Minireview

Institutional protocols for the oral administration (gavage) of chemicals and microscopic microbial communities to mice: Analytical consensus

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Impact statement

Institutional protocols designed for the oral administration of live microbial communities, either complex or microscopic (microcosmic), to mice do not exist. However, this approach is increasingly employed by investigators focusing on the gut microbiome in experimental research. Herein, we propose two analytically Kappa-based consensus protocols to promote reproducibility and standardization in research practices and describe biologically relevant factors in achieving optimal microbial engraftment of communities in germ-free mice.

Abstract

Although there are numerous Institutional Animal Care and Use Committee (IACUC) protocols aimed at standardizing the oral administration (gavage) of liquid chemicals to laboratory rodents, there is ample variability across protocols, and there are no protocols intended for the gavage of microbial communities to mice or rats. The objective of this study was to conduct a scoping review of publically available IACUC protocols from institutions in the United States, identify protocol criteria and deficiencies, and generate an 'analytical consensus' to unify such criteria into two revised protocols: one for chemicals, and another for microbes in mice. Eighteen ($n = 18$) written institutional protocols from prominent universities, and 26 demonstrations videos from various sources (accounting for >155,000 views) were identified in the World Wide Web. Although written protocols listed up to five criteria for consideration (dosing/volume, pregnancy, animal weight), collectively there was major variability and poor statistical agreement across methods (0% Kappa, $p = 0.98$). Because protocols also lacked details relevant for the bypassing and survival of live microorganisms in the gastric (antimicrobial) environment, we compiled two 'analytical (Kappa-based) consensus' protocols from available and new criteria, for the 'gavage of chemicals' and for the 'gavage of delicate microscopic microbial communities' to mice. A major difference lies in the volume of administration, (i.e., 20 mL/kg, without restrictions, after 4-6 h of fasting, for microbes), which was graphically illustrated simulating the predicted impact of administering large and small volumes on microbial distribution of simple microscopic communities. In conclusion, publicly available IACUC gavage protocols are highly variable, and do not reflect the need to adjust the dose volumes to ensure the rapid bypassing of the gastric environment which would impact the survival of microbes, especially if composing delicate microscopic communities. An 'analytical consensus' of IACUC-approved protocols is herein presented as a unifying baseline protocol for consideration.

Keywords: Gavage, gnotobiotic, mouse models, microbiome, inflammatory bowel disease, fecal matter transplant

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Introduction

Because confounding factors often render study conclusions false or invalid,[1,2] great interest exists on improving research methodologies to control for confounding factors in murine research, which is critically important for the discovery of biological features mediated by the gut microbiome in human diseases.[3] Although well-controlled forms of studying environmental heterogeneity can be

beneficial to draw 'externally valid' inferences (instead of 'internally valid,' which applies to the mice studied), spurious variables due to irreproducible methods may also result in the drawing of unreliable conclusions or in irreproducible results.[4–6] Variable criteria used in Institutional Animal Care and Use Committee (IACUC) protocols may also introduce variability. This study examines existing IACUC protocols for the oral administration via an oral–esophageal–gastric cannula (gavage) of mice which were proposed decades ago (before the 'microbiome era' started in the 2000s) to standardize the administration of liquid chemical substances to laboratory mice. Institutional gavage protocols (IGPs) designed for the gavage of live microbial communities (e.g. complex gut microbiotas; simple microscopic communities from intestinal villi, or microscopic lesions) to mice do not exist.[7–9]

To contribute to improving 'rigor and reproducibility in research,'[10] as mandated since 2014 for grant applicants seeking funds from the National Institutes of Health,[11] here we illustrate that IACUC protocols are an important source of methodological variability.

According to the National Association for Biomedical Research (founded in 1979, www.nabr.org) which provides the unified voice for the scientific community on legislative and regulatory matters affecting laboratory animal research, an IACUC is a local working group that research facilities must appoint in accordance with the Animal Welfare Act and PHS Policy on Humane Care and Use of Laboratory Animals.[12] At least one member of the IACUC must be a veterinarian providing care to animals used in research and one must be a public member not affiliated with the institution. In addition of reviewing and approving experiments proposed, the IACUC also ensures that experiments do not result in unnecessary pain or stress to animals or that proposed projects are not unnecessarily duplicated. In an effort to standardize research, IACUCs often publicize step-wise protocols online of common procedures to streamline the review process. Thus, IACUC protocols/guidelines are local mandates and a reflection of the IACUC members' opinions as a consensus statement of the group on numerous abiding issues.

A variety of approaches, such as the coating of the gavage needles (flexible or rigid) with palatable solutions,[13,14] offering the dose in a flavored formulation that is voluntarily consumed by the rodent, including peanut butter or similar,[15,16] palatable pills,[17] honey,[18] and apple juice[19] have been proposed to facilitate the administration of substances or medications to mice. However, the majority of palatability-based methods may not be used for the administration of live microorganisms. That is because some flavored solutions have potent antimicrobial effects.[20,21] Further, when microorganisms are to be transferred, due to the strict anaerobic nature of several species, they have to be transferred rapidly into the animal digestive tract. These microorganisms, therefore, cannot stay indefinitely exposed to room air in the environmental cage/palatable vehicle. Restrain practices, complications, or aseptic procedures or cannula types used for the gavage of mice have been extensively described.[13,22–25]

The objectives of this study were (i) to identify the sources of protocol variability in publicly available IGPs, and to (ii) analytically construct one analytical consensus protocol for the gavage of chemicals (Consensus IGP for Chemicals) and one protocol with expanded applicability to the oral administration of live microorganisms from microscopic communities to mice (Consensus IGP for Microbial Organisms). We conducted a scoping review of publically available protocols for oral gavage of mice in academic research institutions in the United States, and then used 'agreement statistics' to construct the consensus to improve method reproducibility. We also graphically illustrated the relevance of adjusting volume of administration to promote the distribution and colonization of microbial micro-communities in the intestinal tract of mice, which is relevant to various models of intestinal diseases in humans and mice.[8,9]

Methods

Search strategy and impact

For a rapid screening of methods publicly available on the World Wide Web we used Google (citation and web) and PubMed, and the non-exhaustive keyword combination 'oral gavage of mice (+ iacuc or + protocol).' A rapid scoping review was conducted using simplified strategy following the principles of systematic reviews implemented (i) for the screening of protocol and identification of protocol variables relevant to the dosing and frequency of substances to mice; and (ii) to determine if protocols considered the administration of live microorganisms and the antimicrobial effects of the gastric and intestinal secretions. We conducted the search of both 'videos' and 'written documents' platform in Google web. The search was conducted to identify demonstration videos linked to institutional protocols (i.e. university/research center) or journals, excluding videos that were not in compliance with basic standards expected for laboratory rodent research in the USA on aseptic techniques and animal welfare as per IACUC guidelines.[26] We provide impact statistics based on number of viewers of the demonstration videos.

Matrix table of parameters for the analysis and proposal of consensus protocols

Following the identification of written IACUC protocols (keyword search strategy 'oral gavage mice IACUC'), we compiled all original protocols/documents as an extensive and permanent PDF source and provision for the readers in Supplementary File 1. Protocols were deemed institutional because they were available from HTML addresses associated with university domain servers. Since most IACUC protocols refer to comparable criteria (parameters), but since not all had the same number of criteria described, we created a matrix table to present the methods for all the criteria across all protocols listed for analytical consensus. Exclusion criteria included documents such as power point presentations or similar, and protocols that require exclusive password-protected access to institutional websites. If a research institution

had several regional branches, the protocol that was higher on the list of hits was selected for the matrix table. The protocols listed here represent the most discoverable protocols among a total of 98 entries displayed by Google using Safari search engine on 28 October 2018.

Statistics

As described in analytical consensus theory, the notion of consensus is essentially intuitive.[27] A consensus specifies the conditions under which agreement among people can be seen as a sign of knowledge or 'getting it right.'[27,28] Several epistemological schemes are based on the interdependence between agreement and truth[28]; a tangible example of the power of collective opinions is the court system in some countries, which disregards the prosecutors' claims as true unless a jury of independent people agrees.[28] Although each IACUC protocol herein reviewed reflects the consensus among IACUC members, it is unknown the actual number of individuals that made part of each IACUC committee and that agreed or disagreed to each of the listed protocol criteria. Since knowing the number of individuals agreeing to each of the parameters is unknown, as it is also the level of influence that executive members had on each committee, in this study, the level of analytical consensus across the methods in the matrix table was assessed by computing the level of agreement between the factors relevant for gavage across the selected protocols by using Multilevel Kappa statistics[29,30] considering each IACUC as an individual thinking collective entity. For this purpose, the criteria listed in the protocols were categorized as 'agree' or 'disagree' as a measurement of consensus for 5 of the criteria that had at least one concept described in the guideline (e.g. protocols describing that the maximum volume to be administered was 10 mL/kg of body weight were categorized as 'agree'; other alternatives or the lack of this description was deemed as 'disagree,' if the protocol stated that the max. vol. was 20 mL/kg). Linear regression models included this agreement data, year of last update, a regional category designation, and the ranking number across global universities using the Best Global Universities 2018 US News Report.[31] Analyses were conducted using the STATA software (v15.1, College Station, TX) and R (Vienna, Austria; http://www.R-project.org/).

Results

Videos illustrate protocol variability and demand of information on gavage techniques

In total, we identified 26 demonstration videos on YouTube from various parts of the world illustrating the oral gavage of mice, with a similar number of videos describing the procedure for rats. In mice, the total number of views accounted for >155,000 views since 2014 (estimated 100 views/day, observed 189 views/day). Most videos were non-official and had unrestricted access; only three institutional videos in the USA required viewer's validation and contained a 'content warning' message. The duration of videos ranged from 5 s (75 views in three years) to the longest with 14:11 min (a non-official video[32] of a peer-reviewed publication[25]; 13,607 views in two years; 19 views/day). For the latter video, there was an increase of number of views from 13,697 in 28 October 2018 to 17,199 in 14 February 2019, with an average of 34.5 visitors/day, which almost doubled the daily demand of information compared to a previous average of 19 views/day. This growing impact statistics supports the demand and need of information on the subject.

Selection of demonstration videos to promote animal welfare

Several videos intended to illustrate how to administer drugs to pet mice in veterinary centers, while others illustrated the protocols for research settings. None of the videos highlighted or discussed appropriate scientific references or dosing regimens, which is the purpose of this manuscript. Protocol data presented in demonstration videos were not extracted to create the analytical consensus across IACUCs as described in this manuscript, because publically available videos on private online media channels are not reliable sources for scientific reproducibility, lack the long-term peer-reviewed validation, users are free to remove them from the Internet, or they infringe animal welfare recommendations.

Although considerations on instrumentation, material, restrain, and animal welfare have been described elsewhere,[25,33] herein we highlight the importance of *adequate restraining of animals* by selecting some instructional videos. Relevant to our interest in promoting IACUC compliant procedures on animal *restrain* and *welfare* mandated by the USDA and NIH,[26] demonstration videos included two academic videos from universities (Pennsylvania State University; Newcastle University), one from a peer-reviewed journal,[25] and one from a manufacturer of gavage cannulas for mice (see Table 1). Note that the selected videos highlight restrain strategies, but they do not address the problematic of gavage protocol variability, or *dosing* regimes which we propose as 'analytical consensus' in the sections below.

Written IACUC protocols emphasize criteria for monitoring complications in mice

We identified 18 publicly available IACUC written protocols from institutions in the USA out of a total of 98 hits for the keywords '*oral gavage mice IACUC*' (Google web search engine, Safari; 24 October 2018). The same search strategy yielded only one (n = 1) hit in PubMed; however, the search keywords '*oral gavage mice protocol*' identified 46 papers from 1987 to 2018. After screening the title and manuscript content, none of the PubMed manuscripts were institutional protocols. The sections and analyses below are thus based on written protocols available on Internet. The overall layout and appearance of IACUC guidelines or protocols was highly diverse, and thus were the lists of references cited in each protocol (original formats are compiled in Supplementary File 1). Herein, we compiled the list of parameters to monitor and, based on those parameters, we constructed a table for the dosing and frequency of IGP descriptions and estimated kappa agreement statistics.

Table 1. Parameters to be observed to include appropriate animals for a gavage-based study and to be observed after the gavage to ensure identification of procedural complications.*

Observation parameter	Prior to gavage (to select and include or exclude proper animals for experiment)	During gavage (to minimize risk of mouth, larynx, lung, esophageal, and stomach trauma)	After gavage† (to determine the need to adjust the protocol for other animals if needed, and to euthanize suffering mice)
Detect physical abnormalities	Yes	Yes	Passive naso-gastric reflux if excessive material administered. Pain or distress, bleeding or frothing at the mouth, or bluish mucous membrane color in albino mice
Body condition score	Yes	–	–
Behavior	Yes	–	Signs of abdominal pain – kicking their belly, lethargic response
Respiratory pattern and rate	Yes	Yes (insert the needle and wait for few seconds, look for cyanosis and respiratory pattern)	Yes (look for cyanosis, gasping for breath, increased respiratory distress)
Nose or oral bleeding	Yes	Yes (indicated mucosal trauma – can be life threatening)	Yes (indicated mucosal trauma – can be life threatening)
‡Selected videos on demonstration technique	Commercial mice – https://www.youtube.com/watch?v=oYCmKIhveFY&feature=youtube Commercial rats – https://www.youtube.com/watch?v=TO3i_q74ftM Academic mice – https://www.research.psu.edu/arp/training/videos/oral-gavage-in-mice.html Academic mice – http://www.procedureswithcare.org.uk/oral-gavage-in-the-mouse/		

*Notice that mice/and rodents in general have a very strong cardias tone therefore excessive amounts of fluid can lead to over distension of the stomach. As in horses, due to a strong tone in the cardias, vomit occurs very rarely to alleviate over distension. If noticed, it must be assigned to excessive volume. As in horses (and unlike dogs, cats, and humans), mice tend to have stomach rupture, before they have esophageal reflux, although the latter is a rare event in research. Notice that apparent reflux can also be due to the administration of liquid into the trachea. In general, the risk of complications may be decreased by using soft (flexible) gavage tubing (e.g. elastomer tips), rather than stainless steel dosing needles; however, what is more important is the technique and gentle strategy and animal restrain.

†Monitor animals immediately after gavage (for no less than 15 min), and again at least once within the next 12–24 h after the procedure. Contact veterinarian for assistance. A mouse that shows signs of progressive respiratory distress (due to stomach perforation or lung administration) must be euthanized.

‡The set of most viewed videos were the ones made and released by the manufacturer of gavage cannulas in the USA (Instech Laboratories Inc. and Veterinary Biosciences Institute, USA; 103,368 views since 26 August 2014; 171 views/day, 24 October 2018), which was also cited by the University of Pennsylvania program. The second most viewed set of videos was from the '*Journal of Visualized Experiments.*'[25]

Comparing the amount of information dedicated to descriptions for 'Dosing sections' in all protocols (word count per section), it is evident that most protocols extensively promote animal welfare, as required by IACUCs, and emphasize the importance of monitoring the mice in order to prevent complications. However, the word count dedicated to the description of doses for administration was very limited and comparatively minimal.

In this report, we compiled a list of monitoring guidelines for considerations before, during, and after gavage in Table 1. We also emphasize that, although not explained in most IGPs, if serial gavages are to be conducted (e.g. weekly, daily, or several times daily), it is important to inspect the animals and monitor the same observation parameters (Table 1), before each gavage, because complications are more likely to occur due to the accumulation of risk probabilities. Although the use of an incorrect technique has been reported in IGPs as a factor that leads to scarring and narrowing of the esophagus and/or gastric openings and esophageal or gastric rupture, recent studies showed that repeated daily gavage for 18 days, if properly conducted, induces no histologically detectable changes in the esophagus of mice physically restrained, which was comparable to the histological findings in a protocol where gavage was conducted with mice under general anesthesia (isoflurane). The low rate of complications with repeated gavage also indicates that the use of isofluorane is unnecessary when repeated gavages are needed,[34] which is in agreement with the statements in some IGP protocols. In general, repeated gavages are well tolerated in mice, if a proper protocol is used. One of the reviewed IACUC protocols states that 'Proficient oral gavage should result in no significant animal losses (>95% survival rate)' (Supplementary File 1), but in our studies this safe procedure has an attributed mortality rate that is even lower than that (<1%, 1/∼800; over six years).[35,36]

Institutional protocols have poor agreement (0%) on recommended dosing regimens

Analysis of the protocols revealed that the methodology is specifically illustrated and designed for the administration of substances with pharmacological and toxicological purposes in research, and none of the protocols mentioned the usefulness of the guidelines for the administration of microorganisms to mice. Furthermore, none of the 18 protocols identified considered biological factors associated with the species-dependent susceptibility of various microbial species to gastric acidity or bile acids in the upper gastrointestinal tract. Table 2 summarizes the protocol descriptions for seven factors deemed important for oral gavage of mice by examining protocols from the USA and elsewhere, for instance Australia, Singapore, and Europe (Supplementary File 1). None of the identified protocols used in the USA request animals to be fasted, even as an alternative to reduce the risk of gavage-associated complications. One protocol from Flinders University in Australia, described 2 h of fasting prior gavaging to minimize such complications (Supplementary File 1).

As it is evident in Table 2, kappa agreement statistics following the categorization of the protocol descriptors across five variables (selected if there was at least one protocol describing the restriction or guideline) revealed a 'Statistical Kappa Agreement' of 0% across the IACUC methods identified, which was even lower than the 'Expected Agreement' of 16.05% (Kappa, $-0.1912 \pm$ S. Error 0.0932; $Z = -2.05$; Prob>Z $p = 0.98$). Using unsupervised multivariable cluster analysis (57 'agree' and 33 'disagree' data points, total $n = 90$) to illustrate which institutions are more alike, we generated two major protocol clusters that appear to be independent of geographical location but significantly depend on the global ranking of the academic institutions.[31] Hierarchical multivariable cluster analysis, Pearson correlation coefficients, and multivariate linear regression showed that the poor agreement between consensus guideline parameters across institutional protocols for the gavage of mice is more pronounced in high-ranking research institutions, while it does not depend on geographical region or the year of 'last protocol update' (Figure 1).

Analytical consensus protocols for the gavage of chemicals and microbes to mice

Considering that each institutional protocol reflects the consensus perspective of IACUC members, herein we used those agreed-upon guidelines described in the reviewed publicly available protocols to build an 'Analytical consensus protocol for the gavage of chemicals' based on the maximum allowed criteria for the collective descriptions, and the available literature discussed here.

Based on that analysis we then proposed a few adjustments in a new proposed 'protocol for the oral administration of live microbial communities to mice.' Table 2 contains at the bottom of the table a succinct description of the parameters proposed in the consensus for chemical products, while Table 3 emphasizes the rationale for consideration for the consensus for dosing regimen for the administration of microbes. To promote research reproducibility on dosage volume and regimen, we collated the information extracted from the protocols to propose a consensus agreement protocol and to revise the criteria relevant for the gavage of animals at various age and reproductive stages.

In short, although the volume for administration may vary for chemical substances depending on pharmacokinetics of the chemical and goal of the experiment, we propose to always gavage animals with 20 mL/kg of body weight for microbiome studies, without restrictions on maximum volumes, except when there is evidence of advanced pregnancy (see sections below and Table 2). To further minimize the risk of complications, which are rare at such doses, we also recommend a period of fasting prior to gavage, also because diet itself has an antimicrobial effect on certain microbes.

Fasting and short periods of acute fasting in mice are a critical physiological variable that determine the gastric content and acidity, gastrointestinal emptying and motility, and glycemic indexes and blood cortisol levels.[39] This in turn influences the survival and establishment of microbes in the digestive tract, thus we purposefully included fasting

Table 2. Publicly available protocols for the oral gavage in mice from academic institutions in the United States: Analytical consensus protocol for the administration of chemicals to mice.

Institution	Max volume determined by mouse weight	Max volume restricted regardless of mouse weight	The lower the volume the better	Fasting period described	Restriction during pregnancy	Repeated doses	Newborn mice	Year updated
Western University Health Sci.	1–2% of body weight (10–20 mL/kg)	Unrestricted	NR	NR	NR	NR	NR	2008
University of Washington	250 μL	250 μL	NR	NR	NR	NR	NR	2009
George Washington University	Yes. 10 mL/kg (0.1 mL/10 g)	Yes, restricted to 250 μL	NR	NR	NR	If needed, max. 1/every 8 h	NR	2010
U of California San Francisco	Yes. 10 mL/kg	Unrestricted	NR	NR	Yes, less but not specified	NR	NR	2011
Oregon State University	Yes. 10 mL/kg	Unrestricted	NR	NR	Yes, less but not specified	If needed, max. 3 over 24 h	NR	2011
Rutgers University Medicine	'Typical': 10 mL/kg **'Maximum': 50 mL/kg**	Dosing table: 1.75 mL in a 35 g mouse	NR	NR	NR	NR	NR	2012
U of North Carolina Chapel Hill	Yes (5 mL/kg)	Yes, restricted to 125 μL for a 25 g mouse	NR	NR	NR	NR	NR	2012
Boston University	Not specified As a function of body weight	0.125–0.25 mL in 25 g mouse	NR	NR	NR	NR	NR	2014
U of Minnesota	Yes (10 mL/kg) in 2017 volume changed to (5 mL/kg)	Unspecified, but stated that >5 mL/kg cause distress	NR	NR	NR	NR	NR	2014
U of Delaware	Yes. 10 mL/kg	Yes, restricted to 250 μL	NR	NR	Yes, 25% of max. Irrespective of stage	If needed, max. 1/every 8 h, 3 over 24 h	NR	2015
Washington State University	Yes. 10 mL/kg	Unrestricted	NR	NR	Yes, less but not specified	If needed, max. 3 over 24 h	NR	2015
Florida State University	Yes. 10 mL/kg	300 μL	NR	NR	Yes, 25% of max. Irrespective of stage	Up to three times within 24 h	NR	2016
Indiana University	**20 mL/kg, NR in guidelines but in Policy**	200 μL, sole option listed (contradicts policy)	Yes <5 mL/kg or smallest vol. promoted	NR	NR	NR	NR	2016
Georgia State Univ.	Yes. 10 mL/kg	Unrestricted	NR	NR	NR	NR	NR	2016
Pennsylvania State University	Yes. 10 mL/kg	Unrestricted	NR	NR	NR	NR	NR	2016
U of Colorado Denver	Yes. 10 mL/kg	Unrestricted	NR	NR	NR	Up to four times/day every 6 h	NR	2017

(continued)

Table 2. Continued

Institution	Max volume determined by mouse weight	Max volume restricted regardless of mouse weight	The lower the volume the better	Fasting period described	Restriction during pregnancy	Repeated doses	Newborn mice	Year updated
Virginia Tech	Yes. 10 mL/kg	Unrestricted	NR	NR	NR	Up to three times within 24 h	NR	2017
University of Georgia	Yes. 10 mL/kg	Unrestricted	NR	NR	NR	NR	NR	NR
Analytical Consensus Protocol To Gavage <u>CHEMICAL</u> Substances in Mice (Rationale in Table 3)	**10 mL/kg** (100 µL/10 g) Unless pharmacokinetics (PK) of experiments requires it For gavage of **<u>MICROBES</u>** use 20 mL/kg see Table 3	**<u>Unrestricted</u>** (If restrictions are variably allowed in institutional protocols, method reproducibility will be lower)	Min. vol. **not** encouraged Justified only if PK requires it. Consult vet.	**6±1 h fasting <u>during day</u>** to prevent gastric distension if it does not interfere with PK	**<u>10 mL/kg</u>** Plan pregnancy/ experiments; see text for detail criteria on pregnancy.	**Up to 3–4 times/day** every 6 h See Table 3 for PK, gavage interference with fasting	**10 mL/kg** (10 µL/g) Use stereomicroscopy. Consult vet for handling	2019 See text and Table 3 for refs and scientific rationale

NR: Not reported.
With the lack of scientific peer-reviewed evidence, currently scientists would have a difficult time to justify the increase of doses. This paper seeks to illustrate the need for science-based policies that may influence the quality of research in microbiome fields. At UNC the same document has contradicting doses (10 versus 5, but the policy states 5 mL/kg; see **Supplementary File 1** for detailed annotation of references used in each protocol and a copy of the actual institutional protocol).

as a factor in Table 2 to highlight the need for considering that variable in our revised consensus protocol. Similarly, we noticed that no guidelines existed for gavage of lactating or newborn animals, thus we also included that factor in Table 2 to raise awareness. The majority of protocols did not cite scientific publications to support the guidelines on dosing and frequencies. From those that did, the most common citation corresponded to Turner *et al.*,[22,23] which provides generic parameters for dose volumes across laboratory animals of 20 mL/kg as maximum.

On pregnancy, there was also lack of consistency and reproducible criteria across protocols (Table 2). Here, we proposed in the revised analytical protocol (i) that the reproductive status for oral gavage must me known by scientists, and therefore (ii) animals should not be entered into an experiment unless the investigator knows the date to conception/last mating event. If unknown, (iii) abdominal palpation or ultrasound examination of the abdomen must be conducted to determine if animals have advanced pregnancy or any other abdominal structures that may prevent the comfortable expansion of the stomach as gavage fluids are infused. Lastly, the (iv) expected rate of fertility and litter sizes must be known by researchers to know what to expect. For instance, SAMP 1/YitFc mouse line is a poor breeder compared to other healthy mouse lines (1 pup/6 dams in SAMP1/YitFc, compared to C57BL/6J, or Swiss Webster mice, the latter which can produce ~11 pups per dam).[36] With different fertility rates, the gavage of a given volume in advanced gestation cannot be made universal or restricted for the administration of microbial communities as it was described in the past. Therefore, our analytical consensus protocol must cite these criteria for consideration, and preferred large volumes of fluid must be combined with short-term acute fasting to ensure animals can be gavaged with the 20 mL/kg volume suggested. Here, we proposed that animals in advanced pregnancy and large litter sizes be gavaged 10 mL/kg in the first attempt, then observe the mice for 5–10 min to monitor for signs of discomfort (e.g. kicking their abdomen, looking at their flank, pacing in the cage). If none present, then the remaining half of the volume 10 mL/kg can be administered gently. Following 5 min of additional observation, food can be reintroduced to the animals immediately.

Graphical effect of gavage volume on distribution of microbial communities in the gut

The rapid bypassing of the upper segments of the gastrointestinal tract in mammals and the prevention of exposure to highly inhibitory gastric and duodenal bile acids increase the chance of microbial survival of a complex community that contains susceptible low abundant species. It is also mechanically advantageous to administer higher volumes of fluid in a partly emptied stomach (following fasting) to accelerate gastric emptying. To graphically visualize the physical benefits of gastric bypassing as a function of the volume of fluids administered, we prepared an illustration representing the distance of travel and expected local density of bacterial single celled communities (Figure 2). We illustrate that such factors modify the expected

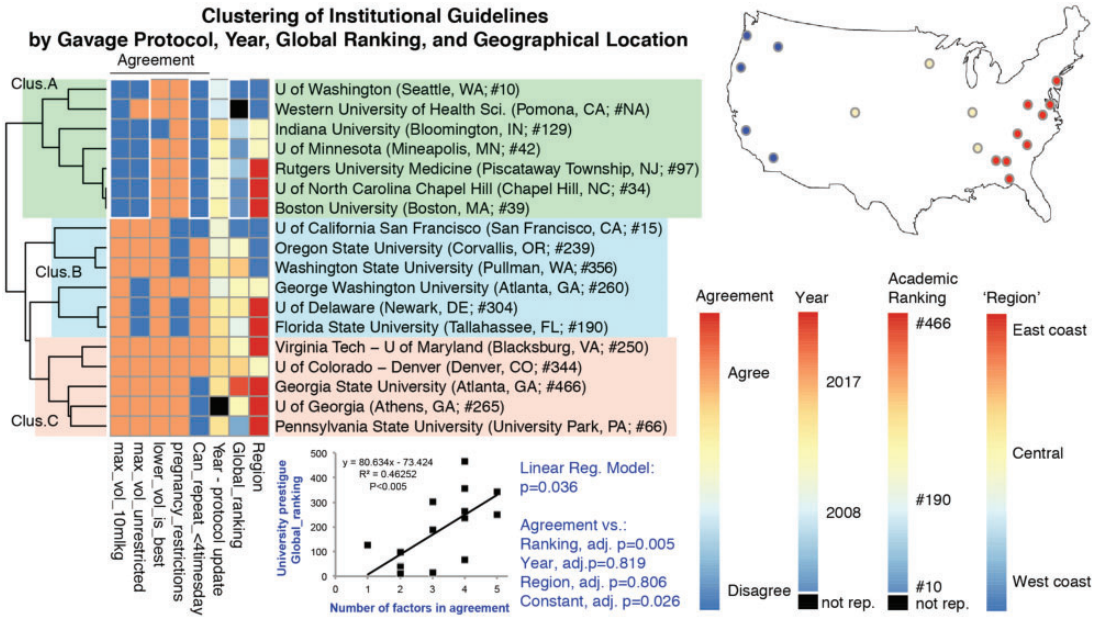

Figure 1. The poor agreement between consensus guideline parameters across institutional protocols for the gavage of mice is more pronounced in high-ranking research institutions. Notice the presence of at least three types of distinctive institutional protocols. They cluster primarily depending on the extent of parameter agreement, and ranking of the institution, and not on the geographical region or year of 'last protocol update.' Protocols in Cluster 'Clus.A' have more guide parameters in disagreement or are incompletely described, and protocols in clusters 'Clus.B' and 'Clus.C' have more guide parameters in agreement or better described. Scatter (correlation) plot highlights the univariate nature of the association between the prestige of the university (high ranking place in the Global Academic Ranking System) and the number of guide parameters in agreement. Multivariable linear regression confirms that the more highly ranked the institution is, the more likely it is to have a protocol that is in disagreement or partially described compared to the pool of all available protocols (consensus agreement).

growth competition and residence times of microbes in the gut lumen and allows for a more even distribution of the probability of survival for each cell in a simulated tubular system (Figure 2).

In short, we illustrate that if the volumes of administration are large, the bacteria in suspension will be more likely to reach faster and farther down the intestinal tract and achieve the contact with a larger surface area over the gastrointestinal mucosa following a more homogeneous distribution patterns. Larger volumes of fluids in a well-hydrated yet fasted individual is expected to result in intestinal peristalses rates that move bacteria aborally faster (compared to low volumes in a dehydrated over fed mouse), protecting them from the harsh effects of the intestinal enzymes and pH changes in the upper digestive tract (areas characterized by the high concentrations of gastric, pancreatic, and hepatic enzymes). If the volume is minimal, bacteria will be exposed to the mucosal surface in the most proximal areas of the intestinal tract, promoting higher bacterial densities, and bacterial competition. If assessed based on cell density and generation rates, highly dense environments will unevenly favor species that survive the proximal intestinal environment and reproduce at faster rates in the presence of primarily undigested diets (as observed *in vitro* with *Escherichia coli*, *Enterococcus faecalis*, and *Lactobacillus reuteri*),[36] compared to microbes that grow slowly and/or that are inhibited by the diet and digestive secretions.

Discussion

That people agree on any given method primarily reflects that the majority of users in any given field typically accept the practice, but it does not necessarily make/imply that such practice is perfect or truthful. Despite the profound benefit of IACUC regulations on animal welfare, here we highlight major variability and inconsistencies in the description on gavage doses (e.g. volumes) across IGPs from prominent institutions in the USA. In the literature, there are reports describing as little as 100 µL for C57BL/6 mice, without justification of the method,[40] which is less than 10% of the body weight of an adult mice, as recommended in most IGPs. Here we identified factors that, according to the collected peer-reviewed evidence, may arguably have deleterious effect on the humanized murine intestinal microbiota, including small volumes of administration. We propose an analytical consensus strategy to systematically cover critical parameters for further study and consideration by local IACUCs. The ultimate goal of a gavage method is to increase the survivability of microbial species of the FMT in the GF mouse model across various human diseases.

The external validity of our consensus inference is quite vast at least for the USA. Based on a global ranking system of academic quality (which closely parallels that of research quality), the institutions that made their IGPs public, which we reviewed in this manuscript, covered a span of at least the top ~500 most reputable universities worldwide. As needed, we referred to IGPs from other countries to illustrate the need to update the collective body of available IGPs/guidelines. There is the evidence based on the use of biological-like Bovine 2-microglobulin as a surrogate for protein feeding that 'gavaging mice to assess absorption of orally ingested proteins can lead to artifacts not seen when the protein is consumed under natural

Table 3. Analytical consensus protocol for the gavage and oral administration of live 'delicate' microbial communities to mice: Proposed parameters and scientific rationale.

Parameters	Problem and warning	Criteria description and rationale
Volume of dose (20 mL/kg; 0.2 mL/10 g of body weight); use PBS (phosphate buffered saline; do not use water or 0.9% NaCl saline alone since their pH is acidic [~5.5] and cannot buffer the gastric pH). Larger volumes (50 ml/kg) could also be be considered as shown in Table 1; but conduct pilot studies for local validation to promote animal welfare.	Current protocols have variable use and definition for typical, ideal, maximum, and absolute maximum volume for the administration of substances via gavage. *Note*: This consensus protocol is revised keeping into consideration the survival of microorganisms from microscopic live communities via gavage. We do not encourage the use of small volumes.	Since actual rodent diets commercially available to mice in USA have been shown to have an inhibitory selection on some bacteria (e.g. *L. reuteri* and *E. coli*), but promote others (e.g. *E. faecalis*),[36] the administration of live microbial communities especially from microcosmic communities could be conducted after 4-6 h of fasting to prevent selection bias driven by the diet with appropriately large volumes of buffered physiological saline to neutralize the effect of the gastric acidity and bile acids as biased inhibitory selection force on individual microbes and microbial communities.[37,38] Since 20 mL/kg of body weight has been validated in animals, here we proposed to administer a microorganism community using 20 mL/kg of phosphate buffered saline to mice following 5±0.5 h of fasting, which has been shown to induce no stress in mice, especially if fasting starts in the morning hours.[39]
Fasting period Remove feed from cages in the morning, and gavage 6 h later during the day. Do not fast overnight, or gavage in the morning.	*Note*: Unless it interferes with medications or toxicological parameters, this part of a protocol has to be considered carefully for the administration of chemicals since some medications may promote gastritis after the fasting period.	To minimize stress on animals, changes in physiological aggression, cortisol blood concentrations, and to minimize the risk of gastric over distension or potential discomfort with the volume infused, it is necessary to remove the feed from the mouse cages early in the morning and plan the gavage of mice before the end of the working day. Fasting at night is more difficult to control as it will exceed 6 h of fasting. Also, nocturnal fasting elicits more profound effect on mice due to their increased activity during the day. Fasting reduces the gastric pH, therefore we suggest using PBS. Short-term fasting may not have a major effect on intestinal motility, but if extended fasting occurs, the pharmacokinetics of chemical compounds and their bioavailability will be affected.[39]
Repeated doses per day	*Note*: The repeated administration of microbial communities to mice for health-related studies is usually not needed. Data indicate that one single dose will be sufficient to colonize GF mice effectively.[35]	Current protocols are designed for the administration of pharmacological or toxicological compounds, which require repeated doses to achieve steady states and reach pharmacologic potency due to the intrinsic absorption and clearance rates. If used for those purposes, this protocol allows for the administration of up to three times a day using the volumes established. However, since fasting may interfere with daily feeding, it is necessary to consult with the veterinarian/IACUC for specific permissions and/or conduct pilot studies as needed. The same indication applies if gavage is needed to administer microbial communities. Since the gavage of microorganisms is conducted using PBS as the vehicle, there will be no hypotonic overhydration of mice over a short-term period. However, if gavage administration is required for a longer periods of time, e.g. >2–3 days, renal washout and electrolyte imbalances may arise especially if 6 h fasting was maintained.

IACUC: Institutional Animal Care and Use Committee.
See further concepts in the 'Discussion' section.

circumstances.'[41] Thus, it is important to improve quality of guidelines for the gavage of mice. Although the body of data assessing the impact of gavage practices on microbiome research and the survival of the members of microbial communities within a given microbiome is remarkably vast, the implementation of standard IGPs protocols is critical, especially for the gavage of live microcommunities isolated from microscopic environments.

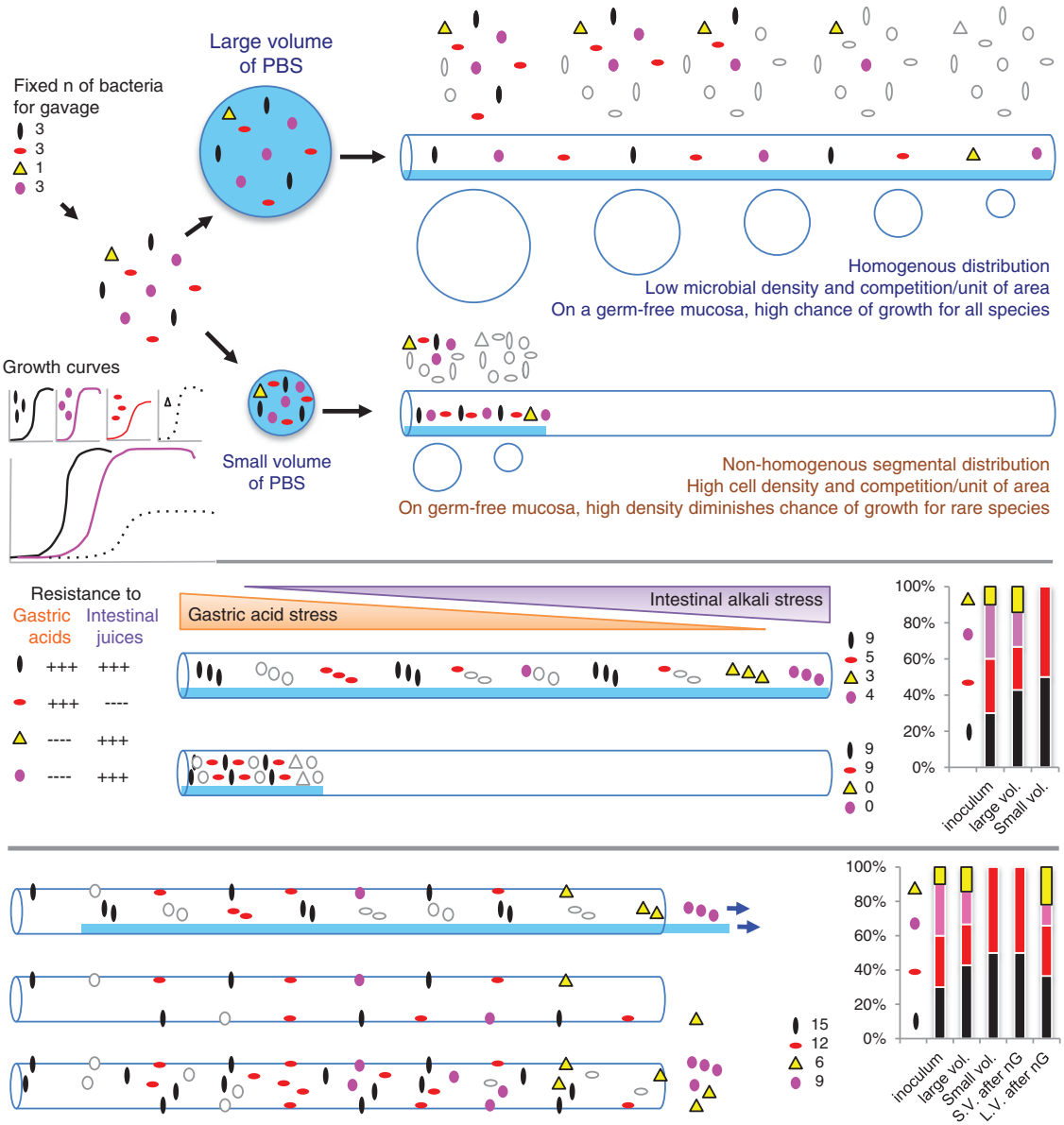

Figure 2. Theoretical probabilities for the distribution of microbial micro-communities in the intestinal tract as a function of the volume of administration. The scenarios represent the spatial transit, distribution, adherence to the gut wall, and overflow of microorganisms suspended in either a large or a small volume of fluid, and expected microbiome profiles, and the drops transit over time. Note the expected counts of a simple community of microbes that would survive and be detected depend on the area of distribution along the tubular space and their intrinsic hypothetical susceptibilities to gastric or intestinal secretions. PBS: phosphate buffered saline.

This has been especially critical in our center as we are determining the role of microbial communities from intramural cavernous fistulous tracts[8] (IM-CavFT, or 'miCaves') lesions in Crohn's Disease.

Using agreement statistics, we quantified that the methods do not agree as a collective. Using epidemiologically valid principles, we then applied the listed criteria already validated by IACUCs as minimum non-harmful practices to the mice in order to develop 'Analytical consensus IGPs for the administration of chemicals' and 'Analytical consensus IGPs for the administration of microbial organisms.' Based on internal medicine and clinical pharmacology principles, clinical and experimental microbiology, and peer-reviewed literature we aimed at eliminating method variability by proposing the use of the highest IACUC-approved volume and dosing regimens available in the reviewed IGPs, and proposed scientifically supported new dose thresholds. The major change is our proposition to use a unique (reproducible dose of) 20 mL/kg of body weight, without restrictions, and accompanied with a 6 h period of fasting. This is comparable to the time that humans have to wait between lunch and dinner, for instance. This time has also been shown to not bear deleterious effects for the mouse biology.[39] Although the dose regimens in the IGPs are not well supported within the scientific literature, and there are contradicting estimates of maximum stomach capacity,[42] the 20 mL/kg volume for microbiome purposes is among the practices approved by some of the reviewed IACUCs. The manuscript proposing such dose[22] has been extensively cited. Even larger

volumes are listed within the Rutgers University protocol (i.e. a dosing table describes that up to 50 mL/kg of fluid could be administered orally via gavage to a mouse with the body weight exceeding 35 g, Supplementary File 1). A consortium has stated that the ideal dosage is 10 mL/kg with a max volume of 20 mL/kg, but this approach reflects common practice and does not necessarily take into account the described maximum volumes tolerated by animals.[43]

Studies of the effect of gastric pH on microbial survival have been well documented in the literature, and microbial survivability in the gastric juice has been even a criterion for the selection of probiotic strains.[37] Not all microbes will survive gastric acidity. Since it would be highly research intensive to validate that the volume has an effect on each microbial species for every particular donor sample, it is advisable to follow criteria that are less likely to have a selection bias, as the bacteria mixture bypasses the upper parts of the intestinal tract. Therefore, we propose to use the maximum volume allowed to favor the rapid transit of the microbes to the more pH neutral intestinal milieu. This can be achieved by using large volumes of pH buffered physiological solutions (specifically, phosphate buffered saline) instead of water, which cannot buffer the gastric acidity or intestinal alkalinity.

In conclusion, publicly available IACUC protocols for the oral gavage in mice have variably described parameters and do not take into consideration potential adjustments needed for the administration of live microbial communities. A unified consensus protocols with a list of biological factors for consideration relevant to live microbiota in microbiome studies are described, which would be better suited for the study of villous-associated microcosmic microbiotas from intestinal micro-pathologies in humans and mice, especially those that could explain chronic onset and progressive inflammatory[8] or immunological diseases, including Crohn's Disease.

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DECLARATION OF CONFLICTING INTERESTS

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REFERENCES

1. Ioannidis JP. Why most published research findings are false. *PLoS Med* 2005;**2**:e124
2. Ioannidis JP. Why most published research findings are false: author's reply to Goodman and Greenland. *PLoS Med* 2007;**4**:e215
3. Nguyen TL, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? *Dis Model Mech* 2015;**8**:1–16
4. Paylor R. Questioning standardization in science. *Nat Methods* 2009;**6**:253–4
5. Richter SH, Garner JP, Würbel H. Environmental standardization: cure or cause of poor reproducibility in animal experiments? *Nat Methods* 2009;**6**:257–61
6. Boulinier T, Nichols J, Sauer J, Hines J, Pollock K. Estimating species richness: the importance of heterogeneity in species detectability. *Ecology* 1998;**79**:1018–1028
7. Rodriguez-Palacios A, Kaydo L, Pietropaoli D, Cominelli F. Stereomicroscopy in pre-clinical and immunological studies of acute and chronic intestinal inflammation. *Gastroenterology* 2014;**146**: S523–4 (Abstr)
8. Rodriguez-Palacios A, Tomohiro K, Kaydo L, Pietropaoli D, Corridoni D, Howell S, Katz J, Xin W, Pizarro T, Cominelli F. Stereomicroscopic 3D-pattern profiling of murine and human intestinal inflammation reveals unique structural phenotypes. *Nat Commun* 2015;**6**:7577
9. Rodriguez-Palacios A, Buttó L, Bederman I, Haller D, Cominelli F. 3D-stereomicroscopic, microbial and metabolic characterization of intestinal villous erosions and ulcerations in mice. *Gastroenterology* 2016;**150**:S578
10. Collins FS, Tabak LA. Policy: NIH plans to enhance reproducibility. *Nature* 2014;**505**:612–3
11. NIH. National Institutes of Health. *Enhancing reproducibility through rigor and transparency,* https://grants.nih.gov/reproducibility/index.htm (2018, accessed 23 October 2018)
12. NABR. National Association for Biomedical Research, Functions of the IACUC, https://www.nabr.org/animal-welfare-2/animal-welfare-in-practice/functions-of-the-iacuc/ (2019, accessed 19 February 2019)
13. Hoggatt AF, Hoggatt J, Honerlaw M, Pelus LM. A spoonful of sugar helps the medicine go down: a novel technique to improve oral gavage in mice. *J Am Assoc Lab Anim Sci* 2010;**49**:329–34
14. Wheatley JL. A gavage dosing apparatus with flexible catheter provides a less stressful gavage technique in the rat. *Lab Anim* 2002;**31**:53–6
15. Diogo LN, Faustino IV, Afonso RA, Pereira SA, Monteiro EC, Santos AI. Voluntary oral administration of Losartan in rats. *J Am Assoc Lab Anim Sci* 2015;**54**:549–56
16. Gonzales C, Zaleska MM, Riddell DR, Atchison KP, Robshaw A, Zhou H, Sukoff Rizzo SJ. Alternative method of oral administration by peanut butter pellet formulation results in target engagement of BACE1 and attenuation of gavage-induced stress responses in mice. *Pharmacol Biochem Behav* 2014;**126**:28–35
17. Walker MK, Boberg JR, Walsh MT, Wolf V, Trujillo A, Duke MS, Palme R, Felton LA. A less stressful alternative to oral gavage for pharmacological and toxicological studies in mice. *Toxicol Appl Pharmacol* 2012;**260**:65–9
18. Küster T, Zumkehr B, Hermann C, Theurillat R, Thormann W, Gottstein B, Hemphill A. Voluntary ingestion of antiparasitic drugs emulsified in

honey represents an alternative to gavage in mice. *J Am Assoc Lab Anim Sci* 2012;**51**:219–23
19. Wheeler TL, Eppolito AK, Smith LN, Huff TB, Smith RF. A novel method for oral stimulant administration in the neonate rat and similar species. *J Neurosci Methods* 2007;**159**:282–5
20. Almasaudi SB, Al-Nahari AAM, Abd El-Ghany ESM, Barbour E, Al Muhayawi SM, Al-Jaouni S, Azhar E, Qari M, Qari YA, Harakeh S. Antimicrobial effect of different types of honey on Staphylococcus aureus. *Saudi J Biol Sci* 2017;**24**:1255–61
21. Piotrowski M, Karpiński P, Pituch H, van Belkum A, Obuch-Woszczatyński P. Antimicrobial effects of Manuka honey on in vitro biofilm formation by *Clostridium difcile*. *Eur J Clin Microbiol Infect Dis* 2017;**36**:1661–4
22. Turner PV, Brabb T, Pekow C, Vasbinder MA. Administration of substances to laboratory animals: routes of administration and factors to consider. *J Am Assoc Lab Anim Sci* 2011;**50**:600–13
23. Turner PV, Pekow C, Vasbinder MA, Brabb T. Administration of substances to laboratory animals: equipment considerations, vehicle selection, and solute preparation. *J Am Assoc Lab Anim Sci* 2011;**50**:614–27
24. JoVE. JoVE science education database. Lab animal research. Compound administration II. JoVE-Cambridge, MA. *J Vis Exp* 2018. https://www.jove.com/science-education/10388/compound-administration-ii
25. Machholz E, Mulder G, Ruiz C, Corning BF, Pritchett-Corning KR. Manual restraint and common compound administration routes in mice and rats. *J Vis Exp* 2012;**67**:e2771.
26. Hawkins P, Morton DB, Burman O, Dennison N, Honess P, Jennings M, Lane S, Middleton V, Roughan JV, Wells S, Westwood K; UK Joint Working Group on Refinement BVAAWF/FRAME/RSPCA/UFAW. A guide to defining and implementing protocols for the welfare assessment of laboratory animals: eleventh report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. *Lab Anim* 2011;**45**:1–13
27. Donegan H, Dodd F. An analytical approach to consensus. *Appl Math Lett* 1991;**4**:21–4
28. Borgatti S, Halgin D. Consensus analysis. In: Kronenfeld D, DeMunck V, Fischer M, Bennardo G (eds) *Blackwell's companion to cognitive anthropology*. Oxford: Blackwell, 2011. Available at: http://steveborgatti.com/papers/BHConsensus.pdf (accessed 20 March 2019).
29. Dohoo I, Martin W, Stryhn H. In: McPike SM (ed) *Veterinary epidemiologic research*. Charlottetown: AVC, Inc., 2003, 799pp.
30. McHugh ML. Interrater reliability: the kappa statistic. *Biochem Med* 2012;**22**:276–82
31. US-News. *Best Global Universities 2018 US News Report*, https://www.usnews.com/education/best-global-universities (2018, accessed 20 October 2018)
32. Machholz E. e A. *Manual restraint and common compound administration routes in… JoVE Video*, https://www.youtube.com/watch?v=s9skgg7dHIA (2016, accessed 14 February 2019, 2012)
33. Turner PV, Vaughn E, Sunohara-Neilson J, Ovari J, Leri F. Oral gavage in rats: animal welfare evaluation. *J Am Assoc Lab Anim Sci* 2012;**51**:25–30
34. Jones CP, Boyd KL, Wallace JM. Evaluation of mice undergoing serial oral gavage while awake or anesthetized. *J Am Assoc Lab Anim Sci* 2016;**55**:805–10
35. Basson A, Abigail R. Basson, Adrian Gomez-Nguyen, Ludovica Butto, Paola Menghini, Luca Di Martino, Minh Iam, Natalia Aladyshkina, Alexander Rodriguez-Palacios, Fabio Cominelli. A human-associated SAMP1/YITFC (SAMP) fecal transplantation mouse model to study the functionality of the gut microbiome. *Gastroenterology* 2018;**154**:S-25
36. Rodriguez-Palacios A, Aladyshkina N, Ezeji JC, Erkkila HL, Conger M, Ward J, Webster J, Cominelli F. 'Cyclical Bias' in microbiome research revealed by a portable germ-free housing system using nested isolation. *Sci Rep* 2018;**8**:3801
37. Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS. Isolation of bovine intestinal *Lactobacillus plantarum* and *Pediococcus acidilactici* with inhibitory activity against *Escherichia coli* O157 and F5. *J Appl Microbiol* 2009;**106**:393–401
38. Sheehan VM, Sleator RD, Hill C, Fitzgerald GF. Improving gastric transit, gastrointestinal persistence and therapeutic efficacy of the probiotic strain *Bidobacterium breve* UCC2003. *Microbiology* 2007;**153**:3563–71
39. Smith A. *Fasting in rodents*. Oslo: Norecopa-Veterinærinstituttet, https://norecopa.no/media/8122/food-deprivation.pdf (2009, accessed 15 February 2019)
40. Staley C, Kaiser T, Beura LK, Hamilton MJ, Weingarden AR, Bobr A, Kang J, Masopust D, Sadowsky MJ, Khoruts A. Stable engraftment of human microbiota into mice with a single oral gavage following antibiotic conditioning. *Microbiome* 2017;**5**:87
41. Craig MA, Elliott JF. Mice fed radiolabeled protein by gavage show sporadic passage of large quantities of intact material into the blood, an artifact not associated with voluntary feeding. *Contemp Top Lab Anim Sci* 1999;**38**:18–23
42. McConnell EL, Basit AW, Murdan S. Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. *J Pharm Pharmacol* 2008;**60**:63–70
43. Hull RM. Guideline limit volumes for dosing animals in the preclinical stage of safety evaluation. Toxicology Subcommittee of the Association of the British Pharmaceutical Industry. *Hum Exp Toxicol* 1995;**14**:305–7