhythmicity tends to occur between morning and evening within about 20% of the microbial species.[57,94] Therefore, it is possible that changes in the gut microbiome may be misinterpreted when arbitrarily collected (am vs. pm) throughout an experiment, including at time of sacrifice [59] in that it is advisable to sample intestinal fecal samples systematically across groups (*i.e.,* sacrificing one mouse per experimental group until complete rather than experimental groups 'in batch). Furthermore, diseases that are modeled and studied in mice that affect the proximal GI tract should be sampled more proximally than the feces, which best reflects bacteria of the distal colon.[95]

3.3 Cage Bedding Material. Bedding material is a fundamental component of husbandry. Many varieties are available to laboratory facilities such as paper products, wood chips and corn cob, each with its advantages and disadvantages in terms of cost, urine absorbency and ammonia build-up although the latter is factor of cage type; static vs ventilated [96-98] Corncob is one of the most frequently used bedding materials due to its low cost and high absorbency, but can contribute to discrepancies in feed conversion (mice consume corncob bedding) and can thus interference with diet-microbiome studies.[61] A series of papers have also demonstrated differences in the presence of estrogenic compounds[99] and endotoxin concentrations,[100] between different bedding types, with corncob reported to directly or indirectly alter estrogen signaling and reproductive behavior in mice and rats.[101] For instance, significant differences between Corncob, hardwood and paper bedding in coliform count (corncob highest, paper lowest) and lipopolysaccharide (LSP) levels (hardwood and corncob significantly higher levels compared to paper) may explain mucosal immune response including IgA production.[100,102]

Of equal importance for consideration is the reported interaction between cage ventilation and various non-edible cage bedding alternatives (paper vs wood chips), which, dependent on the strain and immuno-competence of the animal, has various effects on gut microbiota composition (particularly cecal content).[59 65] It is possible that interaction between bedding and ventilation reflects a variable presence of bedding-derived, unknown aromatic or volatile compounds (exogenous from bedding material or host-derived; urinary ammonia) and that these are removed from the a ventilated, but not static cage.[59]

3.4 Environmental temperature and humidity. Cage temperature and humidity can be affected by house design/material, presence or type of housing/nesting material, presence of filter top, type of bedding and how often it is changed, number, age, and sex of mice within, and the presence of forced ventilation. Changes in temperature and humidity can disrupt the 'normal' mouse environment, causing stress, inhibition of small intestine motility (downregulating CCK and upregulating VIP), and consequent changes in gut bacteria.[2,3] Specifically, higher temperature and humidity levels promote inter-cage ammonia levels and microbial overgrowth in cage bedding (increases as cage bedding dirtiness increases), both important elements that can influence experimental outcomes. ***Cage temperature.*** Individual cage temperature has been shown to differ to that of the external environment by as much as 5°F,[60] with fluctuation resulting from the interaction between day and bedding volume.[66] Increased exposure to cold temperatures can cause adaptive changes in the gut microbiome and brown adipocyte metabolism, making metabolism less efficient and leaking energy in the form of heat.[103,104] For instance,

changes to gut bacteria, including increased *Firmicutes* and *Deferribacteres* and a decrease of *Bacteroidetes* and *Verrucomicrobia*, can be introduced within a couple days of exposure to colder temperatures of 6 degrees Celsius.[103] ***Cage humidity.*** Current guidelines for rodents recommend maintaining a relative microenvironment humidity between 30% to 70%,[105] however significant variations of up to 11% higher have been reported as a result of bedding volume [60] and static mouse caging with levels exceeding 35% can dramatically promote ammonia generation. [66,89]

3.5 Cage Ventilation. The purpose of ventilation is to provide appropriate air quality and a stable environment. Cage ventilation is a factor of air changes per hour [ACPH= (flowmeter reading (in L/min)/cage volume in Litres) x 60min/hr] that largely determines individual cage measurements of ammonia levels, and humidity and temperature, and thus serves as an important aspect in deciding acceptable cage sanitation intervals. ***Ventilated cages*** typically employ exhaust air recycled into HVAC systems to prevent cross-contamination including airborne animal pathogens of travel of fomites and use a provision of 10-15 fresh air changes/hr as an acceptable guideline. Inlet air velocity but not exhaust design has been shown to affect intra-cage air velocity distribution, with static isolator cages having lower air velocities and higher humidity compared to mechanically ventilated cages.[106] ***Static isolation caging*** (without forced ventilation) restricts ventilation, and it is important to compensate by adjusting animal husbandry practices such as cage-change frequency, bedding selection, animal density, placement of cages in a secondary enclosure and macroenvironment adjustments to temperature and humidity.

Since ventilated cages remove compounds from the air more easily, static cages may differ in the presence of aromatic or volatile compounds like ammonia. Cage ventilation also affects moisture levels in bedding material (results in increased bacterial growth),[1] with the interaction between the type of bedding material (aspen vs paperchip) and cage ventilation (static vs ventilated micro isolator cages) recently shown to modulate the gut microbiota isolated from various intestinal regions,[59] thereby impacting reproducibility of animal models. On the other hand, ventilated cages can be colder or exhibit within-cage temperature disparities, which can lead to murine stress in the mice and gut microbiome changes.[59,65,107]

3.6 Cage Type. Divergence in descriptive aspects of bacterial communities (alpha, beta diversity) has been reported for different cage types such as barrier access vs open cages.[108] Type of cage material (*e.g.,* plastic flexible film, plexiglass, polypropylene) and can influence inter-cage temperature and humidity levels.

Suppl. on Recommendation 4: Repeating experiments in different seasons.

Changes to the structure and community composition of the gut microbiome with seasonal variation have been reported by our laboratory[76] and by others in rats.[76]. Specifically, in a metagenome-based analysis comparing pool-based sampling of a mouse colony in August which showed a significant association between ileitis with Helicobacter spp. in SAMP1/YitFc vs. control AKR/J mice, which was not observed when the study was repeated 8 months later and sampling individual breeders (Helicobacter was then observed only in control mice).[76] While it is almost impossible to feasibly control for seasonal variation, both within a long-term experiment or that of multiple experiments spanning over several years, it is important to report details of seasonal variation in the methods.

Suppl. on Recommendation 5: Animal density, clusters, and study power:

The implementability grades indicate that Recommendation 5 ('on animal density and individual caging) based on comments and the distribution of the average grades (which are still high and significant with a t-test $p=0.086\pm0.129$ vs. random grades) is likely to continue to be the most heterogeneous form of husbandry methods variability in the literature (**Figure 7B**). The goal of this Recommendation was to investigate the perceptions that exist among leading scientists. Based on external comments, it was clear

that this Recommendation elicited the most heterogeneous responses reflecting the resistance that exists among researchers to house 1 MxCg.

The potential for stress is an arguable concern with indivudually housed mice despite that social stress has been equally demonstrated for single- and socially-housed mice.[62,63] Housing conditions have been shown to modify behavior (aggression, anxiety, distress) and social housing of mice is generally recommended. [105] Under experimental conditions, mice are most commonly housed by gender which is in contrast to the social organization of wild mice,[62,63] with co-housed males subject to dominance and hierarchy.[63] In a study comparing individual and social housing (C57BL/6, BALB/c) neither the male nor female individually housed mice showed stronger signs of stress compared to that of socially-housed (n=3 mice/cage) mice.[62] However, within-cage differences among socially housed mice (hormonal stress response) were observed.[62] To minimize potential stress in individually housed mice enrichment tools can be used.[109,110] See details on '*animal well-being*', and '*social housing*' in **Supplementary Discussion** and **Supplementary Table 5**.

4.1 Housing density and cage effect. Previous animal density assessments did not consider effects of husbandry-related environmental effects (noise, lighting differences, diet, hierarchy) or source of animals (vendor) that may affect the microbiota and contribute to 'cage effect' and thus misinterpretation of experimental findings (**Supplementary Figure 3;** accumulation of feces and animal density).[12,64] Specifically, increasing housing density to increase the number of mice per group is counterintuitive without including a sufficient number of cages per group to avoid 'cage effect'. In addition, the approach of 5 mice/cage is less cost-effective when compared to single housing of mice because of increased need for cage change frequency with higher density cages to minimize bias derived from high organic matter output (see cost calculator). The sections below discuss the primary factors that contribute to cage dirtiness; cage change frequency

4.2 Animal well-being. A recent summary of conclusions of housing density studies (static and ventilated cages) over the last two decades concluded that for most mice strains, a significant decrease in floor space allowance does not negatively impact rodent well-being and thus animals can be kept at twice the recommended levels by the *Guide*.[64] It is important to note however that one study evaluating housing density across 5 inbred mouse strains, reported increased housing density (up to 4 MxCg; 77.4 cm2 for mice between 15 and 25 g) when to significantly and consistently affect certain parameters pertaining to health status, namely kidney weight, adrenal weight, heart rate and percent body fat (increased fat seen in certain mouse strains).[111] Because animal density introduced uncertainty and potential effects ion physiology; it would be easier to conduct balanced experiments (all caged with same number of animals) and low cage animal density.

6. Supplementary References

To identify husbandry factors capable of influencing gut microbiome and study reproducibility we focused on the 172 most recent studies, see references below.[1,112-283]

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