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ORIGINAL ARTICLE

Microstructure of the hoof capsule of pigmented and partial albino buffaloes

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Abstract

In this study, atomic force microscopy (AFM), microtomography (MCT-2D and MCT-3D) and energy-dispersive X-ray fluorescence spectrometry (EDXRF) were used to generate parameters of the microstructure of the hoof capsule of pigmented and partial albino buffaloes. Seventy-two digits of adult pigmented buffaloes and 16 of partial albino buffaloes were used and equally divided into thoracic and pelvic limbs and medial and lateral claws. Fragments of 10 mm × 10 mm of the dorsal wall, abaxial wall and pre-bulbar sole were collected. The parametric assumptions were tested using a Shapiro–Wilk test (normality). The independent *t*-test was used to compare the means at a 5% significance level. AFM demonstrated that the hoof surface of pigmented buffaloes presented with higher average surface roughness (Ra) and root mean square roughness (Rms) ($p < 0.05$) than the hoof surface of partial albino buffaloes. MCT-2D revealed that pigmented buffaloes had extra tubular keratin with a higher density than intratubular keratin. No pores were observed in the hoof capsule of the buffalo digits. MCT-3D demonstrated that pigmented buffaloes have a higher percentage of large and intermediate horn tubules than partial albino buffaloes. However, this difference was not statistically significant. Partial albino buffaloes showed a statistically higher number of horn tubules/mm^2 than pigmented buffaloes ($p < 0.05$). EDXRF revealed a higher amount of sulphur (S) in the hoof capsule of pigmented buffaloes, and the partial albino buffaloes presented a higher number of minerals such as calcium (Ca), potassium (K), zinc (Zn) and copper (Cu).

KEYWORDS
AFM, buffaloes, micro-CT, sulphur, XRF

1 | INTRODUCTION

Buffaloes are more resistant to hoof disorders than crossbred dairy cattle (Sasidharan & Sunilkumar, 2019). The resistance of buffaloes to foot diseases may be attributed to either lower genetic predisposition, lower feeding regimen or different metabolism compared with cattle (De Rosa et al., 2005; Napolitano et al., 2005). However, a study found a prevalence of 17.7% of foot disorders in Mediterranean buffaloes in Italy, in which the most prevalent diseases were hoof overgrowth (17.6%) and corkscrew claw (15.6%) (Guccione et al., 2016). This study also demonstrated the first clinical description of white line disease, interdigital phlegmon, digital dermatitis and interdigital hyperplasia in the hooves of dairy Mediterranean Buffalo (Guccione et al., 2016). These results demonstrated that it is important to

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further study the hoof horn microstructure and the pathogenesis of foot disorders in buffaloes.

Microscopic characterization and mineral quantification represent a substantial innovation in bovine podology research. This is because they help in the understanding of biochemical and cellular events involved in the formation of hoof capsules (Sasidharan & Sunilkumar, 2019). Some techniques have helped in the microscopic characterization and mineral quantification of buffalo (Assis, Silva, Lima, Gouveia et al., 2017; Assis, Vulcani et al., 2017) and cattle (Assis, Silva, Lima, Sant'Ana et al., 2017; Queiroz et al., 2021) hoof capsules. These advances occurred through methods routinely used in materials science, which represent new perspectives for scientific research on the morphology of buffaloes and cattle hooves.

The use of advanced laboratory methods has elucidated some important issues regarding buffaloes' hoof morphology and composition (Assis, Silva, Lima, Gouveia et al., 2017; Assis, Vulcani et al., 2017). However, little is known about albino and partial albino buffaloes hoof. Albinism in buffaloes is an autosomal recessive congenital disorder characterized by the complete or partial absence of melanin in the skin, hair and eyes. This hereditary disease is caused by the absence of the tyrosinase enzyme that catalyses melanin biosynthesis and is associated with high consanguinity in the herd (Damé et al., 2012). The absence of melanin in albino humans and animals can cause several ocular disorders (Kirkwood, 2009) and skin disorders (Kiprono, Chaula & Beltraminelli, 2014). However, we do not know whether albino and partial albino buffaloes have hooves that are more vulnerable to injury than pigmented buffaloes.

Therefore, this study aimed to use atomic force microscopy (AFM), computed microtomography (MCT) and energy-dispersive X-ray fluorescence spectrometry (EDXRF) to evaluate the microstructure and mineral composition of the hoof capsule of pigmented and partial albino buffaloes.

2 | MATERIALS AND METHODS

The study was approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Goiás (UFG) protocol number 20/2014. Seventy-two pigmented buffalo hooves and 16 depigmented hooves of partial albino buffaloes were collected from slaughterhouses under federal inspection and used in the study. All buffaloes were Jafarabadi breed, aged 24–56 months, reared in an extensive system on farms located in the central-west region of Goiás, Brazil. The reduced number of partial albino buffaloes occurred because of the small population of these animals in the region. Furthermore, they are rarely slaughtered because they are exotic. The hooves were equally grouped into fore and hind limbs and inner and outer claws. Then, three 10 × 10 mm hoof fragments were collected from the dorsal and abaxial wall and pre-bulbar sole (Figure 1).

After fragment removal, the attached soft tissue was removed, and the hoof samples were cleaned and degreased with 70% alcohol. The hoof samples were frozen at −20°C and thawed at an ambient temperature of 25°C before analysis. Half of the samples were subjected to atomic force microscopy (AFM) and computed microtomography (MCT). The other half was used for mineral composition assessment by energy-dispersive X-ray fluorescence spectrometry (EDXRF). The samples were collected and prepared according to a previously described methodology (Rabelo et al., 2015).

A scanning probe microscope (SPM) model Park NX-10 (Park Systems®, Suwon, South Korea) in tapping mode was used for AFM analyses. The probe of the AFM device was NCHR type, composed of silicon coated with reflective aluminium (Point Probe®, Nanoworld®, Neuchâtel, Switzerland), a constant force of 42 N/m and a resonance frequency of 320 kHz. The samples were cleaned and fixed to a metallic base with epoxy resin (Araldite Hobby®, TekBond®, Embu das Artes-SP, Brazil) and positioned on the scanning platform. Areas of 25 and 100 μm^2 were scanned. Gwyddion software (version 2.47; Czech Metrology Institute, Jihlava, Czech Republic) was used to obtain the average surface roughness (Ra) and root mean square roughness (Rq).

The 2D and 3D MCT evaluations were performed using a SkyScan 1272 microtomography (Bruker®). Acquisition parameters were a source voltage of 25 kV, source current of 180 µa, number of rows of 180, number of columns of 1224, 17 µm scaled image pixel size and filter Al 0.25 mm. DataViewer (Bruker®) was employed for 2D evaluations, and CTvox, CTan and CTvol (Bruker®) for 3D evaluations. The 2D samples were assessed by interpreting the colorimetric scale produced by the above-mentioned software. The colour scale ranged from dark brown to light green, according to the regions

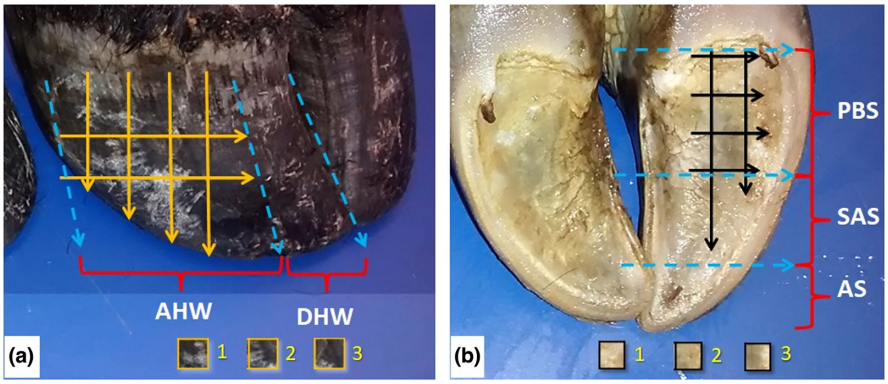

FIGURE 1 Sampling collection of hoof fragments in the buffaloes' hoof capsule. (a) Yellow arrows indicate the direction of cuts and numbers 1, 2 and 3 indicate the fragments obtained from the abaxial wall. AHW: abaxial hoof wall; DHW: dorsal hoof wall. (b) Black arrows indicate the direction of the cuts and the numbers 1, 2 and 3 the fragments obtained from the pre-bulbar sole. AS: apical sole; PBS: pre-bulbar sole; SAS: sub-apical sole

that showed lower and greater X-ray attenuation, respectively (Assis, Silva, Lima, Gouveia et al., 2017). The horn tubules were classified into three diameter ranges to compare the two groups: small diameter (0–25 μm), intermediate diameter (26–100 μm) and large-diameter (>100 μm).

A Ray Ny EDX-720 spectrometer (Shimadzu®, Columbia, EUA) was used to identify the chemical elements located between sodium (Na) and uranium (U) on the periodic table in the hoof samples. The measurements were performed in a vacuum chamber, and liquid nitrogen was used to cool the photon detector. The X-rays were generated by a rhodium (Rh) tube at a voltage of 50 kV and an electric current of 100 μA. The X-ray beam was directed to the sample through a 5.0 mm collimator. The measurement time was 120 s, and the fluorescence spectra were recorded at energies ranging from 0 to 40 keV. The sample was scanned on the inner side of the hoof wall, which was in contact with the laminar corium to avoid contamination by elements such as iron and silicon. The data files provided by the spectrometer were saved in text files (TXT) and transformed into data files (DAT) (Assis, Vulcani et al., 2017).

Statistical analyses were performed using SigmaPlot Version 14 software (Systat Software®, San Jose, USA). The parametric assumptions were tested using the Shapiro–Wilk test (normality), and an independent *t*-test was used to compare the means. Non-parametric data were compared using the Mann–Whitney test. Statistical significance was set at $p < 0.05$.

3 | RESULTS

Atomic force microscopy allowed the reconstruction of three-dimensional images of the superficial topography of keratinocytes (corneocytes) on the surface of the stratum corneum (Figure 2). An independent *t*-test showed that the hoof surface of pigmented buffaloes had a higher average surface roughness (Ra) and root mean square roughness (Rms) ($p < 0,05$) compared with the hoof surface of partial albino buffaloes (Figure 3).

Energy-dispersive X-ray fluorescence spectrometry showed that the sulphur (S) percentage was higher in the pigmented buffalo hooves ($p < 0.05$) compared with partial albino buffaloes. However, partial albino buffaloes presented higher percentages of calcium (Ca), potassium (K), zinc (Zn) and copper (Cu) ($p < 0.05$) compared with pigmented buffaloes (Table 1).

In MCT-2D, we verified that there were no pores in the hoof capsule of the buffalo digits since the extra and intratubular keratin were compact without any spacing between them (Figure 4). A colorimetric scale showed regions of higher density (light green), intermediate density (pink and dark blue) and lower density (brown). This colorimetric scale demonstrated that pigmented buffaloes presented extra tubular keratin with a higher density than intratubular keratin. Conversely, partial albino buffaloes presented intratubular keratin with a higher density than extra tubular keratin (Figure 4).

MCT-3D showed the number and diameter of horn tubules in the hoof capsule of pigmented and partial buffaloes. In this assessment, we found that partial albino buffaloes presented horn tubules with the following diameters and percentages: 15 μm (57.98%), 45 μm (40.69%), 75 μm (1.31%) and 105 μm (0.006%). Pigmented buffaloes presented horn tubules with the following diameters and percentages: 17 μm (40.24%), 51 μm (49.75%), 85 μm (8.46%), 119 μm (1.45%) and 153 μm (0.10%). These values showed that pigmented buffaloes presented a higher percentage of intermediate and large horn tubules than partial albino buffaloes. However, this difference was not statistically significant (Figure 5). Partial albino buffaloes presented a statistically higher number of horn tubules/mm^2 than pigmented buffaloes ($p < 0.05$) (Figure 6).

4 | DISCUSSION

This study revealed important information about the microstructural characterization of the hoof capsule of pigmented and partial albino buffaloes. MCT, AFM and EDXRF allowed for the assessment of the microstructure, density and mineral composition of the buffalo hoof. Thus, demonstrating that these techniques can be applied in the evaluation of other bovids species.

The AFM evaluation demonstrated that the hoof capsule of the pigmented buffaloes presented higher roughness than the partial albino buffalo hoof. The roughness of the buffalo hoof is a little-studied parameter in hooves and apparently does not influence the hoof quality; however it is highly influenced by the environment in which the animals live. We have hypothesized that the greater roughness in the hoof surface of pigmented buffaloes could have been caused by a higher roughness of the ground and higher humidity in the environment where these animals were kept. However, we cannot prove this hypothesis because we did not have access to the farms where the buffaloes were kept. Queiroz et al. (2021), using AFM and confocal laser scanning microscopy (CLSM), verified that biotin supplementation did not influence the roughness of the hoof wall surface. According to these authors, the high irregularity of the hoof surfaces made it difficult for them to scan samples using AFM. The same difficulty was observed in the present study. We believe that AFM is an extremely sensitive examination to assess the roughness of the hoof. Consequently, the probe loses contact with the surface when the grooves are very deep, making this assessment laborious and long. According to Queiroz et al. (2021), CLSM is a more efficient examination for assessing hoof roughness. The influence of surface roughness on the quality and mechanical resistance of the hoof needs to be further investigated in future research.

MCT demonstrated that the hoof of pigmented and partial albino buffaloes consists of intermediate density-keratinized tissue (dark blue and pink) (Figure 4). In all areas, there was some degree of attenuation of the X-rays. Thus, indicating that there were no empty spaces or pores in the evaluated samples. This was also observed in other studies that evaluated cattle (Assis, Silva, Lima, Sant'Ana et al., 2017; Queiroz et al., 2021) and buffalo (Assis, Silva, Lima, Gouveia et al., 2017) hoof capsules by MCT. Conversely, Kasapi and Gosline (1997) evaluated the equine hoof wall morphology using circularly polarized light microscopy

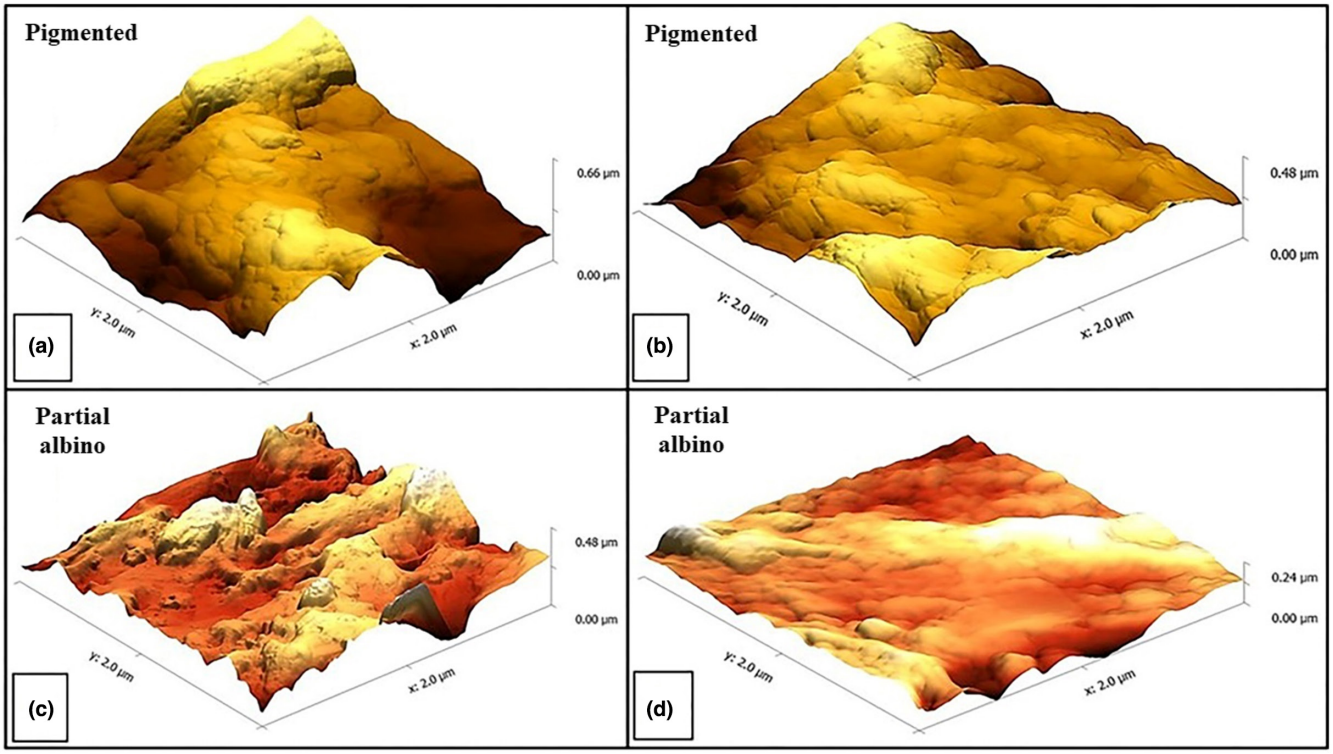

FIGURE 2 Three-dimensional images of the superficial relief of the abaxial hoof wall of the Jafarabadi buffaloes obtained by atomic force microscopy (AFM), showing the variation in the roughness. (a) Keratinocyte surface from the inner claw of the forelimb of pigmented buffaloes. (b) Keratinocyte surface from the outer claw of the hindlimb of pigmented buffaloes. (c) Keratinocyte surface from the inner claw of the forelimb of partial albino buffaloes. (d) Keratinocyte surface from the outer claw of the hindlimb of partial albino buffaloes

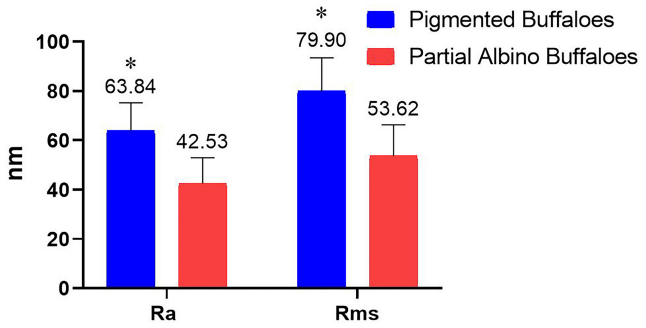

FIGURE 3 Roughness (relief) of the hoof capsule of pigmented and partial albino Jafarabadi buffaloes obtained by atomic force microscopy (AFM). Ra: average surface roughness; rms: root mean square roughness. *indicates a significant difference ($p < 0.05$) according to the independent *t*-test

and found that medullary cavities of the horn tubules were usually devoid of cellular material. The keratinocytes that form the medulla of the horn tubules receive nutrients from the suprapapillary dermis in the central region of the dermal papilla. In this position, keratinization is often incomplete and these keratinocytes disintegrate after a short time, leaving the lumina of the horn tubules empty (Reese et al., 2020, p. 655). The absence of empty regions in the microtomography images can be explained by the position where the samples were obtained in the hoof capsule or by differences in the evaluation techniques used in other studies.

Energy-dispersive X-ray fluorescence spectrometry demonstrated that the hoof capsule of partial albino buffaloes had significantly less S and significantly more Ca, K, Zn and Cu compared with pigmented buffaloes. These differences are difficult to explain, as hoof mineral composition is influenced by several factors, such as feed ratio (Langova et al., 2020) and biochemical events in the horn tissue (Hepburn et al., 2007). The availability of amino acids, macro and micro minerals, and vitamins in the diet significantly affects the growth, quality and structural integrity of the hooves (Langova et al., 2020; Lean et al., 2013; Mülling et al., 1999; Tomlinson et al., 2004). Although buffaloes used in this study were raised on pastures, differences in the type of soil, pasture and mineral supplementation offered to them may have influenced the percentage of minerals present in the hooves.

A possible explanation for the higher concentration of S in pigmented buffalo hooves is the presence of melanin. Melanin is a biological pigment that provides a dark colour to keratinized tissue. Its best-known function is protection against UV radiation. However, it has other functions in the body, such as antioxidant action and immune system modulation (ElObeid et al., 2017). Melanin has a high affinity for substances that have the thioureylene group in its chemical structure (2-thiouracil, methimazole, uracil and thiourea), where S is of crucial importance for the absorption of these substances in melanin (Larsson, 1991; Larsson, 1993). These substances are false precursors for melanin and accumulate in pigmented tissues, where melanin synthesis is high (Olander

TABLE 1 Mean ± standard deviation of the percentage of minerals found in the hoof capsule of pigmented and partial albino Jafarabadi buffaloes obtained by energy-dispersive X-ray fluorescence spectrometry (EDXRF)

BUF	S	Ca	K	P	Zn	Cu
PIG	83.53 ± 2.96a	8.13 ± 1.87b	3.47 ± 1.59b	3.74 ± 0.47a	0.81 ± 0.10b	0.31 ± 0.04b
ALB	72.57 ± 9.31b	16.08 ± 8.5a	4.91 ± 1.79a	3.96 ± 1.34a	1.93 ± 0.51a	0.53 ± 0.09a

Note. Means followed by different letters in the same column indicate a significant difference ($p < 0.05$) by independent *t*-test.

Abbreviations: ALB, partial albino; BUF, buffaloes; Ca, calcium; Cu, copper; K, potassium; P, phosphor; PIG, pigmented; S, sulphur; Zn, zinc.

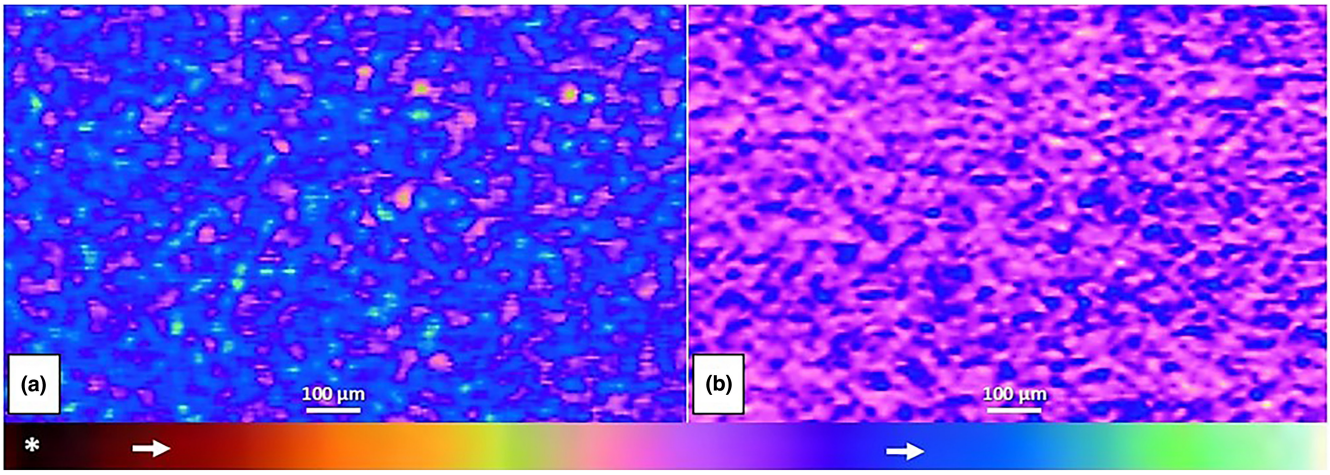

FIGURE 4 Bidimensional microtomography (MCT-2D) of a region of the abaxial hoof wall of pigmented and partial albino Jafarabadi buffaloes. (a) Abaxial hoof wall of pigmented buffaloes, where extra tubular keratin is represented by the dark blue colours and light green, while intratubular keratin is represented by pink. (b) Abaxial hoof wall of partial albino buffaloes, where extra tubular keratin is represented by colour pink and intratubular keratin is represented by dark blue. According to the colorimetric scale indicated at the white (*), the colours brown and yellow represent regions of lower X-ray attenuation, the colours pink and dark blue indicate regions of intermediate X-ray attenuation and the colour light green represents regions of higher X-ray attenuation

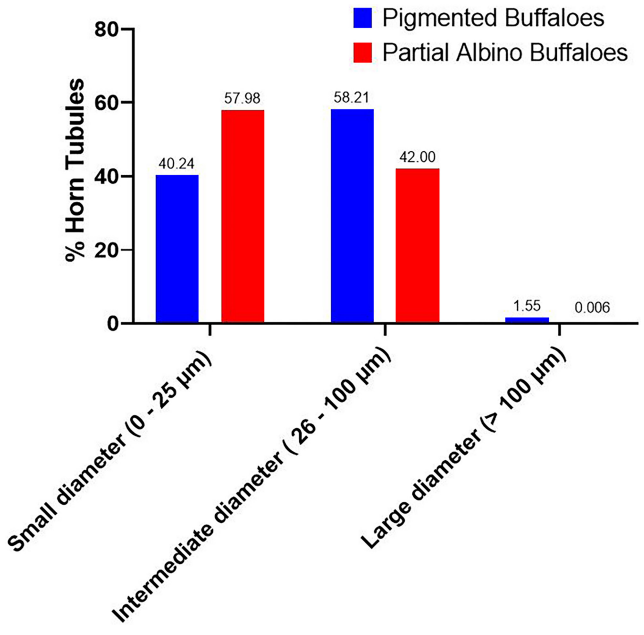

FIGURE 5 Frequency distribution of horn tubule diameters in the hoof capsule of pigmented and partial albino Jafarabadi buffaloes obtained by the microtomography (MCT)

et al., 1983). Therefore, we propose that the highest percentage of S in pigmented hooves may have occurred because of the greater affinity of melanin for substances that have S in their composition. Sulphur (S) is an important mineral for hoof quality because it forms disulphide bridges, which are bonds between sulfhydryl groups responsible for stabilizing the protein structure of keratin (Feng & Coulombe, 2015). However, it is not known whether the S present in the substances that have a thioureylene group can improve the resistance of the hoof capsule. Future research should be carried out to assess whether a higher concentration of S in pigmented hooves may be associated with greater mechanical resistance.

The higher concentration of the minerals Ca, K, Zn and Cu in partial albino buffaloes was an unexpected result. All of these minerals are important in several aspects of keratinization and hoof quality (Langova et al., 2020; Lean et al., 2013). Therefore, it was expected that there would be no differences among the groups or that the pigmented buffaloes would have higher percentages of these minerals. These differences may have occurred because of the greater deposition of these minerals in the basal epidermis during the beginning of keratinization in the hooves of pigmented buffaloes. Moreover, this difference in the percentage of minerals between the groups

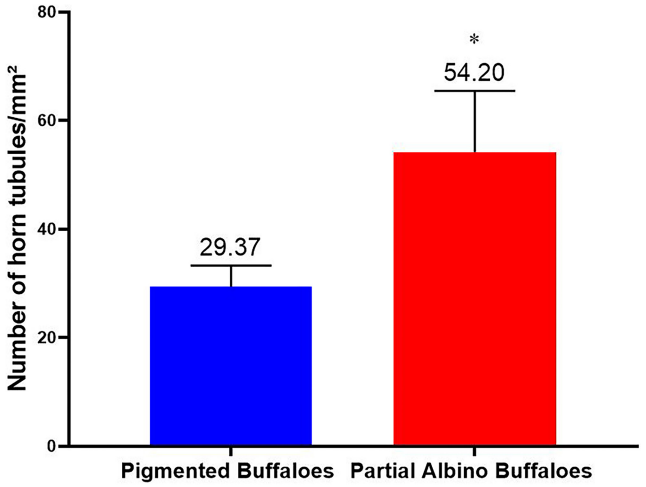

FIGURE 6 Number of horn tubules per mm2 in the hoof capsule of pigmented and partial albino Jafarabadi buffaloes obtained by microtomography (MCT). * indicates a significant difference ($p < 0.05$) according to the independent *t*-test

may explain the difference in density between extra and intratubular keratin found in partial albino buffaloes demonstrated by 2D-MCT (Figure 4).

The lower concentration of Zn in the pigmented buffalo hoof may be explained by the participation of this micromineral in various metabolic pathways for the formation of the horn tissue. This includes the activation of metalloenzymes that act in the differentiation of keratinocytes and the formation of keratin tonofilaments (Lean et al., 2013; Tomlinson et al., 2004; Van Amstel & Shearer, 2006). The participation of Zn in these metabolic pathways during the formation of keratinocytes probably decreases its concentration in the final horn tissue. Thus, the results of this research indicate that pigmented buffaloes probably synthesize more keratin, form more disulphide bridges, and require more Zn to differentiate the keratinocytes compared with partial albino buffaloes.

The percentage of Cu was higher in the hooves of partial albino buffaloes. This result may be explained by the important role of copper in the synthesis of melanin as a cofactor for tyrosinase (Palumbo et al., 1988). Copper also acts as a cofactor for sulfhydryl oxidase enzymes that oxidize thiol groups (-SH) from cysteine residues and form disulphide bridges (Lean et al., 2013). In addition, higher concentrations of Cu are required for the antioxidative activity of the enzyme Cu/Zn superoxide dismutase. This reduces the peroxidation of intercellular cementum lipids (Lean et al., 2013). Therefore, the higher Cu activity in these metabolic pathways in pigmented buffaloes causes less deposition in pigmented horn tissue, which explains the results observed in this study.

The lower Ca concentration in pigmented buffalo hooves can be explained by the function of this mineral in activating epidermal transglutaminases. These enzymes stimulate the formation of cross-links between keratin and other proteins that form keratinized tissues (Yamane et al., 2016). A study demonstrated that Ca and K act synergistically in the release of lamellar bodies rich in lipids, which form intercellular cementum in the skin of rats (Kalinin et al., 2001). This can occur in the hoof of pigmented buffaloes. However, there are no studies that prove the interaction between these minerals in the hoof. Therefore, it is believed that the lower concentrations of Ca, K, Zn and Cu in the hooves of pigmented buffaloes occurred because of the greater role of these minerals in cellular metabolism in the basal layers of the keratinized epidermis.

Partial albino buffaloes showed a higher percentage of horn tubules with smaller diameters (0–25 μm) than pigmented buffaloes. This result suggests that the hooves of partial albino buffaloes are more fragile since the greater deposition of keratin in larger diameter horn tubules is one of the factors that contribute to the resistance of the hoof capsule (McKittrick et al., 2012). However, we did not find clinical studies to support the notion that partial albino buffaloes present higher incidence of hoof disorders than pigmented buffaloes. Therefore, it is recommended that clinical research be conducted to elucidate this issue. In addition, pigmented buffaloes showed a higher concentration of S in the hoof, which justifies the higher density and greater attenuation of X-rays compared with the partial albino buffalo hoof. This can be explained because S has an atomic mass of 32.06 u and forms disulphide bridges. This results in a molecular mass of 62.12 u in the formation of alpha keratin (Post et al., 2020). This protein also forms several disulphide bridges, generating horn tissue with high density, stability and resistance. Sulphur (S) can also bond with Zn (65.41 u) or Cu (63.55 u) (Post et al., 2020). However, in the hoof, these elements are found in a lower percentage and K (39.10 u).

A higher concentration of Ca (40,078 u) was observed in the hoof of partial albino buffaloes. However, as demonstrated in the 2D-MCT (Figure 4), this did not result in greater density in the horn tissue. It is known that in bones, Ca binds with other elements to form hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], which has a molecular weight of 1004.6228 u and is responsible for increasing the attenuation of X-rays in the radiographic examination of bone tissue (Fihri et al., 2017). In the hoof, Ca is found in the isolated form, and despite its greater atomic mass than S, its concentration is 10 times lower in the hoof of pigmented buffaloes (Table 1). Therefore, S is the element that provides the greatest attenuation of X-rays in the buffalo hoof. However, the intrinsic characteristics of the hooves of the partial albino buffaloes need to be better studied, because they are genetically different from pigmented ones.

In conclusion, buffalo hoof had no pores. The hooves of pigmented buffaloes have greater roughness (relief) and a higher density of intratubular and extra tubular keratin compared with the hooves of partial albino buffaloes. Partial albino buffaloes have higher number of horn tubules/mm^2 than pigmented buffaloes. Pigmented buffalo hooves have higher S content than the partial albino buffalo hoof. However, partial albino buffalo hooves present higher concentrations of Ca, K, Zn and Cu.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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