Journal of the American Association for Laboratory Animal Science
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Vol 62, No 3
May 2023
Pages 212–221

Effects of Extended Cage Component Sanitation Interval on the Microenvironment, Health, and Gastrointestinal Microbiome of Rats (*Rattus norvegicus*)

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Washing and sanitizing rodent cage components requires costly equipment, significant personnel effort, and use of natural resources. The benchmark frequency for sanitation of individually ventilated caging (IVC) has traditionally been every 2 wk. In this study, we investigated the effects of extending this interval on the cage microenvironment, basic markers of health, and the gastrointestinal microbiota of rats. We compared our institutional standard of changing the sanitation interval for rat cage lids, box feeders, and enrichment devices from every 4 wk to an interval of 12 wk. The cage bottom and bedding continued to be changed every 2 wk for both groups. We hypothesized that we would find no significant difference between our current practice of 4 wks and continuous use for 12 wk. Our data showed that intracage ammonia levels remained below 5 ppm for most cages in both groups, with the exception of cages that experienced a cage flood. We found no significant difference between groups in bacterial colony forming units (CFU) on cage components. We used 3 novel methods of assessing cleanliness of enrichment devices and found no significant effect of continuous use for 12 wk on the number of CFU. In addition, we found no significant differences between groups for animal weight, routine blood work, or fecal and cecal microbiomes. These data indicate that a sanitation interval of up to 12 wk for components of rat IVC caging has no significant effects on the microenvironment or health of rats. Using the longer interval will improve efficiency, reduce the use of natural resources, and decrease costs while maintaining high-quality animal care.

Abbreviations and Acronyms: CFU, colony forming units; CM, cecal microbiome; FM, fecal microbiome; GI, gastrointestinal tract; GM, gastrointestinal (gut) microbiome; IVC, individually ventilated cage

DOI: 10.30802/AALAS-JAALAS-22-000113

Introduction

Rodent cage sanitation practices are rooted in engineering standards briefly outlined in the *Guide for the Care and Use of Laboratory Animals*. For ventilated rack systems, the *Guide* recommends sanitizing rodent microenvironments at least once every 2 wk with wash and rinse water of 143 to 180 °F (62 to 82 °C).[33] The *Guide* defines the microenvironment as the primary enclosure that contains all the resources that animals contact directly such as the cage lid, box feeders, enrichment, bedding, and cage bottom. A performance standard can influence the cage sanitation interval if the housing system and animal density necessitate either more or less frequent cage changes. Similarly, a longer sanitation interval may be justified if the microenvironment is not compromised as measured by factors such as intracage ammonia, bacterial load on surfaces, and appearance of cage surfaces for example.[33]

Submitted: 07 Dec 2022. Revision requested: 10 Jan 2023. Accepted: 20 Feb 2023.
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Advanced automation in cage wash areas has substantially increased efficiency and reduced labor. However, the upfront costs of the area, including the mechanical, electrical, and plumbing demands, automated equipment, and ongoing costs for service agreements and labor are substantial. Furthermore, cage sanitation is a natural-resource intense process due to the need for hot water and sterilization with either dry heat or steam.[22] Combined, these factors make cage washing one of the most expensive areas to create and operate per square foot in a vivarium. By extending the sanitation interval of cage components, the amount of material that goes through cage wash decreases proportionally, leading to a reduction in cost and natural resource consumption.

Multiple studies have explored extending cage sanitation intervals for rodents. Most of the recent studies have focused on mice and consistently support the extension of cage component sanitation intervals with no significant effects on the microenvironment.[1,12,41] These data are not easily extrapolated to rats. While rats and mice have similar anatomy and physiology, rats are larger than mice, and they are housed in larger cages at a lower housing density than are mice. Several studies have attempted to address cage component sanitation intervals for rats.[8,13,39] However, the variation in experimental design and conclusions of these studies has not resulted in a broadly acceptable extension in cage sanitation interval for rat cages or cage components.

To increase efficiency at our institution, we identify 2 types of components of rodent cages and use different sanitation intervals for each. The cage bottom and bedding material of rat cages are replaced every 2 wk. The rest of the cage components including the cage lid, box feeders, and enrichment are replaced every 4 wk. This schedule was adopted based on the simple observation that the bedding and cage bottom become soiled with urine and feces while the remaining cage components appear less soiled. However, we wanted to validate this practice to provide empirical support and to document an acceptable high quality of care. We therefore investigated the quality of the cage microenvironment, basic physiology, and the gastrointestinal microbiota of rats housed in individually ventilated cages (IVCs) with cage component sanitation intervals of 4 or 12 wk. For both groups, the cage bottom was changed every 2 wk. We hypothesized that this increase in sanitation interval for these specific cage components would not negatively impact the microenvironment or the basic physiology of the rats. Parameters that were evaluated included the intracage ammonia concentration, bacterial load on the surfaces of the cage lid, feeders, and tunnel, and a detailed assessment of the appearance of the tunnel. We also evaluated body weight, hematology, some clinical chemistry analytes, and fecal and cecal microbiota.

Materials and Methods

Animals. Thirty-two male, 5-wk-old Sprague–Dawley rats (Crl:SD, stock code #400) were purchased from Charles River Laboratories (Wilmington, MA). Male rats were selected after a pilot study demonstrated a 5-to-10-fold increase in intracage surface bacterial counts for males as compared with females (data not shown). Crl:SD rats were selected because their fecal microbiome (FM) and cecal microbiome (CM) had been investigated previously.[11] All rats were housed in the same room and allowed to acclimate for 1 wk prior to sample collection. The vendor confirmed the rats were free of Sendai virus, pneumonia virus of mice, rat coronavirus, Kilham rat virus, Toolan H-1 virus, rat parvovirus, rat minute virus, reovirus, rat theilovirus, lymphocytic choriomeningitis virus, Hantaan virus, mouse adenovirus, *Bordetella bronchiseptica*, cilia-associated respiratory bacillus, *Corynebacterium kutscheri*, *Helicobacter bilis*, *Helicobacter hepaticus*, *Helicobacter* sp., *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Mycoplasma pulmonis*, *Pasturella multocida*, *Pasturella pneumotropica*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Streptococcus pneumoniae*, *β*-hemolytic *Streptococcus* sp. (groups B and G), *Pneumocystis* spp., Tyzzer's disease, *Encephalitozoon cuniculi*, and all ecto- and endoparasites. This study was approved by the University of Colorado Denver | Anschutz Medical Campus's Institutional Animal Care and Use Committee.

Husbandry. Rats were randomly distributed and pair-housed in individually ventilated cages (IVC, 12 W × 17.5 D × 8 in. H [30 × 44 × 20 cm]) on MicroVent racks (Allentown, NJ), with 40 air changes hourly (ACH) and automatic delivery of reverse-osmosis–purified, hyperchlorinated water (3 to 5 ppm) by water valves (Avidity Science, Waterford, WI). Cages included a cage lid, 2 stainless-steel box feeders, red-tinted polycarbonate Rat Tunnels (Cat# K3325, Bioserv, Flemington, NJ), and 1.27-cm depth aspen chip bedding (7090 Teklad Aspen Sani-Chips, Envigo, Indianapolis, IN). All cages and cage components were fully assembled prior to steam sterilization in bulk autoclaves (AMSCO series, Steris) using a prevacuum cycle at 133 °C (270 °F) for 15 min and a dry time of 10 min. A wire bar lid was not used, so rats had access to all intracage surfaces. Rats were fed ad libitum irradiated rodent chow (2920×, Teklad Envigo). All cage manipulations and sample collection were performed in a HEPA-filtered animal transfer station (ATS, Nuaire, Plymouth, MN). The ATS surface and gloved hands were sprayed with Clidox-S disinfectant (Pharmacal, Naugatuck, CT) mixed at 1:18:1 concentration. Additional personal protective equipment included a hair bonnet, face mask, and disposable isolation gown. Standard room conditions in this facility have a 14:10-h light:dark cycle (lights on from 0600 to 2000), a minimum of 12 room ACH, and a room temperature of 22 ± 1 °C (72 ± 2 °F). Ambient humidity of the macroenvironment ranged between 30% to 50%.

Experimental design. Sixteen cages of pair-housed rats were divided into either control or experimental groups. Control cages were changed according to our facility's standard practices in which the cage lid, box feeders, and enrichment are changed every 4 wk. Experimental cages were changed using the extended sanitation interval of 12 wk for the cage lid, box feeders, and enrichment. The cage bottom, including soiled bedding, was changed at a 2 wk interval for both groups. Control and experimental cages were distributed on the IVC rack in an alternating pattern.

Ammonia monitoring. Ammonia levels were measured in unopened cages every 2 wk (Figure 1), immediately after its removal from the IVC rack. Intracage air was sampled using a test pump (Matheson-Kitagawa : Irving, TX, Model 8014 - 400B) and colorimetric ammonia detection tubes (Kitagawa, Cat# 105SC, 5 to 260 ppm and Cat# 105SD, 0.2 to 20 PPM). The sampling tube was passed through the water valve grommet and held at the rats' nose level. Because the campus is at 5,403 ft above sea level, a 10% correction was applied to each result to correct for altitude as indicated by the manufacturer's instructions.

Assessment of enrichment tunnels. Autoclaved, red-tinted, round, polycarbonate Rat Tunnels (Bioserv) were provided for rat enrichment. A baseline photograph and weight were recorded for new tunnels after autoclaving and before they were placed in the cages. At 0, 2, 4, 8, and 12 wk, the tunnels were weighed with a subset photographed. To quantify the debris

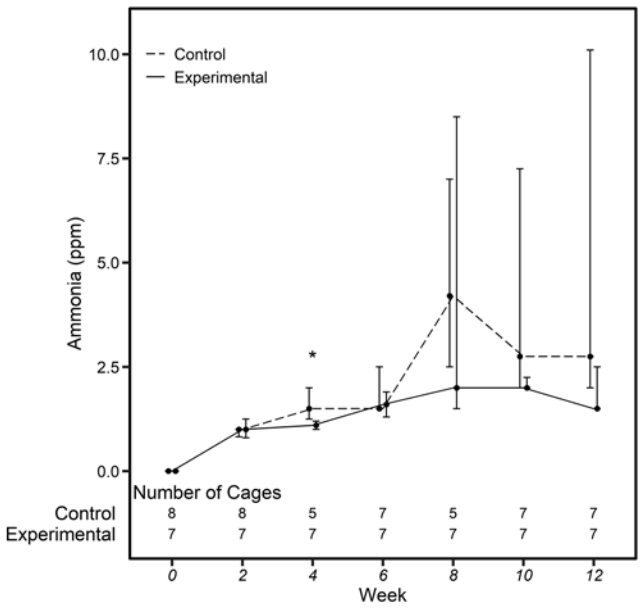

Figure 1. Intracage ammonia concentration (median, IQR) just prior to changing the soiled cage bedding and bottom, performed every 2 wk for both the control and experimental group. The number of cages analyzed at each time point varied due to cage flooding and is provided on the figure. * $P \leq 0.05$

accumulation on their surface, a light meter (MT30 Digital Luminometer) was used to measure light (lux) transmission through the tunnel from 6 cm in the tube. In addition, each external quadrant of the tunnel's external surface was visually scored to quantify the extent of soiling. (Figure 2 and 3 A) Then an area of approximately 30 cm on the external surface of the tunnel was swabbed for quantification of bacterial CFU using standard microbiology methods as described below. At the end of the study, tunnels were weighed, washed, allowed to dry, and reweighed. The difference in weight was considered to be the amount of surface debris accumulation, minus any losses due to chewing.

Microbiology. Samples for assessment of bacterial surface burden on the cage lid, box feeders, and tunnels were collected at 0, 2, 4, 8, and 12 wk using a sterile cotton-tipped applicator (Cat#25 to 806 2WC Lot 6040, Puritan Medical Products, Guilford, ME) moistened in sterile phosphate buffered saline (PBS).[1,39] Samples were collected from an approximate 30 cm total surface area away from the air exhaust ports, the inner cage lid, the lowest face of the box feeder, and the tunnel, as described above (Figure 2). Swab tips were placed in 1 mL of PBS and vortexed for 10 s; 50 to 500 µL of the solution was then distributed over a 5% sheep blood trypticase soy agar plate (Cat# 221621, BD, Franklin Lakes, NJ) by using a glass plate spreader. Plates were incubated at 37 °C for 48 h, and colonies counted to determine CFU/mL. To aid in counting colonies on high density plates, we used a bacterial colony counting app (CountThings, Cupertino, CA) to confirm the number of colonies as compared with a manual count. If bacterial colony density was too numerous to count (TNTC), the sample was given a value of 100,000 CFU/mL. This value was based on the number of colonies that exceeded physical counting with app confirmation because of the size, number, or coalescence of colonies.

Bloodwork. Whole blood was collected immediately before euthanasia by intracardiac puncture under isoflurane anesthesia and was used to perform an automated complete blood count (CBC), manual differential, and a 7 analyte serum chemistry profile. The parameters included white blood cell count (lymphocytes, monocytes, granulocytes percentages, and count, lymphocyte to neutrophil ratio), hematocrit, MCV, RDWa,

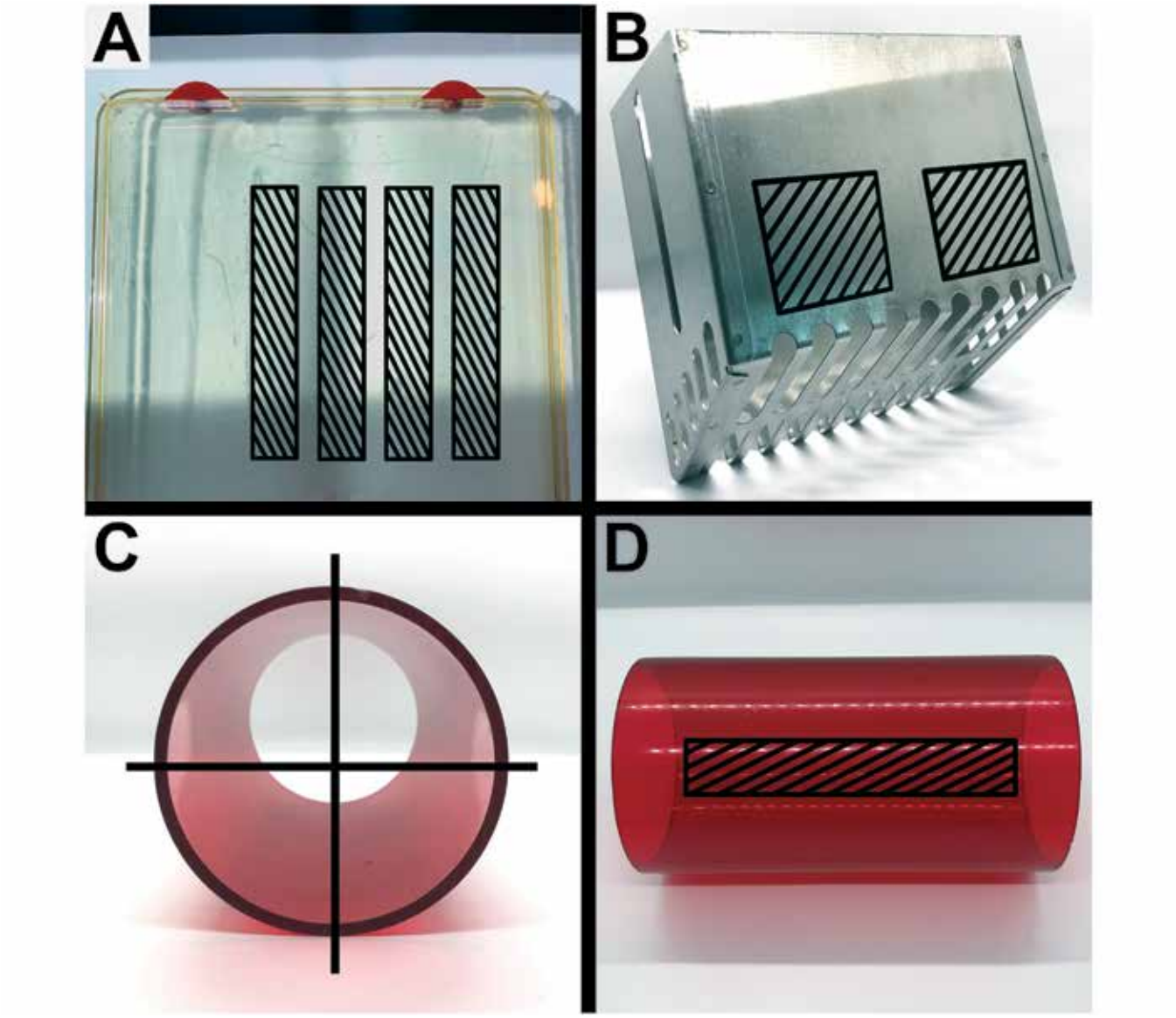

Figure 2. Microbiologic sampling areas for each cage component to determine bacterial surface burden at each time point. Approximately 30 cm of total surface area in direct contact with the rats was swabbed as follows: A) the inner cage lid, B) the lowest face of the box feeder, C and D) areas of the tunnel used for visual scoring and swabbing, respectively.

RDW %, hemoglobin, MCHC, MCH, RBC, PLT, MPV, plus BUN, Creatinine, BUN/Creatinine ratio, Total Protein, Glucose, ALT (GPT), and ALP (HemaTrue and DriChem7000 analyzers, Heska, Loveland, CO). The lymphocyte to neutrophil ratio was then calculated.

Gastrointestinal microbiota sample collection and analysis. A single fecal pellet was collected fresh from each rat at 0, 2, 4, 8, and 12 wk. Rats were placed in an empty autoclaved cage and allowed to defecate normally. If a rat did not spontaneoulsy defecate, the abdomen was gently massaged to stimulate defecation. Fecal pellets were then collected aseptically with a sterile needle and placed in a microcentrifuge tube. These samples were then frozen at −80 °C for fecal microbiome sequencing and analysis. At the end of the 12 wk study, rats were placed under isoflurane anesthesia. After terminal blood collection, the cecal contents were collected as described previously.[12] Briefly, the cecal serosa was incised with a new scalpel blade to allow collection of samples from within the exteriorized cecum. In addition to sampling luminal contents, the mucosa was gently scraped to ensure that mucosa-associated microbes were included in the samples. Cecal samples were approximately 200 µL. After collection, all samples were frozen at −80 °C for submission for microbiome sequencing and analysis. All terminal samples were collected between 0700 and 1100 on 2 consecutive days. After sample collection, rats were euthanized by bilateral pneumothorax under anesthesia.

Fecal and cecal bacterial profiles were determined by broad-range amplification and sequence analysis of 16S rRNA genes using our previously described methods.[28] In brief, amplicons were generated using barcoded[17] primers that target approximately 300 base pairs of the 16S rRNA gene V3V4 variable (338F [5′-ACTCCTACGGGAGGCAGCAG] and 806R [5′ GGACTACHVGGGTWTCTAAT]).[18,26] PCR products were normalized using a SequalPrep™ kit (Invitrogen, Carlsbad, CA), pooled, lyophilized, purified, and concentrated using a DNA Clean and Concentrator Kit (Zymo, Irvine, CA). Pooled amplicons were quantified using Qubit Fluorometer 2.0 (Invitrogen, Carlsbad, CA). The pool was diluted to 4 nM and denatured with 0.2 N NaOH at room temperature. The denatured DNA was diluted to 15 pM and spiked with 25% of the Illumina PhiX control DNA prior to loading the sequencer. Paired-end sequencing was performed on the Illumina MiSeq platform with versions v2.4 of the MiSeq Control Software and MiSeq Reporter, using a 600-cycle version 3 reagent kit.

The paired sequence reads were assembled using phrap[15,16] and pairs that did not assemble were discarded. Assembled sequence ends were trimmed over a moving window of 5 nucleotides until the average quality met or exceeded 20. Trimmed sequences that had more than 1 ambiguity or were shorter than 250 nt were discarded. Potential chimeras identified with Uchime (usearch6.0.203_i86linux32)[10] using Silva reference sequences[38] were removed from subsequent analyses. Assembled sequences were aligned and classified with SINA (1.3.0-r23838)[35] using the 418,497 bacterial sequences in Silva 115NR99[36] configured to yield the Silva taxonomy. The 16S rRNA sequences were grouped into operational taxonomic units (OTUs) based on the lowest common ancestor using SINA/SILVA's default parameters. This process generated a median of 79,859 sequences per sample, IQR: 57,933 to 116,246). All sequence libraries had a Good's coverage score greater than 99%, indicating excellent depth of sequence coverage. Demultiplexed paired end sequence data and accompanying metadata were deposited in the NCBI Sequence Read Archive under BioProject ID PRJNA880293.

Statistical methods/analysis. Group characteristics for ammonia, tunnels, and microbiology were summarized by sanitation schedule using median and interquartile range. The association between group characteristics at each time point and sanitation schedule was evaluated with the Wilcoxon rank-sum test to reduce the influence of outliers in a small sample size and to account for the nonnormal distribution of some of the variables. Within-group comparisons were performed using the Wilcoxon Signed-Rank test. Mixed-effect models were fit using the MIVQUE0 method to examine differences in bloodwork outcomes and to control for correlation within cages due to nonnormality in the data and a compound symmetry covariance matrix. All P values were based on a 2-sided alternative and considered significant if $P < 0.05$. Analysis was performed using SAS version 9.4 (SAS Institute; Cary, NC).

The R (v3.6.3; R Core Team, Vienna, Austria) and Explicet (v2.10.5[37]) software packages were used to visualize and analyze microbiome data. Differences in overall microbiota composition (that is, β-diversity) between groups were assessed by using a permutational multiple analysis of variance (PERMANOVA) test, as implemented by the adonis2 function of the vegan R package.[34] Dissimilarities were measured using the weighted Bray-Curtis and unweighted Jaccard indices, with P values inferred through 10^6 label permutations. The Shannon diversity index (that is, an assessment of α-diversity) was calculated for each sequence library through 1000 replicate samples at a rarefaction point of 17,000 sequences. Between-group differences in Shannon diversity were assessed by the Welch t test.

Results

Ammonia. Intracage ammonia levels were recorded every 2 wk over the 12 wk study. Other than week 4, no statistically significant difference was identified between groups (Figure 1). As seen in Figure 1, the medians of ammonia level were the same or lower in the experimental group (week 12, median = 1.5 ppm, [IQR 1.5, 2.5]) relative to the control group (week 12, median = 2.75 ppm, [IQR 2,10.1], $P = 0.17$) at all time points, never exceeded 4.5 ppm for either group, and were fairly consistent over time. These results indicate that the longer sanitation intervals did not significantly alter ammonia levels in the microenvironment.

Enrichment tunnels. New enrichment tunnels (Figure 3 B) were autoclaved before use, which resulted in a loss of mass (range, 2 to 10 g). Weight, translucence as measured by a lux meter, and visual score of soiling were assessed every 4 wk for the experimental group only. Overall, the rats were observed to heavily use the enrichment tunnels. The levels of light transmission (Figure 3 F, $P = 0.74$) and the change in tunnel weight (Figure 3 G, $P = 0.47$) did not change significantly over the duration of the study. Despite statistically significant differences in the visual scores, the difference was considered to be biologically insignificant (that is, a change from 0 to 0.25, with 0 being a clean new tube and 1 being a light dustiness of the tunnel; $P = 0.03$; Figure 3 E), The most soiled tunnel was found in a flooded cage and was omitted from the statistical analysis of that time point. Tunnel weight loss was attributed to material loss due to rats chewing on the device. These data indicate that continuous use of the tunnel for 12 wk had little impact on several visual indicators of perceived cleanliness.

Microbiology. To determine bacterial load, each cage component was aseptically sampled with sterile PBS moistened swabs at 0, 2, 4, 8, and 12 wk. Bacterial load was determined by counting CFU after 48 h of incubation on 5% sheep blood agar plates. Cages with floods and higher CFU were excluded

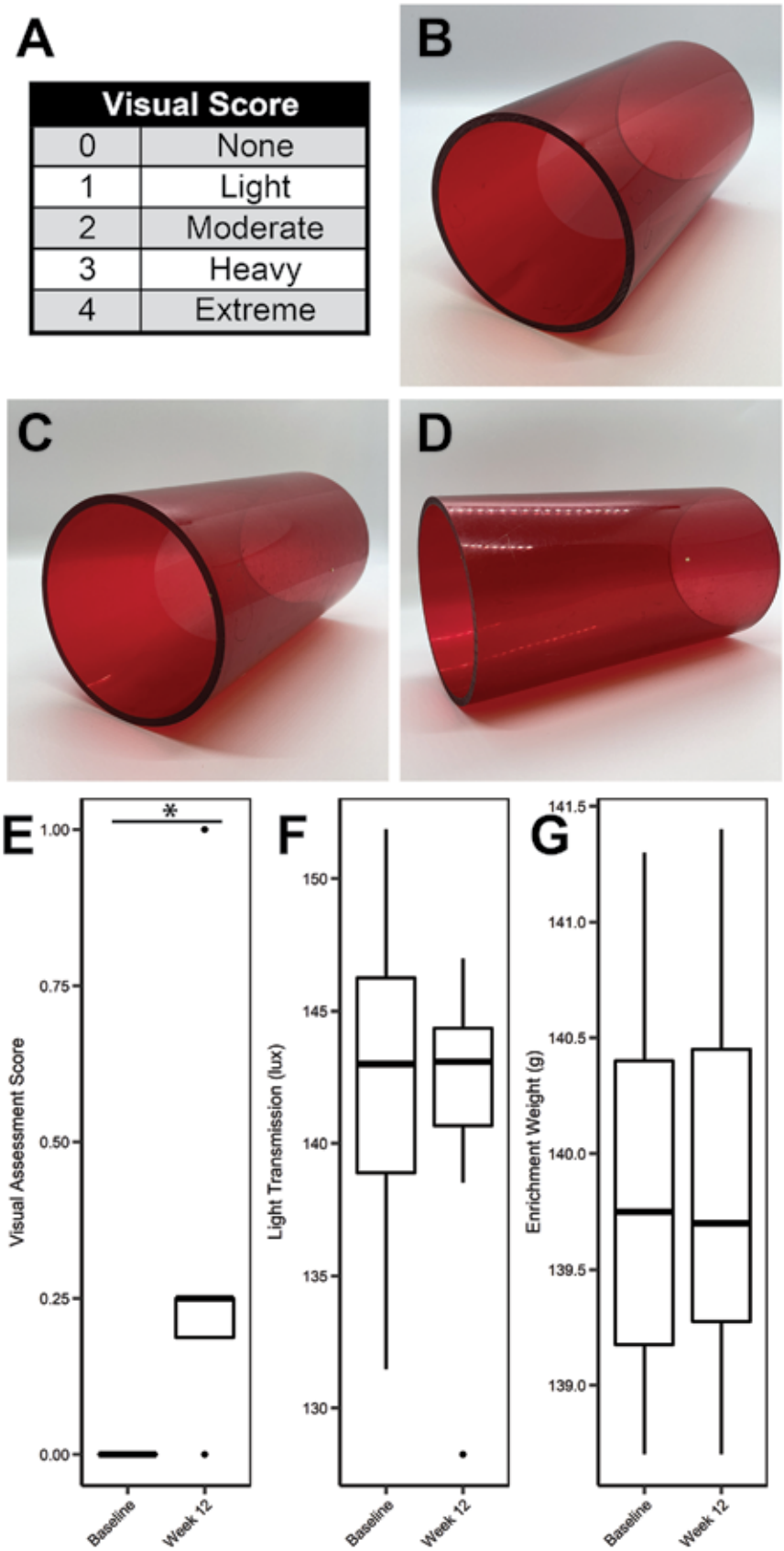

Figure 3. Assessment of tunnel from wk 0 to 12. No significant differences were detected for any of these parameters at any of the 4-wk time points. Therefore, only wk 0 and 12 are shown for comparison. A) visual assessment scale was used to score the levels of debris on the enrichment surface. B) New tunnels (Bioserv) that had been photographed after sterilization by autoclave and were placed in cages at week 0. C and D) Representative photographs of tunnels that had not been sanitized for 12 wk after placement in cages. E) Changes (median, IQR) in the visual assessment score. F) light transmission through tunnel, and G) tunnel weight in the cages of the experimental group at the beginning and end of the study. * $P \leq 0.05$

as described above. CFU from cage component surfaces were not significantly different between the experimental and control groups at any point in time over the 12 wk study (Figure 4). At wk 8, we saw a visual but nonsignificant rise in aerobic bacteria in cultures of all surfaces for both experimental and control groups. By wk 12, CFU were visually but not significantly lower and approached CFU detected at wk 2 and 4. Culture technique controls indicated that contamination did not contribute to the peak at 8 wk. These results suggest that the change in sanitation schedule between groups had little impact on surface bacteria counts except for the excessive bacterial growth related to cage flooding.

Basic Physiology Assessments. Rats were weighed every 2 wk and remained consistent with the vendor's historical body weight data as related to age (data not shown). After 12 wk, the median rat weight of the control and experimental groups was 548 g and 575 g, respectively ($P = 0.87$). In blood collected at the end of the study, the mean values for total WBC and neutrophil, lymphocyte, and monocyte cell populations of all study animals were well within the normal range of healthy rats (Table 1).[5,8,20] In addition, liver enzymes including ALP, ALT, BUN, and creatinine levels were comparable to the age-matched SD rat data provided by the vendor. The ratio of blood lymphocytes and neutrophils were also compared between experimental and control groups as a single indicator of chronic stress.[19] These assessments revealed no statistically significant difference between groups (Table 1). These findings indicate that the change in sanitation schedule between groups had little impact on basic physiologic processes such as weight gain, liver and kidney function, and a single indicator of chronic stress.

Fecal (FM) and cecal microbiota (CM). In total, 128 fecal samples and 32 cecal samples were assessed to characterize differences in the gut microbiota (GM) between the experimental and control groups. Fecal samples were profiled longitudinally, while cecal samples were profiled after animal euthanasia. The bacterial V3V4 region was amplified from all samples via broad-range PCR, and 16S rRNA amplicon Illumina sequencing

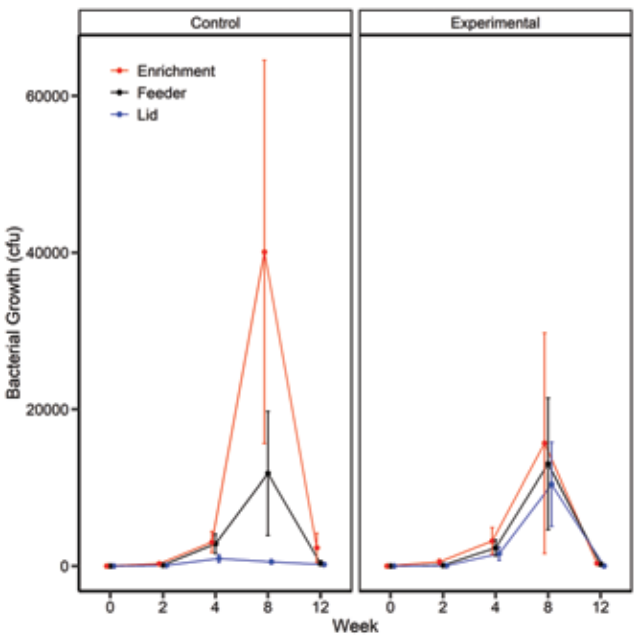

Figure 4. Microbial load reported as CFU (mean ± SEM) measured on intracage components (cage lid, box feeder, and tunnel) at 0, 2, 4, 8, and 12 wk for the control and experimental groups. No significant differences were detected between the 2 groups at any time point.

Table 1. Rat CBC, Select Blood Analytes and NE:LY Ratio after 12 wks*

	WBC	LYM	GRAN	HCT	PLT	BUN	TP	GLU	ALT	ALP	NE:LY
	10^3/uL	%	%	%	10^3/uL	mg/dL	g/dL	mg/dL	U/I	U/I	ratio
Control n = 16	12.4 ± 2.3	79.8 ± 4.1	17.2 ± 3.3	41.1 ± 2.4	490.1 ± 187	18.1 ± 1.46	7.2 ± 0.3	97.63 ± 19.6	29.1 ± 3.36	250.8 ± 102	0.21 ± 0.05
Experimental n = 16	12.8 ± 3.4	79.9 ± 5.5	17.3 ± 4.4	42.9 ± 3.7	591.7 ± 104	17.7 ± 2.1	7.0 ± 0.3	92.5 ± 19.9	31.8 ± 3.8	233.5 ± 57.2	0.22 ± 0.07

*No statistical differences were found between groups for all assessments.

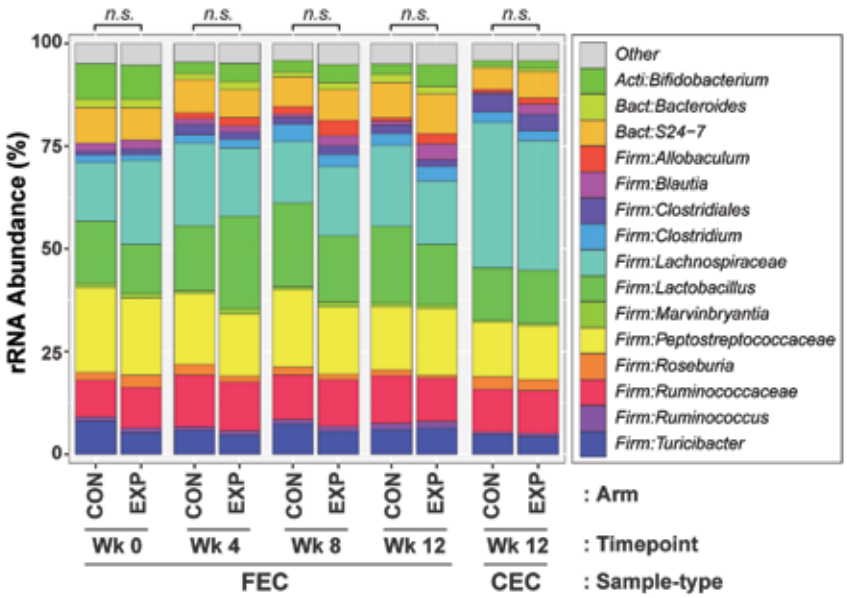

Figure 5. Stacked bar charts showing the relative abundances of genus-level bacterial taxa in the gut microbiota of CRL Sprague–Dawley rats housed in IVC microisolation caging. Bacterial communities were profiled in feces (FEC) and cecum contents (CEC) collected at the indicated timepoints ($n = 16$ rats/group/timepoint). Each stacked bar represents a separate treatment group defined by treatment arm (CON: control arm. EXP: experimental arm) and timepoint. Taxa with % relative abundances < 1% were collapsed into the "Other" group. Between-group differences in microbiota were assessed at each timepoint by permutational ANOVA using both Bray-Curtis (weighted) and Jaccard (unweighted) dissimilarity indices. None of the comparisons were significant (*n.s.*) at a P value cutoff of 0.05.

was performed. Stacked bar charts were generated to visualize the data based on treatment group, time point, and sample type (Figure 5). The phyla Bacteroidetes and Firmicutes dominated both the fecal and cecal microbiotas. The 7 genera in these phyla represent the vast majority of the FM and CM, including Bifidobacterium, *Bacteroides*, S24-7, Lachnospiraceae, *Lactobacillus*, Peptostreptococcaceae, Ruminococcaceae, and Turicibacter. No significant differences were detected between the 2 groups at any time point, as assessed by PERMANOVA test using either weighted (Bray–Curtis) or unweighted (Jaccard) dissimilarity indices (Figure 4). Similarly, no significant differences were detected between the 2 groups at any time point based on the Shannon α-diversity index (Figure 6). Furthermore, no taxa differed in relative abundance between treatment groups at any time point (FDR-corrected $P > 0.05$ for all taxa). Overall, these results suggest that sanitation had little effect on the overall composition of FM and CM over the course of the study.

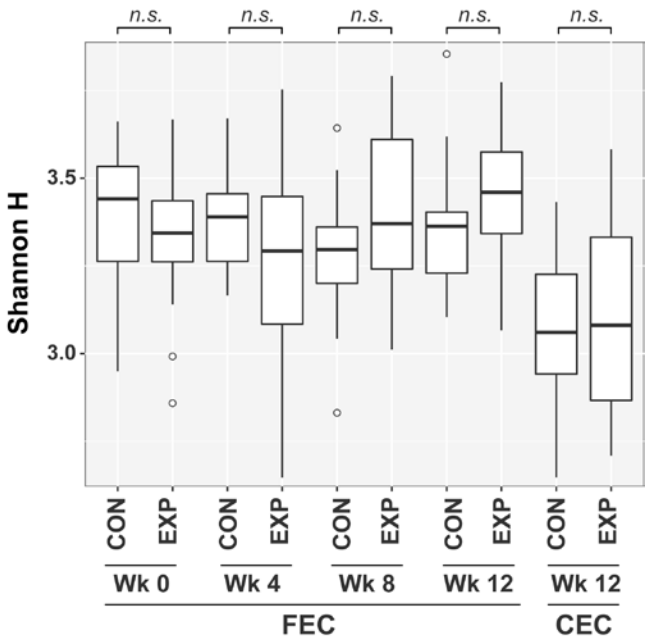

Figure 6. Box plots of Shannon diversity values, stratified by treatment arm (CON: control arm. EXP: experimental arm), timepoint, and sample type (FEC: fecal microbiota. CEC: cecal microbiota). None of the between-group Welch t tests were significant (*n.s.*) at a P value cutoff of 0.05.

Discussion

To achieve greater efficiency while maintaining performance standards, our institution had been using a standard sanitation interval of every 4 wk for rat cage components including the cage lid, box feeders, and enrichment tunnels while maintaining a 2 wk change interval for soiled bedding and cage bottoms. The goal of the current s study was to determine if extending the sanitation interval from 4 to 12 wk for some cage components would result in significant differences in intracage ammonia, bacterial load on cage surfaces, basic animal physiology, or the gastrointestinal microbiota of Crl:SD rats. In short, we found no significant differences in any of the parameters measured between our standard 4 wk practice and the extended 12 wk sanitation interval.

Intracage ammonia is a basic, microenvironmental factor commonly used to assess the need for cage sanitation. In our study, the intracage ammonia was consistent across both groups, gradually increasing with time but staying below the standard threshold of 25 to 50 ppm.[29] For the experimental group, median ammonia concentrations remained below 6 ppm (median 1.5 | QR 1.5, 2.25, $n = 7$) after 12 wk of the study. One subset of rats in the control group cages toggled their water valves, frequently resulting in a higher incidence of cage floods over the course of the study. Cage flooding appeared to be associated with higher levels of intracage ammonia and bacterial counts on all surfaces of these flooded cages. Therefore, any cages that flooded (reported as n in Figure 1) received a full cage change and were removed for the analysis of ammonia, surface bacterial growth,

and enrichment assessment at that specific time point until the next time at which complete cage changes occurred. Only one experimental cage had a known flood; this occurred during wk 2. This cage was completely excluded from all statistical analysis. With flooded cages excluded, ammonia levels did not differ significantly from other studies of intracage ammonia after an extended sanitation interval of 12 wk.[1,29,30,43]

In our study we assessed 3 different components of a full cage unit: the cage lid, box feeder, and enrichment tunnel. In general, the cage lid tended to have the lowest CFU of the cage components tested, and the tunnel the highest, perhaps because of the greater direct contact of the tunnel with the rats and their excrement. We have historically used this rationale to justify different sanitation frequencies for cage components and cage bottoms.[1,39,41] A previous study evaluated dirty bedding mouse sentinels and found that lids were satisfactory for only up to at 2 wk based on CFU.[13] In contrast, we found that extending sanitation intervals from 4 to 12 wk did not produce a significant difference in surface CFU on the lids. The difference between our results and those of the earlier study is potentially due to their use of dirty bedding for sentinels as opposed to our use of clean bedding every 2 wks.[13] In addition, in our study, we observed a 3-fold increase in CFU in both groups at wk 8; this increase was not correlated with increased ammonia. Assay controls allowed us to rule out sample contamination at this time point. Our finding is similar to that of another study that found peaks in CFU on cage surfaces that occurred without a good explanation.[1,8] The unexplained peak at wk 8 did not seem to affect any other parameters and ultimately returned to levels comparable to earlier time points.

We used other novel methods to quantify debris on the enrichment tunnel. We used a light meter to test whether a build-up of debris would block light transmission through translucent plastic commonly used to make rodent enrichment tunnels. To ensure consistency in readings when using a light meter, the user must carefully duplicate the light source and distance from the light for each reading. Our light meter results are supported by the minimal accumulation of debris reported using the visual assessment scale. Previous studies in rats have also demonstrated minimal debris accumulation on enrichment shelters used for up to 12 wk.[8] While these results support an extended sanitation interval for rat enrichment devices, routine visual assessment allows prompt replacement of these devices after excessive accumulation of bedding, urine, or fecal material.

One of the goals of this study was to determine if prolonged sanitation intervals affect the rats' physiology. While limited in sensitivity, basic parameters such as body weight and blood analysis have been used previously for this type of assessment[8] and can be considered part of a multiparameter analysis. Body weight is often used to indicate health status because it can be affected by stress or disease, but can also vary due to diet, sex, age, or strain.[32] Rat body weights in both groups of our study were consistent with the vendor's reported growth curve for this age and strain.[5] We also performed an analysis of serum analytes and electrolytes and a CBC to evaluate the overall health and function of individual organ systems. A previous study also assessed blood parameters to specifically evaluate the interval of enrichment sanitation.[8] Similar to their findings, our blood chemistry data showed no significant differences between groups. The CBC showed few cell populations that were out of the normal range, with no values significantly different between the control and experimental groups. We also used the neutrophil and lymphocyte (NE:LY) ratio as an indicator of chronic stress.[19,42] The previous studies showed that chronic exposure to stressors, whether environmental or pharmaceutical, can lead to an increase in this ratio due to neutrophilia with or without concurrent lymphopenia.[19] The NE:LY ratio is reportedly not influenced by acute stressors, such as handling or anesthesia used to obtain blood samples.[19] This ratio is also resistant to potentially confounding variables such as time of day, appetence, and sex.[6,14] As with the serum chemistry and CBC results, we found no statistically significant difference in the NE:LY ratio between groups in our study.

Comparisons of gut microbiota have also been used to assess basic animal husbandry practices. Several studies have found that the type of caging, cage bedding, diet, type of water, and disinfectant used can influence its composition.[2,12,13,40] Similarly, multiple studies show that stress-induced changes in food consumption and gastrointestinal (GI) transit of rats can affect the microbiome.[3,6,7,19] A previous study evaluated the microbiomes of different segments of the mouse GI tract and determined that found a difference in microbiota responsiveness with regard to environmental changes.[12] In addition, the ceca showed a greater shift in bacterial community prevalence than did feces, indicating that the cecum was more sensitive to changes in husbandry practices.[12] In our current study, we collected fresh feces every 4 wk and cecal contents at the 12 wk endpoint to compare groups. Neither the feces nor the cecal contents showed significant changes in their microbiota, indicating that the extended cage sanitation interval does not alter the FM or CM of these rats.

Modification of husbandry practices such as sanitation intervals require consideration of the many different factors that limit its widespread application. Such factors include type of caging, bedding, and accessories, and the species, number of animals, genetic background, health status, size, age, sex, and diet.[1,2,11,12,23,27,45] We conducted our study using IVCs, which are the most common caging type used for rats in our facility. Our results are consistent with a previous report that the type of IVC system had little effect on bacterial loads on the inner lid surface.[13] However, a different study using static cages found an extensive microbial load on intracage surfaces after only 1 wk.[21] The housing density in our study was 2 adult rats, which allowed a comparison with results from a previous study.[13] However, bacterial load could differ at higher or lower housing densities. Furthermore, renal, hepatic, or other metabolic dysfunctions, like diabetes, may affect an animal's waste production and necessitate more frequent cage changes.[24,43] These types of metabolic diseases can affect the parameters we measured in this study such that extension of the sanitation interval should only be considered for relatively healthy rats.[11,45] The type of food may also change the amount of excretions and should be considered if using special diets such as high-fat or medication-infused.[4,12,31,44] Inanimate objects in the microenvironment such as the type of bedding or the enrichment materials should also be considered. The type of material in these cage components could affect their absorbent properties, which could lead to differences in ammonia or number of bacteria held by or wicked into the structure.[9,12,25,41] Before implementation of new housing practices, the microenvironment and the animals should always be assessed to avoid changes that might have adverse effects.

The goal of this study was determine whether an extended sanitation interval would affect the intracage microenvironment and rat welfare. We concluded that sanitizing the cage components at an interval of 12 wk, as compared with our current institutional standard of 4 wk, could be implemented without adverse effects on rat health. Multifaceted analysis

of the intracage ammonia, enrichment device, cage microbial load, and rat physiology and microbiome revealed comparable values for experimental and control cages. Our data showed that a 12-wk sanitation interval for specific rat cage components meets our performance standard while improving efficiency in use of time, and natural resources.

Acknowledgments

We thank the staff of the Office of Laboratory Animal Resources at the University of Colorado Anschutz Medical Campus for all their help. Specifically, we thank Isaac Carnahan, Genna Nault, Leanna Chaffee, and Caterina Quartararo for their technical support. Statistical support was received from the University of Colorado Cancer Center Support Grant (P30CA046934). DNF is supported, in part, by funding from the CU Denver GI and Liver Innate Immunity Program. The Office of Laboratory Animal Resources provided funding for this project as part of the University of Colorado Anschutz Medical Campus Internship in Laboratory Animal Medicine.

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