






Identification of *Malassezia* species as part of normal skin and ear canal microbiota in horses

Identificación de especies de Malassezia como microbiota normal en piel y canal auditivo externo de equinos

Identificação de espécies de Malassezia como microbiota normal em pele e canal auditivo externo de cavalos

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Abstract

Background: The yeasts of the genus *Malassezia* are considered part of the normal skin microbiota in humans and animals. In horses, several species of the genus *Malassezia* have been reported in different areas of the skin and ear canal. **Objective:** Isolate, characterize and identify the different species belonging to the genus *Malassezia* isolated from the ear canal and skin of equine patients with no dermatological lesions that were referred to the large animal clinic of veterinary teaching hospital at the National University of Colombia. **Methods:** 22 horses were evaluated and sampled. Eighty-two samples were obtained by swabbing either the ear canals (left and right), skin areas of prepuce, mammary gland and inguinal region. The samples were examined by cytological evaluation and were cultured on modified Dixon's agar and phenotypic and molecular identification were performed for yeast colonies. **Results:** Fourteen yeast isolates were obtained from the 82 samples. Biochemical identification determined that 50% (n=7) were *Malassezia* spp., 35.7% (n=5) were identified as *Candida* spp. and 14.3% (n=2) as *Cryptococcus* spp.. Using molecular tests, the *Malassezia* species were *M. slooffiae* (28.6%) and *M. nana* (57.1%); only one isolate was classified as *Trichosporo asahii*. **Conclusion:** *M. nana* and *M. slooffiae* were identified as part of the normal ear canal and skin microbiota in the evaluated horses. The observed prevalence of *Malassezia* spp. was 18.2% (n=4/22) in this study sample.

Keywords: culture; ear canal; horses; identification; *Malassezia*; normal microbiota; PCR; skin; yeast.

Resumen

Antecedentes: Las levaduras del género *Malassezia* hacen parte de la microbiota normal cutánea de humanos y animales. En equinos se han reportado diferentes especies de *Malassezia* aisladas de varias regiones de piel y canal auditivo externo. **Objetivo:** Aislar, caracterizar e identificar las especies del género *Malassezia* spp. a partir de canal auditivo externo y piel de equinos sin lesiones dermatológicas, remitidos a la Clínica de Grandes Animales de la Facultad de Medicina Veterinaria y de Zootecnia de la Universidad Nacional de Colombia. **Metodología:** Se evaluaron 22 equinos, a partir de los cuales se obtuvieron 82 muestras entre hisopados de canal auditivo externo (izquierdo y derecho) y diferentes regiones de piel (prepucio, glándula mamaria e ingle). Las muestras fueron procesadas mediante examen directo y cultivo en agar Dixon modificado. A partir de los aislamientos en los que se observaron colonias morfológicamente compatibles con *Malassezia* spp. se realizó la identificación fenotípica y molecular. **Resultados:** De las 82 muestras procesadas se obtuvieron 14 aislamientos de levaduras, de las cuales mediante identificación bioquímica el 50% (n=7) correspondió a *Malassezia* spp., el 35,7% (n=5) a *Candida* spp., y el 14,3% (n=2) a *Cryptococcus* spp. Luego mediante pruebas moleculares se identificaron las especies del género *Malassezia* como: *M. slooffiae* (28,6%) y *M. nana* (57,1%); y un aislamiento correspondió a *Trichosporon asahii*. **Conclusión:** Se logró identificar las especies *M. nana* y *M. slooffiae* como microbiota normal de la piel y el canal auditivo en los equinos evaluados. La prevalencia de *Malassezia* spp. para la población evaluada fue de 18,2% (n=4/22).

Palabras clave: canal auditivo; cultivo; equinos; identificación; levaduras; *Malassezia*; microbiota normal; PCR; piel.

Resumo

Antecedentes: As leveduras do género *Malassezia* fazem parte da microbiota cutânea normal de humanos e animais. Em cavalos, diferentes espécies de *Malassezia* isoladas de várias regiões da pele e do canal auditivo externo foram reproduzidas. **Objetivo:** Isolar, caracterizar e identificar as espécies do género *Malassezia* spp. do canal auditivo externo e pele equinos sem lesões cutâneas, referiu-se à Clínica de Grandes Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade Nacional da Colômbia. **Métodos:** 22 equinos foram avaliadas a partir dos quais 82 amostras a partir de esfregaços do canal auditivo externo (esquerda e direita) e diferentes regiões da pele (prepúcio, glândula mamaria e virilha) foram obtidos. As amostras foram processadas por exame direto e cultura em ágar Dixon modificado. Dos isolados nos quais as colônias foram observadas morfológicamente compatíveis com *Malassezia* spp. identificação fenotípica e molecular foi realizada. **Resultados:** Das 82 amostras processadas 14 isolados de levedura, que foram obtidos por identificação bioquímica de 50% (n=7) correspondia a *Malassezia* spp., 35,7% (n=5) a *Candida* spp., e 14,3% (n=2) para *Cryptococcus* spp.. Em seguida, usando o teste molecular espécie *Malassezia* foram identificadas como *M. slooffiae* (28,6%) e *M. nana* (57,1%); e um isolamento correspondia a *Trichosporon asahii*. **Conclusão:** As espécies *M. nana* e *M. slooffiae* foram identificadas como microbiota de pele normal e do canal auditivo nos equídeos avaliados. A prevalência de *Malassezia* spp. para a população avaliada foi 18,2% (n=4/22).

Palavras-chave: canal auditivo; cavalos; cultivo; identificação; levedura, *Malassezia*; microbiota normal; PCR; pele.

Introduction

The yeasts of the genus *Malassezia* are classified in the phylum basidiomycota, order Malasseziales, family Malasseziaceae (Crespo *et al.*, 2008a; Gaitanis *et al.*, 2012). These yeasts are considered part of the normal skin microbiota in humans and animals (Hernández, 2005). This genus is physiologically characterized for being lipid-dependent due to its incapacity to synthesize saturated fatty acids that is manifested by requiring an exogenous source of these fatty acids to grow; however, *M. pachydermatis* is an exception given the lack of need for lipids to grow in cultures (Giusiano, 2006). Based on these characteristics, the media commonly used are Dixon's and Leeming, and Notman agars that allow an adequate isolation of these yeasts (Hernández, 2005; Kaneko *et al.*, 2007).

Seventeen *Malassezia* species have been described so far, being lipid-dependent the following ones: *M. furfur*, *M. globosa*, *M. obtusa*, *M. restricta*, *M. slooffiae*, *M. sympodialis*, *M. dermatis*, *M. japonica*, *M. yamatoensis*, *M. nana*, *M. caprae*, *M. equina*, *M. cuniculi*, *M. brasiliensis*, *M. psittaci* and *M. vespertilionis* (Guého *et al.*, 1996; Sugita *et al.*, 2003; Hirai *et al.*, 2004; Cabañes *et al.*, 2007; Cabañes *et al.*, 2011), with the exception of *M. pachydermatis* (Giusiano, 2006; Lorch *et al.* 2018) as already mentioned.

In animals, *M. slooffiae*, *M. globosa*, *M. sympodialis* and *M. pachydermatis* have been reported in the ear canal of dogs and cats (Pulido *et al.*, 2010; Salah *et al.*, 2010); *M. nana* in cattle with or without external otitis and also in the ear canal of healthy horses (Hirai *et al.*, 2004; Aldrovandi *et al.*, 2016), and *M. sympodialis*, *M. slooffiae*, *M. furfur* and *M. pachydermatis* in the ear canal of pigs (Nardoni *et al.*, 2010).

There have been few studies on the presence of *Malassezia* spp. as part of the normal microbiota in horse skin; however some reports have shown them to be present in different areas of the skin surface, such as the inguinal area, back, perineum and ear canal, where species such

as: *M. pachydermatis*, *M. furfur*, *M. restricta*, *M. slooffiae*, *M. obtusa* and *M. globosa* have been isolated from; in addition, *M. furfur*, *M. restricta*, *M. sympodialis* and *M. globosa* have been isolated from the axillar region (Zia *et al.*, 2014; White *et al.*, 2006; Crespo *et al.*, 2002), and *M. equina* from the udder cleft of mares as well as from the preputial fossa of stallions and geldings (Cabañes *et al.*, 2007; White *et al.*, 2006).

Given the limited number of studies worldwide and the lack of studies on *Malassezia* spp. as part of the normal equine skin microbiota in tropical regions as Colombia, the main objective in this study was to isolate, identify and characterize these yeasts from different body regions of horses with no dermatological diseases.

Materials and Methods

Ethical Considerations

This research project was approved by the Bioethical committee of the science faculty of the Pontificia Universidad Javeriana by resolution No 14 on 08-10-14. It was also approved by the University Animal Care committee, by resolution C-023-14 on 01-10-14.

Sampling population

During a study period of 4 months, 22 horses, mares, geldings and stallions with ages that ranged between 26 months and 12 years, admitted to the Large Animal Clinic-Veterinary Teaching Hospital at the National University of Colombia were studied. These horses were presented with various clinical diseases none involving the skin, but these diseases did not represent a risk factor for yeast colonization of the skin or ear canal. The patients did not have any antibiotic, antimycotic nor corticosteroid treatments.

Sampling

The skin of the twenty-two horses was sampled using swabs. Samples were collected from the preputial area (n=10), mammary gland (n=12), inguinal area (n=16) and both ear canals (n=44). Eighty-two samples were collected and transported in sterile tubes at room temperature to the microbiology laboratory at the Pontificia Universidad Javeriana within a two hours-range after sampling.

Sample processing

Each sample was examined directly by making a slide smears using Gram staining to identify any yeast-like structure, oval or rounded blastoconidia of different sizes, filament like structure and bacteria. All samples were cultured on modified Dixon's agar (malt extract 36 g, Peptone 6 g, Ox bile 20 g, Tween 40-10 ml, Glycerol 2 ml, Oleic acid 2 ml, Agar 12 g, deionized water 1,000 ml) (Resusta *et al.*, 2007), and Sabouraud agar (Oxoid Hampshire, United Kingdom) supplemented with chloramphenicol (Sigma-Aldrich – St. Louis, MO, USA), and were incubated at 32 °C during 5 days (Hernández, 2005; Ashbee 2007; Pulido *et al.*, 2010; Cafarchia *et al.*, 2011; Aldrovandi *et al.*, 2016).

All colonies morphologically compatible with *Malassezia* spp. were plated again to obtain pure colonies and describe them macroscopically along with the different morphological characteristics such as size, texture, color, shape, as well as the margin and surface of minimum 10 colonies. In order to describe adequately the microscopic appearance, 30 cells were used to measure length and width; such measurements were made using light microscopy (software Leica Microsystems®.DM 100 LED version 2.1.0.).

The colonies were considered to be *Malassezia* spp. (positive to Urea), underwent a series of biochemical and physiological tests that included assimilation of lipid supplements as Cremophor-EL (Sigma-Aldrich St. Louis,

MO, USA), and Tweens (Merck Darmstadt, Germany/Sigma-Aldrich St. Louis, MO, USA), growth at 37 and 40 °C, enzymatic tests such catalase and β -glucosidase (Cafarchia and Otranto, 2004; Kindo *et al.*, 2004; Ashbee, 2007; Crespo *et al.*, 2008b; Guého-Kellermann *et al.*, 2010). Additionally, phospholipase activity was evaluated using 10% egg-yolk Sabouraud agar (Oxoid Hampshire, United Kingdom) on 4 points plating manner. The results were evaluated after 21 days using the averaging of the Pz values (Cafarchia and Otranto, 2004; Coutinho, 2005; Hurtado-Suarez *et al.*, 2016).

The colonies that resulted morphologically and biochemically compatible with *Candida* spp. (Urea negative) were confirmed by chromogenic Chromagar Candida® (Becton Dickinson GmbH Heidelberg, Germany).

Culture quality control and strain storage

Reference strains of *M. furfur* CBS 7019, *M. pachydermatis* CBS 1879, *M. sympodialis* CBS 7222, *M. slooffiae* CBS 7956 and *M. globosa* CBS 7966 were used. The control strains *Candida albicans* ATCC 90028, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as positive controls for phospholipase activity. All strains, including the isolated ones, were preserved in skim milk at -20°C.

Molecular identification

DNA extraction was performed using the fungi/yeast genomic DNA kit (Norgen® Thorold, ON, Canadá). The extracted DNAs were treated with RNase (Promega, Madison, USA), 2 mg/mL at 37 °C for 4 hours (Gemmer *et al.*, 2002), followed by amplification of region 5.8S DNAr, using primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gaitanis *et al.*, 2002; Hernández, 2005). The amplification was done by using PCR at reaction volumes of 50 μ L having 45 μ L of PCR SuperMix (1.1X) (Invitrogen CA, USA), 1 μ L

of each one of the primers (10 pmol/ μ L) and 3 μ L of genomic DNA (20 ng/mL). The conditions of the reaction were an initial denaturation cycle at 95°C for 5 minutes, 30 cycles at 95 °C for 1 minute, 55 °C for 1 minute and at 72 °C for 1.5 minutes, and an extension final cycle at 72 °C for 5 minutes (Gaitanis *et al.*, 2002; Hernández, 2005). The amplification products were detected by 1.5% agarose gel electrophoresis (Promega Madison, USA) with buffer TBE 1X (Promega Madison, USA), they were stained with ethidium bromide (Invitrogen CA, USA). The gen 5.8S RNAr amplification products were sent for purification and sequencing to Macrogen® Inc (Korea). The sequences were analyzed using BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Results

Cytological examination of the direct Gram stained smears showed yeasts of different forms and sizes in 17.07% (n=14) of all swab samples. The macroscopic morphology of the colonies that were identified as *Malassezia* spp. were cream in color, flat and small with smooth margins with diameters between 1-2.1 mm. These yeasts were isolated mainly from left ear canals (n=5) and udders (n=2). The microscopic measurements showed different sizes of cells that varied from 1.5–2.4 μ m wide and 2.2–3.2 μ m in length.

Biochemical identification test allowed to determine that 50% (n=7) of the yeast isolates belonged to the *Malassezia* genus. The species that were identified included *M. pachydermatis*, *M. globosa*, *M. obtusa*, *M. slooffiae* and *M. sympodialis* in 14.3% (n=1) for each isolate. The remaining two isolates were only identified as belonging to the *Malassezia* genus and were reported as *Malassezia* spp. (see Table

1). However, the molecular identification tests did not agree with the biochemical tests. The molecular tests identified *M. nana* as the most frequent species being 57.1% (n=4) of the isolates, followed by *M. slooffiae* with 28.6% (n=2) of the isolates; the remaining isolate was identified as belonging to a different genus *Trichosporon asahii* (see Table 1). The observed agreement between the two tests was 85.7% when genus identification was tested, but only 14.3% agreement regarding species identification. The rest of the yeasts isolated were determined to be *Candida* spp. in 35.7% (n=5), which were isolated from the preputial and inguinal areas, and *Cryptococcus* spp. was isolated from ear canal in 14.3% (n=2) of the cases.

On the other hand, *Malassezia* spp. was only determined to be present in only 4 out of 22 horses, showing a prevalence of 18.2% in this sample.

In this present study, 28.57% (n=2) of the isolates were phospholipase positive, which was very high for *M. nana* and high for *M. slooffiae*. The remaining 57.1% (n=4) of the isolates did not present this activity.

Candida spp. was isolated in 5 samples presenting negative urease activity and grew in Sabouraud agar. When using Medio CHROMagar Candida BD Becton-Dickinson® media *Candida glabrata* was determined in 80% (n=4) of these isolates and *Candida tropicalis* in 20% (n=1) of the cases. Finally, two samples yielded yeast belonging to genus *Cryptococcus*, basing their classification on positive urease activity and the presence of capsule, when stained with indian ink (Resusta *et al.*, 2007).

Due to the small numbers of isolates, it was not possible to establish any association between isolates and variables such as age, sex or breed.

Table 1. Biochemical and molecular identification of the *Malassezia* spp. isolates from ear canal and udder.

Equine ID	Sample (Anatomical site)	Metabolic and physiologic characteristics of the isolates										Phenotypic identification	Molecular identification			
		Catalase	Urea	Esculin	Growthon Sabouraud	Growth 40 °C**	Growth 37 °C**	Tweens			Cremophor - EL			Phospholipase Activity	Species	Species
							20	40	60	80						
1	EC	+++	+++	-	-	+++	-	+	+	-	-	-	-	+	Malassezia slooffiae	Malassezia slooffiae
		+++	+++	+	-	+	-	-	-	-	-	-	-	NG	Malassezia obtusa	Malassezia nana
2	EC	+++	+++	-	-	+	-	-	-	-	-	-	-	NG	Malassezia spp.	Malassezia nana
		+++	+++	+ weak	-	+	+	+	-	-	-	-	-	NG	Malassezia spp	Malassezia nana
3	EC	+++	+++	-	-	+	-	-	-	-	-	-	-	+	Malassezia globosa	Malassezia nana
		+++	+++	+	+++	+	+	+	+	+	+	+	+	Pz=0,40	Malassezia pachydermatis	Trichosporon asahii
4	U	+++	+++	+	-	+	-	+	+	+	+	+	+	Pz=0,70	Malassezia sympodialis	Malassezia slooffiae

EC: Ear canal

U: Udder

**Grow on Dixon agar at different incubation temperatures. Phospholipase activity: Very high: Pz<0,64. High: Pz between >0,64 and <1. Null: Pz =1. NG: No growth on 10% egg yolk Sabouraud agar.

Discussion

Microscopic characteristics of the colonies identified as *Malassezia* spp. that included shape, margin, size, volume and texture are in accordance with the characteristics reported by several authors (Ashbee, 2007; Arenas, 2008; Crespo *et al.*, 2008a; Guého-Kellermann *et al.*, 2010; Hurtado-Suárez *et al.*, 2016); however, there were differences in colony sizes, which could be attributed to possible limitations when measuring the organisms due to the distribution of the colonies on the agar. Regarding the microscopic characteristics based on the observed differences in the cell length, they were also partially similar to what is reported. This latter observation could also be due to limitations in the measuring as consequence of the cell distribution on the smear. These observations clearly suggest that both methodologies have a limited value when these yeasts are to be identified (Guého *et al.*, 1996; Sugita *et al.*, 2003; Hirai *et al.*, 2004; Hernández, 2005; Cabañes *et al.*, 2007; Crespo *et al.*, 2008b).

The *Malassezia* isolates were determined to be *M. pachydermatis*, *M. globosa*, *M. obtusa*, *M. slooffiae* and *M. sympodialis* with prevalence of 14.3% (n=1) for each one of them. These species have also been reported by other studies to be commonly isolated from normal equine skin (White *et al.*, 2006; Crespo *et al.*, 2002); however, isolation of *M. sympodialis* and *M. pachydermatis* from mammary gland contrasts with the reports by White (2005) and White *et al.* (2006), who isolated *M. slooffiae* and/or *M. equina* from this same anatomical site of healthy mares. According to biochemical test results, the *Malassezia* species was not identified (2.43%) in two isolates, because their results did not coincide with what has been reported in the literature for the different species (Guého *et al.*, 1996).

The identification of one of the isolates as *M. pachydermatis* was based on its ability to grow on Sabouraud Agar at 32 °C, and to be able to assimilate all the Tweens (Guého-Kellermann

et al., 2010; Cafarchia *et al.*, 2011). Despite the fact that *M. globosa* and *M. obtusa* share similar characteristics such as their incapacity to grow at 40 °C and the ability to assimilate tweens, the β -glucosidase test allowed to differentiate one from the other due to the lack of the enzyme by *M. globosa* (Hernández, 2005; Giusiano, 2006). In a similar manner, *M. sympodialis* was differentiated from *M. slooffiae* because it has the β -glucosidase enzyme, and also due to the capacity to assimilate Tweens 40, 60 and 80, while *M. slooffiae* assimilates Tweens 20, 40 and 60, according to the biochemical characteristics reported, it was possible to determine the species of the isolates (Guého *et al.*, 1996; Sugita *et al.*, 2003; Hirai *et al.*, 2004; Giusiano, 2006; Kaneko *et al.*, 2007; Salah *et al.*, 2010).

In order to identify *M. pachydermatis*, the phospholipase activity was evaluated given that it has been reported to be present in higher levels in either isolates from healthy skin or with skin lesions. This previous test and the biochemical characteristic allowed identifying this species (Cafarchia & Otranto, 2004; Juntachai *et al.*, 2009; Pini & Faggi, 2011; Ortiz *et al.*, 2013). In the case of *M. obtusa*, *M. slooffiae*, *M. globosa* and *M. restricta*, it has been reported that they do not show phospholipase activity in healthy animals (Pini & Faggi 2011). In the present study, *M. slooffiae* and *M. globosa* showed phospholipase activity in contrast to what has been reported. The presence of this activity could not be regarded as a virulence factor given that they came from healthy equine skin samples.

M. slooffiae, *M. obtusa*, *M. globosa*, *M. pachydermatis* and *M. sympodialis* were the isolated species from the healthy skin in horses using phenotypical and biochemical tests. These findings are similar to several studies that have reported them as the most common species isolated as normal microbiota of the skin of healthy horses (Crespo *et al.*, 2002; Giusiano, 2006; White *et al.*, 2006).

It is important to highlight that the phenotypical and the molecular identification methods agreed in only one isolate. The

molecular identification technique was able to identify the species of the two isolates that were classified only as *Malassezia* spp. by phenotype classification. These low agreement level could be due to the variations in the results of the biochemical test as seen in the isolates from subject 2 (see Table 1), suggesting the need to use molecular techniques to identify the *Malassezia* species (Gaitanis *et al.*, 2006; Ko *et al.*, 2011; Zia *et al.*, 2015).

Based on the molecular tests used, *M. nana* (80%) was the most frequent species isolated from the ear canal of the horses in the study; it was followed in occurrence by *M. slooffiae* (20%). Similar findings were reported by Aldrovandi *et al.* (2016) and Crespo *et al.* (2002). They reported the same species from this anatomical site, but it is different from the findings by Shokri (2016), who isolated *M. pachydermatis* from the ear canal. An interesting finding was that *Malassezia* spp. was only isolated from ear canals and udders; however, it was not isolated from the other anatomical sample sites despite reports indicating that it has been isolated from those areas in other studies (Crespo *et al.*, 2002; Paterson 2002; White *et al.*, 2006; Shokri, 2016).

The frequency of isolation observed in this study was 18.2% and only one species for each horse was detected. Comparing with other studies, this frequency was lower than the reported by Crespo *et al.* (2002), White *et al.*, (2006) and Shokri (2016), that found prevalences of 34,9, 54 and 60%, respectively, in healthy horses.

Candida spp. was also isolated from some of the study subjects. This yeast is considered as part of the normal microbiota of both the skin of horses and its environment (Sgorbini *et al.*, 2008; Cafarchia *et al.*, 2013; Rózański *et al.*, 2013). This genus may produce opportunistic infections, despite being part of the normal microbiota, in patients with compromise of their immunological status or alteration of the normal microbiota (Cafarchia *et al.*, 2013). It has been also reported that *Cryptococcus* spp. has been detected in the environment where horses are

kept, which may explain why it was isolated in this study (Rózański *et al.*, 2013); however, it has been implicated in mycotic pneumonia in horses (Higgins & Pusterla, 2006).

Malassezia spp. is considered a normal inhabitant of the horse skin, but it has the potential to become a pathogen in cases of abnormal skin microenvironment or cutaneous infections (Paterson, 2002). In conclusion, the prevalence of *Malassezia* spp. in the studied population was 18.2% (4/22), and the species *M. nana* and *M. slooffiae* were identified as part of the skin microbiota in these horses using molecular tests. This is the first report that identifies *Malassezia* spp. as part of normal horse skin microbiota in Colombia.

Declarations

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Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report

Author contributions

Rubiela Castañeda-Salazar: who was responsible for the design or conception of the study, administered the project, coordinated the research, process the samples and wrote the paper.

Diana M Rodriguez-Sandoval: who was responsible for processing the samples and collected the data; participated in the writing of the paper.

Adriana P Pulido-Villamarín: who helped to process the samples, administered the project, and participated in the writing of the paper.

Melva Y Linares-Linares: who helped to process the samples and participated in the writing of the paper.

Olimpo J Oliver-Espinosa: who took the samples, participated in the writing of the paper and reviewed and did a critical reading and editing of the paper.

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