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LUIZ MARCIO DA SILVA

AVALIAÇÃO DA PRESENÇA DE CONTAMINANTES FÚNGICOS E BACTERIANOS NAÁGUA E NO AR DE BIOTÉRIO PARA ROEDORES UTILIZADO PARA PESQUISAS CIENTÍFICAS

UBERLÂNDIA
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Orientador: Prof. Dr. Carlos Henrique Gomes Martins

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ATA DE DEFESA - PÓS-GRADUAÇÃO

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Reuniu-se, de forma Virtual, a Banca Examinadora designada pelo Colegiado do Programa de Pós-graduação em Biologia Celular e Estrutural Aplicadas, da Universidade Federal de Uberlândia, assim composta: **Dra. Loyane Bertagnolli Coutinho – UFU; Profa. Dra. Regina Helena Pires – UNIFRAN e Prof. Dr. Carlos Henrique Gomes Martins**; orientador(a) do(a) candidato(a).

Iniciando os trabalhos o(a) presidente da mesa, **Prof. Dr. Carlos Henrique Gomes Martins**, apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as), que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

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O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

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RESUMO

A contaminação microbiana em biotérios é perigosa para humanos e animais. Portanto, os padrões sanitários devem sempre ser mantidos para evitar a contaminação microbiana nessas instalações. Este estudo teve como objetivo avaliar o nível de contaminação do ar e da água e identificar o microrganismo isolado de um biotério de roedores localizado na Universidade Federal de Uberlândia, Minas Gerais, Brasil. Unidades formadoras de colônias (UFC) por mililitro foram usadas para monitorar quantitativamente a água, enquanto UFC por metro cúbico foi usado para o ar. A identificação microbiana de colônias isoladas foi realizada para monitoramento qualitativo. O número médio total de microrganismos na água variou de 0,015 ± 0,02 a 0,999 ± 0,91 UFC/mL. O número de microrganismos no ar variou de 9,1 ± 4,6 a 351,56 ± 158,2 UFC/m³. Quarenta e um microrganismos identificados nas amostras do biotério de roedores eram potencialmente patogênicos e/ou oportunistas tanto para animais quanto para humanos. Concluindo que tanto as amostras de água quanto de ar apresentaram contaminação de microrganismos potencialmente patogênicos e oportunistas que podem prejudicar tanto roedores quanto humanos. Com base em nossas observações, padrões sanitários específicos adequados para essas instalações devem ser desenvolvidos para controlar a contaminação microbiana, o que evitará zoonoses e garantirá confiabilidade nos resultados científicos dos experimentos com animais.

Palavras-chave: água, ar, biotério, roedores, fungos, bactérias, micróbios

ABSTRACT

Microbial contamination in animal facilities is hazardous for both humans and animals. Hence, sanitary standards should always be maintained to prevent microbial contamination in these facilities. This study aimed to evaluate the level of air and water contamination and identify the isolated microbial of rodent facility located at the Federal University of Uberlandia, Minas Gerais, Brazil. Colony forming units (CFU) per milliliter was used for quantitatively monitoring water, while CFU per cubic meter was used for air. Microbial identification of isolated colonies was performed for qualitative monitoring. The total mean number of microorganism in water ranged from 0.015 ± 0.02 to 0.999 ± 0.91 CFU/mL. The number of microorganism in air ranged from 9.1 ± 4.6 to 351.56 ± 158.2 CFU/m³. Forty-one microorganisms identified in the samples of the rodent facility were potentially pathogenic and/or opportunistic for both animals and humans. Concluding that both water and air samples showed contamination of potentially pathogenic and opportunistic microorganisms that can harm both rodents and humans. On the basis of our observations, specific sanitary standards suitable for these facilities should be developed for controlling microbial contamination, which will prevent zoonosis and ensure reliability in scientific results of animal experiments.

Keywords: waterborne, airborne, rodent facility, fungi, bacteria, microbial

LISTA DE ABREVIATURA E SIGLAS

CONCEA	Conselho Nacional de Controle da Experimentação Animal
Ciurca	Sistema de Cadastro de Uso Científico de Animais
CEUAs	Comissões de Éticas em Uso de Animais
NBA-1	Nível de Biossegurança Animal 1
NBA-2	Nível de Biossegurança Animal 2
NBA-3	Nível de Biossegurança Animal 3
NBA-4	Nível de Biossegurança Animal 4
Felasa	*Federation of European Laboratory Animal Science Associations*
REBIR	Rede de Biotério de Roedores
UFU	Universidade Federal de Uberlândia
UFC	Unidade Formadoras de Colônias

Sumário

Capitulo 1. Fundamentação Teórica

BIOTERISMO

Por mais de um século o bioterismo, prática do uso de modelos animais em pesquisas científicas, ocorre devido a constante modernização e evolução das ciências biomédicas, humana e veterinária. Contudo, por muito tempo essa prática ocorreu sem que houvesse a devida preocupação ética e de qualidade sanitária, onde os animais eram utilizados como instrumentos de trabalho, não havendo a estrutura adequada, condições higiênicas ou treinamento para realização das atividades (ANDRADE; PINTO; OLIVEIRA, 2002).

No Brasil, o marco que regulamentou a utilização de animais em pesquisas científicas ocorreu através da Lei Arouca, Lei nº 11.794/2008 de 15 de julho de 2009. O Decreto presidencial dispõe sobre a criação e a utilização de animais em atividades de ensino e pesquisas cientificas em todo o território nacional. Para garantir o cumprimento das diretrizes da Lei Arouca foram criados os órgãos: o Conselho Nacional de Controle da Experimentação Animal (CONCEA); e o Sistema de Cadastro das Instituições de Uso Científico de Animais (Ciuca). Foram também definidas as normas para funcionamento das Comissões de Éticas em Uso de Animais (CEUAs), com objetivo de garantir o atendimento ético e humanitário do uso de animais para fins científicos (BRASIL, 2008).

Atualmente, animais de laboratório são aqueles criados e produzidos sob condições ideais e mantidos em um ambiente controlado, onde ocorre acompanhamento genético e microbiológico. Os animais na natureza não satisfazem as exigências necessárias para experimentação biológica, pois não são submetidos a nenhum tipo de controle (ANDRADE; PINTO; OLIVEIRA, 2002).

A única forma de garantia da manutenção dos padrões de boa qualidade sanitária é a constante vigilância, visando o bem estar do funcionário de um biotério e, consequentemente, do animal por ele manipulado, e avanços confiáveis nas pesquisas que utilizam esses animais. Por isso biotérios, como os de roedores, são planejados e projetados obedecendo regulamentos, legislações e padrões criados para suprir as necessidades dos animais e pesquisas cientificas lá conduzidas, como instalações e equipamentos (MAJEROWICZ, 2019; POLITI; MAJEROWICZ; CARDOSO; PIETRO *et al.*, 2008).

Portanto, resumindo, um biotério é uma instalação com características próprias, que atende às exigências dos animais onde são criados ou mantidos, proporcionando bem-estar e saúde para que os animais possam se desenvolver e reproduzir, respondendo satisfatoriamente aos testes neles realizados (ANDRADE; PINTO; OLIVEIRA, 2002).

CONTAMINANTES AMBIENTAIS MICROBIOLÓGICOS

A contaminação biológica é um fator que obstaculiza a obtenção da boa qualidade laboratorial (ANVISA, 2019), a forma com que a contaminação pro microrganismos e suas toxinas ocorre no ambiente varia, e quando detectada necessita de investigação. Quando relacionado a contaminação do ar, o predomínio encontrado é a ocorrência por bioaerossóis, gerados no ambiente externo e carreadas para o interior pelo fluxo de pessoas, sistemas de ventilações ou portas e janelas (MIRSKAYA; AGRANOVSKI, 2018; ZHOU; LAI; TONG; LEUNG *et al.*, 2020).

A exposição contínua dos funcionários e animais a possíveis endotoxinas e exotoxinas bacterianas compromete o sistema imune, causando doenças respiratórias e gastrointestinais como enfisema pulmonar e inflamações intestinais, dentre outras, portanto, a presença de bactérias também deve ser investigada (LAI; HANG; ZHANG; SUN *et al.*, 2016). Os microrganismos mais comuns encontrados em amostras de ar são as bactérias *Bacillus* spp., *Staphylococcus* spp., *Micrococcus* spp., *Pseudomonas* spp. e *Enterococcus* spp., e os fungos *Penicillium* spp., *Aspergillus* spp., *Cladosporium* spp. e *Fusarium* spp. (VIANI; COLUCCI; PERGREFFI; ROSSI *et al.*, 2020).

Uma outra fonte ambiental que é afetada por contaminação microbiológica é a água, quando contaminada com fezes pode causar graves doenças ao homem e aos animais como cólera e febre tifoide, devido a isso a ausência de coliformes fecais se tornou um marcador para verificar a segurança da água (LECLERC; MOSSEL; EDBERG; STRUIJK, 2001; NOWICKI; DELAURENT; DE VILLIERS; GITHINJI *et al.*, 2021). No entanto, a presença de outras bactérias e fungos na água não pode ser ignorado, pois também possuem potencial patogênico, principalmente em organismos imunossuprimidos. A capacidade que algumas bactérias e fungos tem de formar biofilmes e se estabelecer mesmo em condições pobres de nutrientes é uma das principais características responsáveis pela contaminação na água (EDSTROM; CURRAN, 2003; KAUFFMANN–LACROIX; COSTA; IMBERT, 2016).

BIOSSEGURANÇA EM BIOTÉRIOS

O desenvolvimento das atividades em biotérios deve sempre ser realizada de acordo com as boas práticas, normas e biossegurança. Sendo um conjunto de medidas e procedimentos voltados para prevenção, controle, minimização ou eliminação de riscos provenientes das atividades que possam comprometer a saúde do homem, do animal, do meio ambiente ou da qualidade das pesquisas realizadas (TEIXEIRA; VALLE, 2010). Biotérios são classificados de acordo com a classe de risco dos agentes biológicos que são manipulados ali, sendo eles nível

de biossegurança animal 1 (NBA-1) onde o trabalho envolve agentes biológicos caracterizados e conhecidos por não provocarem doenças em humanos sadios; nível de biossegurança animal 2 (NBA-2) que usa agentes biológicos com risco individual moderado e baixo risco para a população; nível de biossegurança animal 3 (NBA-3) que utiliza agentes que apresentam potencial elevado de transmissão por aerossóis e risco de provocar doenças fatais; e nível de biossegurança animal 4 (NBA-4) com agente biológicos de potencial patogênico desconhecido representando alto risco individual e coletivo (BRASIL, 2006).

Além das normas de biossegurança a serem seguidas existem recomendações de controle de qualidade para biotérios, como da "*Federation of European Laboratory Animal Science Associations*" (FELASA), onde o monitoramento microbiológico da saúde dos animais é recomendado para assegurar validez e reprodutibilidade dos dados (FELASA, 2014). No entanto não existe nenhum tipo de regulamentação ou lei para controle de qualidade do ambiente dos biotérios no Brasil.

OBJETIVOS

Objetivo geral

Avaliar a presença de contaminantes bacteriológicos e fúngicos no ar e na água da Rede de Biotério de Roedores (REBIR) da Universidade Federal de Uberlândia (UFU).

Objetivos específicos

- Coletar amostras de água e ar do REBIR.
- Determinar a quantidade de Unidades Formadoras de Colônias (UFC) por mL de bactérias e fungos presentes na água da rede de abastecimento, filtrada e estéril do REBIR.
- Determinar a quantidade de Unidades Formadoras de Colônias (UFC) por m² de bactérias e fungos presentes no ar das salas de criação e alojamento do REBIR.
- Isolar e identificar os fungos e bactérias encontradas.

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Capitulo 2.

Monitoring of Waterborne and Airborne Microorganisms in a Rodent Facility

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Monitoring of Waterborne and Airborne Microorganisms in a Rodent Facility

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Running Title: Microbial contamination in rodent facility.

Abstract

Microbial contamination in animal facilities is hazardous for both humans and animals. Hence, sanitary standards should always be maintained to prevent microbial contamination in these facilities. This study aimed to evaluate the level of air and water contamination and identify the isolated microbial of rodent facility located at the Federal University of Uberlandia, Minas Gerais, Brazil. Colony forming units (CFU) per milliliter was used for quantitatively monitoring water, while CFU per cubic meter was used for air. Microbial identification of isolated colonies was performed for qualitative monitoring. The total mean number of microorganism in water ranged from 0.015 ± 0.02 to 0.999 ± 0.91 CFU/mL. The number of microorganism in air ranged from 9.1 ± 4.6 to 351.56 ± 158.2 CFU/m³. Forty-one microorganisms identified in the samples of the rodent facility were potentially pathogenic and/or opportunistic for both animals and humans. Concluding that both water and air samples showed contamination of potentially pathogenic and opportunistic microorganisms that can harm both rodents and humans. On the basis of our observations, specific sanitary standards suitable for these facilities should be developed for controlling microbial contamination, which will prevent zoonosis and ensure reliability in scientific results of animal experiments.

Keywords: waterborne, airborne, rodent facility, fungi, bacteria, microbial.

Introduction

Biological contamination in facilities used for housing animals intended for scientific research hinders the achievement and maintenance of good laboratory quality. (Majerowicz 2019) Constant vigilance of microbial presence is required, as the presence of microorganisms in these environments can cause serious diseases in humans and animals, and may negatively affect the quality of research conducted using these animals. (Felasa 2014, Majerowicz 2019) Therefore, Brazilian animal facilities are designed to comply with regulations, legislation, and standards created to meet the needs of animal and scientific research. (Politi et al. 2008, Brasil 2008)

Quality control programs aimed at maintaining animal health have been designed, (Felasa 2014) however, the environment of animal facilities also has to comply with the quality control standards, as water and air are sources of microbiological contamination. (Cincinelli and Martellini 2017, Dawson and Sartory 2000, Kauffmann–Lacroix et al. 2016, Rao et al. 1996, Strickland and Shi 2021, Zhou et al. 2020) Although known to be sources of contamination, Brazilian legislation does not define specific standards for environmental quality control of animal facilities.

Indoor air contamination occurs via bioaerosols, which, in most cases, are generated in the external environment and carried inside by people, ventilation systems, or doors and windows. (Mirskaya and Agranovski 2018, Zhou et al. 2020) The continuous exposure of employees and animals to possible bacterial endotoxins and exotoxins compromises the immune system, causing respiratory and gastrointestinal diseases such as pulmonar y emphysema and intestinal inflammation. (Lai et al. 2016)

Feces-contaminated water can cause serious diseases in humans and animals, such as cholera and typhoid fever. Hence, the absence of fecal coliforms is used as a marker to verify the safety of water. (Boelee et al. 2019, Leclerc et al. 2001, Nowicki et al. 2021) However, the

presence of fungi in water cannot be ignored, as they may be pathogenic, especially in immunosuppressed organisms. The ability to form biofilms on surfaces and thrive even in nutrient-poor places are primarily responsible for bacterial and fungal contamination in water. (Kauffmann–Lacroix et al. 2016, Edstrom and Curran 2003) Fungi belonging to the genus *Aspergillus* spp. are mainly responsible for disease outbreaks, and its dissemination is related not only to transmission by contaminated water, but also via air through bioaerosols. (Kauffmann–Lacroix et al. 2016)

Several studies have reported the identification of pathogenic and other fungi and bacteria in animal facilities for rodents, (Carriquiriborde et al. 2020, Na et al. 2010, Kunstyr et al. 1997) and quality control recommendations for ensuring the health of rodents have been implemented; however, these parameters do not cover the entire environment of the animal facilities. (Felasa 2014, Na et al. 2010, Mailhiot et al. 2020) Information regarding monitoring and maintenance of the microbiological quality of air and water is currently available, (Dawson and Sartory 2000, Kim et al. 2018, Leclerc et al. 2001, Edstrom and Curran 2003, Westall et al. 2015) which is essential for formulating ways of detecting and controlling infections caused by pathogenic and opportunistic microorganisms in animal facilities. (Schlapp et al. 2018, Cincinelli and Martellini 2017, Mansfield et al. 2010, K unstyr et al. 1997, Ooms et al. 2008, Westall et al. 2015)

For minimizing occupational risks, ensuring scientific quality, and identifying possible contamination points, we aimed to evaluate the presence of bacteriological and funga l contaminants in the air and water of a rodent facility used for scientific research in this study.

Materials and Methods

Characterization of the rodent facility

The rodent facility in the present study has a total area of 733 m², divided into several rooms. The floor plan of the evaluated rooms and their surroundings are shown in Figure 1. Air circulation in the rooms is through common air conditioning. Employees circulating in the breeding area wear disposable and sterile clothing, gloves and caps. The animals are kept in individual ventilation racks with HEPA filter (Tecniplast SpA, Buguggiate, Varese, Italy), which are changed annually, at 40-60% humidity, 20±1°C, and light and dark (12/12) cycles. The rodent's cage is sterilized together with the wood shavings, and they receive feed and filtered and sterilized water ad libitum. Cleaning and changing the cages is carried out weekly.

The cleaning and disinfection of the facility is carried out in cycles of 6 months using Virkon's solution (Antec International, Sudbury, Suffolk, United Kingdom) and 6 months using hypochlorite (Uzzi Quimica Ltda, Uberlândia, Minas Gerais, Brazil) in order to avoid microbial resistance.

Collection and sampling locations

All samples were obtained from the Central Animal Facility of the Animal Facility Network of the Federal University of Uberlandia, Minas Gerais, Brazil. Air samples were collected from three 6 × 6 m² breeding rooms, which were designated in this study as breeding room 1 (BR1), BR2, and BR3. In addition, they were also collected from two 6 × 4 m² housing rooms (HR), HR1 and HR2 (Fig. 1). External atmospheric air samples were used as external control (EC) and were collected from the surroundings of the rodent facility. Water samples were collected at specific points as follows: point (1) water supply system (drinking water, DW), (2) post-filtration water (filtered water, FW), and (3) water sterilized by saturated steam

under pressure (sterile water, SW). All air and water samples were assessed in triplicate and at three different time points, once a month for three months.

Microbiological evaluation of water

The microbiological content of water was evaluated using the total count of bacteria and fungi per milliliter according to the methodology recommended by "*American Public Health Association*". (APHA 2012) For evaluating bacterial presence, 500 mL water was collected from each collection point in sterile flasks and subsequently filtered using a 0.22 μm pore membrane (Millipore®). The membrane was placed in a Petri dish containing Reasoner's 2A agar (R2A, Difco, Detroit, MI, USA) for determining the total count of aerobic bacteria, mannitol agar (MA, Difco) for the recovery of *Staphylococcus* sp, cetrimide agar (CA, Difco) for *Pseudomonas* sp, and MacConkey Agar (MCA, Difco) for enterobacteria, and incubated at 37°C for 24 h in a bacteriological incubator. After the incubation period, the colony forming units per milliliter (CFU/mL) was determined (R2A).

The presence of yeasts was evaluated by collecting 1000 mL of water from each collection point and filtering with a 0.45 μm pore membrane (Millipore®). The membrane was placed in a Petri dish containing Sabouraud dextrose agar (SDA, (Difco) supplemented with chloramphenicol (30 mg/100 mL) (Sigma, St. Louis, MO, USA). The plates were incubated in a biochemical oxygen demand (BOD) incubator at 30°C for 48 h for fungal growth and subsequent enumeration of CFU/mL and re- incubated for 7 days to verify the development of yeasts.

The presence of filamentous fungi was evaluated in a similar way to that carried out for yeasts, the only change being the incubation time. After filtration the plates were incubated in BOD for two to three weeks, being examined from the second day, to verify the development

of filamentous fungi. The samples obtained from point 1 (water supply network) were treated with 1 mL 1.8% sodium thiosulfate per liter to remove residual chlorine. (APHA 2012)

Microbiological assessment of air

Microbiological quality of air was evaluated using the total count of bacteria and fungi per cubic meter. Briefly, a one-stage air sampler (MAS-100, Merck KGaA, Darmstadt, Germany) was placed in the center of all the five rooms evaluated (BR1, BR2, BR3, HR1, and HR2) at a height of approximately 1.5 m from the ground, and the sampler was programmed to collect 500 L of air for 5 min after impacting the Petri plates (90 mm), as the device allows aspiration of particles with diameter greater than 1 μm.

Tryptic soy agar (TSA, Difco) was used for determining the total count of aerobic bacteria, MA (Difco) for the recovery of *Staphylococcus* sp, CA (Difco) for *Pseudomonas* sp, and MCA (Difco) for enterobacteria. After the plates were incubated for 24 h at 37°C in a bacteriological incubator, the CFU/m³ was determined using the conversion table of the device used, as recommended by the manufacturer.

The air collection procedure for determining the presence of fungi in air was the same as that used for bacteria, except that SDA (Difco) supplemented with chloramphenicol (30 mg/100 mL) (Sigma) was used. For yeast the plates were incubated in BOD incubator at 30°C for 48 h for fungal growth and subsequent enumeration of CFU/m³ and re- incubated for 7 days to verify the development of yeasts. For filamentous fungi the plates were incubated in BOD for two to three weeks, being examined from the second day, to verify the development of filamentous fungi. The device's conversion table was used to determine the CFU/m³.

Identification of isolated microorganis ms

The bacterial and yeasts strains found in the air and water samples were isolated and later identified using matrix assisted laser desorption-ionization-time of flight mass

spectrometry (MALDI-TOF MS). Briefly, the microbial culture was suspended in 300 µL distilled water, to which 900 µL of 99.5% alcohol was added and centrifuged at 13,000 rpm for 2 min. After centrifugation, the supernatant was discarded, 20 µL of 70% formic acid was added, and the solution was vortexed. After vortexing, 20 µL of acetonitrile was added and centrifuged for two min at 13,000 rpm. Then, aliquots of the supernatant were analyzed using mass spectrometry (MALDI- TOF, Bruker MALDI Biotyper 4.0). The criteria established for identification were: ≥ 2.0 for species and ≤ 1.7 for genus. (Tarumoto et al. 2016)

The isolated filamentous fungi were identified based on the morphological observation of the colony using giant and microscopic colony techniques, as well as microculture on potato agar (Difco). (Campbell et al. 2013, Larone 2011, Samson et al. 2007, Silva et al. 2011)

Statistical analysis

Statistical analyzes were based on microbial count (CFU/mL). All results are expressed as mean ± standard deviation (SD) of the three collections performed. Data were analyzed using jamovi software (version 2.0) and R: A Language and environment for statistical computing (version 4.0). The data obtained were analyzed by negative binomial regression followed by post-hoc comparisons of groups using Bonferroni corrections, where $P < 0.05$ was considered statistically significant, with the level of significance set at $\alpha = 0.05$.

Results

The results of the quantitative evaluation of microorganisms in water are show n in Figure 2. The means of the number of bacteria present in DW, FW, and SW ranged from 0.223 ± 0.05 to 0.999 ± 0.91 CFU/mL, while that fungi ranged from 0.015 ± 0.02 to 0.147 ± 0.21 CFU/mL. No statistical difference was found regarding the count of microorganisms between the DW, FW and SW, nor between the bacteria and fungi (Table 1).

The results of the quantitative evaluation of microorganisms in air are shown in Figure 3. The means of the number of bacteria present in air (Figure 3 A) were 132.00 ± 99, 130.11 ± 77.0, and 351.56 ± 158.2 CFU/m³ in BR1, BR2 and BRE3, respectively, and 51.44 ± 15.01 and 41.00 ± 10.5 CFU/m³ in HR1 and HR2, respectively. The mean number of fungi in the air of BR1, BR2, BR3, HR1, and HR2 were 12.56 ± 8.9, 12.78 ± 8.6, 36.33 ± 25.8, 10.78 ± 7.7, and 9.11 ± 4.6 CFU/m³ respectively (Figure 3 B). In the EC, 72.11 ± 10.04 CFU/m³ bacteria (Figure 3 A) and 13.11 ± 4.47 CFU/m³ fungi (Figure 3 B) were detected.

More bacteria than fungi were found in the air of the animal facility ($P = 0.001$). In the air was a significant increase in microorganisms in the 3 breeding rooms (BR1, BR2 and BR3) compared to rooms HR1 and HR2 ($P = < 0.05$), when compared to the other rooms, BR3 had an increase in relation to the others (BR1, BR2, HR1 and HR2 - $P = < 0.05$), and rooms BR3 ($P = 0.001$) HR1 ($P = 0.049$) and HR2 ($P = 0.001$) had an increase in microorganisms compared to EC (Table 2).

In total, 44 species, including bacteria, yeasts, and filamentous fungi, were isolated and identified in this study (Table 3). Among these, 14 species were found only in water samples, 20 in air samples, and 10 were found in both water and air samples. Fifty-three microorganisms were found in water and 79 in air.

Discussion

In the present study, we analyzed the presence of microorganisms in three types of water used in the animal facility for rodents: DW, FW, and SW. As Brazilian legislation has not set specific standards for the microbiological quality of water in animal facilities, the legal criteria defining the control and surveillance of water quality for human consumption and its standard of potability were considered for analyzing the data for drinking water obtained from the water supply system in this study. (Brasil 2011) Owing to the biomedical character of the facility, the criteria of the Brazilian Pharmacopoeia were used for analyzing FW and SW. (ANVISA 2019)

In total, 0.996 ± 0.91 CFU/mL bacteria and 0.147 ± 0.20 CFU/mL fungi was detected in the quantitative analysis of DW. The Brazilian legislation for DW uses the absence of fecal coliform markers as a parameter for microbiological analysis of water, (Brasil 2011) because of which it impossible to determine whether the quantitative data obtained in this study are in accordance with normality. However, although no specific method for detecting the presence of fecal coliforms was used in this analysis, we did not detect fecalcoliforms in any DW sample in the MALDI-TOF-based identification of microorganisms in this study.

We detected 0.554 ± 0.19 CFU/mL bacteria and 0.096 ± 0.10 CFU/mL fungi in FW and 0.223 ± 0.05 CFU/mL bacteria and 0.015 ± 0.02 CFU/mL fungi in SW. According to the Brazilian Pharmacopoeia, FW and SW are considered purified water (produced from DW, without the addition of any substance), and hence, the recommended monitoring value in which the total bacterial count is ≤ 100 CFU/mL was used. (ANVISA 2019) Therefore, the data obtained in the present study for FW and SW are within the expected normal standard. None of the Brazilian regulations used in this study allowed us to determine whether the data regarding the quantitative evaluation of fungi present in water were in accordance with normal standard values.

However, although present in low quantities allowed by the Brazilian legislation (Brasil 2011, ANVISA 2019), 24 microorganisms were isolated and identified in the water samples (Table 3). Eight of these 24 microorganisms are of clinical importance for humans and animals, (Quinn et al. 2001, Hirsh and Zee 2003, Carroll et al. 2019) namely, *Aspergillus fumigatus* (found in DW and FW), *Aspergillus* spp. (found in FW and SW), *Corynebacterium* spp. (found in FW), *Enterobacter cloacae* (found in DW and FW), *Escherichia coli* (found in SW), *Fusarium* spp. (found in DW), *Staphylococcus aureus* (found in DW and FW) and *S. epidermidis* (found in SW and FW).

Raynor et al. (1984) evaluated the FW of an animal facility used for scientific research and identified three bacteria: *Delftia acidovorans* (*Pseudomonas acidovorans*), *Achromobacter* spp,. and *Cupriavidus pauculus* (CDC Group IV C-2). In the present study, *D. acidovorans* was also found in the FW of the rodent facility, which corroborates the results of the previous study.

Similar to that for water of animal facilities, specific Brazilian legislation for microbiological analysis of the air of animal facilities is lacking. Therefore, two criteria defined by the National Agency of Sanitary Monitoring (ANVISA) were used in this study. The first criterion establishes the reference standard of indoor air quality in artificially air-conditioned environments for public and collective use and standardizes the maximum recommended values for fungal presence in air. (Brasil 2003) The second criterion is applicable for biomedical facilities and acts as a guide for monitoring air quality in the pharmaceutical industry; this criteria is used for classification of clean areas based on the maximum limit of microorganisms in air. (ANVISA 2013)

According to ANVISA's criteria for indoor air quality, the maximum recommended value for fungi is $\leq$ 750 CFU/m³ for an I/E ratio $\leq$ 1.5, where I represents the indoor environment and E the external control. (Brasil 2003) In the present study, < 750 CFU/m³ of fungi was found in all five rooms evaluated, as recommended by ANVISA. However, the I/E ratio in BR3 was 2.7, which is higher than that allowed. The I/E ratio was < 1.5 in the other four rooms (BR1, BR2, HR1, and HR2) (Fig. 2B).

The comparison made between BR3 and EC (P = 0.001) corroborates the result found in the I/E ratio. However, multiple comparisons also found an increase in the count of microorganisms in HR1 and HR2 compared to EC (P = 0.049 and 0.001, respectively) and when performed the I/E ratio of these rooms, they were within the recommended values. This can be explained by exp(B) BR3 3.403 HR1 0.722 HR2 0.62. The incidence rate of microorganisms

in BR3 is 3.403 times higher than EC and 0.722 and 0,623 times smaller than EC in HR1 and HR2, respectively.

The identification of pathogenic fungi such as *Aspergillus* sp. in the air is also outside the standard recommended by ANVISA. (Brasil 2003) In the present study, four strains of *Aspergillus* sp. were isolated and identified within the rodent facility (Table 3), namely, *A. clavatus* (BR1 and BR2), *A. flavus* (BR1, BR2, HR1 and HR2), *A. fumigatus* (BR1 and BR2), and *Aspergillus* spp. (BR2, BR3 and HR1). In the EC samples, strains of *A. flavus* (which were also found in the rooms described above) and *A. niger* (which were not found in the rooms of the rodent facility) were detected. These findings suggested that the external environment does not contribute to *Aspergillus* sp contamination within the rodent facility, as the strains found in the internal and external environments differed.

In the present study, the fungi, *Paecilomyces variotii,* was isolated and identified in the air of rooms BR2 and BR3. *P. variotii* is pathogenic for humans (Carroll et al. 2019) and opportunistic for animals. (Quinn et al. 2001, Hirsh and Zee 2003) K unstyr et al., (1997) reported *P. variotii* in the internal organs of animals, including rodents, used for scientific research. The findings of this study corroborate these findings.

Literature reviews show that the Brazilian parameter used for defining the presence of fungi as an air quality marker is also used by other countries, although consensus regarding the maximum values of fungi permissible in indoor environments is lacking; some guidelines also emphasize assessment of bacterial presence. (Kim et al. 2018, Rao et al. 1996) In this study, 22 species of bacteria were isolated and identified in air samples from the rooms (Table 3). Among these, six are potentially pathogenic and opportunistic for humans and animals and are of clinical significance, namely, *Acinetobacter* spp. (BR3), *Bacillus cereus* (BR1, BR2 and HR2), *B. pumilus* (BR1, BR3, HR1 and HR2), *E. cloacae* (BR2), *Serratia marcescens* (BR2), and *S.*

aureus (BR1). These findings suggested that airborne bacterial contamination is a potential health hazard for humans and rodents.

Microbiological contamination limit is one of the criteria used by ANVISA for accreditation of clean areas in grades A, B, C, and D in the pharmaceutical industry. This limit is less than 1 CFU/m³ for grade A, 10 CFU/m³ for grade B, 100 CFU/m³ for grade C, and 200 CFU/m³ for grade D. (ANVISA 2013) According to these values and the mean results obtained in this study, the rooms BR1 (132.0 ± 99.2 CFU/m³), BR2 (130.11 ± 77.0 CFU/³) and BR3 (351.56 ± 158 .24 CFU/m³) of the rodent facility were classified as grade D clean area, while rooms HR1 (51.44 ± 15.0 CFU/m³) and HR2 (41.0 ± 10.5 CFU/m³) were grade C clean area.

An Argentinian study monitored microbiological contamination in the blood and internal organs of rats and mice in a facility used for scientific research during 2012−2016, where most of the strains identified were *Proteus* spp. and *Pseudomonas aeruginosa*. During the study period, the authors did not detect any strain of *S. aureus*; however, *Corynebacterium kutscheri* was detected in 12.97% mice and 21.54% rats (Carriquiriborde et al. 2020). In the present study, bacteria of the same genus, *Pseudomonas* sp, were identified; *P. nitroreducens* was detected in DW and SW, and *P. oryzihabitans* (BR2), *P. putida* (HR2), and *P. stutzeri* (BR2) were detected in the air samples. Strains of *S. aureus* were found in DW and FW and in air samples from BR2. A strain of *Corynebacterium* spp. was also found in the FW in the present study.

The Federation of European Laboratory Animal Science Associations (FELASA) recommends that the animal health monitoring program for rodents should investigate the presence of certain microorganisms, including *S. aureus* and *C. kutscheri*. (Felasa 2014) In the present study, as mentioned above, *S. aureus* and *Corynebacterium* spp. were detected in water samples. Although rodents were not monitored in this study, the presence of these bacteria in water represents a high risk of infection.

During an immunological study in mice, Mayeux et al. (1995) discovered *A. fumigatus* contamination in animals, which hindered execution of the study. Later, the source of fungal contamination was tracked to the bedding used; furthermore, 80 CFU/g of the yeast, *Rhodotorula* sp, was found in rodent chow. In the present study, *A. fumigatus* was found in air samples (BR1 and BR2) and water samples (DW and FW) of the rodent facility, which are in agreement with the observations of the previous study. In addition, other strains of *Aspergillus* sp were detected in water, namely, *Aspergillus* spp. (FW and SW) and *Aspergillus terreus* (DW). Three strains of *Rhodotorula mucilaginosa* (DW) were also identified.

Among the 44 species identified in the present study, only one is non-pathogenic and/or opportunistic for humans and/or animals. *Bacillus atrophaeus*, found in the air of BR2 (Table 3), is a spore- forming bacteria widely used in biotechnological processes, mainly as a biological indicator of disinfection and sterilization processes. (Sella et al. 2015) Two microorganisms, *A. niger* and *Lysinibacillus boronitolerans*, were found only in the air outside the facility. Therefore, in the present study, 41 species of clinically important microorganisms were identified in the water and air inside the installation of rodents, which are pathogenic and/or opportunistic microorganisms capable of causing diseases in humans and animals.

Accurate and reproducible data are the cornerstone of scientific research. Animals are often used to obtain data, which is vital for research. The immune system ofanimals maintained under laboratory conditions are sometimes compromised, which renders them susceptible to pathogenic and opportunistic microorganisms. Infected animals may change the results obtained and affect scientific research. (Mansfield et al. 2010)

Although Brazilian legislation has specified parameters defining the microbiological quality of water for human consumption, similar legislation for animal facilities is lacking. Currently, advice regarding indoor air in climate-controlled environments and those for water and air in the pharmaceutical industry are applied to animal facilities. (Brasil 2003, Brasil 2011,

ANVISA 2013, ANVISA 2019) However, scientific facilities, such as the rodent facility in the present study, need extra vigilance for containing microbial contamination, and standards and control routines based on their specific requirements have to be developed (Politi et al. 2008, Straumfors et al. 2018). In this context, this is the first Brazilian study that aimed to quantitatively and qualitatively assess the environmental quality of air and water in a rodent facility used for scientific experimentation.

The identification of potentially pathogenic and opportunistic microorganisms in this study highlights the need for creating monitoring norms and standards specific for environments of animal experimentation. These environments must be reliable and safe for humans, which will prevent zoonosis and enable generation of reliable scientific results. This study contributes to the debate on sanitary standards and norms necessary for good practices that will enable researchers obtain data of desirable quality using animals and ensure that employees and users of animal facilities are safe.

Conclusion

Despite the lack of Brazilian legislation regarding the microbiological quality of water and air in animal facilities, we concluded, based on the data obtained in this study, that the sanitary standards used by the rodent facility at the Federal University of Uberlândia were effective in maintaining good microbiological quality of water. However, according to Brazilian legislation for pharmaceutical industries, the microbiological quality of the air in BR3 did not meet the standards of indoor and climate-controlled environments, and the rodent facility rooms, BR1, BR2, and BR3, were considered to be of cleaning grade D, while HR1 and HR2 were of grade C. In total, 41 microorganisms that were identified in the water and air of the rodent facility were considered potentially pathogenic and/or opportunistic for both animals and humans. Further investigations are required to define the sources of air and water

contamination in the facility, and specific sanitary standards for these environments should be created, which will enable the adoption of control measures that best suit these facilities.

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Conflicts of interest

There are no conflicts to declare

Author contribution

Luiz M. Silva: survey, writing, review; Mariana B. Santiago: survey, review; Paula Augusta D. F. Aguiar: conceptualization, review; Salvador B. Ramos: discussion, review; Murilo V. Silva: discussion, review; Carlos Henrique G. Martins: conceptualization, writing, discussion, review.

Data availability statement

All the microbiological data used to support the findings of this study are available from the corresponding author upon request.

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Figure legends

Figure 1. Rodent facility floor plan. 1, breeding room 1; 2, breeding room 2; 3, breeding room 3; 4, housing room 1; 5, housing room 2; 6, clean corridors; 7, dirty corridors; 8, area entrance; 9, restrooms and workers' locker room; 10, storage room.

Figure 2. Data presented as mean of colony forming units (CFU) per milliliter of waterborne bacteria and fungi found in the rodent facility. DW, drinking water; FW, filtered water; SW, sterile water.

Figure 3. (A) Data presented as mean of colony forming units (CFU) per cubic meter of airborne bacteria found in the rodent facility. (**B)** Data presented as mean of CFU per cubic meter of airborne fungi found in the rodent facility. EC, external control; BR1, breeding room 1; BR2, breeding room 2. BR3, breeding room 3; HR1, housing room 1; HR2, housing room 2. *Ratio indoor/external (I/E) = $\leq$ 1.5 CFU/m³

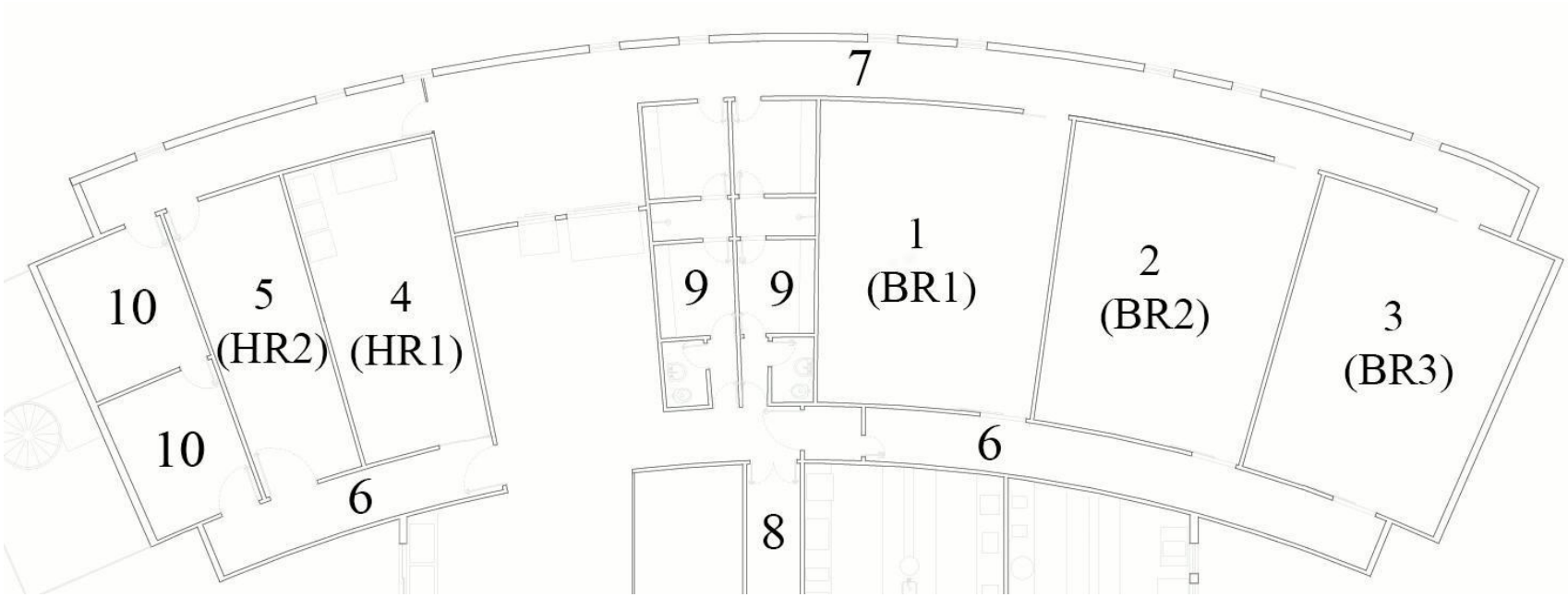

Figure 1.

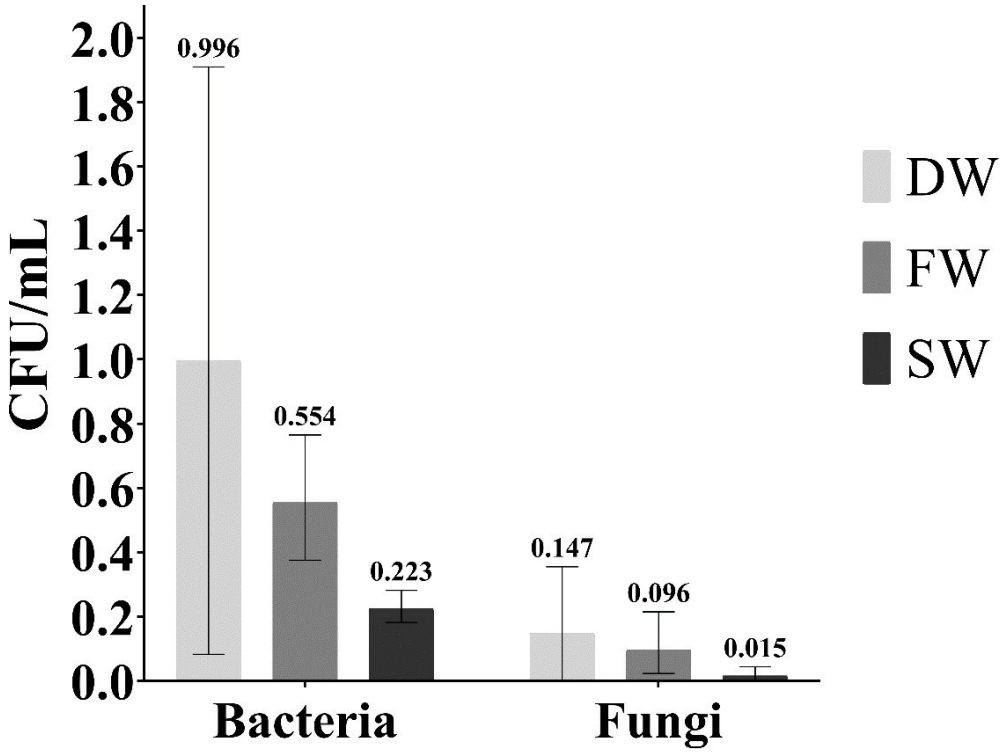
CFU/mL
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.996
0.554
0.223
0.147
0.096
0.015
Bacteria
Fungi
DW
FW
SW

Figure 2.

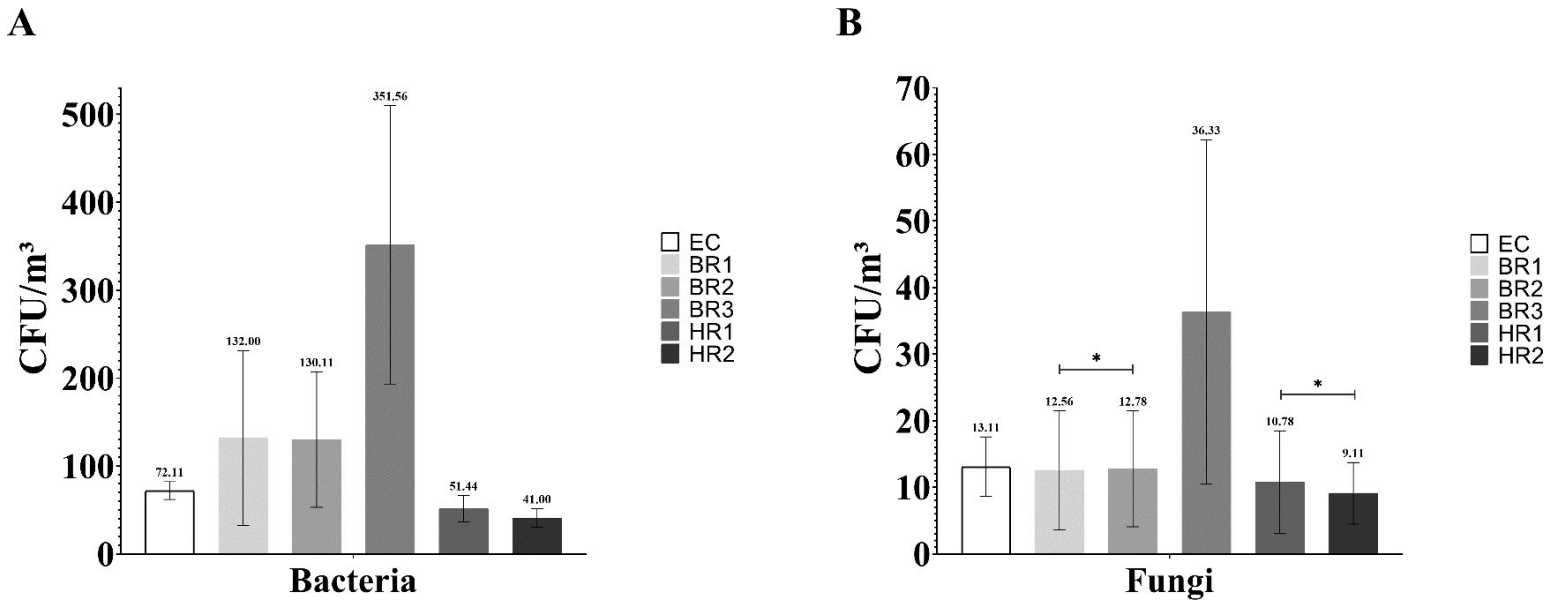
A
CFU/m³
500
400
300
200
100
0
72.11
132.00
130.11
351.56
51.44
41.00
Bacteria
EC
BR1
BR2
BR3
HR1
HR2
B
CFU/m³
70
60
50
40
30
20
10
0
13.11
12.56
12.78
36.33
10.78
9.11
*
*
Fungi
EC
BR1
BR2
BR3
HR1
HR2

Figure 3.

Table 1. Negative binomial regression analysis of microorganism counts from water collections.

Comparisons		exp(B)	SE	z	p
Bacteria	Fungi	1.18e-4	40141.90	-2.25e−4	1.000
DW	FW	0.832	32138.20	-5.71e−6	1.000
DW	SW	9.86e-6	39988.18	-2.88e−4	1.000

Table 2. Analysis of multiple post hoc Bonferroni comparisons of microorganism counts from air samples.

Comparisons		exp(B)	SE	z	p
Bacteria	Fungi	7.27	0.459	31.4	0.001
BR1	BR2	0.944	0.1058	-0.517	1.000
BR1	BR3	0.317	0.0325	-11.218	0 .001
BR1	HR1	1.496	0.1705	3.534	0.006
BR1	HR2	1.734	0.1999	4.774	0.001
BR1	EC	1.080	0.1184	0.701	1.000
BR2	BR3	0.336	0.0341	-10.732	0.001
BR2	HR1	1.585	0.1790	4.081	0.001
BR2	HR2	1.837	0.2103	5.315	0.001
BR2	EC	1.144	0.1246	1.238	1.000
BR3	HR1	4.714	0.4880	14.976	0.001
BR3	HR2	5.464	0.5748	16.142	0.001
BR3	EC	3.403	0.3368	12.372	0.001
HR1	HR2	1.159	0.1349	1.269	1.000
HR1	EC	0.722	0.0800	-2.941	0.049
HR2	EC	0.623	0.0700	-4.215	0.001

Table 3. Microorganisms isolated and identified in the air and water of the rodent facility and their possible pathogenicity for humans and animals.

Microorganisms	Numbers of microorganisms isolated in air and water samples									Pathogenic to humans[1]	Pathogenic to animals[2]
	Water			Air							
	DW	FW	SW	BR1	BR2	BR3	HR1	HR2	EC		
Acinetobacter spp.	0	0	0	0	0	1	0	0	0	Y	O
Aspergillus clavatus	0	0	0	1	1	0	0	0	0	O	O
Aspergillus flavus	0	0	0	1	1	0	1	1	1	Y	O
Aspergillus fumigatus	1	1	0	2	2	0	0	0	0	Y	Y
Aspergillus niger	0	0	0	0	0	0	0	0	2	Y	O
Aspergillus spp.	0	1	2	0	3	2	1	0	0	Y	Y
Aspergillus terreus	1	0	0	0	0	0	0	0	0	Y	O
Bacillus atrophaeus	0	0	0	0	1	0	0	0	0	N	N
Bacillus cereus	2	1	2	1	1	0	0	1	1	O	O
Bacillus circulans	0	0	1	0	0	0	0	0	0	Y	O
Bacillus megaterium	0	0	1	0	0	2	0	0	0	Y	N
Bacillus pumilus	1	2	0	2	0	1	1	1	1	Y	O
Bacillus thuringiensis	0	0	1	0	0	0	0	0	1	O	N
Brevibacillus borstelensis	0	0	1	0	0	0	0	0	0	O	N
Candida parapsilosis	1	0	0	0	0	0	0	0	0	Y	O
Corynebacterium spp.	0	1	0	0	0	0	0	0	0	Y	Y
Delftia acidovorans	0	1	0	0	0	0	0	0	0	O	N

Numbers of microorganisms isolated in air and water samples											
Microorganisms	Water			Air						Pathogenic to humans[1]	Pathogenic to animals[2]
	DW	FW	SW	BR1	BR2	BR3	HR1	HR2	EC		
Enterobacter asburiae	0	1	1	0	0	0	0	0	0	O	O
Enterobacter cloacae	2	2	0	0	1	0	0	0	0	Y	Y
Escherichia coli	0	0	1	0	0	0	0	0	0	Y	Y
Fusarium spp.	1	0	0	0	0	0	0	0	0	Y	Y
Lysinibacillus boronitolerans	0	0	0	0	0	0	0	0	1	O	N
Lysinibacillus sphaericus	0	0	0	0	0	0	0	2	0	O	N
Paecilomyces variotii	0	0	0	0	1	1	0	0	0	Y	O
Pantoea eucrina	0	0	0	1	0	1	0	0	1	O	N
Pantoea septica	0	0	0	0	0	0	0	1	0	O	N
Pseudomonas nitroreducens	1	0	1	0	0	0	0	0	0	N	N
Pseudomonas oryzihabitans	0	0	0	0	1	0	0	0	0	O	N
Pseudomonas putida	0	0	0	0	0	0	0	1	0	O	O
Pseudomonas stutzeri	0	0	0	0	1	0	0	0	0	O	O
Rhodotorula mucilaginosa	3	0	0	0	0	0	0	0	0	Y	N
Serratia marcescens	1	0	2	0	1	0	0	0	0	Y	O
Staphylococcus aureus	1	3	0	1	0	0	0	0	0	Y	Y
Staphylococcus capitis	1	0	0	0	0	0	0	0	0	O	O
Staphylococcus cohnii	0	0	0	1	0	0	0	0	1	O	O

Numbers of microorganisms isolated in air and water samples											
Microorganisms	Water			Air						Pathogenic to humans[1]	Pathogenic to animals[2]
	DW	FW	SW	BR1	BR2	BR3	HR1	HR2	EC		
Staphylococcus epidermidis	0	1	3	0	0	0	0	0	0	Y	Y
Staphylococcus equorum	0	0	0	0	1	0	0	0	0	O	O
Staphylococcus gallinarum	1	2	1	0	0	0	0	0	0	O	O
Staphylococcus lentus	0	1	0	2	2	3	2	2	1	O	O
Staphylococcus nepalensis	0	0	0	1	1	0	1	1	0	O	O
Staphylococcus sciuri	0	0	0	0	1	3	0	2	0	O	O
Staphylococcus succinus	0	0	0	0	0	1	0	0	0	O	O
Staphylococcus warneri	2	0	0	0	1	0	0	0	0	O	O
Staphylococcus xylosus	0	0	0	1	0	0	0	0	2	O	O

DW, drinking water; FW, filtered water; SW, sterile water; BR1, breeding room 1; BR2, breeding room 2; BR3, breeding room 3; HR1, housing room 1; HR2, housing room 2; EC, external control. [1] according to Carrol et al.,(2019). [2] according to Hirsh and Zee, (2003) and Quinn et al., (2001). Y, yes. O, opportunistic. N, no.