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Variation in Bacterial Contamination of Microisolation Cage Tops According to Rodent Species and Housing System

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The *Guide* recommends sanitizing cage components, including microisolation cage tops (MCT), at a minimum of every 2 wk. Previously published data demonstrated that mouse MCT microbial loads do not increase until at least 2 wk and that sanitation can be delayed past 2 wk. How microbial loads differ on mouse compared with rat MCT, as well as across different ventilation systems, remains unclear. We hypothesized that MCT microbial loads would be higher in tops from rats compared with mice and would differ according to IVC ventilation system. We evaluated bacterial loads on MCT at serial time points to 90 d from static cages housing mice or rats and from rat and mouse cages on several ventilation systems (mice, 6; rats, 4). MCT were determined to have sufficiently elevated bacterial loads to necessitate changing based on either statistically significant changes in bacterial loads or values greater than 50 cfu. Across all ventilation systems, bacterial counts at 14 d were significantly higher on rat MCT compared with mouse MCT. Across the ventilation systems examined, rat MCT cfu remained similarly elevated from 14 d through 90 d. Mouse MCT total cfu were also stable across multiple ventilation systems yet remained lower than 50 cfu until at least 90 d. Patterns of bacterial species isolated from rat MCT were relatively consistent over time and ventilation system, whereas mice showed greater variability in both contexts. We found that 14 d is an appropriate sanitization time point for rat MCT, whereas the interval at which mouse MCT are cleaned can be extended to 90 d at least. Our data highlight interspecies differences in the accumulation of bacteria on MCT and that mouse MCT sanitation intervals for several housing systems can be extended beyond 14 d.

Abbreviations: MCT, microisolation cage top; RLU, relative light units

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According to the *Guide for the Care and Use of Laboratory Animals*, cages, racks, and accessories (for example, cage tops) should be sanitized at least once every 2 wk.[7] However, there are few data to support this recommendation pertaining to accessory cage components. The appropriate sanitation frequency for rodent cages and their components is a common topic of research and debate in our field. Changing cage tops more frequently than may be necessary might affect management practices, including increasing labor costs associated with housing research rodents and decreasing equipment lifespan. The development of institutional performance standards showing that the cage microenvironment is not compromised and does not pose an adverse risk to the animals may serve as justification to decrease sanitation frequency.[7,8]

Performance standards have been developed previously for deviations from earlier versions of the *Guide* in regard to cage changing frequency. Bedding changes for mice housed in ventilated caging can be done in 2-wk[9,11] or as long as 3-wk[10] intervals without adversely affecting the health of their mice. For mice and rats in open, wire-top caging, complete cage sanitation every 4 wk has been shown to be sufficient.[6] Optimal times for microisolation cage top (MCT) sanitation remain an area of interest, with previous studies showing that change frequencies for mouse and rat cage lids can be extended to anywhere from 6 wk to 6 mo.[2,13] Another study found that the majority of filter tops from mouse cages had bacterial loads of less than 50 cfu at 4 wk.[14] Many factors likely affect the accumulation of bacteria on MCT for rats and mice. One factor that has not been evaluated in the current literature is the combination of rack ventilation systems. In addition, the only published data regarding rat MCT suggest a potential difference between rodent species regarding the number of organisms present.[13]

In the current study, we hypothesized that bacterial contamination on MCT depends on the rodent species housed and type of rack ventilation system. We evaluated bacterial load and species prevalence on MCT of mouse and rat sentinels. Animals were housed in IVC on several different ventilation systems for a period of 90 d to determine optimal times for changing cages. We found that times to increased accumulation of bacterial loads on MCT varied between rats and mice. Furthermore, we found that total MCT bacterial loads remained fairly consistent across different types of ventilation systems within each rodent species. Taken together, these data help provide further information pertaining to factors that influence MCT bacterial load accumulation.

Materials and Methods

Animals. Female Sprague–Dawley rats (Hsd:Sprague–Dawley; age, 4 wk) and female CD1 mice (Crl:CD1(ICR); age, 5 wk) in use as sentinel animals were housed in an AAALAC-accredited animal facility at the University of Michigan. All components

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of the experiment were approved by the University of Michigan IACUC. Temperature- and humidity-controlled rooms were maintained on a 12:12-h light:dark cycle with 10 to 15 room air changes hourly. According to the health surveillance program, mice and rats in this facility were consistently negative for the following excluded agents: lymphocytic choriomeningitis virus, mouse adenovirus, *Mycoplasma pulmonis*, pinworms, Theiler murine encephalomyelitis virus, pneumonia virus of mice, reovirus, and Sendai virus. In addition, mice were consistently negative for mouse hepatitis virus, minute virus of mice, mouse parvovirus, epizootic diarrhea of infant mice virus, ectromelia virus, polyomavirus, and fur mites. In addition, rats were consistently negative for Kilham rat virus, rat parvovirus, sialodacryoadenitis virus, and rat minute virus. None of these pathogenic organisms were detected on during routine screening on any of the mouse or rat sentinels throughout the duration of these studies.

Rats and mice were pair-housed in each cage. All animals had unrestricted access to water and food (5LOD irradiated rodent chow, LabDiet, St Louis, MO). Food was provided in hanging feeders for mouse IVC or in wire bar feeders for static and rat IVC. Water was provided by using automated watering systems (Avidity Science, Waterford, WI) for IVC and water bottles for static cages. Water was either filtered or reverse-osmosis–treated prior to being made available to animals. All rodents were housed in IVC (Allentown Caging, Allentown, NJ) filled with either 1/4-in. or a mix of 1/4-in. and 1/8-in.corncob bedding (Bed-o'-Cobs, Anderson, Maumee, OH), whereas static cages were filled with 1/8-in.corncob bedding and included wire lids with integrated steel food hoppers and filter tops. Mice were housed in IVC (n = 10 cages per ventilation system) or static cages (n = 3 cages per ventilation system). For IVC, ventilation rate was consistent across ventilation systems at 50 to 70 cage-volume air changes hourly. We examined several types of ventilation systems, including self-contained blower units and room ventilation-based systems. Mice were housed on racks with 6 different ventilation systems, including 3 different self-contained blower units (blower 1 through 3) and 3 different room-level ventilation systems (room vent 1 through 3). Rats were housed on racks with 4 different ventilation systems including one self-contained blower unit (blower 1) and 3 different room-level ventilation systems (room vent 1 through 3).

At day 0, mice and rats were placed in cages containing soiled bedding with MCT that had been previously sanitized by exposure to acid treatment and 180 °F water treatment for 5 min through cage wash. Rat and static MCT were washed individually, whereas mouse IVC MCT were stacked prior to being sanitized. IVC were handled at least once every 2 wk during the normal cage changing for the rack, whereas static cages were changed at least once weekly. Roughly 1 tablespoon of soiled bedding from all cages on the rack was placed into a single empty cage until all cages had been sampled and the cage was full. A single sentinel cage received bedding from a variable number of cages, with a maximum of 70 cages contributing to a single sentinel cage. Sentinel animals as well as the MCT were then transferred to the cage containing soiled bedding. Rats in all housing conditions and mice in static cages had wire bars that prevented animals from accessing the MCT; mice housed in IVC were fed by using hanging feeders and therefore had the ability to contact the MCT. All cages used in this study were handled in a vertical laminar flow hood that was sprayed with disinfectant (Spor–klenz, Steris Life Sciences, Mentor, OH) prior to opening the cages. In addition, caretakers' gloved hands were dipped into the disinfectant after touching any external surface of the cage, before handling the internal surfaces, such as wire feeders.

Microbiologic sampling. At each time point, MCT were sampled for the presence of bacteria by using 2 methods, ATP assessment and RODAC contact plates (diameter, 65 mm; Tryptic Soy Agar with Lecithin and Polysorbate 80, Pharmacal Research Laboratories, Waterbury, CT). Samples for ATP measurements (relative light units, RLU) were collected by using swabs and analyzed (novaLUM 2, Charm Sciences, Lawrence, MA). Each MCT was first sampled by using a single contact plate by pressing the plate against a consistent solid surface on the water bottle holder of the MCT (Figure 1). A single swab was then used to sample the entire MCT prior to ATP analysis. Mouse and rat MCT were sampled in the same location and manner for both contact plates and ATP analysis. All plates were then submitted to the Michigan State University Diagnostic Center for Population and Animal Health laboratory for colony counting and determination of Gram status. Plates were incubated at 37 °C with 5% CO_2 for as long as 7 d, with counts and organism identification occurring at day 1, 2, and 7 of incubation, with final colony counts and species presence calculated at day 7 on a per-plate basis per the lab recommendations. Unique colonies were enumerated and organisms speciated by using an extended direct transfer isolate preparation with MALDI-TOF mass spectroscopy (Microflex, Bruker, NJ). Michigan State University Diagnostic Center for Population and Animal Health reported at least genus-level information and in many cases genus and species for colonies identified. MCT were repeatedly sampled for each cage at 0, 14, 30, 60, and 90 d after initiation of the study. Total bacterial load was evaluated against standards set by the American Public Health Association[17] and previously used in the laboratory animal setting:[5,13] good, less than 25 cfu; fair, lower than 50 cfu; poor, 50 cfu and greater; and too many to count, 250 cfu or more.

Statistical methods. Visual and statistical analysis was performed by using R version 3.4.3 (Comprehensive R Archive Network [CRAN], https://cran.r-project.org). A P value less than 0.05 was used to define statistical significance. For comparison of ATP measurements with colony counts, we used the rmcorr package to perform repeated-measures correlation on the mouse and rat data sets separately.[1] The Mann–Whitney U test was used to compare day 14 levels of cfu between mice and rats.

We used mixed-effect linear regression in the lme4 package[3] to compare the different effects of the ventilation systems on colony counts over time in mouse MCT lids.[4] The distribution of colony counts was right-shifted, and data subsequently were log2-transformed to create a normal continuous distribution in the data set. We excluded static cages from the analysis due to the low number of cages in the study. Model fixed effects included the time and the interaction of time with the different ventilation systems examined to assess how they altered colony counts. A random effect of cage ID was included to control for time and repeated sampling of the cages.

Results

We observed very few gram-negative bacteria on MCT for mice or rats during the 90-d timeframe of the study. As a result, we evaluated total colony counts to determine the overall bacterial load on MCT, which primarily consisted of gram-positive bacteria. We compared colony counts with RLU measurements to determine whether we could use ATP assessment as a replacement for colony counts. We found a poor correlation between measured ATP levels on the lids and total colony counts for both mouse and rat MCT over time (Figure 2, mice: r = 0.188, P = 0.0014; rats: r = 0.475, $P = 1.60 \times 10^{-6}$) Given this, we used bacterial counts (cfu) as a measure of MCT bacterial loads.

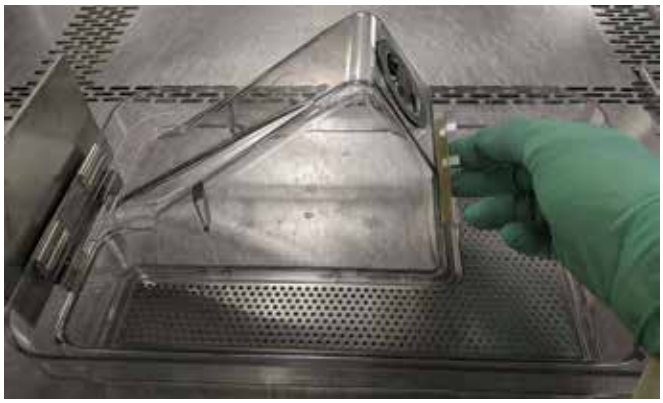

Figure 1. Representation of contact plate sampling of MCT.

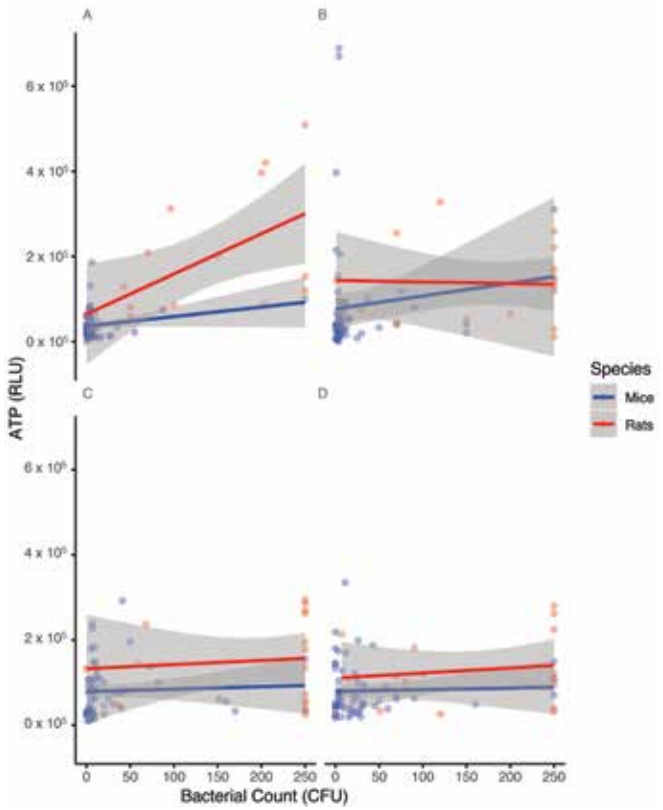

Figure 2. Repeated-measures correlation of ATP level and bacterial colony counts (cfu) measured on MCT from rats (r = 0.475, P = 1.60 × 10^{-6}) and mice (r = 0.188, P = 0.0014) at (A) 14, (B) 30, (C) 60, and (D) 90 d. Lines represent linear regression of data for each species. Shaded areas represent the standard error of the linear regression line. Repeated-measures correlation was used for comparing ATP and cfu levels while controlling for time.

We used a paired approach to determine changes in bacterial load by using both statistical analysis and industry standards. We first compared colony counts on mice and rat tops at the 14-d cage sanitization time point proscribed by the *Guide*.[7] Rats had significantly ($P = 2.207 \times 10^{-6}$) elevated MCT colony counts, compared with mice (Figure 3). In addition, most rat MCT sampled were already at or above the 50 cfu cutoff, whereas all but 2 mouse MCT were below 50 cfu. Given these differences, we examined how the various ventilation systems affected MCT cfu levels in rats to see whether bacterial elevations were consistent over time. Rat MCT had counts at or above the 50 cfu cutoff from 14 d throughout the study (Figure 4). We found a statistically

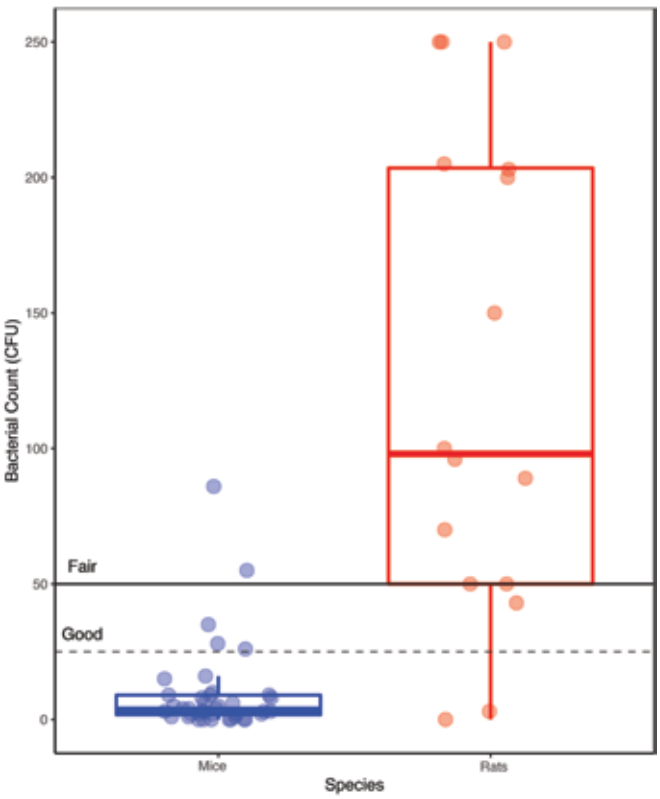

Figure 3. MCT cfu count (median ± interquartile range) for IVC housing mice compared with rats ($P = 2.207 \times 10^{-6}$). The Mann–Whitney U test was used to compare median cfu counts between rats and mice.

significant effect of time ($P = 2.90 \times 10^{-7}$) on rat MCT cfu levels but no effect of the various ventilation systems (Table 1).

We next examined bacterial loads on MCT from mouse cages. In contrast to rat MCT, the median colony count on mouse MCT stayed below 25 cfu for the duration of the experiment (Figure 5). We found that although levels were below the 25- and 50-cfu cutoffs, there was a statistically significant ($P = 5.17 \times 10^{-11}$) effect of time on total MCT cfu levels. Ventilation system did not significantly affect colony counts during the 90-d evaluation (Table 1). We next examined MCT cfu values across various ventilation systems for both mice and rats to assess what percentage of cages fell within each of the cfu cutoffs. Mouse MCT had cfu levels at or below 50 cfu until day 90, and the percentage of cages above that threshold fell over time (Figure 6). The majority of rat MCT cfu were above the 50 cfu cutoff starting at day 14, and this trend remained the same up until day 90. Most of the rat cages had bacterial loads of greater than 250 cfu by day 30, whereas only a few mouse cages that ever reached this threshold.

We next examined the genera and species of bacteria present on MCT over time. A total of 20 unique species of bacteria were identified on MCT from mice, whereas only 11 unique species were identified for rats (Table 2). We found a limited set of bacteria, which was dominated by *Aerococcus* and *Staphylococcus*, on the MCT from rats (Figure 7). Rat MCT bacterial contaminants remained stable over time. Mouse MCT had various patterns of bacterial contamination at the genus level (Figure 8). For some ventilation systems, the bacterial loads were relatively stable over the 90 d, whereas others showed increased variability.

Discussion

Identifying optimal standards for sanitation intervals of different rodent IVC cage components is an ongoing area of

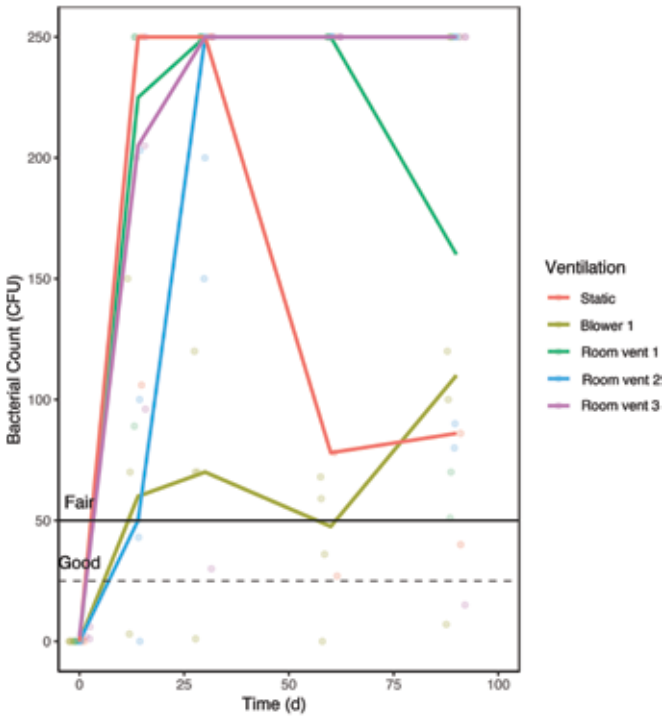

Figure 4. Rats MCT bacterial count cfu counts across ventilation systems and static cages throughout 90 d experiment. Color indicates the ventilation system; lines represent the median value for each ventilation system; data points represent individual cage values, within the respective ventilation cohorts. Time had a significant effect on rat MCT cfu ($P = 2.90 \times 10^{-7}$), whereas ventilation did not (Table 1).

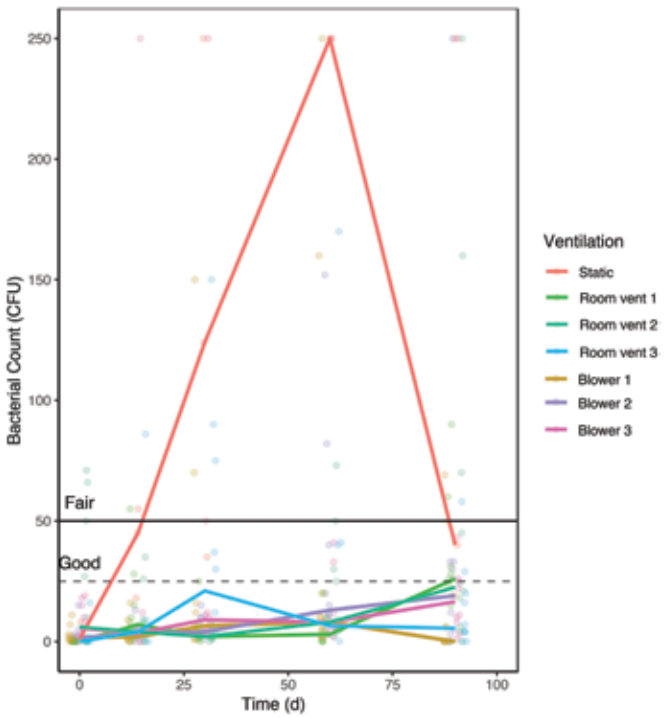

Figure 5. Mice MCT bacterial count cfu counts across ventilation systems and static cages over time. Color represents each ventilation system. Lines represent median values for each ventilation system. Data points represent each cage value within the respective ventilation cohorts. Time had a significant effect on mouse MCT cfu ($P = 5.17 \times 10^{-11}$), whereas ventilation did not (Table 1).

Table 1. Regression statistics for effect of time and ventilation systems on total rat and mouse MCT bacterial loads

	Coefficient	*P*
Rat ventilation model regression table		
Time	NA	2.90×10^{-7}
Blower 1	0.0502	NA
Room vent 1	0.1843	0.133
Room vent 2	0.0059	0.589
Room vent 3	−0.0181	0.121
Mouse ventilation model regression table		
Time	NA	5.17×10^{-11}
Blower 1	0.0208	NA
Blower 2	−0.0064	0.263
Blower 3	0.0029	0.621
Room vent 1	0.0049	0.398
Room vent 2	0.0096	0.094
Room vent 3	−0.0053	0.358

research in husbandry management. We investigated how bacterial accumulation on the IVC MCT top was affected across various rack or ventilation systems by the rodent species housed. We used a paired approach that combined bacterial contamination performance standards[5,13,17] and statistical analysis to determine appropriate MCT change intervals. We show that mice and rats had very different patterns of bacterial contamination of MCT. Furthermore, total levels of bacterial contamination were relatively consistent across multiple ventilation systems within each species.

MCT from mice and rat cages had different total levels of bacterial accumulation. Mice and rats have previously been reported to have differences in MCT and wire bar gram-negative cfu accumulation at 180 d; however the authors did not statistically compare mice and rats.[13] In addition, we found that between days 14 and 90, MCT bacterial loads remained consistent for each of the bacterial species. Although mouse MCT bacterial loads did not change significantly, the genus-level diversity of organisms identified varied across racks and over time. Increased sanitization intervals in mice is consistent with other published studies evaluating colony counts or ATP levels on mouse caging accessories.[2,13,14] Furthermore, these previous studies each used different combinations of racks and ventilation equipment. We likewise found that bacterial contamination of MCT was consistent in each of these species across multiple cage–ventilation systems combinations. Our findings underscore the importance of understanding how differences between mice and rats may affect their husbandry and care.

In our current study, we identified very few gram-negative organisms; most organisms identified were gram-positive. It remains unclear what contribution either gram-positive or gram-negative organisms has on adversely affecting the welfare of animals, given that both types of organisms can exist as a portion of the normal flora.[12,15] The majority of our samples had colony counts that were lower than what has previously been published as the limit of detection for bacterial samples by using ATP (as RLU),[16] thus perhaps explaining the poor correlation between ATP levels and colony counts. In addition, American Public Health Association standards relative to total bacterial loads were not originally designed for use in assessment of bacterial loads in laboratory animal management settings and thus may be difficult to interpret in the context of MCT bacterial levels. Anecdotally, all the animals in our study remained healthy throughout its duration, and no incidences of clinically relevant disease were reported. We also found that, in both rats

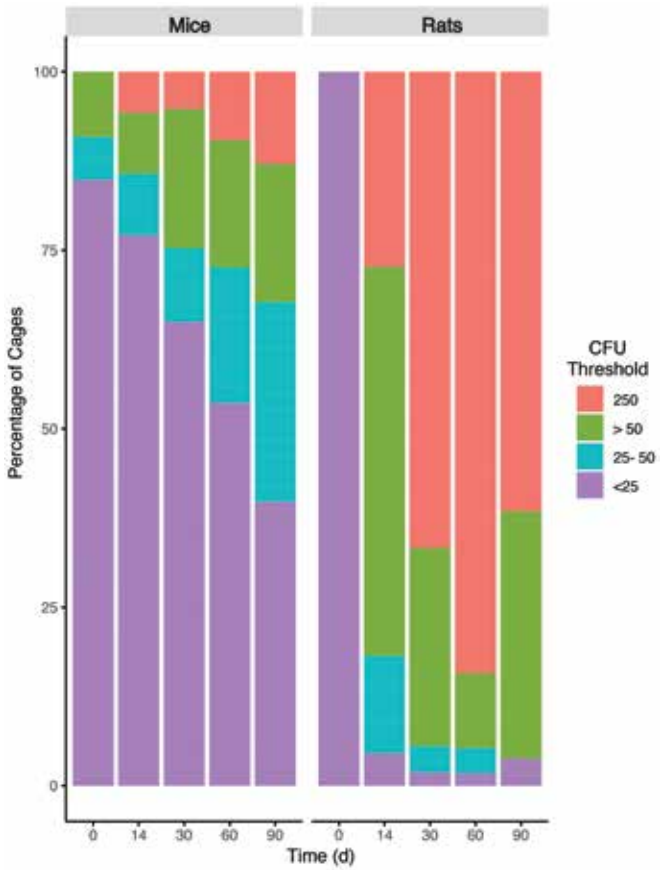

Figure 6. Percentages of cages that fell within the various colony count thresholds (>250 cfu, too numerous to count; >50 cfu, poor; 25 to 50 cfu, fair; <25 cfu, good) for rats and mice over time.

and mice, most of the organisms we identified were commensals that would be unlikely to negatively affect the animals.

We used sentinel animals for this study, given their high exposure to microbiologic load of all animals on a rack and their long-term presence on the rack. However, one limitation to using sentinel cages is they may be handled less frequently than experimental or breeding mouse cages, so further studies analyzing cages that are handled frequently would be practical. We were unable to control the number of animals to which each sentinel was exposed throughout the duration of the study, due to changes in investigator colony size. We cannot account for how this variation might have affected overall MCT cfu levels. In addition, our sentinel cages were housed with 2 animals per cage, so further studies of mice housed 5 to a cage would be warranted. We tracked MCT bacterial contamination only until 90 d, and previous work has shown that contamination remained relatively stable out until 180 d.[13] Further work may be warranted to determine whether mouse MCT contamination remains consistent past 90 d. In addition, we found at that roughly 20% of mouse IVC MCT bacterial counts were greater than 25 cfu prior to being placed on the cage at the initial time point. Mouse IVC MCT are washed in stacks in our facilities, and this practice may have led to initial values that were above the 25-cfu cutoff.

In conclusion, when deciding the frequency of MCT changeout for rodents, it is important to consider differences between species, because rats develop unacceptable levels of bacteria on their MCT more rapidly than mice. In addition, several ventilation systems do not significantly influence the total microbial load in the tested IVC cage types. The most important factor

Table 2. Bacteria identified on rodent microisolation tops

	Identification frequency	
	Mice	Rats
Aerococcus species	5	3
Aerococcus viridans	27	12
Bacillus cereus	7	NI
Bacillus licheniformis	5	NI
Bacillus pumilis	5	NI
Bacillus species	34	3
Enterococcus faecalis	43	1
Enterococcus gallinarum	14	1
Escherichia coli	3	NI
Lactobacillus johnsonii	7	NI
Lactobacillus species	3	NI
Microbacterium species	13	NI
Paenibacillus species	14	5
Proteus mirabilis	2	NI
Sphingomonas species	2	NI
Staphylococcus aureus	1	1
Staphylococcus sciuri	2	3
Staphylococcus species	14	4
Staphylococcus xylosus	32	49
Streptococcus species	4	NI
Staphylococcus intermedius group	NI	1

NI, not identified

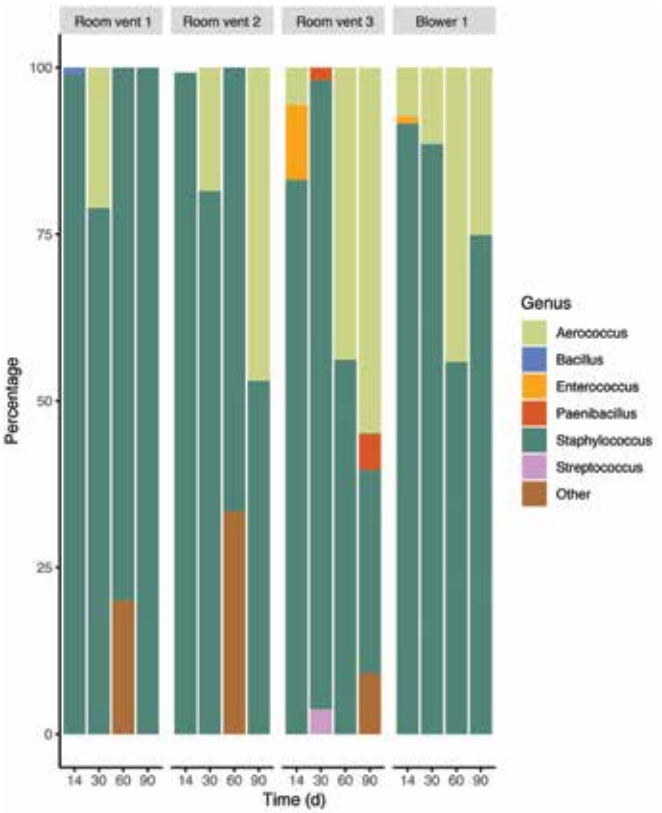

Figure 7. Percentage of bacterial genus representation from the total colony counts for rat MCT across each ventilation system.

to consider in evaluating performance-standard–based sanitation times is the overall effect on the health of the animals. Sanitation intervals for various components of rodent cages can

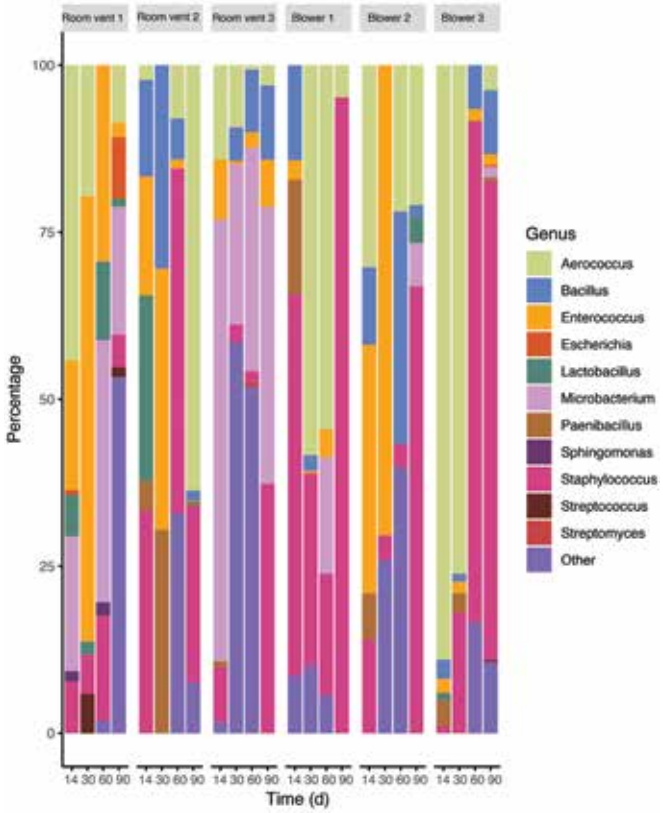

Figure 8. Percentage of bacterial genus representation from the total colony counts for mice MCT across each ventilation system.

affect multiple aspects of husbandry management, including labor costs, efficiency, cage wash resource use, and long-term equipment usage. Taken together, our data support that, across multiple institutions and facilities, mouse MCT bacterial loads are consistent over long periods of time beyond the 14 d recommended in the *Guide*.[7]

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