LUCIANA CINTRA

Triple Aim - Metodologia de gestão para instalações animais: definição de indicadores de bem-estar e saúde em camundongos de laboratório

São Paulo
2022

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Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Doutor em Ciências.

Departamento:

Patologia

Área de concentração:

Patologia Experimental e Comparada

Orientador:

Prof. Dra. Claudia Madalena Cabrera Mori

São Paulo

2022

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

4224
FMVZ

Cintra, Luciana
Triple Aim - Metodologia de gestão para instalações animais: definição de indicadores de bem-estar e saúde em camundongos de laboratório / Luciana Cintra. – 2022.
114 f. : il.

Tese (Doutorado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Patologia, São Paulo, 2022.

Programa de Pós-Graduação: Patologia Experimental e Comparada.

Área de concentração: Patologia Experimental e Comparada.

Orientadora: Profa. Dra. Claudia Madalena Cabrera Mori.

1. Camundongo. 2. Bem-estar. 3. Comportamento de nidificação. 4. Microbioma. I. Título.

Ficha catalográfica elaborada pela bibliotecária Camila Molgara Gamba, CRB 7070-8, da FMVZ/USP.

CERTIFICADO

Certificamos que a proposta intitulada "TRIPLE AIM: METODOLOGIA DE GESTÃO PARA INSTALAÇÕES ANIMAIS", protocolada sob o CEUA nº 1353060218 (ID 005710), sob a responsabilidade de **Cláudia Madalena Cabrera Mori** *e equipe; Luciana Cintra; Sandra Regina Alexandre-Ribeiro; Guilherme Buzon Gregores; Camila Musumecci Guimaraes Azzi; Camila Fernanda Hernandes; Lucas Renan Rodrigues da Silva* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 18/10/2018.

We certify that the proposal "TRIPLE AIM: A NEW MANAGEMENT TOOL FOR ANIMAL FACILITIES", utilizing 40 Isogenics mice (males and females), protocol number CEUA 1353060218 (ID 005710), under the responsibility of **Cláudia Madalena Cabrera Mori** *and team; Luciana Cintra; Sandra Regina Alexandre-Ribeiro; Guilherme Buzon Gregores; Camila Musumecci Guimaraes Azzi; Camila Fernanda Hernandes; Lucas Renan Rodrigues da Silva* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 10/18/2018.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 02/2018 a 12/2021 Área: Patologia Experimental E Comparada

Origem:	Biotério de Camundongos Isogênicos do Departamento de Imunologia do ICB/USP						
Espécie:	Camundongos isogênicos	sexo:	Machos e Fêmeas	idade:	20 a 180 dias	N:	20
Linhagem:	Diversas			Peso:	20 a 40 g		
Origem:	Não aplicável biotério						
Espécie:	Camundongos isogênicos	sexo:	Machos e Fêmeas	idade:	20 a 180 dias	N:	20
Linhagem:	diversas			Peso:	20 a 40 g		

Local do experimento: Centro de Experimentação e Treinamento em Cirurgia - Hospital Albert Einstein (CETEC) Biotério de Camundongos do Departamento de Imunologia do Instituto de Ciências Biomédicas da Universidade de São Paulo (ICB/USP).

São Paulo, 28 de junho de 2022

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Instituto de Ciências Biomédicas
ICB USP Excelência em Ensino e Pesquisa

Universidade de São Paulo
Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "TRIPLE AIM: Metodologia de gestão para instalações animais.", protocolada sob o CEUA nº 8442040522, sob a responsabilidade de **Sandra Regina Alexandre Ribeiro** *e equipe; Luciana Cintra* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Instituto de Ciências Biomédicas (Universidade de São Paulo) (CEUA-ICB/USP) na reunião de 22/06/2022.

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Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: 48 meses Depto/Setor: Imunologia

Origem:	Biotério do Departamento de Imunologia				
Espécie:	Camundongos isogênicos	sexo:	Machos	Idade ou peso:	8 a 10 semanas
Linhagem:	C57BL/6			**N amostral:**	25
Origem:	Biotério do Departamento de Imunologia				
Espécie:	Camundongos isogênicos	sexo:	Fêmeas	Idade ou peso:	8 a 10 semanas
Linhagem:	C57BL/6			**N amostral:**	25

São Paulo, 28 de junho de 2022

Profa. Dra. Luciane Valéria Sita
Coordenadora da Comissão de Ética no Uso de Animais
Instituto de Ciências Biomédicas (Universidade de São Paulo)

Prof. Dr. Francemilson Goulart da Silva
Vice-Coordenador da Comissão de Ética no Uso de Animais
Instituto de Ciências Biomédicas (Universidade de São Paulo)

ALBERT EINSTEIN
INSTITUTO ISRAELITA DE ENSINO E PESQUISA

São Paulo, 28 de maio de 2020

À
Luciana Cintra

Certificamos que a proposta intitulada "*Triple AIM: metodologia de gestão para instalações animais*", protocolo CEUA nº *4037/20*, sob a responsabilidade da pesquisadora *Luciana Cintra* - que envolve a produção, manutenção, e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 08 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho nacional de Controle da Experimentação Animal (CONCEA), foi aprovado pela Comissão de Ética no Uso de Animais da Sociedade Beneficente Israelita Brasileira Albert Einstein (CEUA/Einstein) em *28/05/2020*.

Vigência da Autorização: Maio/2020 a Dezembro/2022

Espécie	Linhagem	Sexo	Idade	Peso	Total	Procedência
Camundongo	Qualquer	Ambos	Várias	Vários	40	CETEC

Aproveitamos a oportunidade para informar que:

- O projeto/treinamento de pesquisa deve ser desenvolvido conforme delineado no processo aprovado;

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ENSINO E PESQUISA

- A CEUA/Einstein deve ser informada de todos os fatos relevantes que alterem o curso normal do projeto/treinamento. É papel do pesquisador responsável assegurar medidas imediatas adequadas frente a eventos não previstos;

- Eventuais modificações ou emendas ao processo devem ser apresentadas à CEUA/Einstein de forma clara e sucinta, identificando a parte a ser modificada e suas justificativas;

- No caso de treinamento, esta aprovação tem validade de 12 meses a partir da data de aprovação pela CEUA/Einstein e para cada evento realizado nesse período, o pesquisador responsável deverá apresentar um relatório das atividades realizadas;

- No caso de projeto de pesquisa, esta aprovação tem validade de acordo com o cronograma proposto no protocolo. Os relatórios deverão ser enviados a cada 6 (seis meses), a partir da data de aprovação da CEUA/Einstein.

Atenciosamente,

Thiago P. A. Aloia

Dr. Thiago Pinheiro Arrais Aloia
Coordenador da Comissão de Ética
no Uso de Animais – CEUA/Einstein

FOLHA DE AVALIAÇÃO

Autor: CINTRA, Luciana

Título: **Triple Aim - Metodologia de gestão para instalações animais**: definição de indicadores de bem-estar e saúde em camundongos de laboratório

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutor em Ciências.

Data: _____/_____/_____

Banca Examinadora

Prof.Dr._____

Instituição:_____ Julgamento:_____

Prof.Dr._____

Instituição:_____ Julgamento:_____

Prof.Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

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Prof. Dr._____

Instituição:_____ Julgamento:_____

DEDICATÓRIA

A educação sempre teve papel importante na minha vida. Hoje, realizo um dos meus sonhos. Gratidão por esta conquista. Essa tese de doutorado é a prova de que nenhum sonho é inalcançável e de que só a educação cria pontes indestrutíveis. Dedico essa tese de doutorado a todas as pessoas que me apoiaram durante esse processo, que me incentivaram a persistir mesmo em meio às diversidades deste tempo tão difícil da pandemia do COVID-19. A meus pais pelo incentivo ao estudo em todas as fases da minha vida. Ao meu marido por todo amor, apoio e compreensão em dias turbulentos. A meus filhos, que tiveram que entender os momentos que precisei estar ausente. A meus amigos, incluindo as novas amizades construídas nesta jornada, por nunca me abandonarem e sempre me ajudarem nos desafios. A minha orientadora, por todo o ensinamento, paciência e compreensão nos momentos de dificuldade. Dedico esse título de doutora a todos vocês com muito amor e com a certeza de que estarão presentes em todas minhas próximas conquistas!

AGRADECIMENTOS

Agradeço...

A Deus por sempre me mostrar novos caminhos e pelos sonhos realizados.

Á Professora Claudia Mori, por todo incentivo, apoio, ensinamentos, motivação e carinho nesta jornada.

Á equipe do Centro de Experimentação e Treinamento em Cirurgia do Hospital Albert Einstein, vocês foram essenciais nesta conquista.

Aos amigos do Laboratório de Modelos Animais, do Biotério do Departamento de Patologia da FMVZ/USP, em especial Dennis, Mariana, Jilma e Silvia.

Ao Biotério do Departamento de Imunologia do ICB/USP, em especial Sandra Regina Alexandre-Ribeiro, uma amiga e apoiadora desde o início deste projeto.

As estagiárias Victória e Daniela, por todo o apoio na coleta de dados.

À Comissão de Ética no Uso de Animais da FMVZ/USP, ICB/USP e Einstein.

Aos colaboradores do serviço de Pós-Graduação da FMVZ/USP.

Aos colaboradores da Biblioteca Virginie Buff D'Ápice da FMVZ/USP.

Aos professores que aceitaram fazer parte da banca examinadora e me ajudaram a idealizar este sonho.

Ao Hospital Israelita Albert Einstein, pelo apoio no meu crescimento profissional e pela confiança, em especial ao Dr. Luiz Vicente Rizzo e meus pares.

E todos que não menciono por serem muitos, mas contribuíram de alguma forma com a realização deste trabalho.

“O período de maior ganho em conhecimento e experiência é o período mais difícil da vida de alguém. ”

Dalai Lama

RESUMO

CINTRA, L. **Triple Aim - Metodologia de gestão para instalações animais**: definição de indicadores de bem-estar e saúde em camundongos de laboratório. 2022. 114 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2022.

Biotérios são locais onde animais de laboratório são criados e mantidos para serem utilizados em experimentos científicos. Essas instalações animais precisam ser adequadamente gerenciadas, com foco no bem-estar dos animais e reprodutibilidade dos resultados experimentais. Quando lidamos com modelos animais, são diversos os fatores que podem influenciar o resultado experimental. A saúde física e mental dos animais de laboratório está entre os fatores determinantes para a qualidade das pesquisas. O Triple Aim é uma estratégia centrada em três dimensões: melhorar a experiência do animal no biotério; melhorar a saúde das populações; e reduzir o custo com o cuidado. Para isso, o objetivo deste estudo foi definir indicadores que sejam acessíveis, coerentes e de fácil execução para serem utilizados como sistema de medição em diferentes instalações de animais. Este estudo analisou a efetividade, semelhanças e divergências de dois indicadores em dois biotérios distintos, que podem ser facilmente avaliados em camundongos de laboratório: escore de construção do ninho na gaiola e predominância bacteriana na microbiota intestinal, especificamente a taxa de *Firmicutes*/*Bacteroidetes* (F/B). O indicador de escore de ninho foi avaliado em uma escala de Likert de 1 a 5 em relação a variáveis macro e microambientais, diferentes linhagens, sexos e idades de camundongos. Os resultados mostram predominância do escore 3 nos camundongos, com variações para escores mais altos dependentes da temperatura, idade dos filhotes nascidos na gaiola, idade das fêmeas e tipo de material de enriquecimento fornecido na gaiola; e escores mais baixos dependentes da umidade, posicionamento do ninho na gaiola, background da linhagem e tempo que o animal foi exposto ao material de nidificação. A microbiota intestinal foi analisada em camundongos C57BL/6J a fim de verificar similaridades nos biotérios e comparar com literatura prévia, verificando a importância de conhecer o padrão da microbiota intestinal desses animais e assim poder utilizar por exemplo da taxa F/B como um indicador de saúde dos camundongos. Com isso, concluímos que esses

indicadores podem ser uma ferramenta importante na avaliação do bem-estar físico e mental dos camundongos de laboratório, podendo servir tanto como um marcador de avaliação individual como populacional dos biotérios, tornando mais ágeis ações de melhoria nos cuidados de camundongos de laboratório, e prevenindo riscos de vieses importantes em resultados experimentais.

Palavras-chave: Camundongo. Bem-estar. Comportamento de nidificação. Microbioma.

ABSTRACT

CINTRA, LC. **Triple Aim - Management methodology for animal facilities**: definition of well-being and health indicators in laboratory mice. 2022. 114 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2022.

An animal facility is a place where laboratory animals are bred and kept for use in scientific experiments. These facilities must be properly managed with emphasis on animal welfare and reproducibility of experimental results. When handling animal models, several factors can affect experimental results. The physical and psychological health of the experimental animals is one of the critical factors in the quality of the studies. Triple Aim is a strategy that focuses on three dimensions: improving the experience of animals in the facility, improving the health of animal populations, and reducing the cost of care. Therefore, the objective of this study was to define indicators that are accessible, coherent, and easy to execute, and that can be used as a measurement system in different animal facilities. This study analyzed the efficacy, similarities, and differences of two indicators in two different animal facilities that are easy to assess in laboratory mice: the nest-building score and bacterial dominance in the gut microbiome, specifically the Firmicutes/Bacteroidetes ratio (F/B ratio). The nest-building indicator was scored on a Likert scale from 1 to 5 depending on macro- and microenvironmental variables, different strains, sex and age of the mice. In mice, nest-building score 3 predominated, with higher scores depending on temperature, age of young born in the cage, age of females, and type of enrichment material provided in the cage, and lower scores depending on humidity, placement of the nest in the cage, strain, and time the animal was exposed to the nesting material. The gut microbiome was analyzed in C57BL/6J mice to verify similarities in animal facilities and to compare with previous literature. This confirms the importance of knowing the pattern of the gut microbiome in these animals and thus being able to use, for example, the F/B rate as an indicator of mouse health. We therefore conclude that these indicators can be an important tool for assessing the physical and mental well-being of laboratory mice. They can serve as markers for both individual and population assessment of animal facilities,

allowing measures to improve the care of mice to be taken more quickly and avoiding the risk of significant bias in experimental results.

Keywords: Mice. Welfare. Nesting behavior. Microbiome.

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1 INTRODUÇÃO

Esforços constantes são empregados na gestão das instalações animais, tentando meticulosamente controlar todas as variáveis que podem distorcer os resultados de estudos, tais como: enriquecimento ambiental, disposição das gaiolas nas racks ventiladas, volumes de cama estéril, ciclos constantes de luz e escuridão, fluxo consistente de ar e temperatura controlados, além de fatores intrínsecos aos animais como a microbiota intestinal (SERVICK, 2016). Pesquisadores surpresos com resultados incongruentes se perguntam quais variáveis ocultas podem se esconder nessas gaiolas de camundongos.

Biotérios do mundo todo apresentam desafios quanto a avaliação preventiva de problemas relacionados ao bem-estar e saúde dos animais de laboratórios, e não há consenso na definição de indicadores que possam demonstrar de maneira ágil se a gestão das instalações está no caminho certo ou há necessidade de revisão dos processos e planos de melhoria.

Nesse estudo propomos avaliar dois indicadores a fim de mensurar a qualidade de vida de animais, sendo um para avaliação de bem-estar (escore de ninho) e outro para saúde (microbiota intestinal) dos camundongos de laboratório, e também destacar os desafios e oportunidades com relação as variáveis de macroambiente (temperatura e umidade), microambiente (enriquecimento ambiental, posição do ninho na gaiola e quantidade de indivíduos na gaiola) e intrínsecas aos animais (linhagem, sexo e idade). Para os gestores de instalações animais que produzem e mantém camundongos de laboratório, há uma cobrança crescente para reconhecer bem-estar e relatar as características de saúde dos animais em suas colônias. Os cientistas precisam ser informados dos fatores que podem influenciar o resultado experimental e ter uma visão de futuro em relação aos modelos de camundongos usados em suas pesquisas. Nesse sentido, o nosso estudo propôs criar subsídios para avaliar a população de animais e cuidados individuais no biotério e conseguir solucionar problemas de maneira organizada com menores custos.

Nessa tese iniciamos com uma breve revisão de literatura, descrevendo uma nova ferramenta de gestão de biotérios (*Triple Aim*) e contextualizando o porquê dos

indicadores na avaliação de bem-estar (experiência do animal no ambiente de biotério) e importância da saúde populacional do biotério como um todo. Posteriormente foram redigidos três capítulos em forma de artigo, os dois primeiros sobre a avaliação do escore de ninho em duas instalações animais distintas (Biotérios de Camundongos do Departamento de Imunologia do Instituto de Ciências Biomédicas da Universidade de São Paulo – ICB-USP e Centro de Experimentação e Treinamento em Cirurgia do Hospital Israelita Albert Einstein) e quais fatores influenciam esse indicador de bem-estar, podendo alertar os gestores sobre o bem-estar físico e mental dos animais de laboratório. No terceiro capítulo, foram apresentados os achados mais relevantes na avaliação da microbiota intestinal e a comparação entre as duas instalações animais, abrindo oportunidades futuras nesta nova área de estudo dos animais de laboratório.

Por fim, na última parte foram apresentadas as considerações finais com os principais pontos levantados durante a realização desse projeto iniciado a quatro anos, além das perspectivas futuras de questões que ainda possam ser estudadas mais profundamente, abrindo uma linha de pesquisa de grande motivação para mim durante esta jornada.

2 REVISÃO DE LITERATURA

2.1 INTRODUÇÃO

A comunidade científica brasileira se defronta com inúmeros entraves relacionados com a produção de modelos animais de qualidade, e o que com relativa frequência, leva a recusa de publicação em periódicos internacionais. O uso de modelos animais sem qualidade, ou seja, sem padrão sanitário definido (Specific Pathogen Free – SPF) e sem padronização de processos, compromete a reprodutibilidade e universalidade experimentais, com consequência para o desenvolvimento de uma pesquisa com pouca expressividade internacional (SALES, 2013).

A variabilidade nos estudos acarreta no aumento de animais utilizados na experimentação para atingir os resultados esperados. E, atualmente, enfrentamos uma redução de recursos pelas agências de fomento para financiamento de projetos no país, sendo prioritário a redução de custos nas instalações animais.

Esses problemas relacionados a gestão de instalações, além de gerar altos custos, desrespeitam valores morais e princípios éticos que norteiam a experimentação animal. Todos esses obstáculos estão relacionados a três pontos em especial: cuidado adequado do animal / indivíduo, controles populacionais como programa de prevenção e manejo populacional, redução de custos no manejo dos animais. Para assegurar este foco triplo, se faz necessário de recurso humano qualificado e dedicado no cuidado e uso de animais de laboratório (FOX *et al.*, 2015).

2.2 FERRAMENTA DE GESTÃO: TRIPLE AIM

O *Triple Aim* foi descrito por Berwick e colaboradores (BERWICK; NOLAN; WHITTINGTON, 2008) como uma metodologia aceita para aperfeiçoar o desempenho do sistema de saúde e melhorar simultaneamente a saúde populacional e experiência individual do cuidado, e reduzir custos per capita dos cuidados na saúde humana (STIEFEL; NOLAN, 2012). As condições para isso incluem identificar a população, desenhar os cuidados primários, gerenciar a saúde populacional e financeira. Também podemos destacar a importância do pessoal envolvido no processo, como um quarto objetivo, sendo que estão associados à insatisfação dos clientes, redução de resultados (baixa produtividade) e aumento de custos, colocando em risco a metodologia como um todo (BODENHEIMER; SINSKY, 2014).

Internacionalmente, ferramentas de gestão são utilizadas para garantir e controlar qualidade e custos nos biotérios (BASSUK; WASHINGTON, 2013, 2014). Como exemplo, temos o Lean Management, filosofia de gestão focada na redução de desperdícios, isto é, excluir o que não tem valor para o cliente e aumentar a produtividade. Também conhecido como Sistema Toyota de Produção, esta ferramenta surgiu na fábrica de automóveis Toyota, no Japão, e tinha como objetivo reduzir custos e aumentar a qualidade e a velocidade de entrega dos produtos aos clientes, com foco

em quatro princípios: filosofia, pessoas, processos e solução de problemas (GRABAN; TOUSSAINT, 2018). A metodologia Lean pode ser usada nos biotérios para melhorar processos relacionados a segurança dos colaboradores, qualidade dos experimentos, redução de custos, com foco nos princípios éticos (por exemplo dos 3Rs – redução, refinamento e substituição) e cuidado adequado dos animais de laboratório, buscando alcançar e sustentar resultados significativos para os clientes (pesquisadores, colaboradores e animais). Os biotérios de experimentação possuem mais similaridades com processos em instalações de produção do que com os tradicionais laboratórios (COSGROVE, 2012).

O uso de ferramentas de gestão de qualidade em biotérios pode contribuir para o engajamento da equipe e usuários, com benefícios aos animais. É nisto que se baseia o termo recentemente utilizado na Ciência de Animais de Laboratório, Cultura do Cuidado (*Culture of Care*), termo também utilizado entre os profissionais de saúde. As ferramentas de gestão oferecem uma visão multinível e abrangente de diferentes metodologias, apresentando detalhes sobre a mentalidade e o comportamento dos colaboradores e as diferentes relações dentro da cultura, permitindo assim o início de projetos de melhoria. Essas ferramentas abordam elementos essenciais de uma cultura cooperativa em termos do que pensamos, o que fazemos e como trabalhamos juntos. Essa mentalidade de abordagem positiva e proativa exigem que a equipe seja capacitada dentro uma estrutura acordada e é um facilitador forte e eficiente para assuntos relacionados ao bem-estar animal, os 3Rs (do inglês redução, refinamento e substituição), além de requisitos legislativos e procedimentos operacionais padrões (BERTELSEN; ØVLISEN, 2021). O uso de indicadores-chave de desempenho nas instalações de animais pode ser uma forma útil para atender abordagens práticas para trabalhar com Cultura de Cuidado de forma fácil e não formalizada. Os indicadores escolhidos neste estudo abordam um resultado com foco no animal, com o propósito de trabalhar com Culture of Care – para garantir a otimização dos 3Rs e bem-estar animal, levando a redução do estresse nos animais e gerando resultados científicos mais robustos.

2.3 INDICADORES

O bem-estar animal se correlaciona diretamente com a adequação das muitas variáveis ambientais, sejam elas físicas, nutricionais ou enriquecimento. O objetivo principal deste estudo foi estabelecer indicadores válidos na gestão de biotérios. Para definir indicadores é importante conhecer a designação e descrição da instalação animal avaliada e ter conhecimento das linhagens presentes.

2.3.1 Indicadores de macro e microambiente

Animais de laboratório são seres complexos que respondem a parâmetros ambientais. Mesmo mudanças sutis e algumas vezes imperceptíveis no ambiente podem levar a variabilidade do resultado experimental e conclusões duvidosas. O controle das variáveis ambientais, juntamente com a provisão de manejo adequado, é crucial para o uso adequado e bem-estar dos animais (BLOOMSMITH *et al.*, 2018).

O microambiente refere-se ao recinto primário de um animal, que inclui a área imediata ao redor do animal (gaiola); e as condições físicas que cercam o microambiente são definidas como o macroambiente (sala). Sistemas de gaiolas ventiladas permitem que diferentes microambientes existam dentro do mesmo macroambiente. O microambiente e o macroambiente devem ser adequados a espécie, idade dos animais e à finalidade para a qual estão sendo utilizados *(BLOOMSMITH et al.*, 2018).

Na rotina dos biotérios, são coletados dados para avaliação do ambiente / sala onde os animais são alojados. Esses dados devem seguir as recomendações legais e ser controlados quanto a variações fora dos parâmetros estabelecidos (CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL, 2015), sendo também exigidas nos guias internacionais (NATIONAL RESEARCH COUNCIL, 2011; GETTAYACAMIN; RETNAM, 2017). Os indicadores úteis e normalmente avaliados nos biotérios são: temperatura e umidade, iluminação e frequência de troca das gaiolas.

Os parâmetros ambientais da sala de animais normalmente são monitorados com a suposição de que o ambiente dentro da gaiola espelha de perto o ambiente da

sala. Apesar dos parâmetros ambientais da gaiola, como umidade e temperatura, serem diferentes da sala, ou seja, a temperatura e umidade geralmente são mais altas do que os níveis observados na sala, o conhecimento das condições ambientais da sala pode ser usado para prever certas condições dentro da gaiola (ROSENBAUM *et al.*, 2010).

A temperatura e a umidade devem ser monitoradas e registradas regularmente, utilizando por exemplo um termohigrômetro. Garantir temperatura e umidade consistentes e adequadas para a espécie animal mantida em uma sala de biotério é fundamental para a termorregulação animal, para manter a fisiologia normal e promover tendências comportamentais naturais. Uma vez que grandes flutuações nas temperaturas podem ser prejudiciais à saúde e bem-estar animal, as temperaturas devem ser mantidas em um ponto de ajuste próximo ao meio da faixa e a variação não deve ser superior ou inferior a dois graus. Flutuações da temperatura no macroambiente e altas taxas de ventilação interna podem ser mitigadas no nível da gaiola, fornecendo materiais de nidificação apropriados que podem ajudar na termorregulação. Esses materiais oferecem uma opção para os animais construírem ninhos, se abrigarem ou se enterrarem na cama em resposta às condições ambientais dentro da gaiola (BLOOMSMITH *et al.*, 2018).

A umidade relativa (UR) é outro componente do macroambiente que pode ter efeitos prejudiciais à saúde animal se não for mantida dentro de faixas toleráveis. Há uma faixa mais ampla de controle de umidade (dados recomendados para camundongos: 30%–70%) do que temperatura, mas manter a consistência na UR é importante para todos os animais usados em pesquisas biomédicas. A umidade relativa extremamente alta também pode influenciar as condições do microambiente, como aumento dos níveis de umidade na cama, condensação da parede da gaiola, temperaturas mais altas da gaiola, deterioração de alimentos e geração bacteriana de amônia (BLOOMSMITH *et al.*, 2018).

A iluminação deve estar em níveis adequados, independentemente da localização do animal na sala. As instalações animais seguem a recomendação de 325 lux aproximadamente 1 m acima do piso para alojamento de animais de rotina (CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL, 2015). Como a maior parte da atividade humana em uma instalação de pesquisa normalmente

ocorre durante o dia, o fotoperíodo mais amplamente utilizado em instalações de pesquisa com animais é a iluminação durante o dia e a escuridão à noite. Ciclos de luz que fornecem 12 a 14 horas de luz diariamente são apropriados para a maioria dos animais de laboratório. É recomendado a mudança gradual em um fotoperíodo aumentando ou diminuindo lentamente os níveis de luz, criando um efeito do crepúsculo ao amanhecer em vez de uma mudança repentina na iluminação. Como interrupções não programadas nos fotoperíodos podem ser devastadoras para os resultados da pesquisa, a iluminação deve ser controlada eletronicamente e monitorada regularmente para garantir que os ciclos permaneçam consistentes. Deve-se também tomar cuidado para evitar a interrupção do ciclo escuro causada pela entrada da luz por frestas ao redor e sob os batentes das portas ou janelas desprotegidas. Displays digitais em ventiladores de rack ventilados ou outros equipamentos mantidos na sala dos animais também podem emitir luz suficiente para interromper um ciclo escuro (BLOOMSMITH *et al.*, 2018).

Outros fatores que precisam ser avaliados na rotina de biotérios são número, idade, linhagem e tamanho dos animais presente na gaiola; ventilação forçada das racks ventiladas; e o material e a frequência de troca das camas. A exposição a grandes flutuações ou extremos de temperatura e umidade pode resultar em mudanças comportamentais, fisiológicas e morfológicas, que podem afetar negativamente o bem-estar animal e o desempenho da pesquisa, bem como os resultados dos protocolos de pesquisa (NATIONAL RESEARCH COUNCIL, 2011).

A avaliação baseada em recursos está relacionada com o uso de indicadores que refletem o ambiente dos animais e como os animais lidam com as mudanças ambientais, preservando suas funções biológicas e psicológicas. Os indicadores incluem índices ambientais relativos ao alojamento e criação dos animais, bem como às atividades de rotina (por exemplo, limpeza das gaiolas) (CAMPOS-LUNA *et al.*, 2019).

2.3.2 Indicador de Bem-estar

Os indicadores de bem-estar são baseados em parâmetros fisiológicos, seguidos por indicadores relativos ao comportamento (normal e anormal), interação social e meio

ambiente (CAMPOS-LUNA *et al.*, 2019). Ou seja, o bem-estar animal é avaliado pela observação ou medição das características físicas ou comportamentais do animal em associação com fatores que determinem a qualidade do ambiente onde é mantido. O ambiente do animal, presença de materiais de enriquecimento, exposição a variações de temperatura ou umidade, ou detalhes de rotinas de criação são alguns exemplos de indicadores importantes de serem mensurados na rotina de cuidados dos animais de laboratório.

A agregação de todos os aspectos do bem-estar do camundongo de laboratório (físico, fisiológico, comportamental e ambiental) em um protocolo de bem-estar é fundamental para fornecer uma avaliação geral.

A inclusão de indicadores de recursos em protocolos de avaliação de bem-estar é importante, pois incluem procedimentos, tratamentos e gerenciamento que podem ter um alto impacto no bem-estar, especialmente em animais de laboratório (por exemplo, temperatura ambiente e enriquecimento ambiental nas gaiolas) (CAMPOS-LUNA *et al.*, 2019). O bem-estar é complexo, por isso geralmente é importante avaliar mais de um indicador para revelar até que ponto a qualidade de vida do animal é realmente adequada, em vez de avaliar apenas um aspecto da biologia ou ambiente do animal (SEJIAN *et al.*, 2011).

Duas perguntas-chave podem ser abordadas para avaliar o bem-estar: 1) O animal é fisicamente saudável? e 2) O animal tem o que deseja? A avaliação da saúde física, pode ser realizado utilizando o diagnóstico clínico, falaremos no próximo tópico desta introdução. Mas, o comportamento pode ser usado para responder a essas duas perguntas (DAWKINS, 2003). Os indicadores comportamentais são importantes, pois são fáceis de medir e mostram as adaptações de um animal às condições ambientais presentes.

Também é importante que os indicadores de bem-estar avaliem os sentimentos do animal, funcionamento físico e/ou naturalidade. Os sentimentos podem ser cruciais para alguns conceitos de bem-estar. Mesmo animais saudáveis podem ter um bem-estar ruim se estiverem ansiosos, entediados ou socialmente estressados. Apesar dos sentimentos serem particulares de cada indivíduo, fica a questão se é possível medir os sinais comportamentais e físicos dessas experiências subjacentes (BROOM, 2008).

Há evidências científicas de que o enriquecimento ambiental tem um efeito positivo na emoção, cognição, comportamento, fisiologia, peso corporal, reprodução, secreção hormonal e desenvolvimento da prole. É também uma etapa necessária para obter resultados com alta precisão e alto nível de reprodutibilidade. Porém, a introdução de enriquecimento ambiental não se restringe apenas a adição de objetos nas gaiolas, abrangendo também o espaço e tipo de gaiola, material de cama, manuseio e contato social (MASSARI *et al.*, 2019).

O maior interesse quando oferecemos enriquecimento ambiental é aumentar o bem-estar animal, proporcionando aos animais estimulação sensorial e motora, por meio de estruturas e recursos que facilitem a expressão de comportamentos típicos da espécie e promovam o bem-estar psicológico por meio de exercícios físicos, atividades manipulativas e desafios cognitivos, de acordo com as características específicas da espécie (NATIONAL RESEARCH COUNCIL, 2011).

Apesar de alguns cientistas expressarem preocupações de que o enriquecimento ambiental pode comprometer a padronização experimental ao introduzir variabilidade, existem estudos que mostram evidências indicando que as condições de alojamento podem ser enriquecidas sem comprometer a precisão ou reprodutibilidade dos resultados experimentais (WOLFER *et al.*, 2004; WÜRBEL, 2007; ANDRÉ *et al.*, 2018).

O único ponto de atenção é que os dispositivos de enriquecimento, como abrigos e material de nidificação, também contribuem para a variação de temperatura e umidade dentro das gaiolas dos camundongos (NATIONAL RESEARCH COUNCIL, 2011).

Para obter efetividade na gestão de biotérios, é essencial garantir o bem-estar dos animais mantidos e produzidos para os estudos experimentais. Para isso é necessário definir um indicador que avalie individualmente as gaiolas.

Não há um padrão-ouro para avaliar a validade das medidas relevantes para o bem-estar em roedores de laboratório. Além disso, a análise do comportamento em camundongos pode ser difícil devido a subjetividade de sistemas de pontuações estabelecidos na literatura (KRAEUTER; GUEST; SARNYAI, 2019). Alguns estudos têm princípio comum para desenvolver um esquema de avaliação do bem-estar para animais de laboratório (LEACH; THORNTON; MAIN, 2008; CAMPOS-LUNA *et al.*,

2019). Esses estudos destacaram o registro de recursos específicos que são fornecidos na gaiola do camundongo e como esses animais o utilizam, tais como a construção de ninho pela disponibilidade de material de nidificação.

2.3.2.1 Escore de ninho como indicador de bem-estar

Alguns autores desenvolveram um esquema de avaliação para medir o bem-estar de camundongos de laboratório através do material do ninho presente nas gaiolas e como os camundongos usam esse material na preparação de seus ninhos (LEACH; THORNTON; MAIN, 2008; CAMPOS-LUNA *et al.*, 2019). O comportamento de construção de ninhos em camundongos de laboratório é relatado como um indicador etologicamente relevante de bem-estar, utilizado para identificar estressores térmicos, gaiolas agressivas, doença e dor. A observação do comportamento de construção de ninhos em colônias de camundongos fornece um refinamento para a avaliação de saúde e bem-estar no dia-a-dia (GASKILL *et al.*, 2013b).

Além disso, a avaliação de ninho é um procedimento simples, barato e de fácil execução que, juntamente com testes de comportamento típico da espécie, é um ensaio sensível para identificar fenótipos comportamentais. Um estudo anterior descreve um sistema de pontuação simples e não invasivo de construção de ninhos, facilmente medido nas gaiolas dos camundongos (DEACON, 2006).

De fato, fornecer material de nidificação é uma estratégia fácil para aumentar o bem-estar dos roedores de laboratório (PIETROPAOLO *et al.*, 2004) e o sistema de pontuação de construção de ninhos pode, ser usado para avaliar a integridade do comportamento e o bem-estar geral (KRAEUTER; GUEST; SARNYAI, 2019).

A construção do ninho é um comportamento inato dos camundongos para conseguir conforto, termoregulação e habitação para seus filhotes (DEACON, 2006). No entanto, a construção do ninho depende de fatores ambientais.

Há outros estudos que demonstram o uso do escore do ninho como indicador para o bem-estar dos camundongos (GASKILL *et al.*, 2013b; SPANGENBERG; KEELING, 2016), porém observa-se diferença no desempenho de construção do ninho influenciado pelo meio ambiente (GASKILL *et al.*, 2013a; MAHER *et al.*, 2015; SPANGENBERG; KEELING, 2016; JOHNSON *et al.*, 2017), linhagem, sexo e idade

(GOTO; OKAYAMA; TOYODA, 2015; XIONG *et al.*, 2021) e a localização do ninho na gaiola (MAKOWSKA *et al.*, 2019). Vários fatores são conhecidos por afetar a qualidade do ninho, incluindo nível de luz, temperatura e alterações fisiológicas e etológicas dos camundongos. MAKOWSKA *et al.* (2019) observaram que o abrigo da luz propiciava ninhos com escore mais alto, e também evidenciaram indiretamente que os camundongos preferem separar os dejetos do local de construção do ninho.

A avaliação do comportamento do camundongo de laboratório pelo uso de material de nidificação pode ser uma ferramenta valiosa para a avaliação do bem-estar, mas requer muita prática e conhecimento do avaliador para usar de forma eficaz e assim os resultados possam ser vistos como confiáveis (CAMPOS-LUNA *et al.*, 2019). O pessoal responsável pelo cuidado e criação de animais deve receber treinamento em biologia comportamental das espécies para monitorar os efeitos do enriquecimento, bem como identificar o desenvolvimento de comportamentos adversos ou anormais (NATIONAL RESEARCH COUNCIL, 2011).

Os enriquecimentos de abrigo, como os rolinhos de papelão, contribuem na divisão de recintos, designando diferentes áreas em seu ambiente, como por exemplo espaço para alimentação e excreção (BAUMANS, 2005). O rolo de papelão além de ser material de abrigo e ninho, permite oportunidades para exploração e locomoção. A combinação de materiais é um dos comportamentos observados em animais, para construção de ninhos mais estruturados e complexos (VAN DE WEERD *et al.*, 1997). Poucos estudos avaliaram a combinação de enriquecimentos ambientes, como materiais de abrigo e ninho, investigando sua preferência e utilização em diferentes fases de vida.

O interesse do nosso estudo foi avaliar o escore de ninho como um indicador de qualidade de vida dos camundongos de laboratório, e identificar fatores que podem influenciar este indicador. Com isso, realizamos a coleta de dados de escore de ninho para comparar entre as seguintes variáveis: temperatura e umidade média diária, tempo de acompanhamento (período de observação: mês e estação do ano), idade média e quantidade de camundongos na gaiola, sexo, presença e idade dos filhotes quando avaliado casais monogâmicos, posição das gaiolas na rack, posição do ninho na gaiola, tipo e uso do enriquecimento ambiental (ex. rolinho de papel).

2.3.3 Indicador de saúde

Observar e relatar sinais clínicos em animais de laboratório é necessário por muitas razões: avaliação do bem-estar animal, conformidade com o princípio dos 3Rs de refinamento, conformidade com a regulamentação nacional e recomendações internacionais e, mais importante, para rastreabilidade do resultado científico. Além disso, a comunicação de sinais clínicos tem alto valor científico e ético. O exame dos animais deve ser efetuado por pessoal competente, de forma sistemática, em intervalos adequados e as observações clínicas devem ser registradas de forma eficaz para permitir que essas informações sejam usadas (FENTENER VAN VLISSINGEN *et al.*, 2015).

A necropsia para avaliação da causa morte e registro de ocorrências, contribuem na tomada de decisão da equipe nos biotérios, adicionado ao monitoramento da saúde dos roedores baseado nas recomendações da FELASA (Federation of European Laboratory Animal Science Associations) (MÄHLER *et al.*, 2014), são indicadores importantes para atender as recomendações internacionais de qualidade sanitária dos animais mantidos no biotério.

O acompanhamento de índices reprodutivos das linhagens também são bons indicadores para ter certeza se as matrizes estão saudáveis. Temos os seguintes indicadores para avaliação reprodutiva: taxa de fertilidade, taxa de natalidade, prolificidade, intervalo entre partos, precocidade, taxa de desmame, mortalidade pré e pós desmame, vida útil reprodutiva (NEVES; MANCINI FILHO; MENEZES, 2013).

Os exames hematológicos e bioquímicos podem apoiar na avaliação de saúde dos animais de laboratório, principalmente para definir um padrão dentro de cada instalação animal. Isto porque os valores de referência divergem entre os biotérios, sendo essencial estabelecer uma base comparativa entre as diferentes instalações e consequentemente para avaliar alterações funcionais nos animais de laboratório mantidos em cada instituição (SANTOS *et al.*, 2016).

A análise de microbiota fecal dos camundongos tem sido uma novidade no padrão de qualidade dos biotérios, uma vez que há relatos de que o microbiota pode influenciar no resultado experimental (LEYSTRA; CLAPPER, 2019; MOORE *et al.*,

2019). Poucos são os biotérios que conhecem a microbiota fecal dos animais mantidos e sabe-se que esta análise pode ter variação entre linhagens e locais de alojamento (NGUYEN *et al.*, 2015; XIAO *et al.*, 2015b; HUGENHOLTZ; VOS, 2018; BOWERMAN *et al.*, 2021).

Há uma percepção crescente de que a microbiota intestinal de modelos de camundongos de laboratório deve ser considerada no contexto da pesquisa biomédica como um todo. Cada vez mais é importante o pesquisador ter informações sobre a microbiota dos camundongos em sua pesquisa e como isso pode influenciar o fenótipo de seu modelo. Com isso em mente, as influências do microbiota estão implicadas na reprodutibilidade dos modelos animais, traduzibilidade nos estudos em seres humanos e descoberta da influência na saúde e doença (ERICSSON; FRANKLIN, 2021).

Nesse contexto, escolhemos o microbiota como um indicador de saúde a ser investigado em nosso estudo, a fim de definir padrões que configure preocupações ou alertas na avaliação dos camundongos a serem utilizados na pesquisa científica.

2.3.3.1 A microbiota intestinal como um indicador de saúde ou doença em camundongos de laboratório

O termo microbioma intestinal refere-se à comunidade de todos os microrganismos, incluindo bactérias, vírus, protozoários, fungos e seu material genético coletivo, que coloniza e existe no intestino de todos os animais. Já, a microbiota é a combinação única de microrganismos que existem em um ambiente específico, como o intestino. Dentro do hospedeiro, a microbiota desempenha um papel crítico no fornecimento de nutrição através do metabolismo de componentes dietéticos e a absorção de minerais, a manutenção da função da barreira intestinal, proteção contra infecção por patógenos e contribuindo para o desenvolvimento do sistema imunológico, metabolismo de drogas e secreção hormonal, todos os quais influenciam a saúde do hospedeiro (CRESCI; IZZO, 2019).

Uma explosão de estudos recentes tanto em animais como seres humanos sugere que os residentes microbianos podem influenciar a suscetibilidade a doenças, predispõem a obesidade ao longo de gerações, e altera a resposta às drogas (SERVICK, 2016).

Como qualquer modelo animal, devemos sempre estar cientes da necessidade de tradução para a condição humana. Isso também é verdade quando consideramos a microbiota intestinal. Embora a fisiologia intestinal seja semelhante entre roedores e humanos, também existem diferenças gritantes. Os roedores e humanos co-evoluíram com seus microrganismos e, embora bastante semelhante tanto funcionalmente quanto no nível taxonômico e nível de gênero, eles raramente compartilham as mesmas espécies.

Os microrganismos residentes dos camundongos são uma preocupação emergente. A microbiota pode mudar por diversas razões, incluindo uma mudança na formulação da ração, fontes de grãos ou proteínas dentro de uma marca (ERICSSON *et al.*, 2018); tratamento da água (BIDOT; ERICSSON; FRANKLIN, 2018), companheiros de gaiola que compartilham microrganismos (BOGATYREV; ROLANDO; ISMAGILOV, 2020). Alguns pesquisadores suspeitam que mesmo o estresse, como a separação da mãe, também pode mudar ecossistema microbiano de um camundongo (KEMP *et al.*, 2021). Em um estudo, a comparação entre as variáveis gaiola, cama e dieta tiveram pouco efeito sobre a composição geral das comunidades bacterianas fecais. Mas verificou-se uma mudança na composição da microbiota fecal ao longo do estudo em todos os camundongos, sugerindo que a composição da microbiota mudou ao longo do tempo à medida que os camundongos envelheceram (ERICSSON *et al.*, 2018).

Atualmente, mais laboratórios estão sequenciando amostras fecais em busca de genes bacterianos. O primeiro passo na definição de uma microbiota que possa ser considerado normal para roedores de laboratório, pode ser analisar as fezes dos camundongos procedentes de diferentes biotérios (SERVICK, 2016).

A coleta do conteúdo luminal cecal sugere ser o melhor indicador de influências ambientais sobre microbiota intestinal, e o uso de amostras fecais pode levar a “falsos negativos” na triagem de efeitos na microbiota. Embora o uso de fezes como amostra representativa forneça vários benefícios óbvios, como aquisição não invasiva, deve-se considerar a coleta de conteúdo luminal cecal em estudos terminais, particularmente aqueles que investigam os efeitos das influências ambientais sobre a microbiota (ERICSSON; FRANKLIN, 2021).

Essas influências trazem várias implicações para a modelagem animal, particularmente no contexto da pesquisa para investigar a microbiota intestinal. As diferenças observadas na microbiota podem ser dependentes de uma interação de duas ou mais variáveis, e isto destaca a complexidade pela qual fatores (por exemplo, linhagem de camundongo, tipo de alojamento/ventilação, cama e localização da amostra) podem influenciar a microbiota intestinal (ERICSSON *et al.*, 2018).

Embora diferentes fenótipos de modelo tenham sido relatados usando camundongos adquiridos de diferentes fornecedores, a composição e a uniformidade da microbiota fecal em camundongos de várias origens genéticas de diferentes fornecedores não são claras. Diferenças significativas na riqueza e diversidade de populações microbianas fecais em camundongos foram observadas em fornecedores comerciais renomados (ZHANG; FRANKLIN; ERICSSON, 2021).

A variação na microbiota intestinal entre colônias de roedores contribui para a variabilidade e, mais importante, para a reprodutibilidade dos modelos de roedores. O que se percebe é que os modelos de doença podem mostrar variação na expressão da doença ao longo do tempo dentro do mesmo laboratório. A gravidade da doença pode variar significativamente, dependendo apenas da microbiota intestinal que herdaram, uma prova de conceito de que a microbiota pode contribuir para diferenças fenotípicas entre colônias de roedores (FRANKLIN; ERICSSON, 2020).

Os estudos da microbiota e seu papel no fenótipo dos modelos de roedores são muito limitados e as interpretações devem ser cuidadosas. Os dados obtidos de sequenciamento alvos podem ser considerados geradores de observação e apoiar na geração de hipóteses. Uma limitação inclui os bancos de dados de sequência usados para anotação (muitas unidades taxonômicas operacionais ou sequência de amplicon variantes só podem ser mapeadas para nível de gênero, família ou mesmo ordem) (FRANKLIN; ERICSSON, 2020). Esses métodos são fortemente dependentes dos bancos de dados de referência e têm um desempenho ruim em conjuntos de dados de microrganismos não humanos associados. Mais da metade das unidades taxonômicas operacionais (OTUs) diferencialmente abundantes não são classificadas mais precisamente do que o nível familiar, limitando as inferências funcionais que possam ser desenhadas (LONG *et al.*, 2021).

A abundância de muitas OTUs, muitas vezes identificadas ao nível de espécie, bem como várias taxas superiores, diferiram de maneiras dependentes do fornecedor e da linhagem. Tais diferenças foram evidentes na microbiota fecal de camundongos desmamados e persistiram durante todo o estudo, até vinte e quatro semanas de idade. Esses dados fornecem a primeira análise aprofundada da trajetória de desenvolvimento da microbiota fecal em camundongos de diferentes fornecedores e um ponto de partida a partir do qual os pesquisadores podem refinar modelos animais afetados por diferenças na microbiota intestinal e, assim, possivelmente reduzir o número de animais necessários para realizar estudos com poder estatístico suficiente (ERICSSON *et al.*, 2015). Há relato que após 12 semanas da chegada ao biotério, a microbiota intestinal dos grupos de camundongos parece se homogeneizar em direção a um novo perfil, talvez refletindo uma resposta aos recursos compartilhados do novo ambiente. Além isso, o sexo também apresenta efeitos variáveis em um amplo conjunto de análises e, portanto, o uso de ambos os sexos em estudos com camundongos continua sendo um elemento essencial de delineamento experimental (LONG *et al.*, 2021).

A composição da microbiota intestinal pode ser afetada pelo estado de saúde do hospedeiro, muitas vezes levando à questão de saber se as diferenças observadas entre indivíduos saudáveis e afetados na composição da microbiota são causadoras ou meramente correlativas. Apesar do objetivo comum de um estado de saúde ideal e eliminação de patógenos, a criação de roedores de laboratório não é, no entanto, padronizada. Existem muitas diferenças sutis dentro do manejo de animais de laboratórios, assim como existem diferenças distintas entre as regiões do trato gastrointestinal em relação à a densidade e composição da microbiota luminal. Portanto, é razoável acreditar que a doença ou os efeitos induzidos pelo tratamento na microbiota pode passar despercebido em estudos baseados puramente em amostras fecais (ERICSSON *et al.*, 2018).

Os roedores da comunidade de pesquisa biomédica também vivem em ambientes comparativamente intocados e mais higiênicos em comparação com os humanos, e nosso sucesso em eliminar patógenos ao longo de várias décadas provavelmente diminuiu a riqueza de sua microbiota a um nível muito inferior ao dos seus homólogos humanos (FRANKLIN; ERICSSON, 2020).

Recentemente, a atenção tem sido focada em camundongos de fontes não laboratoriais (por exemplo, camundongos de estimação ou selvagens) devido a exposição a diferentes antígenos em comparação com camundongos de laboratório tradicionais. Como resultado, camundongos de estimação desenvolvem um sistema imunológico mais semelhante ao humano adulto, enquanto o camundongo de laboratório possui um sistema imunológico infantil, menos desenvolvido (ZHANG; FRANKLIN; ERICSSON, 2021).

Assim, o desejo histórico de eliminar e excluir todos e quaisquer patógenos e oportunistas dos os animais de pesquisa (aumentando ostensivamente a reprodutibilidade às custas de traduzibilidade) é confrontado com evidências crescentes de que o paradigma SPF tradicional pode ser insuficiente ou mesmo inadequado para alguns estudos.

Mesmo que a variabilidade da microbiota possa resultar em fenótipos diferentes e, contribuir para a baixa reprodutibilidade do modelo, há muitos aspectos positivos e há um interesse em avaliar como as diferenças de microbiota afetam a saúde e a doença. Explorar a complexidade existente na microbiota intestinal de roedores deve ser considerada como um complemento importante para os estudos experimentais envolvendo experimentação animal (FRANKLIN; ERICSSON, 2020).

As considerações descritas acima apresentam ambos os desafios e oportunidades, para indivíduos que trabalham em quase todos os níveis de investigação biomédica. Para os gestores de instalações animais que precisam garantir a qualidade dos camundongos produzidos e mantidos no laboratório, há uma necessidade crescente para reconhecer e relatar as características da microbiota intestinal em suas colônias. Com isso, uma colônia de produção deve ser pesquisada via sequenciamento de amplicon de rRNA 16S e relatada aos usuários. Isto custaria pouco mais do que a bateria de testes diagnósticos realizados em camundongos sentinela. Os cientistas precisam estar cientes dos fatores que levam a falta de confiabilidade e reprodutibilidade nos experimentos e a ter uma visão de futuro em relação à modelos de camundongos usados em suas pesquisas.

CAPÍTULO 1

Evaluation of nest building performance in laboratory mice: strain, age, and sex differences

HIGHLIGHTS

- B6J exhibited lower median nest scores compared to obese and nude mouse strains.
- Female nude mice represent 96% of nest scores between 4 and 5 at low temperatures.
- Temperature variation did not influence BTBR obese mice nest scores. Mice that used the cardboard tunnels for sheltering had the lowest nest scores.
- Female B6J and nude mice showed a positive correlation between age and nest score.

ABSTRACT

Nest building by mice is a natural behavior that serves comfort, thermoregulation, and protection. Therefore, evaluation of nest-building performance is indicated to assess animal welfare and behavioral integrity. Nest-building performance can vary among mouse strains, sex, or age and is influenced by environmental factors. The objective of this study was to evaluate nest-building performance of three strains of mice, comparing different age groups and sexes, and to test whether there is a relationship between nest score and the presence of cardboard tunnels. We used a simple, non-invasive scoring system to assess nesting in home cages described previously. B6J, *nude* and BTBR obese strains aged 31 to 90 days of both sexes were compared to analyze nest building behavior at two different temperatures (19 and 24^{o}C). The results show that there was a positive correlation between age and nest quality when B6J and *nude* female mice were compared. Newly weaned mice had the lowest nest score, suggesting that nest quality depends on learning nest-building behavior. There was a significant difference in building performance by strain as a function of temperature: *nude* female mice showed higher nest scores at low temperatures. In addition, providing cardboard tunnels for the mice influenced nest-building behavior. Nest scoring quality was better when *nude* or B6 mice used cardboard tunnels for nest building or defecation and urination (*toilet*). Our

data suggest that nest-building performance depends on the strain of mice and on variations in the macroenvironment (temperature) and microenvironment (enrichment material). An important factor affecting nest-building performance is lower temperatures, and high-scoring nests could indicate thermal discomfort in the mice.

Keywords: Nesting behavior; nude mice; Inbred mice; Obese mice; Animal welfare.

1. INTRODUCTION

The mouse is the most commonly used species in animal studies, and ensuring its welfare is essential to the success of the results and the quality of the projects. Animal welfare indicators are needed to assess the quality of life of laboratory animals and to improve the management of animal facilities. One question is how to evaluate animal welfare objectively and scientifically.

Indicators of animal welfare are based on physiological parameters, followed by indicators related to behavior (normal and abnormal), social interaction, and environment (Campos-Luna *et al.*, 2019). That is, animal welfare is assessed by observing or measuring the physical or behavioral characteristics of the animal in conjunction with factors that determine the quality of the environment in which it is kept. The animal's environment, the presence of enrichment materials, exposure to temperature or humidity fluctuations, or details of husbandry routines are some examples of environmental indicators. Summarizing all aspects of laboratory mouse welfare (physical, physiological, behavioral, and environmental) in a welfare protocol is critical to an overall assessment. Resource-based assessment uses indicators that reflect the animals' environment and how the animals cope with environmental changes while maintaining their biological and psychological functions. Indicators include environmental indices related to housing and husbandry, as well as routine activities (e.g., cage cleaning) (Campos-Luna *et al.*, 2019).

Incorporating resource indicators into welfare assessment protocols is important because they include procedures, treatments, and management that can have a major impact on welfare, especially for laboratory animals (e.g., ambient temperature and environmental enrichment in cages) (Campos-Luna *et al.*, 2019). Because welfare is complex, it is often important to assess more than one indicator to determine the extent of the animal's quality of life, rather than just one aspect of the animal's biology or environment (Sejian *et al.*, 2011). Two key questions can be asked to assess well-being: 1) Is the animal physically healthy and 2) Does the animal have what it needs? Behavior can be used to answer these two questions (Dawkins, 2003). Behavioral indicators are important because they are easy to measure and provide information about an animal's adaptations to current environmental conditions.

It is also important that indicators of well-being assess the animal's emotions, physical functioning, and/or naturalness. Emotions, for example, may be critical to some concepts of well-being. Even healthy animals can have poor well-being if they are anxious, bored, or socially stressed (Broom, 2008).

It is scientifically proven that an enriching environment has a positive impact on emotions, cognition, behavior, physiology, body weight, reproduction, hormone release, and offspring development. This is also a necessary step to obtain results with high precision and a high degree of reproducibility. However, environmental enrichment is not limited to the addition of objects in the cages, but also includes the space and type of cage, bedding material, handling, and social contact (Massari *et al.*, 2019).

The greatest interest in environmental enrichment is to improve animal welfare by providing sensory and motor stimulation through structures and resources that facilitate the expression of species-typical behaviors and promote psychological well-being through physical exercise, manipulative activities, and cognitive challenges, depending on the specific characteristics of the species (National Research Council, 2011). Although some researchers have expressed concerns that environmental enrichment may compromise the standardization of experiments by introducing variability, there are studies that support this view and show that enclosure conditions can be enriched

without compromising the accuracy or reproducibility of experimental results (André *et al.*, 2018; Wolfer *et al.*, 2004; Würbel, 2007).

The only thing to note is that enrichment devices such as shelters and nesting materials contribute to temperature and humidity fluctuations in the mice cage. Other factors that may contribute to these fluctuations include the number, age, species and size of animals in the cage, forced ventilation of ventilated racks, and the type and frequency of cage changes. Exposing animals to large fluctuations or extremes in temperature and humidity can result in behavioral, physiological, and morphological changes that can negatively affect animal welfare and research performance, as well as the results of research protocols (National Research Council, 2011).

Some authors have developed an assessment scheme to measure the welfare of laboratory mice based on the nesting material present in the cages and how the mice use this material in preparing their nests (Campos-Luna *et al.*, 2019; Leach *et al.*, 2008). Nesting behavior in laboratory mice is considered an ethologically relevant indicator of animal welfare and is used to detect thermal stressors, aggressive caging, disease, and pain. Observation of nesting behavior in mouse colonies provides refinement for assessing daily health and well-being (Gaskill *et al.*, 2013).

In addition, nest assessment is a simple, inexpensive, and easy-to-perform test that, along with other species-specific behavioral tests, is a sensitive test for identifying behavioral phenotypes. A previous study described a simple and noninvasive nest assessment system that can be easily measured in mouse cages (Deacon, 2006).

Providing nesting material is a simple strategy to improve the welfare of laboratory rodents (Pietropaolo *et al.*, 2004), and the nest assessment system can indeed be used to assess nest integrity, behavior and general well-being (Kraeuter *et al.*, 2019). However, nest building depends on environmental factors. Several factors affect nest quality: Light intensity, location of the nest in the cage, temperature (mice housed at lower temperatures build higher quality nests), and well-being (mice with impaired well-being build lower quality nests) (Gaskill *et al.*, 2013; Goto *et al.*, 2015;

Johnson *et al.*, 2017; Maher *et al.*, 2015; Makowska *et al.*, 2019; Spangenberg and Keeling, 2016; Xiong *et al.*, 2021).

Assessing the behavior of laboratory mice using nesting material can be a valuable tool for evaluating animal welfare, but requires a great deal of practice and knowledge on the part of the assessor for the results to be considered reliable (Campos-Luna *et al.*, 2019). Personnel responsible for the care and rearing of animals should be trained in the behavioral biology of the species to monitor the effects of enrichment and detect the development of negative or abnormal behaviors (National Research Council, 2011).

Some environmental enrichments, such as cardboard rolls, help to divide enclosures by allocating different areas for animals, such as space for feeding and elimination (Baumans, 2005). The cardboard roll not only serves as shelter and nesting material, but also provides opportunities for exploration and locomotion. Combining materials is one of the behaviors observed in animals to build better structured and more complex nests (Van de Weerd *et al.*, 1997). Few studies have investigated the combination of environmental enrichments such as shelter and nesting materials and examined their preference and use at different life stages.

In our study, we examined the nesting behavior of C57BL/6J (B6J), CByJ.Cg-Foxn1nu/J (nude), and BTBR.Cg-Lepob/WiscJ (obese BTBR) mice of different ages (weanlings, juveniles, and adults) and sexes. Mice were studied during laboratory routines in their own experimental housing. Nest-building score was associated with a number of measurements, including temperature and enrichment material (paper roll). The objective of this study was to determine if nest-building score can be a valid, reliable, and practical indicator for routine daily assessment of laboratory animal welfare. In this study, we used an approach to examine the extent to which various factors come together to provide evidence that the nest-building score is an indicator of welfare assessment.

2. METHODS

2.1. ANIMALS AND HOUSING

Male and female inbred mice of strains C57BL/6J (B6J), mutant CByJ.Cg-*Foxn1*^{nu}/J (*nude*), and BTBR.Cg-Lep^{ob}/WiscJ (BTBR obese) were obtained from the breeding colony of the Center for Experimentation and Surgical Training (Sociedade Beneficente Israelita Albert Einstein), an AAALAC accredited institution. The mice were specific pathogen-free (SPF) for ectromelia virus, lymphocytic choriomeningitis virus, mouse minute virus, mouse hepatitis virus, mouse parvovirus, mouse pneumonia virus, reovirus, Sendai virus, Theiler murine encephalomyelitis virus, hantaviruses, cilia-associated respiratory bacilli, *Clostridium piliforme, Klebsiella pneumonia, Mycoplasma pulmonis, Pasteurella multocida, Pasteurella pneumotropica, Pseudomonas aeruginosa, Salmonella spp, Staphylococcus aureus, Streptobacillus moniliformis*, β-hemolytic *Streptococcus spp, Streptococcus pneumoniae*, endoparasites, and ectoparasites. We check for viruses and bacteria every six months and for endoparasites and ectoparasites every two months. The animal facility is just positive for *Helicobacter sp*. Groups of 3 to 5 mice of the same sex were housed in individually ventilated cages with a ventilation rate between 45 to 60 air changes per hour. B6J and *nude* mice were housed in Alesco® cages (32x20x21cm) and BTBR obese in Tecniplast® cages (37x20x13cm), with solid floor and wood flake bedding (Good Life™, Granja RG, SP, Brazil). To improve the environment, we offered cardboard tunnels (Relax™, Granja RG, SP, Brazil). Pellet feed (irradiated, Nuvilab CR1™, Quimtia, PR, Brazil) and autoclaved water were offered *ad libitum*.

Temperature and humidity were controlled by a dedicated air conditioning system and measured with a digital thermal hygrometer. The room was monitored daily by technicians and an online system also monitored the temperature every 5 minutes; the daily average temperature was considered for this study. Room temperature ranged from 18.3 to 25.4ºC, according to the minimum and maximum temperatures measured during experiments (figure S1). Based on monthly average temperatures, the three coldest months averaging 19.6 degrees Celsius (July, August, and September) and the

three months with the highest temperatures averaging 23.9 degrees Celsius (December, January, and February) were selected for this study.

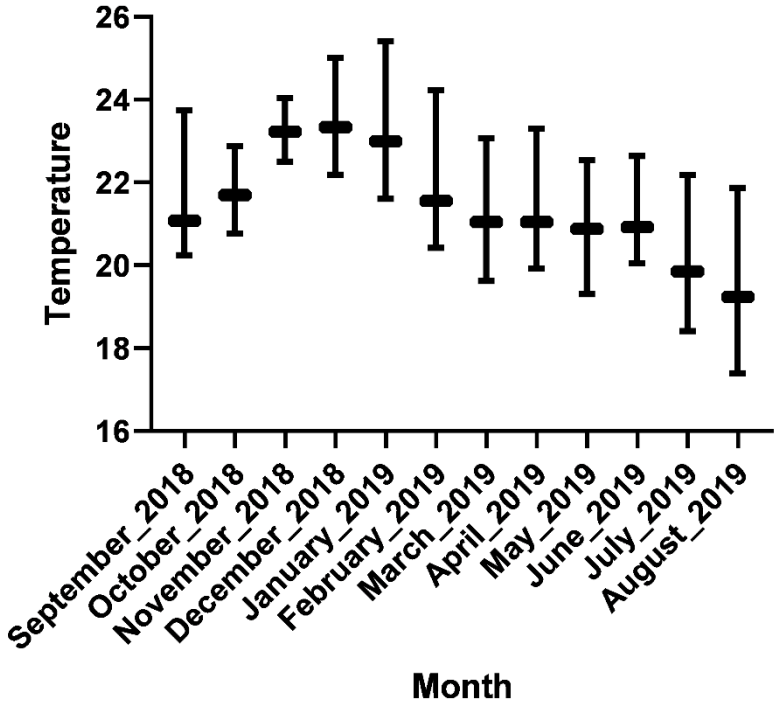

Figure S1. Monthly average temperatures of the animal facility.

Humidity ranged from 41 to 65% and ventilation system used 100% fresh air and temperature was controlled (direct expansion unitary system) by an internal thermostat. The room was on a 12:12-h light-dark cycle (lights on from 07:00 to 19:00).

Nesting material consisted of two pieces of 100 × 20 cm^2 white paper towels, weighing approximately 6g (Kleenex^{TM}, Kimberly-Clark, TX, US).

2.2. EXPERIMENTAL DESIGN

Our observational study involved 14 to 26 mouse cages, separated by strain (B6J, *nude,* and BTBR obese) and sex (male or female) (Table 1).

Table 1. Number of cages and observation per strain and sex.

Description	B6 female	B6 male	nude female	nude male	obese female	obese male
Number of observations	58	133	94	185	81	89
Number of cages	16	22	14	26	14	17

Age varied from 21 to 109 days and the animals were divided into the following age groups: newly weaned (up to 30 days of age), young (31 to 45 days of age), and adult (over 46 days of age).

A scoring criteria sheet was adapted to evaluate the nests based on three different scoring systems as described by Deacon (2006), Gaskill *et al.* (2013), and Kraeuter *et al.* (2019) as shown in figure 1. Nest building evaluations occurred from September 2018 to August 2019. The nests were evaluated once a week at 8:00 am, during cage changing, to avoid additional stress. Each cage was taken out of the rack and transferred to the workbench. In this way mice had one week to handle the enrichment material. The lid was removed, and animals gently transferred to clean cages containing one autoclaved cardboard tunnel and two autoclaved sheets of paper towel and placed back to the rack. Three technicians analyzed and scored the nests. They were trained for three weeks before the beginning of the study, followed the descriptions of each score and scored all nests. Nest score was determined only after at least two technicians agreed.

Score	Picture	Adapted from Deacon (2006), Gaskill (2013) and Kraueter (2018)
1		Nesting material poorly handled (more than 90% intact). The material has spread through the cage, and it has not been enough moved or torn.
2		Nesting material partially torn, a flat nest.
3		A cup nest, the nesting material may sometimes be in a broadly defined nest area. 50-90% has been shredded.
4		Nesting material is gathered into a nest within a quarter of the cage floor area. Nest like doughnut, but incomplete dome.
5		Perfect nest; more than 90% of the nesting material is torn and the nest is a crater; complete and enclosed dome.

Figure 1. Scoring criteria: nest building performance evaluation.

This study was approved by the University of São Paulo Animal Care Committee (CEUA Protocol Number: 1353060218) and the Albert Einstein Hospital Animal Care and Use Committee (CEUA Protocol Number: 4037-20). Procedures followed the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and the Brazilian Legislation (Conselho Nacional de Controle de Experimentação Animal – CONCEA*). This experiment was conducted according to Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) and the Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines. *https://antigo.mctic.gov.br/mctic/export/sites/institucional/institucional/concea/arquivos/publicacoes/Fasciculo-02.-Roedores-e-Lagomorfos-2019.pdf

2.3. STATISTICAL ANALYSIS

We conducted all analyses using Action Stat Pro, statistical software for excel. Rank data for multigroup comparisons were analyzed by nonparametric statistical tests (Mann-Whitney U test and Kruskal-Wallis). For statistical analysis by chi-square, the groups were divided into the following ages: newly weaned (up to 30 days of age), young (31 to 45 days of age), and adults (over 46 days of age). Spearman test was used for correlation between age and nest score and Kruskal-Wallis test for effects of age, sex and use of cardboard tunnels for the nonparametric nest building data. A value of $p< 0.05$ was considered statistically significant.

3. RESULTS

3.1. B6J mice

There was no association between age and nest score for B6J males ($p=0.39$). However, an association was found for B6J females ($p<0.01$), which exhibited a positive correlation between age and nest score ($p<0.01$, figure 2 below).

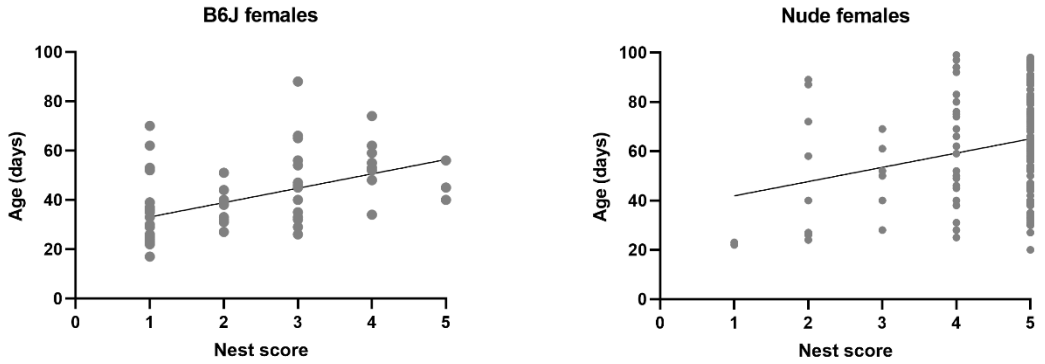

Figure 2. Correlation rate of nest score x age of B6J and nude females mice.

There was statistical difference in the nest score in males when comparing the use of cardboard tunnels for housing or disposal of waste (*toilet*) ($p<0.01$, figure 3). Mice that used the tunnels to urinate and defecate built higher nest score when compared with mice that used the tunnels for sheltering. Females used tunnels for sheltering in most analyses (87%). The median nest score for B6J was 2 for females and 3 for males.

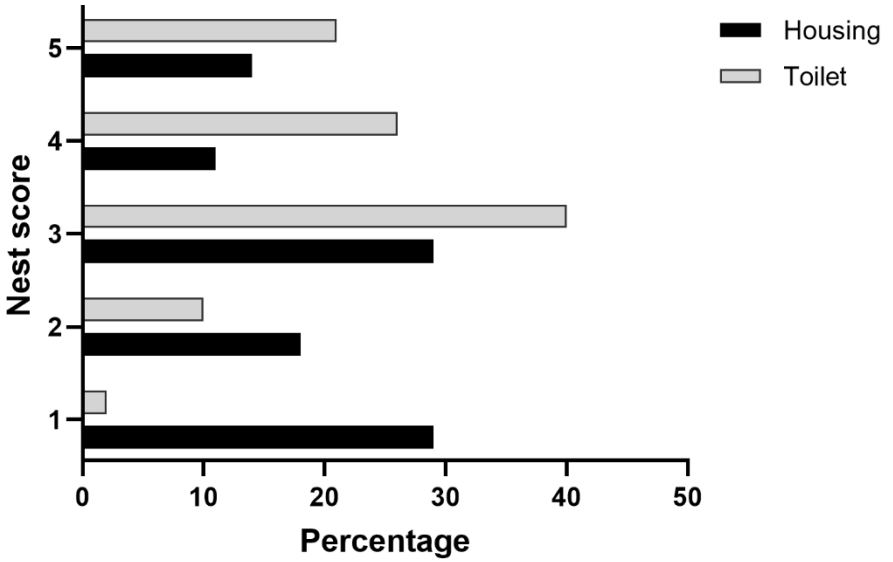

Figure 3. Nest score when comparing the use of cardboard tunnels for housing or for disposal of waste (toilet) in males B6J mice.

3.2. *Nude* mice

In contrast to observations of B6J mice, an association between age and nest score for both males and females (p= and p=0,7721) was identified. Nest scores of 1 and 2 were obtained by *nude* males with a mean age of 26 days (SD: 12 days). We also observed a correlation (p=0.033) between age and nest score for female *nude* mice, as shown in figure 2, previously.

There was statistical difference in the nest score when assessing the use of the tunnels for sheltering or nest construction by males and females (p<0.01), see figure 4.

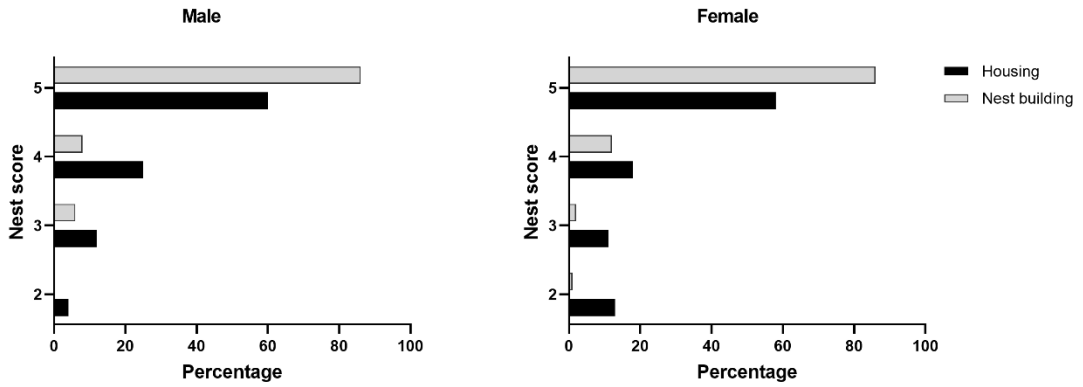

Figure 4. Percentage of nest score in relation to the use of cardboard tunnels in male and female nude mice.

Statistical difference between males and females was only found in analyses of data collected in August when the average temperature was 19.6ºC. The median nest score was 5 for both sexes.

3.3. BTBR obese mice

The median nest score for BTBR obese was 4 for females and 3 for males. There was no association between age and nest score in males (p=0.1386) or females (p=0.5276) (see percentage per age and strain - Table 2). In addition, there was no

statistical difference when evaluating the nest score and the use of the tunnels (p=0.8722).

Table 2. Percentage of nest score by age group, gender and strain.

Age Group	**Newly weaned**					**Young**					**Adult**				
Nest Score	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
B6 male (%)	27	11	33	17	11	28	13	23	26	10	13	21	27	17	21
B6 female (%)	81	6	13	0	0	25	38	25	4	8	20	5	35	35	5
Nude male (%)	29	29	14	7	21	0	3	13	17	67	0	1	12	31	56
Nude female (%)	15	23	8	15	38	0	4	5	18	74	0	2	3	19	77
Ob/Ob male (%)	23	12	24	29	12	9	22	27	21	21	7	13	25	26	29
Ob/Ob female (%)	9	9	27	45	9	11	11	23	22	33	8	7	21	30	34

3.4. Comparison between B6J x *nude* x BTBR obese strains

We compared nest scores for both sexes of B6J, *nude*, and BTBR obese mice, aged 31 to 90 days, living at average room temperatures of 23.9ºC or 19.6ºC. The results are shown in figure 5.

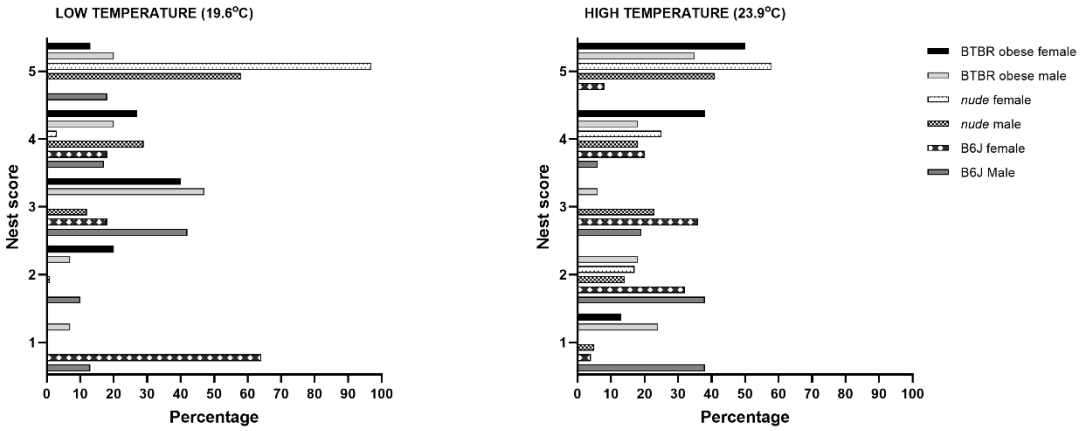

Figure 5. Comparison of the percentage of nest score at the high (mean 23.9oC) and low (mean 19.6oC) temperatures for the B6J, nude and BTBR obese strains, both sexes.

At 23.9ºC, the three mouse strains showed no statistically significant difference in nest scores of either male or female. In the lower temperature, just nude females showed lower nest scores when compared with males (p=0.0180).

B6J and BTBR obese males showed significant difference in the nest score at 23.9ºC average temperature (p=0.0475). However, there was no significant difference in the nest score at the same average temperature (p=1) for BTBR obese and *nude* females.

B6J and *nude* mice, aged between 31 and 90 days, showed statistical differences in the nest scoring, regardless of temperature and sex.

When comparing the same sex to the two temperature scenarios (19.6ºC and 23.9ºC), statistical difference was only found for the B6J males (p= 0.015).

4. DISCUSSION

Nest-building is a behavior naturally observed in mice. According to a previous study (Deacon, 2006), B6J mice are generally reliable nesters. Also, most mouse strains will build nests with scores between 3.5 and 4.5. Deacon (2006) conducted his study providing wood-chip bedding and 0.5g of unshredded cotton squares (nestlet), a type of nest material. We provided the minimum amount of nesting material recommended by Makowska *et al.* (2019) to reduce thermal distress under typical animal room temperatures (~18-23ºC). Moreover, we gave the animals an additional enrichment (cardboard tunnels), and the median nest score for B6J was 2 for females and 3 for males. As found by Gaskill *et al.* (2013), male mice built higher quality nests than females. However, in our study, B6J females used the cardboard tunnels for sheltering, suggesting this could be one reason to lower nest scores. There were sex-dependent responses to tunnels: B6J male mice did not use tunnels for sleeping/sheltering. Instead, the B6J males used the tunnels as an area to discharge feces and urine, showing better nests than the ones that used the tunnels for sheltering. Future studies could consider the nest place in the cage, as well. There is also indirect evidence that

mice prefer to separate elimination behavior from nesting activity (Makowska *et al.*, 2019).

Pietropaolo *et al.* (2004) demonstrated that single-housed laboratory rodents continue performing nest-building activities even in the presence of tubes, and the use of the tunnels did not interfere with the nest-building performance. In our study, the cardboard tubes were an additional relevant nesting material that stimulated their nesting behaviors of gathering, burrowing, and sorting, and they improved their nest-building performance.

Hess *et al.* (2008) demonstrated that mice build a more complex nest using shredded paper strips than compressed cotton squares (nestlets) and paper handkerchief (facial tissues). Other studies showed that bedding is also essential for high-quality nests because mice can incorporate it into their nests (Kraeuter *et al.*, 2019). We observed that mice could shred the cardboard tunnels and combine them with the paper towel building better nests; therefore, contributing to the nesting performance of the *nude*.

The nest score depends on the strain, and the interaction between sex also yielded differences in nest scores as observed by Gaskill *et al.* (2013b). A significant interaction between strain and sex also affected nest score means, but the B6J mice showed the lowest scores.

Gaskill *et al.* (2013b) found a negative linear correlation between nest score and radiated temperature means, independent of sex or strain. Therefore, any mouse that builds a more dome-like nest will lose less heat. The *nude* appears to be more strongly impacted than others. They built more dome-like nests, probably to decrease the amount of radiated heat. Higher scoring nests are related to lower body heat loss (Gaskill *et al.*, 2013).

Mice must use either behavioral or autonomic processes to maintain homeothermy. Nest material into the mice cage is crucial, because they are generally housed with a thin layer of bedding material, which harms insulation, and they cannot construct igloo-like nests from it. Nesting material allows mice to alleviate cold stress by controlling their thermal microenvironment within the cage, despite it may also decrease body temperature variability and normalize metabolic rates (Gaskill *et al.*, 2013).

Nesting materials help rodents to thermoregulate in the unnatural and cold laboratory cage environment. In typical laboratory environments, mice are housed with an average room temperature of 21ºC. Fischer *et al.* (2018) showed that mice without access to nests had an average energy expenditure three times greater than the resting natural metabolic rate, when housed at 21ºC.Thus, standard laboratory housing conditions undoubtedly impose metabolic stress on mice and may affect many metabolic (and other) parameters under such conditions.

Our data showed no difference in nesting performance when the animals of both sexes from the three strains were maintained at 24ºC. Different mouse strains seem to have different behavior patterns to thermoregulation and different physiological strategies to maintain a constant body temperature under standard laboratory ambient temperatures (Gaskill *et al.*, 2013). We also demonstrated that in our study as the *nude* had higher nest score quality at 20ºC. The B6J males had different nest scores when comparing temperatures of 20 and 24ºC. Knowing that the mice housed at lower temperatures build higher quality nests, there is a positive correlation of thermal discomfort and nesting performance. The standard animal house conditions for mice are not thermoneutrality. According to Fischer *et al.* (2018) and Reitman (2018), when B6J mice were housed at temperatures ranging from 26 to 29ºC, metabolic studies have more reliable data to compare with humans.

Our observational study assessed the nesting performance of three mouse strains. The purpose of choosing these strains was not only because they comprise the most used inbred (B6J) and mutant strains (*nude* and BTBR obese) research mice but also because they require specific environmental conditions. Popularly used mouse strains possess distinct thermoregulatory characteristics (Gordon, 1993). Obese mice are probably more susceptible to heat. *Nude* and hairless mice probably need higher temperatures for comfort because they have no hair.

Analyzing BTBR obese mice, we observed that the nesting performance was the same in lower temperatures. A study discusses the development of genetic rodent models for an obesity study led to the fortuitous discovery of a correlation between thermoregulatory dysfunction and obesity (Gordon, 1993). Thermoregulation and caloric balance are intimately related. Therefore, the obese mouse showed a marked inability to

thermoregulate when exposed to cold temperatures. In addition, facultative thermogenesis in the obese mouse is defective. At ambient temperatures below thermoneutrality, obese mice have a reduced metabolic rate and a core temperature 1-2°C below that of lean animals – both young and adult obese mice display reduced motor activity (Carlisle and Dubuc, 1984). That is one of the reasons to believe that the BTBR obese mice did not build better nests at lower temperatures in our study.

Cold-induced thermogenesis in brown adipose tissue (BAT) is the most relevant thermoregulatory mechanism in adult mice. It is further potentiated in nude and hairless strains mainly due to a diminished insulating capacity of the hairless skin. The noradrenalin concentration is more than twice as high in interscapular BAT of *nude* at 22ºC compared to 28ºC. The reduction of energy stores (white adipose tissue) impairment of actual in the *nude* at 22ºC explains the thermogenic effect of BAT with resulting hypothermia in this mutant. This result explains the insufficient thermogenic activity of BAT in the *nude* and, consequently, the down-regulation of body temperature (Funda *et al.*, 1998).

The B6J and *nude* mice nest performance are associated with age: newly weaned mice have the lowest score nests compared to young and adult mice. We believe that this data relates to the animals learning how to build nests. A recent study by Xiong *et al.* (2018) demonstrated a decline in nest-building performance in aged adult mice when comparing 25-month old mice with 7-month-old mice. The authors also did not observe significant differences for sex in nest building of 7-month-old mice.

Our study found variation in the nest performance on three strains and differences between B6J male and female mice. *Nude* mice were considered better nest builders and maybe more thermally stressed than B6J. It is unknown if B6J is less driven to build a nest because they do not feel as cold as other mice or are not as skilled as the other strains in building nests. Although, B6J females has used the tunnels for sheltering, this could be a reason for not building the nest. Our research team will focus on conducting further studies to understand more factors that influence nesting performance and therefore, improve the quality of life of mice in the laboratory.

5. CONCLUSIONS

Nest-building performance of mice depends on the macro- and micro-environment in which the animals are kept. Mice change the quality of their nests as a function of temperature. Therefore, nest building material is one of the most important elements of the environment in the mouse cage and is critical to the longevity of the mice. Nesting material helps mice regulate their body temperature and gives them some control over their environment. Therefore, it can be considered as an indispensable material for the cage as it contributes to positive welfare and the nesting score can be used as an indicator for routine daily assessment of the welfare of laboratory animals.

Declarations of interest: none

Acknowledgments

This study was supported by Sociedade Beneficente Israelita Albert Einstein and Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES), Brasil – Finance Code 001. The authors are most grateful to Angelica da Purificação, the animal technician responsible for improving animal welfare and the quality of the experimental data.

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CAPÍTULO 2

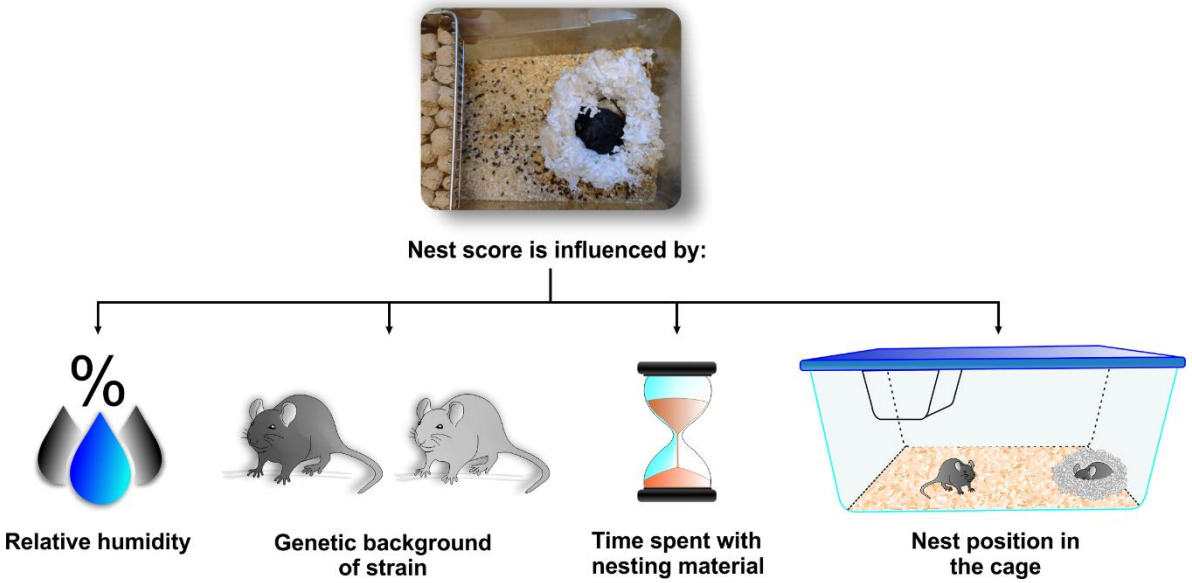
Nest building in breeding mice: impact of macro- and micro-environment

ABSTRACT

The housing and management conditions of laboratory mice must be strictly controlled in order to reduce the impact of pathophysiological changes that affect animal health and welfare, possibly resulting in increased variability within experimental results. One way to improve the activity and survival of laboratory mice is to enrich the environment by providing nesting material and shelter. The objective of this study was to determine if nest-building quality could be used to detect changes in mouse mating behavior in a rodent facility under controlled conditions. Nest-building scores from 847 cages containing monogamous pairs of mice were assigned according to 18 pre-defined indicators. The effects on nest quality were evaluated using descriptive data analysis, correspondence analysis and ordinal logistic model fitting. The results showed a strong relationship between nest quality and nest position. Humidity, genetic background, cage change, number and age of pups in the cage, and death in the cage affected the nest-building score. The most important indicators were cage change and relative humidity, both of which exerted significant negative effects on nest-building quality. Even though the criteria were well defined, the observer could still influence nest score appraisal. However, in a long-term observational study, observers could improve their assessment by training and acquiring greater experience in score assignment. Nest-building score is easy to assess, incurs low operating costs and is a valid indicator of the health and well-being of laboratory mice. We believe that nest score can provide valuable support in the management of animal facilities.

Keywords: Animal welfare, mice, nesting behavior, genetic background, humidity

GRAPHICAL ABSTRACT



INTRODUCTION

Laboratory housing affects the health and welfare of experimental animals, while inappropriate environments can jeopardize research and scientific studies by fostering poor management techniques that may reduce the reproducibility of experiments. Any increase in variability in the results obtained inevitably leads to higher costs, since larger numbers of animals are required to participate in the experiment in order to achieve reliable outcomes.[1]

Nesting, which is an innate and motivated behavior in both male and female mice, represents a strategy by which to improve the activity and survival of the animals and, thereby, maintain their warmth, reproductive activity and protection even when they are raised in the laboratory. Nesting behavior is influenced by a variety of environmental factors, including temperature variation and physiological challenges such as cognitive decline.[2] In this context, environmental enrichment through the provision of nesting material is routinely employed in mouse facilities to ensure the health and well-being of the animals and to meet the physiological and ethological needs of the species. According to the literature, the supply of nesting material to improve the welfare of

laboratory animals does not compromise experimental results,[3] and many researchers have applied this technique in the evaluation of animal behavior.

In the present study, we collected data from a routine breeding colony in order to establish whether the nest-building score was a reliable indicator of well-being in laboratory mice and to determine which factors might affect nesting performance.

MATERIALS AND METHODS

This observational study was approved by the Ethics Committee on the Use of Animals of the Institute of Biomedical Sciences, University of Sao Paulo (CEUA-ICB/USP protocol No. 8442040522). The experiments were conducted in accordance with the guidelines of the Conselho Nacional de Controle de Experimentação Animal (CONCEA), Ministério da Ciência e Tecnologia, Brasília, DF, Brazil. Data were collected between August 2018 and November 2019 in a breeding colony at the Experimental Mice Facility of the Immunology Department at ICB/USP.

Animals and experimental design

The strains of mice employed in the study were classified according to their genetic background and comprised 33 strains with C57BL/6 (B6), BALB/c or 129 backgrounds, and 10 strains with miscellaneous backgrounds (Table 1). The animals were housed in 847 individual ventilated cages (IVC) held in one of ten ventilated Alesco™ racks (ALBR Industria e Comercio Ltda, Monte Mor, SP, Brazil) that were distributed at six locations within the animal facility. Each microisolator cage had a floor area of 451 cm^2 and served a monogamous mating system and the respective litters. The cages contained approximately 150 g (2.5 cm cage height) of flake bedding (Good Life™, Granja RG, São Paulo, SP, Brazil), irradiated pelleted feed (Nuvilab CR1™, Quimtia, Colombo, PR, Brazil), filtered and sterilized acidified (pH 2.5-3.5) drinking water, and two sheets (*circa* 6 g) of paper towels (Kleenex™, Kimberly-Clark, Irving, TX, USA) as nesting material. Previous studies have shown that mice prefer cages with

whole paper towels compared to paper strips,[4] and have suggested that 6 g is an appropriate amount of paper to provide per cage.[5]

Animals were maintained under specific-pathogen-free (SPF) conditions under a dark-light cycle of 12:12 hours with light intensity between 130 and 325 lux, a temperature of 20 to 26 °C, relative humidity of 30 to 70%, and 20 fresh air changes per hour. The routine of the care providers remained unchanged throughout the entire study period and comprised day 1 cage change, including the rear of the cage with bedding, food and water, and day 7 replacement of the water-drinking bottle and food. On the other hand, there were changes in the colony during the observational period as new pairs were formed and old pairs removed.

Analysis of nests and data collection

Nests were observed in the mornings (between 08.00 and 10.00 h) of the 3rd and 14th day after a cage change. In order to appraise the nests, cages were removed from the ventilated racks and the lids were opened in changing stations. The quality of nests was assessed using a 5-point Likert scale adopted from the literature,[6–8] where 1 denoted that the paper towel had been barely used and 5 indicated that the nest was perfect and had a "donut" shape. A total of 6540 observations were recorded during the study with data being collected by two different observers, the first from August 2018 to July 2019 and the second from August to November 2019. For each of the observations, the following variables were recorded: 1 - the observer responsible for evaluating the nest; 2 - week during which the nest was evaluated; 3 – strain classified by genetic background (B6, BALB/c or 129); 4 - number of pups in the cage; 5 - age of the pups when the nest was evaluated (considering four groups, namely without pups, with pups aged up to 7 days, with pups aged 8 to 15 days, or with pups aged 16 to 21 days); 6 - position of the nest in the cage (front, middle or back); 7 – position (column and row) of the cage in the ventilated rack (supplementary file Figure S1); 8 - rack identification; 9 – location of rack; 10 - presence of litter (indicating whether or not the cage had litter during the observed week); 11 - cage change (binary variable indicating whether or not the cage had been changed on the day of observation); 12 - room temperature on the

day of observation; and 13 - relative humidity on the day of observation. During nest evaluation, additional binary evaluations were conducted to determine if there were any occurrences during the cage change as, for example, 14 - male removal (indicating whether or not the male was removed from the cage); 15 – barbering; 16 – cannibalism; 17 – death in the cage; and 18 - inflammatory processes (conjunctivitis, dermatitis or other obvious inflammatory processes).

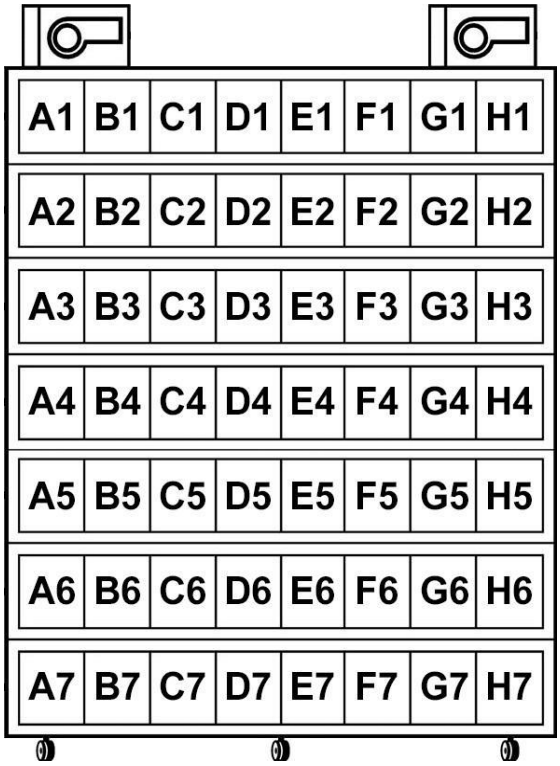


Figure S1. Diagram demonstrating cage location (column and row) in an individually ventilated cage (IVC) system.

Statistical analysis

Statistical analyses were performed with the aid of the following computer programs for Windows: R version 4.1.1, RStudio version 1.4, PowerBI version 2.96.1061.0 and IBM SPSS Statistics version 20. Each of the 18 variables listed above was correlated with the quality of nest construction by assigning a score to the nest on a 5-point Likert scale that ranged from 1 (no nest built) to 5 (nest well-built/structured).

McNemar's test was used to compare the nest score immediately before and after cage change and birth of pups.

An ordinal logistic model was fitted to the categorical nature of the response variables in order to assess the combined effects of the variables. The explanatory variables were included under the proportional odds assumption, and the Akaike Information Criterion (AIC) was used to select predictor variables to identify indicators of nest quality (stepwise algorithm). A partial proportional odds model was considered with variables not meeting the above assumption having non-proportional odds. The initial and final models are presented in the supplementary material (Tables S1 and S2), along with exponentiated coefficients, p-values and the lower and upper limits of the 95 percent confidence intervals.

Table 1. Strains, number of cages and observations of nest-building quality relating to this study.

Group	Strain	Common name	Room number	IVC number	Number of cages	Number of observations
129	129 SvE	129 SvE	16	4	31	262
	129S2-Alox15^{tm1Fun}/J	129 ALOX 5	16; corridor	4; 5	36	369
	129S2-Ifnar1^{tm1Agt}/Mmjax	129 ABR	16; corridor	4; 5	27	164
Totals for 129 background	**3**				**94**	**795**
B6	B6.129-Ahr ^{tm1Bra}/J	AHR null	15	6	34	207
	B6.129-Ido1^{tm1Alm}/J	IDO KO	corridor	5	33	273
	B6.129P2-Aim2$^{Gt\ (CSG445)Byg}$/J	Aim2	17	1	11	86
	B6.129P2-il10^{tmCgn}/J	B6 IL10	16	4	22	205
	B6.129P2-P2rx7^{tm1Gab}/J	B6 P2RX7	17	1	27	319
	B6.129S2-Cd28^{tmMak}/J	B6 CD28	17	2	20	233
	B6.129S2-*Cd8a^{tm1Mak}*/J	CD8 KO	corridor	5	32	346
	B6.129S2-Cd4^{tm1Mak}/J	B6 CD4	17	2	31	272
	B6.129S2-Ighm^{tm1Cgn}/J	B6 B KO	17	1	17	149
	B6.129S4- Grin1^{tm2Stl}/ J	fNR1, NR1 flox	17; 16; 12	1; 3	24	211
	B6.129S6-Clec7a^{tm1Gdb}/J	B6 Dectin-1	17; 16	2; 3	25	125
	B6.129S7-Ifng^{tm1Ts}/J	B6 INFG	15	6	21	232
	B6.129S7-Rag1tm1Mom/J	B6 Rag1	corridor	5	18	188
	B6; 129- Xpc^{tm1Ecf}/ J	XPC	13	9; 10	9	34
	B6; 129S4- C3 tm1Crr / J	B6 C3 J	17	1	24	305
	B6.Cg-Tg(TcraTcrb)425Cbn/J	B6 OTII	12	8	21	135

	C57BL/6	B6	17; 16; 15	1; 3; 6; 7	161	1061
	B6; 129S4- Ptgs1$^{tm1.1Hahe}$/ J	Cox-1 flox, 129 S4 Cox-1	17	1	1	9
	A.B6-Hc1	A/J C5+	17; 16	2; 3	12	123
	B6.129 Xpa^{tm1Hvs}/J	XPA	13	9; 10	11	31
Totals for B6 background	**20**				**554**	**4544**
BALB/c	BALB/cJ	BALB/c	17	2	45	424
	C.Cg-Tg(DO11.10)10Dlo/J	DO11.10	12	8	14	248
	BALB/cJ-Kmt2d^{bapa} (MGI:6442583)	bate palmas	13	9; 10	15	113
	carc (MGI:5987005)	careca	13	9; 10	4	18
	crup (MGI:5704085)	cruza as perna	12	8	41	157
	eqlb (MGI:5987007)	equilibrio	13	9; 10	5	24
	frqz (MGI:5987008)	fraqueza	13	9; 10	6	19
	Otop1^{mlh} (MGI:103206)	mergulhador	13	9; 10	14	63
	Pcdh15^{roda} (MGI:5987014)	rodador	13	9; 10	5	22
	Sacc (MGI:5987015)	sacudidor de cabeça	13	9; 10	3	18
Totals for BALB/c background	**10**				**152**	**1106**
Others strains	10		17; 16	1; 3	47	95
Totals for all strains	**43**				**847**	**6540**

Table S1. Initial logistic model.

Variables	Estimate	Standard error	*p* value
Group strain B6	0.474	0.096	< 0.001
Group strain 129	0.834	0.133	< 0.001
Other group strains	0.326	0.188	0.083
Rack row	-0.182	0.162	0.262
Number of pups	-0.168	0.220	0.443
Change of cage (Yes)	-0.555	0.047	< 0.001
Room (12)	-0.069	0.122	0.57
Room (15)	-0.246	0.127	0.053
Room (16)	-0.026	0.128	0.841
Room (17)	0.112	0.112	0.318
Room (corridor)	0.073	0.129	0.571
Barbering	-0.205	0.275	0.455
Canibalism	0.491	0.295	0.096
Removal of male	0.191	0.335	0.569
Inflammatory processes	-0.331	0.558	0.553
Temperature	-0.170	0.011	< 0.001
Humidity	-0.041	0.004	< 0.001
Week of observation	-0.014	0.003	< 0.001
Death in the cage	0.292	0.097	0.003
Age of pups (< 7 days)	0.523	0.105	< 0.001
Age of pups (7 to 15 days)	0.048	0.098	0.626
Age of pups (15 to 21 days)	-0.392	0.100	< 0.001
Rack row * Temperature	0.008	0.007	0.239
Number of pups * Temperature	0.004	0.009	0.669
Strain group B6 * Number of pups	-0.028	0.024	0.25
Strain group 129 * Number of pups	-0.036	0.031	0.252
Other strain groups * Number of pups	-0.021	0.056	0.704

Table S2. Final logistic model considering 95% confidence interval limits for the exponentiated coefficients.

Variables	Estimates	Standard error	Exponentiated coefficient	*p* value	Lower limit	Upper limit
Strain group B6: 1	-1.238	0.238	0.290	< 0.001	0.182	0.462
Strain group B6: 2	-0.588	0.098	0.556	< 0.001	0.458	0.673
Strain group B6: 3	-0.284	0.102	0.753	0.005	0.617	0.919
Strain group B6: 4	-0.219	0.161	0.803	0.173	0.586	1.100
Strain group 129: 1	-1.350	0.358	0.259	< 0.001	0.129	0.523
Strain group 129: 2	-0.865	0.141	0.421	< 0.001	0.319	0.556
Strain group 129: 3	-0.606	0.138	0.546	< 0.001	0.416	0.715
Strain group 129: 4	-0.719	0.205	0.487	< 0.001	0.326	0.728
Other strain groups: 1	-0.795	0.401	0.452	0.048	0.206	0.992
Other strain groups: 2	-0.472	0.193	0.624	0.014	0.427	0.910
Other strain groups: 3	-0.293	0.186	0.746	0.115	0.519	1.074
Other strain groups: 4	0.490	0.355	1.632	0.167	0.814	3.271
Number of pups	0.107	0.015	1.113	< 0.001	1.080	1.146
Change of cage (Yes)	0.553	0.047	1.738	< 0.001	1.586	1.905
Temperature	0.118	0.032	1.125	< 0.001	1.056	1.199
Humidity: 1	0.012	0.010	1.012	0.220	0.993	1.032
Humidity: 2	0.036	0.004	1.037	< 0.001	1.028	1.046
Humidity: 3	0.050	0.004	1.051	< 0.001	1.042	1.060
Humidity: 4	0.054	0.006	1.056	< 0.001	1.043	1.069
Week: 1	-0.019	0.013	0.981	0.156	0.956	1.007
Week: 2	0.013	0.004	1.013	< 0.001	1.006	1.020
Week: 3	0.015	0.004	1.015	< 0.001	1.008	1.022
Week: 4	0.025	0.006	1.026	< 0.001	1.015	1.037
Death in the cage	-0.289	0.097	0.749	0.003	0.620	0.906
Age of pups (< 7 days)	-0.560	0.105	0.571	< 0.001	0.465	0.701
Age of pups (7 to 15 days)	-0.068	0.098	0.935	0.489	0.771	1.132
Age of pups (15 to 21 days)	0.374	0.099	1.453	< 0.001	1.197	1.764

RESULTS

The number of cages and observations relating to each of the studied strains, along with the locations and identities of the corresponding IVC systems, are presented in Table 1. Distribution of the 6540 observations according to month of data collection (Figure 1A) reveals gaps in December 2018 and January 2019. Figure 1B displays the ratio of cages followed-up per week over the study period and the Kaplan-Meier estimated curve of follow-up time, from which it may be observed that there was a decrease over time in the number of cages followed-up and that the mean follow-up time was 10 weeks. The distribution of observations according to nest-building score was symmetrical with a modal value of 3 (Figure 1C), while the mean nest score was also 3 when data collected throughout the study period were analyzed together (Figure 1D). The distribution and modal values of nest-building scores assigned by the two observers are shown in supplementary file Figure S2.

Observer 1 was found to award scores of 1 and 5 more frequently than observer 2 as reflected by a p-value < 0.001 in the X^2 test for homogeneity.

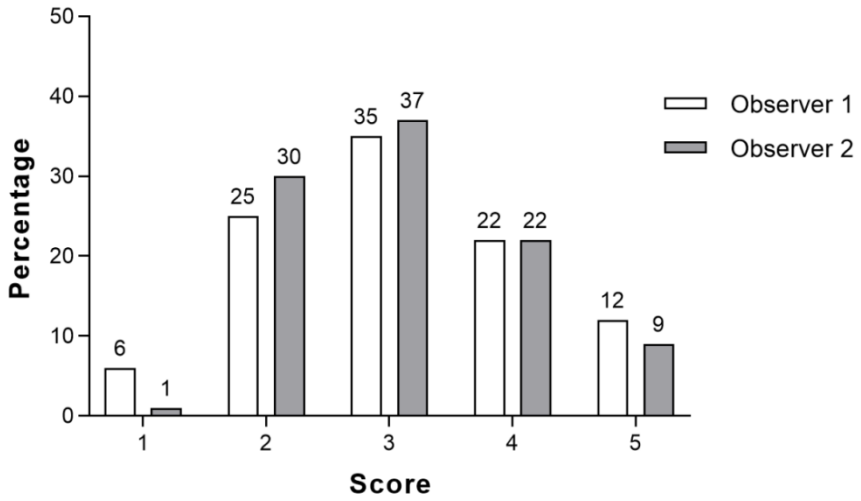


Figure S2. Score distribution by observer: Observer 1 – Total number of observations = 3596; score 1 (219) to score 5 (420); and Observer 2 – Total number of observations = 2944; score 1 (18) to score 5 (274).

There was significant dependence between the location of the nest in the cage (i.e. front, middle or back) and the nest-building score according to the X^2 test for independence ($p < 0.001$). As can be seen in Figure 2A, nests built in the middle of the cage typically presented lower scores than those located in the front or back, while analysis of the correspondence map of nest locations and nest-building scores (Figure 2B) confirmed that nests at the front of the cage were assigned the highest scores with those in the middle were attributed the lowest. The distribution of nest locations according to clustered strains (Figure 2C) revealed strong associations of the BALB/c group with nests located in the back of the cage and of the B6 group with back-built nests, while the correspondence map (Figure 2D) revealed the slightly superior performance of the 129 group strains and a marked difference in cage behavior of mutant strains with the BALB/c background, which had the lowest scores.

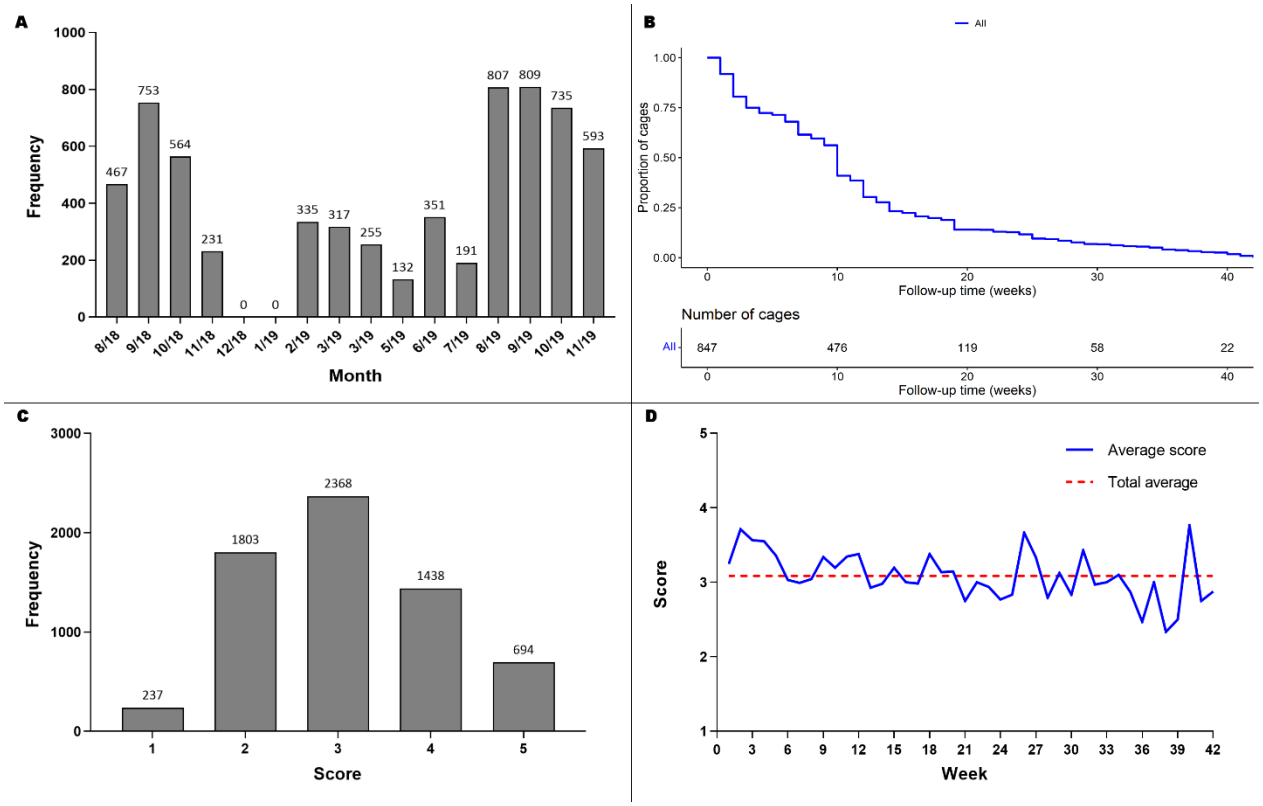


Figure 1. Distribution of nest-building observations during the study period. (**A**) observations per month; (**B**) proportion of cages accompanied per week; (**C**) distribution of scores over the complete study period; and (**D**) average score per week of observations in which the cage was not changed.

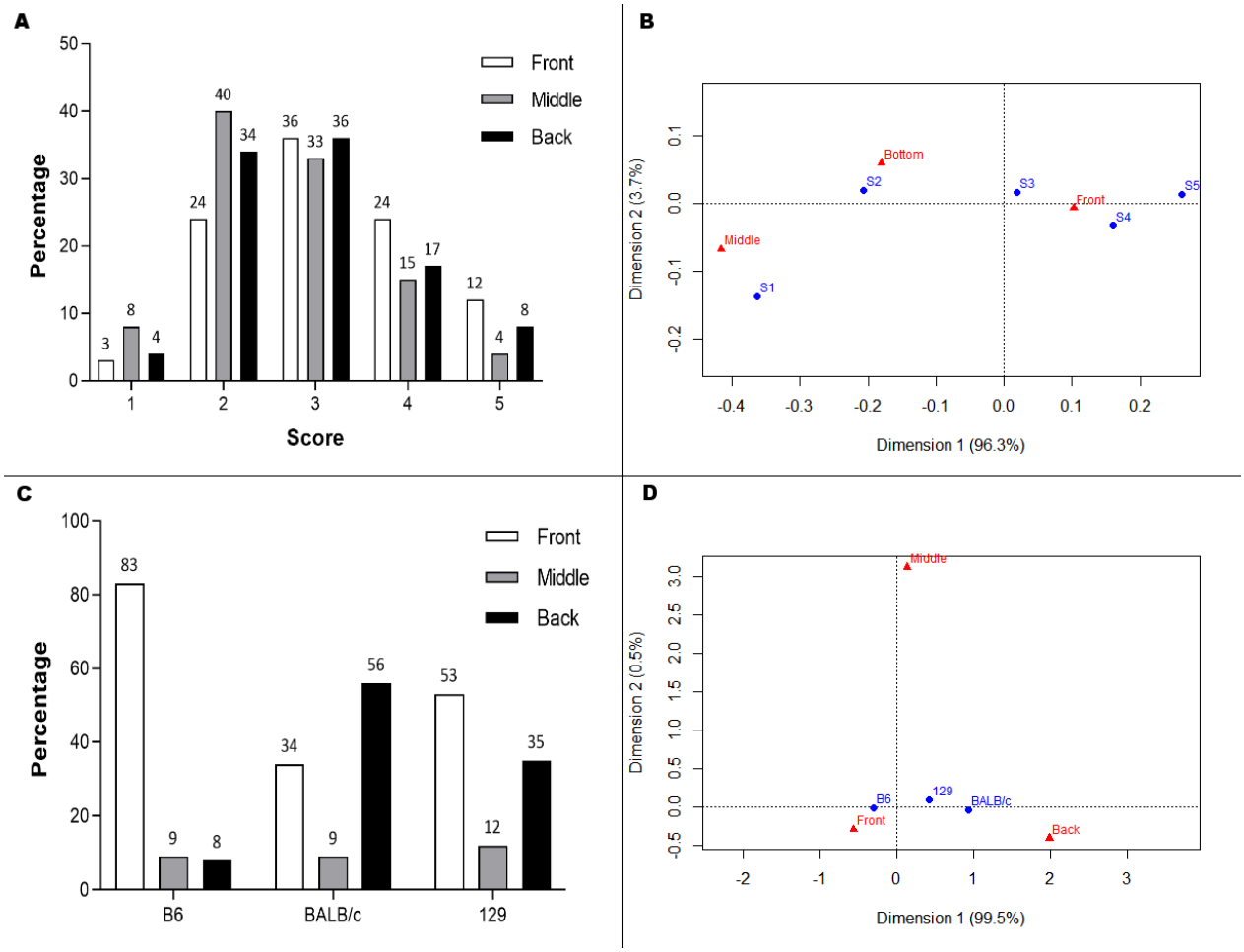


Figure 2. Nest-building scores in relation to categories of nest location (front, middle or back of cage) and genetic background (B6, BALB/c or 129). (**A**) Nest scores distributed by nest location in the cage ($p < 0.001$ for all scores except score 3 for which $p = 0.308$); (**B**) Correspondence map showing nest location within the cage categories in red and nest score indicators in blue (S1 = score 1, S2 = score 2, S3 = score 3, S4 = score 4, and S5 = score 5); (**C**) Distribution of nest location in the cage according to genetic background; and (**D**) Correspondence map showing categories of nest location in the cage in red and clustered lineage (genetic background) in blue.

With respect to the variable column and row position of a cage within the IVC system, it was not possible to find a systematic pattern of differences associated with specific locations. It is important to note, however, that the cages could change their position from one week to another. Comparison of the distribution of scores as functions of the variables occurrence or non-occurrence of barbering, cannibalism, inflammatory processes and male removed from the cage, showed that these events had no effect on nest-building scores. In contrast, a death in the cage resulted in a significant increase ($p = 0.003$) in score in comparison with cages in which there were no deaths. However, death events occurred only occasionally with only 6% of all cages encountering the death of an animal.

Application of the X^2 test for homogeneity to compare nest scores according to whether the cage was changed or not revealed a significant difference ($p < 0.001$) in score distribution, with the mean score for cage change being 2.9 while the corresponding score for no cage change was 3.3. Moreover, comparison by McNemar´s test of nest scores immediately before and after a cage change confirmed a significant effect of this variable ($p < 0.001$) (Table 2). On the other hand, when nest scores determined immediately before and after the birth of a litter were compared using McNemar´s test, no significant effect ($p = 0.901$) was found (Table 3). However, several trends were detected in that scores of 3 and 4 were most frequent in cages without pups, while a score of 3 was common in cages with pups up to 7 days old, and scores of 2 and 3 were more usual in cages with pups aged 7 to 21 days. These observations are confirmed by the closeness of the scores in the correspondence map of score by age of pups in the cage (Figure 3A) and in the distribution of nest score by profile of the pups (Figure 3B) in which cages with 16 to 21-day-old hatchlings exhibited scores closer to 2.

Table 2. Distribution of scores immediately after cage change as a function of score immediately before the event.

Score prior to cage change	Score after cage change				
	1	2	3	4	5
	Number of observations				
1	12	25	33	15	4
2	26	275	306	122	46
3	32	372	411	186	74
4	25	191	272	155	59
5	15	78	101	89	48

Table 3. Distribution of scores immediately after the birth of pups as a function of score immediately before the event.

Score prior to birth of pups	Score after birth of pups				
	1	2	3	4	5
	Number of observations				
1	8	22	22	13	8
2	18	134	178	104	37
3	23	204	259	142	53
4	18	101	148	91	37
5	7	46	55	44	32

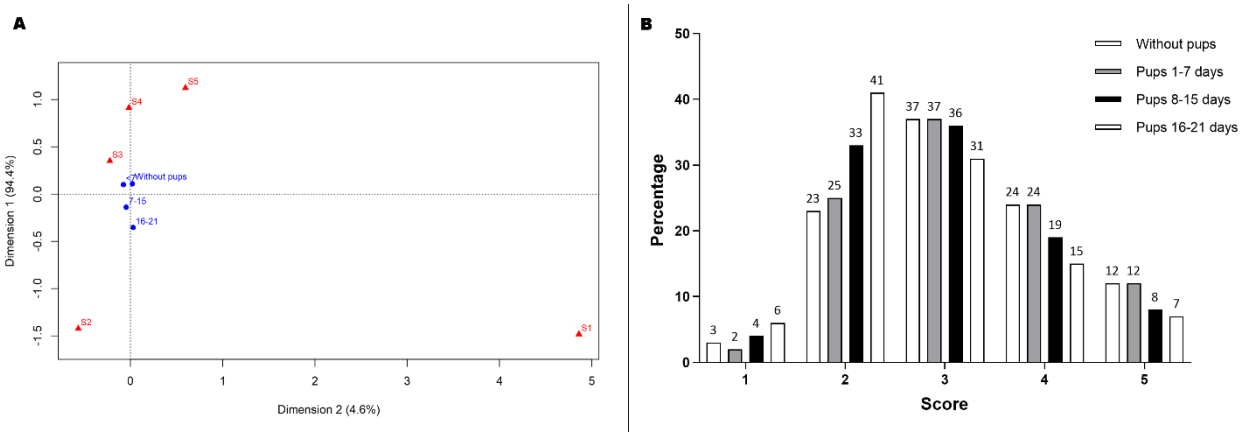


Figure 3. Nest-building scores in relation to the ages of pups in the cage. (**A**) Correspondence map showing nest score indicators in red (S1 = score 1, S2 = score 2, S3 = score 3, S4 = score 4, and S5 = score 5) and age of pups (grouped according to age) in blue; and (**B**) Distribution of nest scores by absence or presence of pups (grouped according to age). For all scores $p < 0.005$.

Based on logistic model analysis, it is clear that the probability of a lower nest score increases as the relative humidity in the environment increases. In order to interpret the results of the fitted model, we considered the exponentiated coefficients shown in the supplementary file (Tables S1 and S2), the values of which were obtained from estimates of the cumulative odds ratios.

DISCUSSION

Nesting is an evolutionarily important behavior in rodents and assessment of nest building has been used to evaluate daily life activity and overall well-being in laboratory mice.[2] In managing an animal facility, it is important to select and provide valid reliable nesting materials that are both freely available and readily stored. According to Neely *et al.*,[2] protocols involving shredded paper are more reliable than those incorporating other materials when evaluated by different researchers. However, an earlier study had demonstrated that mice prefer nesting material in the form of sheets, such as paper towels or tissues, rather than material provided in strips.[4] Therefore, in the present investigation, paper towel sheets were selected as the standardized form of environment enrichment.

In our observational study, nest scores were assessed as part of the regular laboratory routine, and there was a gap in data collection between December 2018 and January 2019 coinciding with staff vacations. Although the mean nest score values assigned by the two observers involved in the study were very close, fluctuations were detected between them. The problem of subjective nest

classification has been highlighted by other authors,[9] and the requirement to use more observers in order to calculate a mean of multiple ratings and, thereby, reduce bias in qualitative ratings, has been highlighted.[2] However, this strategy would require more time and personnel to score the nests and would increase costs incurred by the animal facility.

Although nest-building scores recorded in this study tended towards 3 regardless of mouse strain, analysis of the strains by genetic background revealed differences in nesting performance, with higher scores for those in the 129 group and lower scores for mutant strains with the BALB/c background. This finding contrasts with that of an earlier report,[10] which claimed that the interaction between strain and nesting material was not significant for nesting performance. On the other hand, a more recent study provided confirmatory evidence for the effect of genetic background by demonstrating that nest building varied between mice strains with different genetic make-up.[2] Mice with the BALB/c background were found to be shallower builders, although the difference between strains was not due to the transgene of interest.

Our novel analysis of the relationship between strains clustered by genetic background and nest position in the cage showed a strong association of the BALB/c group with back-built nests and of the B6 group with front-built nests. It has been assumed in the literature that the position of the nest in the cage is related to cold stress and aversion to IVC ventilation.[11] However, Wirf [12] found no differences in nest positions when three modified IVC systems were employed in which the air supplies were located in different positions. In this study, the back of the cage was reported as the most popular place for nesting by nude (NMRI-Foxn1nu) and B6 (C57BL/6N) mice in all IVC systems tested. In the present study, different behaviors were observed within the gene clusters even when mice were housed in the same IVC system and under the same environmental conditions. An alternative explanation for the observed differences in nest positions is that the upper rows in the IVC system may be closer to the light, and this might influence the evaluation in some way. This hypothesis was refuted, however, because no systematic pattern of differences was observed between nest scoring and the location of the cage rows on the IVC.

The finding in the present study that increasing relative humidity has a negative effect on nesting confirms a previous report by Wirf.[12] According to this author, increased humidity can cause the temperature to appear higher than it

actually is, thus resulting in a low nest score because the female does not then require a compact and insulated nest. Variations in cage humidity may be related to the IVC system[13] or to the volume of bedding available in the cage.[14] A further important aspect of high humidity is that it may give rise to an increase in the incidence of mortality in pups prior to weaning.[15] It is believed that high relative humidity (75% and above) can significantly affect ammonia emission interactions between mouse strain and cage materials,[16] although another study suggests that temperature and humidity are not significant predictors of ammonia concentration.[14] Nevertheless, it is our view that increased concentrations of ammonia may exert a negative effect on animal welfare, and this may give rise to a decrease in nesting scores. We propose that, in future studies, relative humidity in the cage is measured and correlated with ammonia concentration and litter volume in order to test the hypothesis. In addition, the effect of animal room temperature on the nesting behavior of mice requires further consideration since it is known from previous studies that nest scores decrease as temperature increases and the smaller nests result in increased heat loss.[17] In general, IVC systems have higher temperatures than animal rooms, and the higher the temperature the more likely females are to build nests that are of somewhat lower quality.[12]

According to Martin *et al.*,[18] breeding mice with pups tend to build higher quality nests than those without pups. This behavior is consistent with the expectations of nest construction by rodents since females are highly motivated to build nests to aid in thermoregulation and to protect their pups. In the present study, we observed that cages housing breeding pairs with recent litters exhibited the highest mean nest complexity with nests in cages with pups aged up to 7 days having scores close to 3, while cages with pups aged 16 to 21 days were assigned lower scores. This finding is in agreement with an earlier study involving nude and C57 strains of mice in which nest scores decreased as the pups grew.[12] Cages with pups that are close to weaning tend to be messier, while older pups disrupt nesting because of the increase in the number of animals housed in the cage. Clearly, the number of pups born per litter must also be considered since a higher population density generates more heat and ammonia in the cage and this can affect animal welfare.

Nest building is essential component of parental care, hence changes in nest structure or nest-building behavior are likely to affect survival and reproduction.

When female mice have access to nesting material and are able to build nests in the first few days after parturition, maternal stress is reduced and nest building is related positively to the number of pups born and weaned.[12] Adequate nesting material allows mice to reduce heat loss, thereby releasing energy for reproduction, lactation and subsequent pup growth, resulting in an increase in the number of pups born and weaned, a reduction in pup mortality (number of pups weaned/born), and an overall improvement in reproductive rates.[19] However, analysis of nest scores determined immediately before and after the birth of a litter revealed that there were no significant effects related to the event. Interestingly, nest-building behavior appeared to be maintained even in the absence of young.

The ability to nest is relatively conserved within a genus, but species prioritize nest-building behavior differently.[20] The present study employed immunodeficient strains that are, in general, more sensitive to handling and environmental factors. In this context, the provision of nesting material early in life can improve the well-being of all mice in a colony by reducing stress and strengthening their ability to care for the pups. Moreover, giving the animals an element of control over their environment serves to protect them from stressful situations, which can help prevent stereotypic behaviors.[18,21] However, the occurrence of abnormal behaviors such as barbering and cannibalism was so low in our study that it was not possible to determine whether these events influenced nest-building scores. According to Moody *et al.*,[22] the use of nesting material that causes the mouse to spend more time nesting may help reduce barbering behavior. Another factor that may be relevant in determining the quality of nest build is the amount of time that the animal is exposed to the nesting material. Martin *et al.*[18] reported that nest scores improved when mice were given additional time (48 h) to construct their nests using small, short, rolled-up pieces of paper as nesting material. Thus, in contrast to other studies that assessed nesting 24 h after a cage change,[2] we chose to assess nests three and fourteen days after the change. An improvement in nest score was observed when the animal spent more time with the nesting material, and a significant effect on nest score was observed after cage change.

In the present study, nesting outcomes were observed to differ significantly between cages with mortalities and those without such incidents. Although the mortality rate encountered in our study (6.24%) was considered reasonable[19,23] and the number of deaths was low, the data obtained has implications for nest evaluation

and highlights the importance of nest score as an indicator of mouse health. Nesting behavior has been described as an ethologically promising indicator for assessing welfare without affecting animal behavior and serves as a guide for the detection stress in mice, whether from cage aggressors or disease.[7] Moreover, some researchers have reported that mice can be given up to 6 g of paper nesting material without affecting the ability of care providers to identify mice that require veterinary assistance during daily inspection.[5]

The relevant and previously unpublished data on nest building by different strains of laboratory mice presented in this study demonstrate that nesting behavior is sensitive to macro- and micro-environmental changes. A significant correlation between nest-building score and nest position was discovered, highlighting the fact that different genetic backgrounds perform differently in terms of quality of nest construction. Cage change provoked one of the most significant negative effects on nest score, while an increase in the relative humidity of the environment resulted in lower nest scores. It is concluded that score-based nest classification methodology, when used consistently in routine management, is a valid parameter for determining the quality of life of the mice.

Acknowledgements

We are grateful for assistance from Mr. Dourival Ferreira Macarenhas at the Experimental Mice Facility of the Immunology Department ICB/USP.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was funded by Hospital Israelita Albert Einstein and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; grant code 001).

Data availability

The data that support the findings of this study are available from the corresponding author (LC) upon reasonable request.

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CAPÍTULO 3

Gut Microbiota profile of C57BL/6J mice derived from different breeding facilities

ABSTRACT

The fecal microbiome of twenty C57BL/6J mice from two Brazilian animal facilities was analyzed using 16s RNA gene clone libraries for analyzing the gut microbiota diversity of laboratory mice. This study is a comparable representation of resultant microbial diversity existing in different animal facility mice populations and that the representative gut microbiota of an animal facility can be monitored using this methodology. *Lachnospiraceae*, *Muribaculaceae,* and *Oscillospiraceae* were the most abundant family in 16S rRNA profiling based on amplicon sequencing and shotgun sequencing data for both animal facilities. Differences between the phylum could be statistically significant when comparing *Firmicutes* and *Bacterioidetes* group, but the Firmicutes/Bacteroidetes ratio was not statistically significant comparing the microbiome bacterial of the two facilities. The purpose of this study is also to understand if the Firmicutes/Bacteroidetes ratio could determine health status in mice. Our study shows there are variations in the intestinal microbiome of the animals comparing the facilities: *Intestinimonas* and *Pseudoflavonifractor* are the bacterial genus statistically significant differences; *Lachnospiraceae*, *Muribaculaceae,* and *Oscillospiraceae* are the bacterial families with a statistically significant difference. Both animal facilities were confirmed positive for *Helicobacter spp*. Comparing gender, the microbiome of males has more quantity of *Bacterioidales* than females: the significant difference was the family *Prevotellaceae* in the microbiome of the Animal Facility 1, and the specie *Muribaculum intestinale* in the microbiome of the Animal Facility 2. Considering this study, the gut microbiome of mice needs to be evaluated in other strains and sex to understand the variations between the laboratory mice themselves and environmental and management factors.

Keywords: Gut Microbiome; Mice; 16S rRNA; Mouse Intestinal Bacteria

INTRODUCTION

There are several effects that can make replication of experiments difficult. Most often, a study is not tenable because the researcher is unaware that he or she's conducting a slightly different experiment. Increasingly, researchers are questioning the potential impact of the microbiome on research. Variability in the composition of the gut microbiota of mice may play an important role in experimental models [1].

Any environmental factor, including psychological stress, can alter experimental outcomes. A related reading of the "reproducibility crisis" affecting biomedical research suggests that it's actually two separate crises, characterized by low experimental reproducibility within and between laboratories, but also by the reproducibility of scientific results between different species, including translatability to humans. While certain factors related to experimental design and statistical analysis have been identified as a cause of low reproducibility, a wealth of data suggests that the gut microbiome is a critical factor in the reproducibility and translatability of research results obtained with animal models [2].

The gut microbiome, a complex community of bacteria, viruses, protozoa, and fungi found in the gut of humans and animals, plays an important role in host health and disease. Biomedical research often uses animal models to study human disease, and the microbiome can change due to the effects of many factors. In particular, variations in the microbiome can contribute to differences in the phenotypes of disease models [3].

Much of today's biomedical research is conducted in rodents, which have complex and often uncharacterized microbiomes. This potential source of variability is increasingly drawing attention to the question of how best to care for laboratory animals. The gut microbiome varies widely and can be modulated by environmental variables [4]. The list of factors that can influence the microbiome of laboratory animals is growing and includes animal origin [5,6], diet and cage type [7], bedding type [7,8], water treatment [8], transport [9], housing density [10], sex [11,12], and genetics [6,13,14], among other factors. Even in apparently identical batches of mice - same strain, same supplier - and under the same conditions (same cage type, same bedding, same room), differences in gut microbiome composition have been observed [15].

It's known that these microbial communities often exist in a complicated balance that, when disturbed (i.e., dysbiosis), promotes susceptibility to disease. Due to numerous functional redundancies, the composition of these colonies can vary

dramatically in healthy individuals. Thus, the question arises as to how we can account for the microbiota when designing experiments and modeling reproducibility, and how we can take advantage of the extensive variation in laboratory mouse research [4].

With the advent and advancement of state-of-the-art sequencing technology, it's possible to characterize this complex microbiological ecosystem without the need for culturing, which has led to an explosion of information and new challenges in interpreting the data obtained. It's important to know the gut bacteria of laboratory mice, and this can be done by determining operational taxonomic units (OTUs) using 16s ribosomal RNA sequencing [16]. Previous studies describe microbial diversity in different strains [17,18] and also as a function of sex [19] and age [17].

However, the identification of microorganisms from laboratory mice can be extremely difficult. Species are constantly changing, making standardization completely impossible and largely unmeasured. Complicating matters further, some of these bacteria are critical to the health and immune response and help to ensure that research is robust and meaningful [15].

A possible starting point for such standardization could be the definition of the microbiome of the major rodent producers. Although the composition may change slightly after shipment to other facilities, these changes are minimal compared to the differences between colonies. In experiments where the gut microbiome is of interest, monitoring should be conducted throughout the study to avoid unforeseen circumstances during the experiment. The microbiome can be maintained in rodent colonies for many generations if appropriate management techniques are used. These include: ventilated racks, exchange of cages in disinfected biosafety cabinets, exchange of personal protective equipment between animals (especially personal protective equipment such as gloves and sleeves that come into contact with the animals), and use of irradiated or autoclaved food, water, and bedding [4].

The use of sentinels in laboratory animal facilities may aid in the control of important mouse pathogens that are already known to confound results. However, it is suspected that the zeal for cleanliness may have eliminated some of the most complex microorganisms that make mice useful models for human disease. Variations in the microbiome can skew results, but exposure to microorganisms could be key to some studies and make the mouse a more realistic model of the human immune system. But we are still getting used to all this, learning about the universe of

the gut microbiome, along with a hard-won culture of cleanliness and the notion that tightly controlled mice make research more reproducible. It is anticipated that the fecal microbiome will be included in the materials and methods section of articles in the coming years as an important parameter for reproducibility of studies with experimental animals [15].

The Firmicutes/Bacteroidetes (F/B) ratio is widely accepted to have an important influence on the maintenance of normal intestinal homeostasis in humans [20]. In mice, some studies evaluate the F/B ratio as an indicator of intestinal dysbiosis in obese models [21], different diets [22] and the relationship with animal weight [23].

Current data also suggest that cecal samples may be a better indicator of environmental influences on the gut microbiome, and the use of fecal samples may lead to “false negatives” in screening for effects on the microbiome [7].

Therefore, the objective of our study was to determine and compare the composition of the cecal intestinal microbiome of C57BL/6J mice and the F/B ratio from two different animal facilities, using a methodology based on 16S rRNA. Our interest is to try to define indicators such as the F/B rate and microbiome patterns under similar management conditions.

MATERIALS AND METHODS

Animals and Housing

Male and Female C57BL/6J mice were housed in two different breeder locations: Center for Experimentation and Surgery Training of Hospital Israelita Albert Einstein (1), an AAALAC-certified facility, and the Institute of Biomedical Sciences, University of São Paulo (2). All animal procedures described in this study were performed according to the Brazilian guidelines for animal care (CONCEA) and approved by IACUC: CEUA_Einstein number # 4037-20 and CEUA_ICB # 8442040522. Mice have housed in individually ventilated Alesco® cages, with a ventilation rate between 40-45 air changes per hour, and bedding with wood flakes (Good Life™, Granja RG, SP, Brazil). Cardboard tunnels were used to enrich the environment (Relax™, Granja RG, SP, Brazil). Pellet food (irradiated food, Nuvilab CR1™, Quimtia, PR, Brazil) and autoclaved-only (1) or acidified and autoclaved (2) drinking water was offered *ad libitum*. Room temperature varied between 19 and 25ºC, humidity ranged from 41 to 65%, and rooms were on a 12:12 hour light-dark cycle (lights on from 07:00 to 19:00).

All mice were specific pathogen-free (SPF) for ectromelia virus, lymphocytic choriomeningitis virus, murine minute virus, mouse hepatitis virus, mouse parvovirus, murine pneumonia virus, reovirus, Sendai virus, Theiler murine encephalomyelitis virus, hantaviruses, cilia-associated respiratory bacillus, *Clostridium piliforme*, *Klebsiella pneumonia*, *Mycoplasma pulmonis*, *Pasteurella multocida*, *Pasteurella pneumotropica*, *Pseudomonas aeruginosa*, *Salmonella spp*, *Staphylococcus aureus*, *Streptobacillus moniliformis*, β-hemolytic *Streptococcus spp*, *Streptococcus pneumoniae*, endoparasites, and ectoparasites.

The C57BL/6 (B6) mouse is widely used in scientific research. In this study we sought to understand the extent of similarities and differences between mice from two different production sites in the same C57BL/6J strain. We evaluated the composition of the fecal microbiota in the mice as soon as they were received in the animal facility.

The microbiome of 20 eight-week-old mice was analyzed; five females and five males from Animal Facility 1 and four females and six males from Animal Facility 2. Both B6J came from the Jackson Lab to begin breeding in their respective animal facilities.

Mice were randomly assigned, euthanized with overdose anesthesia (ketamine 300mg/kg and xylazine 30mg/kg) and luminal contents of the cecum were collected during necropsy in the morning, following recommendations by ERICSSON & FRANKLIN (2021). Microbial communities were characterized via 16S rRNA amplicon sequencing using the Illumina MiSeq platform.

Isolation of intestinal bacterial DNA, PCR amplification of 16S rRNA gene sequences, cloning, and sequencing

Total DNA was isolated from the caecum. Samples for determining OTU sequences were obtained following the best practices of sample collection, including the appropriate transport conditions to preserve the bacterial DNA, and were sent to Neoprospecta®(Brazil) for processing. After the DNA extraction, V3-V4 primers were chosen using the following conditions: the first PCR primers contain the Illumina sequences based on TruSeq structure adaptor (Illumina, San Diego, CA), allowing for the second PCR with indexing sequences. PCR reactions were always carried out in triplicate using Platinum Taq (Invitrogen, USA) under the following conditions: 95ºC for 5 min, 25 cycles of 95ºC for 4s, 55ºC for 30s and 72ºC for 45s, and a final

extension of 72ºC for 2 min for PCR 1. For PCR 2 the conditions were: 95ºC for 5 min, 10 cycles of 95ºC for 45s, 66ºC for 30s and 72ºC for 45s, and a final extension of 72ºC for 2 min. For comparison, the standard Illumina 16S protocol was used as described (Illumina Technical Note 15044223 Rev.B). The final PCR reaction was cleaned up using AMPureXP beads (Beckman Coulter, Brea, CA) and samples were pooled into sequencing libraries for quantification and processing. Amplicon estimations were performed using Picogreen dsDNA assays (Invitrogen, USA). Pooled libraries were diluted for accurate qPCR quantification using the KAPA Library Quantification Kit Illumina platform (KAPA Biosystems, Woburn, MA). Libraries were sequenced using the MiSeq system and standard Illumina primers provided in the kit. Usually, a single end 300nt run was performed. After sequencing, a bioinformatic pipeline performed sequence demultiplexing, adaptor, and primer trimming. The reads were normalized to 283 pb sizes. Clustered sequences (OTUs) were then classified by comparison with the 16S rRNA database (NeoRefdb, Neoprospecta Microbiome Technologies, Brasil). Sequences with at least 99% identity with the reference database were assigned to their respective OTUs.

Statistical analysis

Once a final set of OTUs was generated, a full description of statistical parameters was performed, including central tendency (mean), variation (standard deviation), or other basic estimates. Statistical analyzes were performed on different samples and the statistical test was used bilaterally to understand the OTUs of each mouse, sex, and site of origin. The relative contribution of relevant metadata traits and their interactions to the overall explanation of variability in the microbial community was assessed using MANOVA. Repeated measures ANOVA were used to assess changes in diversity. Statistical analysis was performed using the Jasp software, and OTUs with adjusted p-values < 0.05 were considered significant.

RESULTS

There are statistical differences between the presence of *Firmicutes* and *Bacteroidetes* comparing the microbiome of the two animal facilities ($p<0.01$). Figure 1 represents mean percentage of bacteria per phylum of mice from each animal facility. Although, the *Firmicutes* and *Bacteroidetes* ratio (FB ratio) was not statistical significant comparing the microbiome per gender of the two animal facilities ($p=0.07$; figure 2 and table 1).

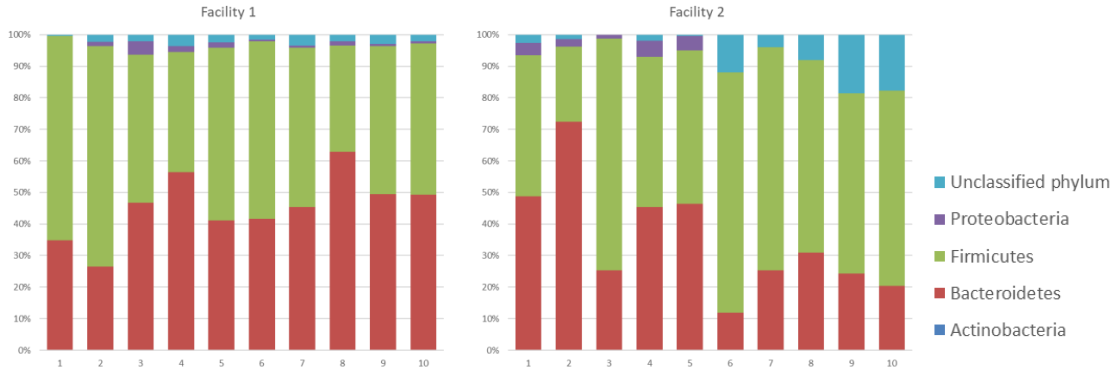

Figure 1. Percentage of phylum bacterial comparing the microbiome of the animal facilities.

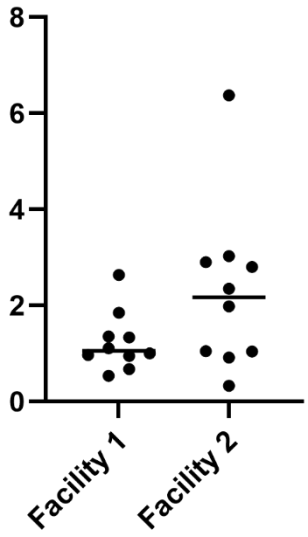

Figure 2. Firmicutes and Bacteroidetes ratio (F/B ratio) of the intestinal microbiome mice analyzing each animal facility.

Table 1. Descriptive Statistics of gender and animal facility comparing F/B ratio.

Descriptive Statistics	Female Einstein	Female ICB	Male Einstein	Male ICB
Sample number	5	4	5	6
Mean	1.501	1.299	0.986	2.932
Median	1.335	0.983	0.971	2.576
Std. Deviation	0.770	1.114	0.298	1.827
Shapiro-Wilk	0.960	0.862	0.955	0.847
P-value of Shapiro-Wilk	0.810	0.266	0.772	0.150
Minimum	0.675	0.327	0.537	1.045
Maximum	2.637	2.903	1.356	6.378

Lachnospiraceae, *Muribaculaceae,* and *Oscillospiraceae* were the most abundant family for both animal facilities. *Lachnospiraceae* corresponds to 25% of Animal Facility 1 and 21% of Animal Facility 2 microbiome colonies; *Muribaculaceae* to 23% of mice microbiome of Animal Facility 1 and 19% of Animal Facility 2; *Oscillospiraceae* to 9% of the mice microbiome of Animal Facility 1 and 6% of Animal Facility 2.

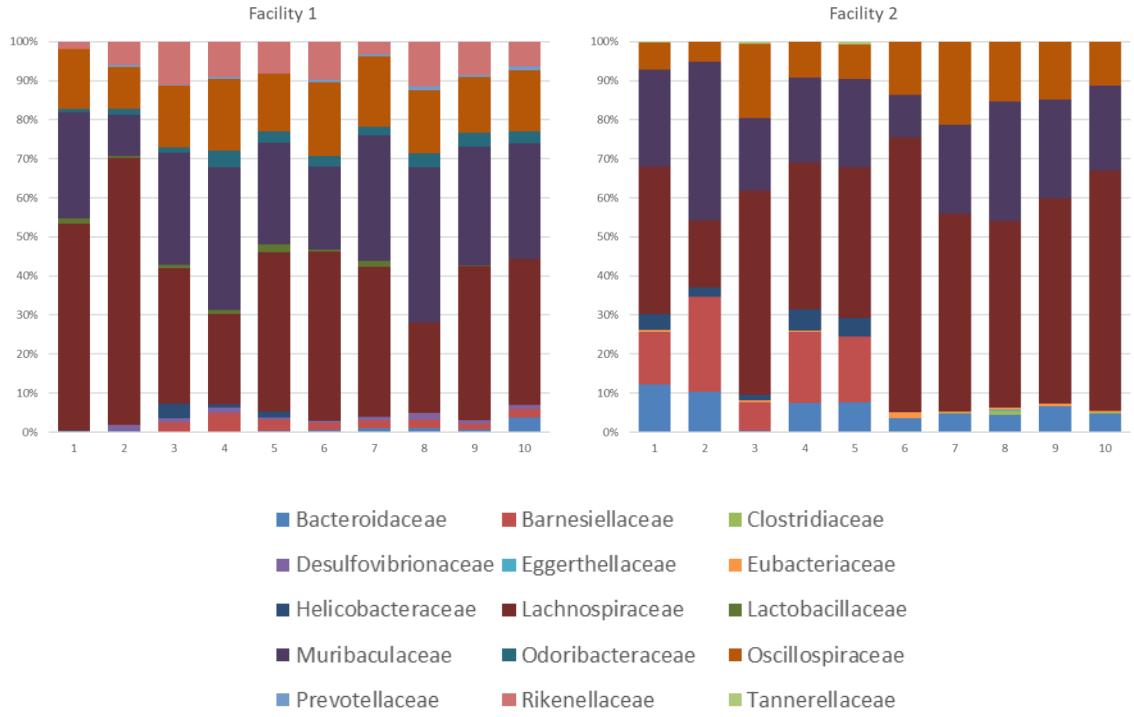

Figure 3. Percentage of each animal's bacterial family compared to the microbiome of the animal's facilities.

The main difference between the mice intestinal microbiome of the two animal facilities was the presence of genus bacterial *Intestinimonas* (p=0.023) and *Pseudoflavonifractor* (p=0.009)*;* and of families bacterial *Lachnospiraceae* (p<0.001), *Muribaculaceae* (p=0.001) and *Oscillospiraceae* (p<0.001). *Helicobacter sp.* was detected in both animal facilities.

Comparison of samples from gender significant differences were found for the order *Bacteroidales* (p=0.026). In the Animal Facility 1, there is more quantity of the family *Prevotellaceae* (p=0.023) in the intestinal microbiome male mice (1% of the microbiome) than females; and, in the Animal Facility 2, there is more quantity of the specie *Muribaculum intestinale* (p=0.019) in the microbiome of the male mice (4% of the microbiome) than females.

DISCUSSION

As with any animal model, we must always be aware of translatability to humans. This is also true when we consider the gut microbiome. While the gut physiology of rodents and humans is similar, there are also striking differences. Rodents and humans have co-evolved microbiomes, and although they are quite similar both functionally and at the taxonomic and genus levels, they rarely share the same species [4].

Like the human gut microbiota, the mouse microbiome is mainly composed of two dominant bacterial phyla, Firmicutes and Bacteroidetes, which account for more than 90% of the total community [24–28]. However, the species richness of a mouse can vary from animal facility to animal facility, as can the abundance of certain microorganisms [14]. This difference in abundance was observed among the animal facilities examined in our studies with variation among the genera *Intestinimonas* and *Pseudoflavonifractor* and the families *Lachnospiraceae*, *Muribaculaceae*, and *Oscillospiraceae*. Thus, we can highlight the supplier as a crucial factor in the variation of the microbiome.

The resident microbiota of mice is a new problem. The microbiome can change for a variety of reasons, including diet and cage type [7], bedding [7,8], water treatment [8], transport [9], housing density [10], sex [11,12]. Some researchers suggest that stress, such as separation from the mother, can also alter the microbial ecosystem of a mouse. Today, more and more laboratories are sequencing fecal samples for bacterial genes. The first step in defining a "normal" microbiome of laboratory rodents may be to analyze the microbiome of your suppliers' mice [15].

Current data suggest that the contents of the cecum may be a better indicator of environmental influences on the gut microbiome, and this was the main reason we sampled only the cecum of the animals analyzed in our study. Although the use of feces as a representative sample offers several obvious advantages, such as noninvasive collection (and thus the possibility of longitudinal studies with repeated measurements), collection and analysis of cecal content should also be considered in end-stage studies, especially those examining the effects of environmental influences on the microbiome [7]. We can also emphasize the importance of knowing precisely the gut microbiome from which the strains used are derived by periodically creating

banks of stool samples that are stored in a freezer at -80°C to provide a historical record of the microbiome in each colony as well as a potential source for reinoculation if needed [2].

The composition of the gut microbiome can be influenced by the health status of the host, which often leads to the question of whether the observed differences in microbiome composition between healthy and diseased individuals are causative or merely correlative. However, despite the common goal of optimal health and pathogen elimination, the rearing of laboratory rodents is not standardized. There are many subtle differences in laboratory animal husbandry, including but not limited to bedding, water treatment, cage ventilation, and diet. There are also significant differences between regions of the gastrointestinal tract in terms of the density and composition of the luminal microbiota. Therefore, it is reasonable to assume that disease- or treatment-related effects on the microbiome may go unnoticed in studies based solely on fecal samples [7].

The main difference between the two facilities examined in our study is the type of water treatment, one being sterilized and the other acidified. Another study reported that evaluation of the composition of cecal samples revealed a pattern dependent on water treatment in the most common OTUs, e.g., the families *Bacteroidales* and *Lachnospiraceae* [8]. We also found this pattern in our study between facilities, with higher average OTUs for the family *Bacteroidales* in Tier 2 and higher average OTUs for the family *Lachnospiraceae* in Tier 1. In contrast to the results of BIDOT, ERICSSON, and FRANKLIN (2018), *Akkermansia sp*, *Peptococcaceae*, and *Anaerostipes* bacteria were not found in the facility where animals received autoclaved water, and neither *Enterorhabdus* nor *Shuttleworthia* bacteria were found in the facility where animals received acidified water.

In a recent study, the microbiome of SPF mice from the Jackson Laboratory (Jax) and Envigo was found to be characterized by the same dominant colonizers (e.g., *Muribaculaceae*) but showed striking differences in the relative abundance of many other families, including *Prevotellaceae*, *Ruminococcaceae*, and *Erysipelotrichaceae* [3]. The provider-specific composition of the gut microbiome and provider-dependent differences in the abundance of different OTUs persisted for up to 24 weeks, especially in the *Bacteriodetes* family [29].

Consistent with our results, *Muribaculaceae* and *Lachnospiraceae* were the predominant families identified in the phyla *Bacteroidetes* and *Firmicutes* and related

to the normal mouse gut microbiome [30]. Moreover, two families, *Lachnonospiraceae* and *Oscillospiraceae*, which dominate *Firmicutes*, showed high abundance in our mouse microbiome and also in the human microbiome [28]. On the other hand, the family *Muribaculaceae*, which dominates the *Bacterioidetes*, was also abundant in our mouse microbiome, but it is smaller compared to the human microbiome [28]. High diversity of *Lachnospiraceae* has been found in other studies [18,21,24]. It is difficult to predict the functional role of the abundant *Lachnospiraceae* family, which may promote intestinal homeostasis [31]. Abundance of this bacterium has been associated with a group of gut health [21], on the other hand, it has also been associated with stress in mice [32].

Recent studies have begun to characterize the differences between the microbiomes of mice from different suppliers and different genetic backgrounds, as well as mice bred using different breeding methods, and how these factors contribute to microbial changes when mice are placed in a different animal facility [6,9,14,33]. These studies show that differences in community composition profiles reflect a complex interplay of known and undetermined factors, highlighting the importance of ongoing efforts to understand baseline variations in mouse microbial profiles.

Regarding variation in the gut microbiome among rodent colonies and its contribution to the reproducibility of rodent models, it is perceived that disease models within the same laboratory may exhibit differential disease expression over time. Disease severity can vary significantly depending on inherited gut microbiota alone, providing evidence that microbiota can contribute to phenotypic differences between rodent colonies [4]. A previous study suggests that provider-dependent differences in the microbiome exert model-specific influences depending on the disease mechanism. This study compared different providers, analyzed the microbiome and disease severity in mouse models, and found significant changes in disease manifestation, independent of background genetics [34].

Although different model phenotypes have been reported in mice from different providers, the composition and uniformity of the fecal microbiota in mice of different genetic backgrounds from different providers is unclear. The abundance of many operational taxonomic units, often identified at the species level, and several higher taxa differed depending on the supplier and lineage. These differences were evident in the fecal microbiota of weaned mice and persisted throughout the study up to twenty-four weeks of age. These data provide the first in-depth analysis of the

developmental course of the fecal microbiota in mice from different suppliers and a starting point from which researchers can refine animal models affected by differences in the gut microbiota [14].

Notable among these are the segmented filamentous bacterium (SFB), which plays a role in the development of Th17 immunity in the intestinal mucosa, and rodent *Helicobacter*, which have been shown to serve as low-grade inflammatory triggers against "commensal" gut microbiota. The effects of *Helicobacter* and SFB colonization in disease models are not yet known, but given their prevalence in rodent colonies, they are prime candidates for phenotype confounding and should be considered in the absence of reproducibility [4].

We detected Helicobacter spp. in the two animal facilities examined in our study. Although Helicobacter is on the FELASA exclusion list [35], it is a common colonizer of research mice in research facilities around the world and remains clinically undetectable in most mouse strains. However, this bacterium is often thought to induce immune responses against the background mucosal microbiome in genetically susceptible hosts. In contrast to the nonspecific Th17 immune response elicited by SFB, Helicobacter spp. have been associated with Th1 immune responses in the past. Interestingly, as with many other Proteobacteria, their colonization may depend on the presence or absence of SFB [2].

In mice, sex-specific differences in the overall profile of the gut microbiome between males and females have been observed in several studies, although these depend on the particular experimental setup and it may still be difficult to separate them from environmental influences (e.g., housing). As with many studies of the mouse microbiome, baseline microbial communities may differ between facilities. Another study showed that the composition of the gut microbiota in male mice differed significantly from that of female mice, regardless of diet. Similar to another study, C57BL/6 mice showed a higher relative abundance of *Muribaculaceae* in males compared to females [36]. The order *Bacteroidales* was more abundant in male C57BL/6 mice, consistent with another study [37]. An almost opposite result was observed in wild-type B6.129S mice at another institution, where males had higher relative abundance of the gut-typical *Ruminococcaceae* and *Anaerostipes*, while females had higher relative abundance of the less characteristic *Peptostreptococcaceae* [12]. In a larger experiment with 89 different strains of mice, some differences in the gut microbiome were consistent between the sexes, but most

differences were host-specific and therefore possibly environmentally dependent [11]. The *Clostridiaceae* and *Lachnospiraceae* families were most abundant in female mice - a remarkable mix of clades that in some cases are not even present in the human gut but are present in mice [38]. Sex also affects the richness and diversity of the gut microbiome, so the use of both sexes in mouse studies remains an essential element of experimental design [29].

Abundant RNA-based phyla (*Firmicutes* and *Bacteroidetes*) correlated strongly with differences in housing conditions, with significant individual associations for access barriers, food handling, presence of other mice, and housing type [23]. The ratio of *Firmicutes* to *Bacteroidetes* (F/B ratio) has been extensively studied for the gut microbiota of humans and mice. It is generally accepted that the *Firmicutes/Bacteroidetes* (F/B) ratio has an important impact on the maintenance of normal gut homeostasis. Increased or decreased F/B ratios are considered dysbiosis, with the former generally observed in obesity and the latter in inflammatory bowel disease (IBD). Data from animal models show consistent differences in the two major bacterial phyla, with a significant increase in *Firmicutes* and a decrease in *Bacteroidetes* in obese mice compared with wild-type mice, although their diet and activity levels are similar [39]. Because the F/B ratio has been associated with the obesity phenotype [39], RAUSCH *et al.* (2016) examined the relationship between the F/B ratio and a minimum body weight of 30 g in DNA-based samples, and the results were significant in mice. These results imply a non-trivial relationship between animal weight and broad taxonomic composition, which is only present in permanent (DNA-based) microbial communities [23]. Our study showed a variation of F/B ratio from 1.5 to 3.0, suggesting that the mean value of this indicator in each animal facility can be defined as a marker of health status.

The considerations outlined above highlight the challenges and opportunities for the management to identify and report on the characteristics of the gut microbiome in their colonies. Specifically, a production colony could be routinely examined using 16S rRNA amplicon sequencing to identify differences in long-term experimental outcomes. Assessment of the microbiome could be integrated into health surveillance programs in which mice serve as historical controls and sentinels over time. It is also important for scientists to understand that when using animals from different providers, they will be working with models with different microbiomes.

CONCLUSION

16s RNA sequencing can be used to determine the microbial diversity in the gut of mice used in experimental and preclinical research. Mice housed in different facilities and bred using different breeding methods may have different compositions of their gut microbiome. Because we know that the gut microbiota can contribute to the variability of experimental results obtained in mouse models, it is important to have a monitoring program to detect pathogens and understand the microbiome pattern in each animal facility. We believe that the F/B ratio can be monitored as an indicator of mouse health and help reduce variability in experimental research.

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3 CONSIDERAÇÕES FINAIS

Considerando os aspectos éticos e o incentivo por medidas para melhorar a reprodutibilidade da pesquisa empregando modelos animais, nosso estudo demonstra a necessidade de considerar os indicadores, escore de ninho e microbiota intestinal, na gestão de instalações de camundongos de laboratório.

Diferenças entre as instalações de camundongos, genética das linhagens e protocolos de manejo têm demonstrado contribuir para a variação nos fenótipos e para a não reprodutibilidade dos resultados experimentais.

As considerações descritas neste trabalho apresentam ambos os desafios e oportunidades, para as pessoas que trabalham com os animais de laboratório.

O bem-estar animal reflete positivamente no resultado das pesquisas científicas. O estresse pode acarretar diversas consequências relevantes à fidedignidade dos resultados experimentais. E como avaliar se os animais de laboratório estão em bem-estar, até mesmo relacionado as suas emoções? Como reconhecer o estresse, antes de qualquer manifestação clínica? O escore de ninho foi uma proposta neste estudo, inicialmente a fim de avaliar o seu uso pelos camundongos de laboratório, e entender seu comportamento frente a diversas variações do ambiente onde o animal é alojado. O objetivo final foi averiguar um padrão de construção de ninho nos camundongos de laboratório, e torna-lo uma ferramenta na rotina do bioterista, por exemplo durante as trocas de gaiolas, para avaliar o bem-estar dos camundongos de forma individual por gaiola nas diferentes linhagens ou sexos, e até mesmo entre os casais com ou sem filhotes.

Em um esforço para descrever e explicar a variação na construção de ninho nas instalações de animais foi realizada este trabalho com camundongos de duas instalações de camundongos diferentes localizadas em uma instituição pública e outra em uma instituição privada por um período de aproximadamente quatro anos. O escore de ninho 3 foi um padrão predominante em todas as linhagens, segundo a escala adaptada pelo nosso grupo de estudo; e avaliamos os efeitos em relação a diversas variáveis: tipo de gaiola, temperatura e umidade, observações ao longo do ano (estação do ano e meses), linhagem, idade, quantidade e sexo dos animais presente na gaiola, posição da gaiola na rack ventilada, posição do ninho dentro da gaiola, tipo de enriquecimento ambiental oferecido na gaiola e seu uso como material de nidificação, refúgio ou excreção.

As nossas conclusões são que linhagem, idade, posição da gaiola e posição do ninho na rack ventilada têm influencias sobre o escore. Entre os enriquecimentos ambientais, o uso do rolinho influencia o escore de ninho, dependente do modo que é utilizado pelo animal. Não encontramos efeito significativo da quantidade de animais na gaiola. Achados interessantes incluem a umidade como uma variação considerável na qualidade de construção do ninho. Compatível com dados de literatura, observamos ainda escores de ninho maiores em temperaturas mais baixas (inferior a 20ºC).

Os gestores de biotérios são responsáveis por garantir a produção e manutenção de camundongos de laboratório a fim de fornecer aos pesquisadores animais de qualidade para a pesquisa. Existe a necessidade de treinar os técnicos que cuidam dos animais, para estarem aptos a reconhecer o bem-estar animal, utilizando por exemplo o escore de ninho. Isso traria informações praticas ao gestor sobre qualquer influência na colônia (como variações de temperatura ou alterações cognitivas).

Os gestores também precisam relatar as características da microbiota intestinal e patógenos presentes em suas colônias. Especificamente, uma colônia pode ser pesquisada via sequenciamento de amplicon de rRNA 16S a fim de relatar aos pesquisadores a microbiota das linhagens mantidas no biotério, e isto custaria pouco mais do que a bateria de exames de controle sanitário realizados em camundongos sentinela. Em outro contexto, os cientistas precisam estar cientes dos fatores discutidos neste estudo e a ter uma visão de futuro em relação à modelos de camundongos usados em suas pesquisas.

Em nosso estudo, o que avaliamos foi que qualquer mudança no manejo, como o tratamento da água, pode influenciar na composição da microbiota. Também destacamos a taxa de *Firmicutes* e *Bacteroides*, como um provável indicador de saúde. Não chegamos a um valor de referência da taxa F/B nos camundongos, que possa refletir uma microbiota saudável. Mas fica uma motivação para estudos futuros aprofundarem este indicador na gestão da saúde de animais de laboratório, agregando na documentação do controle sanitário preventivo de animais de laboratório.

4 PERSPECTIVAS FUTURAS

Há a necessidade de esforços contínuos para considerar as fontes de variação em modelos animais e entender como elas contribuem para a reprodutibilidade experimental.

A composição da microbiota tendo ou não impacto nos efeitos dos fenótipos do modelo animal e reprodutibilidade do estudo deverá ser considerada pelos pesquisadores e determinada nos indivíduos de animais estudados, como um indicador de saúde, além do controle sanitário, um indicador populacional do biotério.

Controlar e documentar fatores ambientais é importante para reduzir fontes de variação imprevistas. A variação entre animais pode ser extensa, mesmo quando controlado por outros fatores, e combinado com diversos outros efeitos relacionados ao próprio animal e ambiente onde é mantido.

Inicialmente, em nosso estudo acreditávamos que existiria um padrão de microbiota entre os camundongos, mesmo quando mantidos com o mesmo manejo. Os estudos ainda precisam ser continuados, mas o que percebemos é que a microbiota pode variar ao longo do ano, dentro do mesmo biotério, e um interesse é avaliar a microbiota em relação as variáveis macro e microambientais, como realizado para o escore de ninho.

Já se sabe que os filos abundantes (*Firmicutes, Bacteroidetes, Proteobacteria*) correlacionam-se fortemente com as diferenças nas condições de manejo, com associações individuais significativas para barreira de acesso, tratamento de comida, presença de outros camundongos e tipo de alojamento (RAUSCH *et al.*, 2016). Nosso interesse agora é avaliar a microbiota ao longo do ano, e verificar suas modificações em relação a temperatura e umidade, estação do ano e provavelmente correlacionar com o escore do ninho, podendo estabelecer uma relação entre bem-estar e saúde, ou seja, avaliar o equilíbrio físico e mental dos animais de laboratório.

Um outro interesse é avaliar o microbioma ambiental e seu efeito na microbiota intestinal de camundongos de laboratório. As contagens de coliformes e níveis de lipopolissacarídeos (LPS) como metodologia para avaliação de higienização adequada é indicada pelo GUIDE (NATIONAL RESEARCH COUNCIL, 2011). Mas isto não demonstra a complexidade do microbioma ambiental envolvido.

Numerosos estudos investigaram o impacto da densidade de alojamento, frequência de troca de gaiola, sistema de gaiola e ventilação, e outras variáveis relacionadas com a criação do ponto de vista do bem-estar animal, mas muito pouco explorou sobre a influência dessas variáveis na composição da microbiota intestinal dos animais.

Os avanços nas tecnologias de sequenciamento permitiram uma avaliação robusta de microbiota e os modelos animais desempenham um papel crítico no estabelecimento de relações causais entre a microbiota intestinal e a doença. Apesar dos esforços para manter a consistência genética e padronização dos processos de produção do camundongo, a microbiota intestinal do camundongo é sensível à variação ambiental, e as diferenças resultantes podem contribuem para a variação nos resultados, dependendo do design, origem dos animais, condições de alojamento e outros fatores.

Surpreendentemente, o alojamento em gaiolas abertas, sem uso de racks ventiladas, diminui a diversidade de espécies, embora este resultado contraditório possa ser explicado devido altos padrões de higiene presentes nessas barreiras. Características como gaiola não ventilada individualmente, a presença de outras cepas de camundongos e acesso irrestrito às instalações parecem aumentar a variação interindividual de camundongos. (RAUSCH *et al.*, 2016). Muito trabalhos mostram que fatores associados à pesquisa de roedores, incluindo fornecedor, sistema de gaiola, e tipos de cama podem alterar a microbiota intestinal de camundongos de laboratório. Dados de microbiota cecal revelaram mudanças ao comparar os métodos de descontaminação da cama e da água, destacando a complexidade pela qual os fatores ambientais interagem para modular a microbiota (BIDOT; ERICSSON; FRANKLIN, 2018).

Com isso, nossos esforços seguirão nesta linha de pesquisa, na padronização destes indicadores como ferramenta na gestão *Triple Aim* de biotérios, avaliando a experiência individual de camundongos (bem-estar) e saúde populacional dos animais mantidos no laboratório, de maneira ágil e consequentemente reduzindo custos e trazendo qualidade para a experimentação animal.

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APÊNDICES

Apêndice 1. Resultados de controle sanitário conforme recomendações da FELASA - Federation of European Laboratory Animal Science Associations (Berard *et al.*, 2014) das instalações animais analisados durante este trabalho.

	BIOTÉRIO	**ICB**						**Einstein**								**METODOLOGIA**
	DATA	**out/18**		**abr/19**		**set/20**		**fev/18**		**out/18**		**abr/19**		**set/20**		
	Local de realização	**Alchemy**		**FMVZ-USP**		**FMVZ-USP**		**FMVZ-USP**		**Alchemy**		**FMVZ-USP**		**FMVZ-USP**		
	Linhagem	**C57BL/6**		**C57BL/6**		**C57BL/6 CD4**		**Balb/C; *nude***		**Balb/C**		**B6; MTMG**		**Balb/C**		
ENDOPARASITAS	Aspiculuris tetráptera	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Chilomastix bettencourti	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Cryptosporidium spp.	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Entamoeba spp.	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Giardia muris	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Hymenolepsis diminuta	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Hymenolepsis nana	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Syphacia spp.	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Spironucleus muris	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia / PCR
	Tritrichomonas muris	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
ECTOPARASITAS	Demodex musculi	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Myobia musculi	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Myocoptes musculinus	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Notoedres muris	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Ornitonyssus bacoti	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Polyplax spp.	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Psorergates simplex	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Radfordia spp.	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
	Trichoecius rombotsi	Neg.	0/2	Neg.	0/1	Neg.	0/2	Neg.	0/5	Neg.	0/2	Neg.	0/2	Neg.	0/3	Microscopia
VÍRUS	Mouse hepatitis virus (MHV)	Neg.	0/4	Neg.	0/1	Neg.	0/2	Neg.	0/3	Neg.	0/3	Neg.	0/2	Neg.	0/3	Elisa
	Mouse rotavirus (EDIM)	Neg.	0/4	Neg.	0/1	Neg.	0/2	Neg.	0/3	Neg.	0/3	Neg.	0/2	Neg.	0/3	Elisa
	Murine norovirus (MNV)	Neg.	0/4	Neg.	0/1	Neg.	0/2	+	1/3	+	1/3	+	2/2	+	2/3	Elisa
	Minute virus of mice (MVM)	Neg.	0/4	Neg.	0/1	Neg.	0/2	Neg.	0/3	Neg.	0/3	Neg.	0/2	Neg.	0/3	Elisa

	Mouse parvovirus (MPV)	Neg.	0/4	Neg.	0/1	Neg.	0/2	Neg.	0/3	Neg.	0/3	Neg.	0/2	Neg.	0/3	Elisa
	Theiler's murine encephalomyelitis virus (TMEV)	Neg.	0/4	Neg.	0/1	Neg.	0/2	Neg.	0/3	Neg.	0/3	Neg.	0/2	Neg.	0/3	Elisa
	Lymphocytic choriomeningitis virus (LCMV)	Neg.	0/4	Neg.	0/1	Neg.	0/2	Neg.	0/3	Neg.	0/3	Neg.	0/2	Neg.	0/3	Elisa
	Mouse adenovirus type 1 (FL)	Neg.	0/4	Neg.	0/1	Neg.	0/2	Neg.	0/3	Neg.	0/3	Neg.	0/2	Neg.	0/3	Elisa
	Mousepox (ectromelia) vírus	Neg.	0/4	Neg.	0/1	Neg.	0/2	Neg.	0/3	Neg.	0/3	Neg.	0/2	Neg.	0/3	Elisa
	Pneumonia virus of mice (PVM)	Neg.	0/4	Neg.	0/1	Neg.	0/2	Neg.	0/3	Neg.	0/3	Neg.	0/2	Neg.	0/3	Elisa
	Reovirus type 3 (REO)	Neg.	0/4	Neg.	0/1	Neg.	0/2	Neg.	0/3	Neg.	0/3	Neg.	0/2	Neg.	0/3	Elisa
	Sendai virus (SEND)	Neg.	0/4	Neg.	0/1	Neg.	0/2	Neg.	0/3	Neg.	0/3	Neg.	0/2	Neg.	0/3	Elisa
	Hantaan virus (HANT)	NR		Neg.	0/1	Neg.	0/2	Neg.	0/3	NR		Neg.	0/2	Neg.	0/3	Elisa
	Mouse cytomegalovirus (MCMV)	NR		Neg.	0/1	Neg.	0/2	Neg.	0/3	NR		Neg.	0/2	Neg.	0/3	Elisa
	Parvovirus (NS-1)	NR		Neg.	0/1	Neg.	0/2	Neg.	0/3	NR		Neg.	0/2	Neg.	0/3	Elisa
	Mouse pneumonitis virus (K)	NR		Neg.	0/1	Neg.	0/2	Neg.	0/3	NR		Neg.	0/2	Neg.	0/3	Elisa
	Polyoma virusn(POLY)	NR		Neg.	0/1	Neg.	0/2	Neg.	0/3	NR		Neg.	0/2	Neg.	0/3	Elisa
BACTÉRIAS	Bordetella bronchiseptica	NR		Neg.	0/1	Neg.	0/2	Neg.	0/5	NR		Neg.	0/2	Neg.	0/3	PCR
	Corynebacterium kutscheri	NR		Neg.	0/1	Neg.	0/2	NR		NR		Neg.	0/2	Neg.	0/3	PCR
	Mycoplasma pulmonis	NR		Neg.	0/1	Neg.	0/2	Neg.	0/5	NR		Neg.	0/2	Neg.	0/3	PCR
	Pasteurella pneumotropica	NR		Neg.	0/1	Neg.	0/2	Neg.	0/5	NR		Neg.	0/2	Neg.	0/3	PCR
	Streptococcus pneumoniae	NR		Neg.	0/1	Neg.	0/2	Neg.	0/5	NR		Neg.	0/2	Neg.	0/3	PCR
	Salmonella spp.	NR		Neg.	0/1	Neg.	0/2	Neg.	0/5	NR		Neg.	0/2	Neg.	0/3	Cultura
	Proteus mirabilis	NR		Neg.	0/1	Neg.	0/2	NR		NR		Neg.	0/2	Neg.	0/3	Cultura
	Streptobacillus moniliformis	NR		Neg.	0/1	Neg.	0/2	NR		NR		Neg.	0/2	Neg.	0/3	PCR
	Helicobacter spp.	NR		Neg.	0/1	+	2/2	+	5/5	NR		+	2/2	+	3/3	PCR
	Streptococcus pyogenes	NR		Neg.	0/1	Neg.	0/2	Neg.	0/5	NR		Neg.	0/2	Neg.	0/3	PCR
	Citrobacter rodentium	NR		Neg.	0/1	Neg.	0/2	Neg.	0/5	NR		Neg.	0/2	Neg.	0/3	Cultura
	Staphylococcus aureus	NR		Neg.	0/1	Neg.	0/2	Neg.	0/5	NR		Neg.	0/2	Neg.	0/3	Cultura / PCR
	Klebsiella oxytoca	NR		Neg.	0/1	Neg.	0/2	Neg.	0/5	NR		Neg.	0/2	Neg.	0/3	Cultura
	Klebsiella pneumoniae	NR		Neg.	0/1	Neg.	0/2	Neg.	0/5	NR		Neg.	0/2	Neg.	0/3	Cultura
	Pseudomonas aeruginosa	NR		Neg.	0/1	Neg.	0/2	Neg.	0/5	NR		Neg.	0/2	Neg.	0/3	Cultura