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Microbial diversity involved in the etiology of a bovine respiratory disease outbreak in a dairy calf rearing unit

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ABSTRACT

The etiological agents involved in a bovine respiratory disease (BRD) outbreak were investigated in a dairy heifer calf rearing unit from southern Brazil. A battery of PCR assays was performed to detect the most common viruses and bacteria associated with BRD, such as bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine alphaherpesvirus 1 (BoHV-1), bovine coronavirus (BCoV), bovine parainfluenza virus 3 (BPIV-3), *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*. Bronchoalveolar lavage fluid (BALF) samples were taken from 21 heifer calves (symptomatic $n = 15$; asymptomatic $n = 6$) that, during the occurrence of the BDR outbreak, were aged between 6 and 90 days. At least one microorganism was detected in 85.7 % (18/21) of the BALF samples. Mixed infections were more frequent (72.2 %) than single infections (27.7 %). The interactions between viruses and bacteria were the most common in coinfections (55.5 %). The frequencies of BRD agents were 38.1 % for BRSV, 28.6 % for BVDV, 33.3 % for BCoV, 42.85 % for *P. multocida,* 33.3 % for *M. bovis*, and 19 % for *H. somni*. BoHV-1, BPIV-3, and *M. haemolytica* were not identified in any of the 21 BALF samples. Considering that BALF and not nasal swabs were analyzed, these results demonstrate the etiological multiplicity that may be involved in BRD outbreaks in dairy calves.

1. Introduction

Bovine respiratory disease (BRD) in calves represents an important cause of economic losses for the dairy industry worldwide due to costs associated with reduced weight gain, farm labor, treatment, prophylaxis, and high morbidity and mortality rates [1–4]. Bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine alphaherpesvirus 1 (BoHV-1), bovine coronavirus (BCoV), and bovine parainfluenza virus 3 (BPIV-3) are the main viral pathogens infecting the bovine respiratory system [5–7]. *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* are the major bacteria involved in secondary infection of the respiratory tract and are associated with pneumonia in young dairy calves [6,8,9]. These etiological agents may cause a single infection or act in synergy in coinfections, enhancing the severity of the disease [10,11]. Although BRD may affect cattle of different ages, it is more commonly diagnosed in calves up to 3 months, and the peak of the disease usually occurs between 4 and 6 weeks of age [2,12,13].

The calf rearing units have been used for many years in veal calf and cattle feedlots; however, it is currently also being adapted for calves from dairy herds. Calves are transported from different herds of origin shortly after birth [14] or until the second week of age [15,16] to the dairy calf rearing units or veal calf feedlots, while calves for feedlots are transported only after weaning [17,18]. In the specialized heifer calf rearing units, the outbreaks of BRD in calves are commonly reported [15]. Also, adverse conditions in transportation, nutrition, temperature, and sanitary and environmental management may lead to immunosuppression and increased susceptibility to pathogens of the bovine respiratory system [19,20].

In Brazil, BRD reports are limited to specific pathogens and do not completely describe the etiology of the disease. Most of the Brazilian studies are conducted in postmortem examinations of calves, limiting the knowledge regarding possible simultaneous infections by several etiological agents [21–23]. Frequently, treatment with antibiotics and

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supportive therapy is performed, and the etiological agents involved are rarely identified [13,24,25]. However, characterizing the microorganisms associated with BRD is essential to improve health status of the herd, mainly in the dairy calf rearing units.

The present study reports a molecular diagnostic survey for multiple etiological agents during an outbreak of BRD in heifer calves in a Brazilian dairy calf rearing unit.

2. Materials and methods

2.1. Calf rearing unit

The BRD outbreak occurred in a dairy calf rearing unit located in Parana state, southern Brazil. The region has a humid subtropical climate with hot, humid summers and mild winters with an average temperature of 21 °C. The rearing unit maintained approximately 125 mixed-breed heifer calves obtained from 45 small dairy cattle herds for household milk production that were associated with a dairy cooperative.

Data on housing, feeding, and management of the calves were collected through an interview with the veterinarian in charge. Calves arrive at the rearing unit at 2–5 days of age and are housed in 5 group pens (7 × 3 m). Twenty to 25 calves are grouped in each pen until approximately 60 days of age. Calves are fed in an automatic feeder system for each pen with calf milk replacer in a common nipple, and concentrates on pelleted calf feed containing 23 % crude protein are provided *ad libitum*. Thus, heifer calves from diverse origins and with distinct health and immunological status are grouped together in the same rearing unit.

Data about the sanitary status of the original herds of the calves were unknown, except for the compulsory sanitary management practices against bovine brucellosis, tuberculosis, and foot and mouth disease, according to the national program for the control and eradication of these diseases. None of the dairy herds that provided heifer calves for the rearing unit vaccine cows to improve the colostrum quality and thus provide passive protection to calves against the major BRD-causing pathogens. The efficiency of the transfer of passive immunity is also not monitored. In the rearing unit, the calves also do not receive vaccines for BRD control.

2.2. Diseased animals

First, asymptomatic and symptomatic heifer calves with BRD were identified by clinical examination. Typical clinical signs of respiratory disease were not present in calves classified as asymptomatic. Calves classified as symptomatic showed clinical signs of coughing and copious nasal discharge in association with at least two of the following clinical manifestations: rectal temperature above 39.5 °C, prolonged capillary refill time, pale mucous membranes, heart rate above 120 beats/min, respiratory discomfort, and respiratory rate above 40 breaths/min [6,15]. Symptomatic calves with BRD signs were not separated from asymptomatic animals, even during clinical treatment. The treatment of BRD-affected calves was performed with broad-spectrum antibiotics (spectinomycin and tulathromycin) and anti-inflammatory drugs.

2.3. Bronchoalveolar samples

After clinical examination, 21 bronchoalveolar lavage fluid (BALF) samples were collected from asymptomatic (n = 6) and symptomatic (n = 15) untreated calves following the collection procedures previously described [6]. The calves of the calf rearing unit were divided into 3 groups based on age, between 6–30 days, 31–60 days and over 60 days. At least four BALF samples per age group were collected at random including asymptomatic and symptomatic calves. The collection procedures of BALF samples were conducted by veterinarians at the Universidade Estadual de Londrina, Paraná, Brazil, including a trained veterinary surgeon, in a single visit to the rearing unit. The samples were placed in sterile tubes, shipped on ice baths and stored at −80 °C until processing.

2.4. Detection of infectious agents associated with BRD

Nucleic acids were extracted from 500-μL aliquots of BALF samples pretreated with sodium dodecyl sulfate (SDS) and proteinase K incubated at 56 °C for 30 min at a final concentration of 1 % (v/v) and 0.2 mg/mL, respectively. BALF samples were then processed following a silica/guanidine isothiocyanate protocol [26]. The extracted nucleic acid was eluted in 50 μL of ultrapure nuclease-free diethylpyrocarbonate-treated sterile water and stored at −80 °C until used for molecular analysis.

Molecular diagnostic assays (PCR, RT-PCR, and nested PCR) were performed for the detection of the main infectious agents associated with BRD. The techniques were performed separately to amplify each of the infectious agents, a product with 288 bp was amplified of the BVDV 5' UTR gene [27], 371 bp of the BRSV G gene [28], 251 bp of the BCoV N gene [29], 647 bp of the BPIV-3 HN gene [30], 425 bp of the BoHV-1 D gene [31], 460 bp of the *P. multocida* ORF clone KMT1 [32], 408 bp of the *H. somni* 16S gene [33], 385 bp of the *M. haemolytica* lktA-artJ intergenic region [6], and 488 bp of the *M. bovis* 16 S–23 S intergenic region [34].

Aliquots of sterile ultrapure water were included as negative controls in all procedures. Samples previously known as positive for each of the pathogens investigated in this study were included as positive controls as follows: prototype Los Angeles, NADL, A51908, SF4/32, and Mebus strains cell culture (MDBK) adapted for BoHV-1, BVDV, BRSV, BPIV-3, and BCoV, respectively; nucleic acid from previous reports for *H. somni* [35]; and housekeeping samples for *P. multocida*, *M. haemolytica*, and *M. bovis* [36] were also included as positive controls.

Aliquots of 5 μL of the amplified products were analyzed by electrophoresis in 2 % agarose gel in TBE buffer pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA), stained with ethidium bromide (0.5 μg/mL), and visualized under UV light.

2.5. Nucleotide sequence analysis

One positive amplicon with a better quality of each BRD pathogen detected was sequenced to confirm the specificity of the amplified product. The amplicons obtained were purified using PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen® Life Technologies, Carlsbad, CA, USA), and quantified using Qubit® Fluorometer (Invitrogen® Life Technologies, Eugene, OR, USA). Direct sequencing was performed using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Foster City, CA, USA) with the forward and reverse primers, in a 3500 Genetic Analyzer sequencer. Sequence quality analyses and consensus sequences were obtained using Phred/CAP3 software (http://asparagin.cenargen.embrapa.br/phph/). Similarity searches were performed with nucleotide sequences deposited in the GenBank database using the BLAST highly similar tool software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results

The BRD outbreak analyzed in this study lasted approximately 20 days, from the first clinical signs were observed until their complete remission. The morbidity rate of calves with clinical manifestations of respiratory distress reached 42 %. Calves aged up to 30 days were more frequently affected than older calves, and in the last week of the BRD outbreak, seven calves died.

The frequencies of microorganism detection in this study were 28.6 % (6/21) for BVDV, 46.6 % (8/21) for BRSV, 33.3 % (7/21) for BCoV, 42.85 % (9/21) for *P. multocida,* 19 % (4/21) for *H. somni*, and 33.3 % (7/21) for *M. bovis*. All these respiratory pathogens were detected in

Table 1
Microorganisms identified by molecular assays in bronchoalveolar lavage fluid samples from symptomatic and asymptomatic dairy heifer calves in a bovine respiratory disease outbreak.

Microorganisms	Calf group		Total
	Symptomatic (n = 15)	Asymptomatic (n = 6)	
Viruses			
BVDV	5	1	6
BRSV	7	1	8
BCoV	6	1	7
BPI-3	–	–	–
BoHV-1	–	–	–
Bacteria			
M. bovis	5	2	7
H. somni	3	1	4
P. multocida	6	3	9
M. haemolytica	–	–	–

asymptomatic and symptomatic calves (Table 1). The detection of viruses and bacteria occurred in calves of all age groups (6–30 days; 31–60 days; and over 60 days). Three respiratory microorganisms (BPIV-3, BoHV-1, and *M. haemolytica*) were not identified in any of the BALF samples analyzed in this study.

Eighteen (85.7 %) of the 21 calves were infected with at least one infectious agent of BRD. Among the positive animals, mixed infections were more frequent (13/18; 72.2 %) than single infections (5/18; 27.7 %) (Table 2). Single infections were more frequently associated with bacteria (4/18; 22.2 %) than with viruses (1/18; 5.5 %). Associations between viruses and bacteria were the most frequent 10/18 (55.5 %). Other mixed infections presented a frequency of 3/18 (16.6 %) (Table 3).

The identities of the respective detected microorganisms were confirmed by their similarities with other nucleotide sequences deposited in a database using BLAST software. All obtained DNA sequences were clearly readable and have been deposited in GenBank. The accession numbers for samples from the outbreak are as follows: BVDV1d (KM982443), BRSV (KM982441), BCoV (KM982442), *P. multocida* (KM982445), *H. somni* (KM982444), and *M. bovis* (KM982446).

4. Discussion

To the best of our knowledge, the current study is the first molecular-based investigation of the main pathogens involved in BRD during an outbreak in a dairy heifer calf rearing unit. This study also represents the first South American report of molecular detection of BRD mixed infections in calves with clinical signs respiratory disease. The results obtained in the current study add to what was described on pathogens associated with BRD in dairy calves [6], since most studies of BRD infections were conducted in feedlot cattle [2,10,18,37].

In this study, mixed infections of BVDV, BRSV, BCoV, *P. multocida*, *H. somni,* and *M. bovis* were found in calves aged 6–60 days. The great diversity of etiological agents found in these animals from the calf rearing unit is related to a large number of herds of origin, differently of feedlot cattle, where the diversity of origins is less. Probably, this

Table 2
Type of infection (bacterial and viral) identified in a bovine respiratory disease outbreak in dairy heifer calves.

Infection type	Calf group		Total
	Symptomatic (n = 15)	Asymptomatic (n = 6)	
Single (Virus)	3	–	3
Single (Bacteria)	4	3	7
Mix (Virus / Bacteria)	6	2	8
Negative	2	1	3

Table 3
Distribution of microorganisms identified in bronchoalveolar lavage fluid samples in a severe outbreak of bovine respiratory disease in a dairy calf rearing unit according to the type of infection and calf age.

Infection Type	Microorganisms	N° of calves	Age (days)
Single	BVDV	1	55
	P. multocida	1	74
	M. bovis	2	6, 60
	H. somni	1	90
Double	*M. bovis* + *P. multocida*	2	22, 55
	M. bovis + *H. somni*	1	6
Triple	BVDV + BRSV + *P. multocida*	1	60
	BVDV + BRSV + BCoV	2	19, 82
	BRSV + BCoV + *P. multocida*	1	66
	BRSV + BCoV + *H. somni*	2	68, 82
	BRSV + *P. multocida* + *M. bovis*	1	59
	BCoV + *P. multocida* + *M. bovis*	1	54
Quadruple	BVDV + BRSV + BCoV + *P. multocida*	1	19
Total		18	

etiological diversity of microorganisms potentially pathogenic to the respiratory tract may have contributed to the high rate of morbidity (42 %) and the difficulty in treating calves in this outbreak of BRD.

BPI-3, BoHV-1, and *M. haemolytica* were not detected in BALF samples these calves evaluated. In Brazilian cattle herds, BPI-3 was detected in few studies, isolated in a single animal in south of the country [38] and another study using immunohistochemical it was detected in four animals [39], but in the most BRD studies was not found [3,18,22,36]. Possibly, BPI-3 circulates with low frequency in Brazilian cattle herds.

The seroprevalence of BoHV-1 infection in cows of dairy cattle herds in the geographical region of the calf rearing unit is high [40]. BRD associated with BoHV-1 usually occurs in older animals. Thus, it is likely that maternal antibodies acquired by calves against BoHV-1 provided adequate protection in the first months of age [41].

M. haemolytica is a natural inhabitant of the upper respiratory tract of the bovine species, occasionally it may develop BRD outbreaks [42]. In this study, no BALF sample analyzed was positive for this bacterium. Our results are consistent with another study also carried out in rearing unit, with calves from different dairy herds of origin, in which a low number of animals infected by *M. haemolytica* was observed (2 %), however a greater number of positive for bacterial agents, such as *Mycoplasma* sp. and *P. multocida*, and viral agents such as BRSV and BCoV [15].

Considering that the upper airways of asymptomatic calves may be colonized by a variety of bacterial pathogens [43], the present study was performed using BALF as clinical specimens. These samples of the lower respiratory tract are suitable to achieve a more reliable result of microorganisms associated with the etiology of BRD [6]. In addition, the molecular diagnosis adopted in the current study permitted a large number of species-specific tests for each pathogen after molecular assays were standardized.

Diagnostic techniques used in previous investigations of mixed infections in BRD cases in Brazil usually include pathological examination, serology, bacterial cultures and/or virus isolation [21,23,38,40]. However, these techniques may be appropriate for one agent but not for the other due to different sensitivity values [44]. Additionally, serology tests in young cattle may interfere with colostral antibodies and cross-reactions of pathogens with other commensal microorganisms, making interpretation of test results difficult [41]. Classical virus and bacterial isolation methods are laborious and time-consuming [44]. Among the antemortem diagnostic procedures applicable to BRD-affected calves, molecular techniques may be considered the most appropriate due to their fast and reliable results, facilitating actions to define preventive and therapeutic strategies in Brazilian herds [45].

In comparison to pathogens detected in BALF from diseased and

healthy calves, a higher frequency of viral detection was observed in animals with clinical signs of BRD. Calves housed together were the main reservoir of infectious agents to susceptible young cattle [7]; thus, possibly asymptomatic and symptomatic calves may have contributed to pathogen dissemination among the heifer calves. Evaluating bacterial pathogens, there was no increase in detection in disease animals compared to clinically healthy animals in this study. However, a study conducted with a larger number of samples and herds found a higher bacterial frequency in diseased animals [15], this difference in our study may have been due to the small number of samples used.

In Brazil, there are few reports about BRD, and these studies have focused on efforts to detect the involvement of respiratory tract organs by gross and microscopic lesions, serological evaluations, and characterization of specific pathogens [3,18,21–23,35,36,40,46,47].

A retrospective study of 12 years was performed by a university in the south of Brazil, herds with outbreaks of BRD were evaluated and animals that died, the age of the calves varied from 1 day to 12 months. A high morbidity rate was observed reaching up to 100 % of the animals and the mortality rate reached 34 % [22]. However, preventive and control measures are not usually performed in most Brazilian dairy cattle herds, and may have contributed to the high rates of morbidity and mortality [48]. In our study, we found a morbidity rate that reached 42 %; the high rate of infected animals may be due to the non-separate disease calves, increasing load of pathogens in the environment and favoring the infection of susceptible calves [15].

In this study, the findings of 85.7 % positive BALF samples for at least one of the pathogens associated with BRD highlight the importance of this disease in the calf rearing unit, especially of the mixed infections that were more frequent (72.2 %) than single infections (27.7 %). The most frequent mixed infections were between viruses and bacteria (55.5 %), which shows the synergism of these infectious agents in BRD, similar to severe pneumonia in children and puppies [49,50]. Single viruses associated with BRD are rarely lethal to cattle. Severe pneumonia usually occurs when commensal bacteria from the nasopharynx invade the lower airways of cattle after stressful conditions and viral infections. These predisposing factors affect the host defense mechanisms by altering mucosal surface components and decreasing the activity of innate immune system cells, such as T lymphocytes, B-lymphocytes, monocytes, and macrophages, thereby increasing the exposure to pathogens [9].

BRSV had the highest prevalence of this outbreak (46.6 %). This result is in keeping with previous studies conducted in Demark and Finland, which observed that this virus is the most important in BRD of dairy calves [6,15].

The BCoV described in this BRD outbreak was the second report of this virus associated with respiratory symptoms in cattle in Brazil. The first case was detected in a beef cattle feedlot [46]. The role of BCoV in BRD has been recognized recently with the dual enteric and respiratory tropism of some strains [51]. BRD related to BCoV is a potential threat to calves in calf rearing units, especially if the peak of virus shedding is highly short, occurring up to 4 days after arrival at facilities for rearing practices [52].

The immunosuppressive effect of BVDV in the host organism is considered the main factor associated with the development of BRD in cattle of affected herds due to an increased risk of infections by other pathogens [53,54]. Multiple strains of BVDV have been reported in cattle from the same geographic region of the calf rearing unit, such as BVDV1a, BVDV1b, and BVDV1d, associated with reproductive failure in cows [55]. Within these subgenotypes, BVDV1b was suggested be predominantly associated with BRD in calves [56]. However, the phylogenetic analysis performed using 5′UTR sequences of BVDV strains in the BALF samples of the current BRD outbreak clustered with the BVDV1d subgenotype. Furthermore, susceptible calves do not become ill until 5 weeks of age after exposure to BVDV virulent strains when passive immunity is acquired, even supposing that low virus replication may occur in these animals [57]. However, in the present study, BVDV was detected in BALF samples of calves in the first weeks of age. This finding may be due a failure in the transfer passive antibodies to the newborn calves due to colostrum deprivation, low intake or even the lack of immunity of the cow with the different strains of BVDV, this can make the calves susceptible the infection occurs in the first weeks of life. Also, infection of these calves with different BVDV strains of their herds of origin, since cross-protection between different BVDV subgenotypes is incomplete [54,55].

Among the four bacterial pathogens in BRD cases evaluated in this study, only *M. haemolytica* was not detected. *P. multocida*, *M. bovis*, and *H. somni* were detected in single or in mixed infections with other BRD pathogens. These results need to be considered with caution, as *P. multocida* and *M. haemolytica* are eventually listed as a primary agent of BRD, being more often considered opportunistic pathogens, and contributing to the increase of clinical signs in outbreaks [5,15]. Our study differs from another study that evaluated bacteria present in the lower respiratory tract of healthy and diseased calves with BRD in Brazil, in which *Enterobacteriaceae* were the predominant bacteria in BALF samples [21]. However, these bacteria are not associated with outbreaks of BRD. Similar results to this study were obtained by another recent Brazilian study, nasal swabs from cattle with BRD were analyzed and found the presence of *H. somni*, BRSV, BCoV, and *M. haemolytica* in single infections or co-infections [18].

The findings of *P. multocida* as the most prevalent pathogen (42.85 %) in the outbreak are in accordance with other investigations that revealed this bacteria as the most common isolated in dairy calves with BRD [6,58]. The presence of *H. somni* in this study is new evidence of the disease caused by this underdiagnosed bacterium, which has been described only recently in diseased cattle in Brazil associated with systemic disease, reproductive manifestations, thrombotic meningoencephalitis, and respiratory distress [18,35,36]. Diagnoses of *M. bovis* are difficult cultivate due to overgrowth of contaminants in nutritionally complex media after a long period of incubation [34,59]. The current study presents an alternative to detect *M. bovis* specifically in BALF samples by nested PCR and differentiate it from other species of mycoplasmas that are opportunistic and/or commensals of the respiratory tract.

At the time of sampling, the BRD outbreak in the calf rearing unit was not controlled. Antimicrobial drugs used in calves (spectinomycin and tulathromycin) are commonly used in Brazil for BRD treatment. However, these drugs were not used as metaphylaxis and/or therapy in the early stages of BRD, which increased the success of treated calves [24,60]. In association with this condition, potential risk factors for BRD previously described were detected at the rearing unit [12,13,61]. This unit includes automatic milk feeders with a common nipple, large group pens (> 12 calves), older calves housed with younger calves, and calves from various sources with unknown sanitary status.

A decrease in bacterial susceptibility to antibiotics and/or lack of appropriate evaluation of calves under treatment for further therapy are other important causes of failure in the control of BRD, especially for mycoplasmal infections. A study with *M. bovis* isolated from young cattle in France revealed that 100 % of recent strains are resistant to common antibiotics used to control BRD [62]. Thus, it may be suggested that when calves are challenged with so many etiological agents and in the presence of risk factors, simple maternal immunity and broad-spectrum antimicrobial drugs may not be sufficient to control BRD in calves. However, an essential tool for prevention is the use of commercial vaccines against the main pathogens that cause respiratory, bacterial, and viral diseases. These vaccines should be administered mainly to cows in the final period of pregnancy, to promote the transfer of immunoglobulins by colostrum. In addition, it is essential to ensure the intake of an adequate amount of colostrum soon after the calves are born. Finally, all preventive measures together can help to reduce the number of susceptible animals and the excretion of microorganisms, in addition to reducing the risk of BRD occurring mainly in the critical phases, which are transport and grouping in the calf rearing unit

[17,63].

5. Conclusion

In conclusion, the findings of this study revealed the presence of several microorganisms, viruses and bacteria, associated with BRD in a dairy heifer calf rearing unit from Brazil that differs from previous reports carried out with lower respiratory tract samples in the country. The diversity of potentially pathogenic microorganisms for the respiratory tract of calves identified mainly in mixed infections in conjunction with the risk factors present in this calf rearing unit may explain the high morbidity rate and the difficulty in the treatment of calves in this BRD outbreak. The use of a molecular diagnostic platform provided the rapid and reliable identification of several microorganisms involved in the BRD outbreak. Further studies focusing on the characterization of etiological agents detected in BRD are extremely important to clarify the circulating strains in epidemiological studies. The elucidation of the plurality of infectious agents that may be involved in BRD outbreaks in dairy calves highlights the importance of adopting preventive measures for BRD control, particularly in calf-rearing units where heifer calves from diverse origins and with distinct health and immunological status are grouped together in the same rearing system.

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Ethical approval

This study was approved by the Ethics Committee on the Use of Animals in Teaching and Research of the Universidade Estadual de Londrina (UEL) under number 6371.2013.43. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Declaration of Competing Interest

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

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