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Microtomographic Parameters and Nanoindentation of the Hoof of Girolando Cattle

B. M. Assis[1], L. A. F. Silva[2], C. R. O. Lima[3], F. J. F. Sant'Ana[4]*, G. P. Santos[1], V. A. S. Vulcani[1] and R. E. Rabelo[1]

[1] Addresses of authors: Universidade Federal de Goiás, Regional Jataí, BR 364, Km 195, 75800-000, Jataí, Goiás, Brazil;
[2] Escola de Veterinária e Zootecnia, Universidade Federal de Goiás, Campus Samambaia, 74690-900, Goiânia, Goiás, Brazil;
[3] Universidade Estadual de Goiás, Campus Jataí, R. Léo Lince 610, 75800-000, Jataí, Goiás,Brazil;
[4] Laboratório de Diagnóstico Patológico Veterinário, Universidade de Brasília, Hospital Veterinário de Grandes Animais, Granja do Torto, 70636-020, Brasília, Distrito Federal, Brazil

***Correspondence**
Tel.: +55 61 3468-7255;
Fax: +55 61 3468-7255;
e-mail: santanafjf@yahoo.com

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Summary

The aim of this study was to describe the microstructure of the pigmented and depigmented hoof capsule of Girolando cattle by bi- and tridimensional microtomography and nanoindentation, analysing the possible relation between these findings and the susceptibility of such animals to podal diseases. To carry out the microtomography and the nanoindentation, duplicated samples were collected from the dorsal wall, abaxial wall and pre-bulbar sole of the hoof capsule. Material collection was performed in 40 medial digits of thoracic limbs and 40 lateral digits of pelvic limbs. The bidimensional microtomography showed that the dorsal wall of the thoracic and pelvic limbs presented higher density, followed by the abaxial wall, and finally by the sole, with the lowest density. Moreover, the hoof capsule of cows of Girolando breed is a compact, non-porous material, and constituted by extratubular and intratubular keratin. By the tridimensional microtomography, it was possible to measure the angles of the corneal tubules in relation to the periople and the claws in the different regions of the hoof capsule, which were 90° for the dorsal wall, 55° for the abaxial wall and 70° for the sole. The tridimensional microtomography also showed corneal tubules of different diameters: 17, 51, 85, 119 and 153 μm. The nanoindentation test, when performed in different regions of the hoof capsule, did not reveal significant difference of Vickers hardness in the evaluated areas. However, we verified a larger elastic module of these regions on the transversal cut of the corneal tubules compared to the longitudinal cut.

Introduction

Epidemiologic, clinical and therapeutical aspects of podal diseases in dairy cattle have been the target of scientific investigations for decades. However, research of hoof morphology, especially micro and nanostructural characteristics, is scarce. The lack of more complex studies has limited the understanding of physiological events in healthy hoofs, as well as the anatomical structure in different breeds, and their predisposition to podal diseases. In such circumstances, the employment of efficient therapeutical protocols and the implementation of adequate preventive measures are restricted, intensifying the economic losses of the breeders. This reality provides new scenery for scientific research, which implies revisiting the morphological sciences under new perspectives, employing innovative methods. The use of advanced scientific techniques of investigation that have already been used in other domains, for instance, physics, chemistry, geology, mechanical and construction engineering, dentistry and orthopaedics appears as a unique tool for agrarian sciences, especially veterinary medicine. This innovation seems to be promising for the understanding of the microstructure and of the factors related to resistance and quality of the hoof capsule of bovine digits (Assis, 2015; Rabelo et al., 2015).

Bovines hoof is an anatomic structure constituted of keratinized epidermic tissue, divided in wall, heel, sole, heel bulb, white line and claw (Greenough, 2007). The epidermis is responsible for the proliferation of keratinocytes, keratin synthesis, intercellular cement and the maintenance of quality and integrity of the hoof regions (Bragulla et al., 2004; Silva et al., 2008). Studies have shown that concentric compactness of tubular keratin forms horn tubules (Banks, 1991), strongly connected by sulphur bridges and keratinocytes, linked by desmosomes and intercellular cement (Silva et al., 2001; Nosanchuk and Casadevall, 2003; Tomlinson et al., 2004; Greenough, 2007). This support confers stability, resistance and hardness to the hoof (Mulling and Hagen, 2012). However, conventional methods employed to identify these structures, such as histopathology, radiology and tomography, have not been enough to unravel all the physiological events that occur in the hoof (Tombolato et al., 2010; Belge et al., 2012; Silva, 2012). As the bidimensional and the tridimensional microtomographies allow to evaluate the microstructure of several materials and to estimate density variation by differences in X-ray attenuation (Lima et al., 2009; Lopes et al., 2012), preliminary studies have used this tool to investigate the microscopic events of bovine hoof.

Simultaneously, nanoindentation tests measure the resistance to penetration or resistance to deformation of the material. This methodology was used in a pioneering way to measure Vickers hardness on buffalo's hooves (Assis, 2015). The test is based on the use of the mechanical tester equipment that records, in real time, the values of the applied force and the penetration depth, generating a graphic in a particular mathematical model. Therefore, it is possible to extract information about the elastic and plastic properties of the material, resulting in the Vickers hardness and elastic module.

Tridimensional microtomography (TM) and nanoindentation (NH) techniques are innovative, auxiliary and versatile methods of precise diagnosis in osteology (Silva, 2012). Although it is still experimental, it has extreme relevance for bovine podology (Assis, 2015; Rabelo et al., 2015). Therefore, the importance of such basic and innovative research, as well as the belief that the information obtained may determine anatomical parameters, and hence influence the future of podology, supported this study.

In this study, we aimed at describing the microstructure of the pigmented and depigmented hoof capsule of Girolando cattle by bi- and tridimensional microtomography and nanoindentation, analysing the possible relation between the findings and the susceptibility of such animals to podal diseases.

Material and Methods

This research was carried out after the approval by the Ethics Committee for the Use of Animals from UFG, protocol No. 20/2014. Eighty pelvic limbs were collected in federal inspected cold stores, from the distal third of the metacarpal and metatarsal, totalling 40 thoracic and 40 pelvic limbs (being 20 from the left and 20 from the right) of 20 adult cows (24-60 months), of Girolando breed, free from any kind of hoof infirmity. Before the collection at the cold stores, the anatomical pieces were strictly evaluated. Of the 80 pieces analysed, 40 digits were strictly pigmented and 40 digits were strictly depigmented.

After this step, the material was identified and placed under refrigeration in a refrigerator at 4°C (Winkler and Margerison, 2012). Duplicated samples were collected from the dorsal wall, abaxial wall and pre-bulbar sole of the hoof capsule, with approximately 10 × 10 mm, vertically and horizontally to the keratinized tissue, to carry out the microtomography and the nanoindentation. Material was collected from 40 medial digits of thoracic limbs and 40 lateral digits of pelvic limbs. The choice was based on the information that these digits receive greater weight overload and present greater incidence of podal injuries (Tomlinson et al., 2004). The samples were cleaned by the removal of all soft tissue, and then they were placed in plastic packages and frozen at −15°C (Tarlton et al., 2002; Tombolato et al., 2010; Rabelo et al., 2015). At the next step, the material was sent to the Laboratory of Nanotechnology (LNNano) in Campinas, São Paulo State, Brazil, where the microtomographic tests were performed. As for the 2D and 3D microtomographies and the nanoindentation test, the samples were previously defrosted at room temperature. This evaluation aimed at verifying the presence or absence of pores in the hoof capsule keratin, the difference in density in the regions evaluated, as well as quantifying (mm^2) and analysing the morphometry of the horn tubules of bovine hoof. For the nanoindentation test, the samples were sent to the Department of Material Engineering – Engineering School, São Carlos, USP – São Carlos, São Paulo State, Brazil. This test showed Vickers hardness and the elastic module of the material. The samples were analysed according to the methodology described by Fischer-Cripps (2011) and Assis (2015).

The data were analysed by *T*-test for the comparison between means, and ANOVA for three means, at a 5% significance level (Sampaio, 2010).

Results

In the bidimensional microtomography evaluation, the colorimetric scale indicated density differences by the X-

ray attenuation. The colours brown and yellow represented regions with smaller attenuation, characterizing smaller density. On the other hand, the colours pink, blue and green showed greater X-rays attenuation regions, and consequently, higher density. The dorsal wall presented greater density followed by the abaxial wall and finally by the sole, which had the lowest density (Fig. 1).

Using bidimensional microtomography, we verified the hoof capsule of cows of the Girolando breed is a compact, non-porous material, and constituted by extratubular and intratubular keratin (Fig. 1). This characteristic was materialized by the tridimensional analysis. Therefore, by applying a 3D model to the analysis, we verified that there are no pores or spaces between two keratins, revealed by the difference in colouration (Fig. 2).

By evaluating only the intratubular keratin, the structure that allowed the observation of the morphology, angulation and diameter of horn tubules using the tridimensional microtomography, we observed these horn tubules are parallel and helicoidal in all regions of the hoof capsule (Fig. 3). A detailed analysis of the material allowed to measure the angulation of the horny tubules in relation to the periople and claw in different regions of the hoof capsule. The dorsal wall presented 90° angulation, abaxial wall 55° and sole 70° (Fig. 3b,c,d).

The tridimensional microtomography revealed horn tubules of different diameters: 17 μm, 51 μm, 85 μm, 119 μm and 153 μm. The greatest percentage of horn tubules is represented by a structure of 51 μm, followed by tubules with 17, 85, 119 and 153 μm present in the

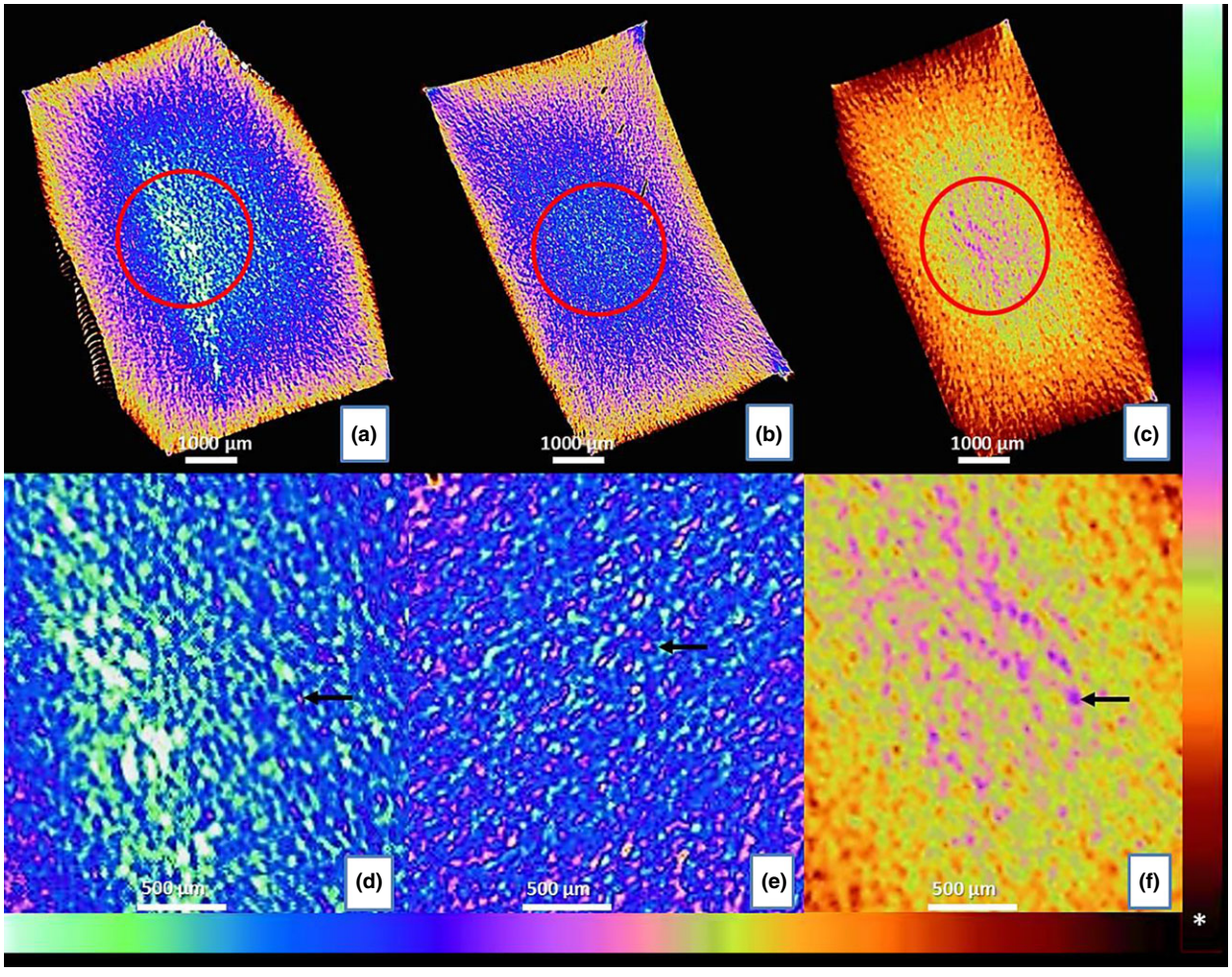

Fig. 1. Bidimensional microtomography of the hoof capsule of Girolando cattle. (a) Dorsal wall; (b) abaxial wall; and (c) sole of the hoof capsule, showing difference in density, red circles. According to the colorimetric scale indicated at the white (*), the colours brown and yellow represent regions of lower density, and the colours pink, blue and light green, greater density. (d) Dorsal wall (black arrow), intratubular keratin (pink/blue) and the extratubular keratin (green). (e) Abaxial wall, intratubular keratin (pink – black arrow) and extratubular keratin (blue and light green), indicating lower density of intratubular keratin in relation to extratubular keratin. (f) Sole region, intratubular keratin (blue/pink – black arrow), and extratubular keratin (yellow), indicating the lowest density of the sole compared to abaxial and dorsal walls.

dorsal wall, abaxial wall and sole, respectively, in both thoracic and pelvic limbs. However, there was no significant statistical difference between the evaluated digits ($P > 0.05$).

By comparing the regions of the hoof capsule, the abaxial wall of the thoracic digits of presented a higher percentage of 85 μm tubules in relation to the dorsal wall and the sole ($P < 0.05$). On the other hand, the dorsal

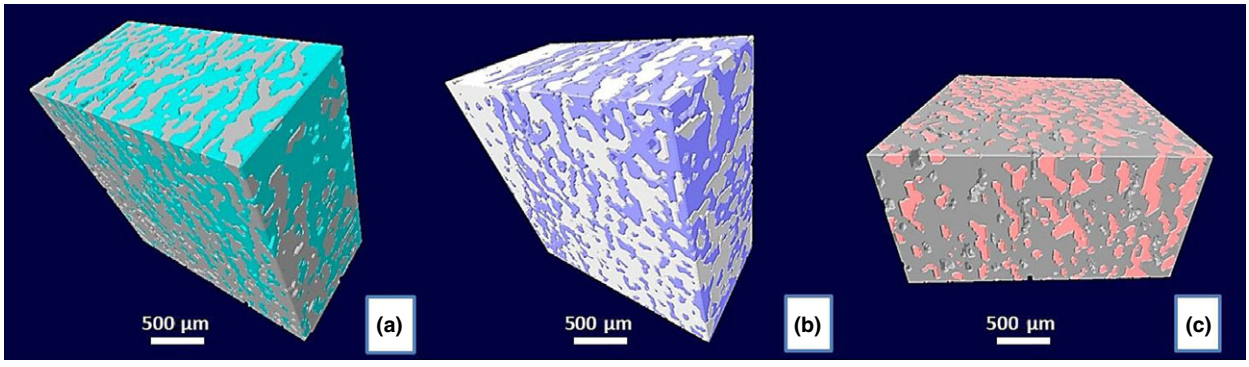

Fig. 2. Tridimensional microtomography of the hoof capsule of the digits of Girolando cattle (3D model, obtained by the software CTvol, of intratubular and extratubular keratin arranged in a compact way without pores). (a) Dorsal wall with the presence of intratubular keratin (green) and extratubular keratin (grey). (b) Abaxial wall, showing intratubular keratin (purple) and extratubular keratin (white). (c) Sole with intratubular keratin (pink) and extratubular keratin (grey).

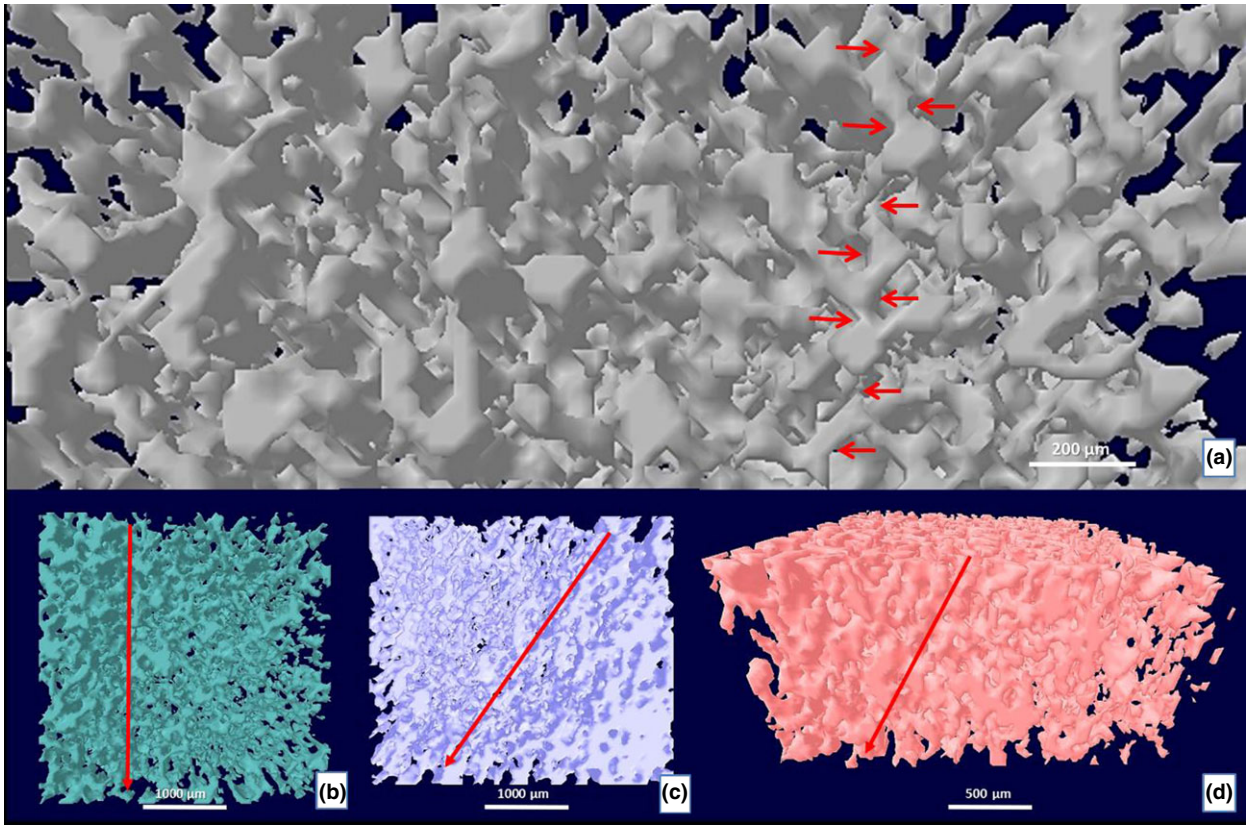

Fig. 3. Tridimensional microtomography (3D model obtained by CTvol) of the hoof capsule of digits of Girolando cattle. (a) Horn tubules in helicoidal shape (red arrows). (b, c and d) Angulation of the corneous tubules: 90° for dorsal wall, 55° for abaxial wall and 70° for sole, respectively (red arrows).

wall and sole showed the greatest percentage of 17 μm horn tubules in relation to the abaxial wall ($P < 0.05$). Regarding the diameter, percentage and number of horn tubules/mm^2 in the evaluated regions, we did not verify statistical difference among these regions ($P > 0.05$), regardless of the digits being pigmented or depigmented (Table 1).

Vickers hardness test and elastic module, when performed in different regions of the hoof capsule, did not present significant difference, regardless of the pigmentation ($P > 0.05$). However, we verified a difference in Vickers hardness and elastic module of such regions regarding the longitudinal and transverse cuts of the corneal tubules ($P < 0.05$) (Table 2).

Discussion

Initially, a general analysis regarding the importance of scientific investigations about the microstructure of the hoof capsule of cattle reinforces the uniqueness of the data herein presented. Although the subject has been investigated by different techniques, including histological evaluation, radiology, tomography, electronic microscopy and venography (Tombolato et al., 2010; Belge et al., 2012; Freitas, 2015), studies employing bidimensional and tridimensional microtomography or Vickers hardness tests are scarce in cattle podology (Assis, 2015; Rabelo et al., 2015). These analyses are innovative and important; nevertheless, macrostructural, epidemiologic and clinical investigations have continuously directed the scientific research on cattle hoof. Therefore, as the traditional methods used to evaluate digit samples have limited the understanding of morphophysiological events that happen in this anatomic structure, many researchers have been searching new perspectives that facilitate the assessment of the hoof capsule. Thus, it is reasonable to study the microstructure of healthy hooves first to better understand the possible influence of the intrinsic mechanisms of each breed on risk factors and the vulnerability of the animals to several podal diseases.

In this manuscript, the difference in density observed among the dorsal wall, the abaxial wall and the sole may be related to the higher or lower overload of body mass on this anatomical structures. The sole needs to be more elastic to decrease the impact during support of the locomotor limb on the ground. Thus, the sole seems to be more vulnerable to certain infirmities such as ulcers, erosions and white line disease, as some authors have already mentioned (Raber et al., 2004; Belge et al., 2012). Other studies have also attributed the presence of greater overload of body mass on dorsal and abaxial walls compared to the sole to the differences in hardness among the regions of cattle hoof (Budras and Mülling, 1998; Tomlinson et al., 2004); however, these findings have not been correlated to a greater susceptibility of the sole to hoof disease. In the other studies, microtomography or Vickers hardness test have not been performed. A study showed higher density of abaxial wall and sole in buffaloes,

Table 1. Diameter, percentage and total number of corneal tubules present in the digits of Girolando cattle by microtomography examination. Each set of data between lines is independent comparisons

DIG/HR/AL	Percentage of corneal tubules					
Diameter	(17 μm)	(51 μm)	(85 μm)	(119 μm)	(153 μm)	NCT/mm2
TL – M	36.76 ± 3.91a	51.73 ± 2.94a	9.95 ± 1.16a	0.16 ± 0.002a	0.00 ± 0.00a	28.48 ± 3.84a
PL – L	36.61 ± 3.54a	48.75 ± 3.65a	11.20 ± 2.81a	0.47 ± 0.023a	0.00 ± 0.00a	35.97 ± 2.87a
DW	36.82 ± 1.35a	51.28 ± 2.99a	10.65 ± 3.13a	0.31 ± 0.012a	0.00 ± 0.00a	28.43 ± 4.31a
AW	36.81 ± 1.53a	50.53 ± 3.13a	10.28 ± 3.15a	0.96 ± 0.062a	0.00 ± 0.00a	35.96 ± 2.49a
SOL	36.44 ± 1.73a	48.85 ± 3.85a	10.78 ± 2.05a	0.03 ± 0.004a	0.00 ± 0.00a	35.15 ± 2.51a
TL – DW	37.72 ± 0.99b	53.49 ± 1.58a	8.61 ± 0.69a	0.16 ± 0.09a	0.00 ± 0.00a	30.36 ± 0.35a
TL – AW	28.93 ± 3.09a	50.47 ± 2.963a	16.14 ± 1.6b	4.00 ± 3.75a	0.00 ± 0.00a	31.02 ± 0.79a
TL – SOL	43.63 ± 3.07b	51.23 ± 4.71a	5.10 ± 1.69a	0.03 ± 0.04a	0.00 ± 0.00a	24.20 ± 1.5a
PL – DW	35.92 ± 1.11a	49.07 ± 5.08a	12.69 ± 3.49a	2.14 ± 2.49a	0.15 ± 0.21a	26.52 ± 0.17a
PL – AW	44.68 ± 8.66a	50.59 ± 4.54a	4.43 ± 3.72a	0.28 ± 0.40a	0.00 ± 0.00a	34.81 ± 0.002a
PL – SOL	29.24 ± 18.69a	46.47 ± 9.79a	16.47 ± 7.43a	6.99 ± 9.88a	0.82 ± 1.16a	48.16 ± 0.76a
TL – PIG	37.98 ± 7.39a	51.69 ± 3.44a	9.79 ± 4.57a	0.52 ± 0.72a	0.00 ± 0.00a	23.81 ± 6.96a
PL – PIG	43.33 ± 7.09a	51.15 ± 3.28a	5.39 ± 4.35a	0.13 ± 0.22a	0.00 ± 0.00a	37.57 ± 3.23a
TL – DEPIG	35.54 ± 7.77a	51.77 ± 3.13a	10.10 ± 6.75a	2.27 ± 3.79a	0.31 ± 0.53a	33.52 ± 3.46a
PL – DEPIG	29.90 ± 12.14a	46.27 ± 7.16a	17.01 ± 9.99a	6.15 ± 6.98a	0.65 ± 0.87a	34.22 ± 2.56a
Mean	36.70	50.15	10.50	1.64	0.13	32.55

Means followed by similar letters in the same column and same variable did not differ among each other ($p > 0.05$).
DIG, digits; HR, Hoof Regions; AL, anatomical localization; TL, thoracic limb; PL, pelvic limb; DW, dorsal wall; AW, abaxial wall; SOL, sole; DEPIG, depigmented; NCT/mm^2, number of corneal tubules per mm^2; L, lateral; M, medial; PIG, pigmented.

Table 2. Vickers hardness values and elastic module of the hoof capsule of Girolando cattle

DIG/HR/LC	VICKERS hardness	Elastic module
TL	29.331 ± 4.872a	4.624 ± 0.367a
PL	29.136 ± 3.851a	4.664 ± 0.652a
DW	30.193 ± 3.375a	4.644 ± 0.466a
AW	30.463 ± 2.947a	4.694 ± 0.676a
SOL	27.044 ± 5.650a	4.593 ± 0.439a
TL – DW	29.950 ± 4.310a	4.652 ± 0.292a
TL – AW	30.649 ± 2.968a	4.639 ± 0.525a
TL – SOL	27.393 ± 6.953a	4.581 ± 0.328a
PL – DW	30.435 ± 2.627a	4.635 ± 0.634a
PL – AW	30.277 ± 3.262a	4.749 ± 0.863a
PL – SOL	26.695 ± 4.816a	4.606 ± 0.571a
TL – DW	29.745 ± 4.110a	4.645 ± 0.396a
TL – LD	28.709 ± 6.218a	4.592 ± 0.353a
PL – DW	29.895 ± 5.410a	4.445 ± 0.473a
PL – LD	28.629 ± 2.636a	4.809 ± 0.738a
TL – PIG	30.827 ± 4.550a	4.563 ± 0.408a
TL – DEPIG	25.666 ± 0.446a	4.794 ± 0.421a
PL – PIG	29.071 ± 1.697a	4.527 ± 0.596a
PL – DEPIG	33.057 ± 1.606a	4.300 ± 0.215a
TC	29.566 ± 2.696a	5.461 ± 0.089b
LC	29.233 ± 4.316a	4.552 ± 0.522a
Mean	29.33	4.65

Means followed by the same letter in the same column did not differ among each other ($p > 0.05$). Means followed by the same letters in the same column and same variable differ among each other. ($p < 0.05$).

LD, lateral digit; MD, medial digit; TL, thoracic limb; PL, pelvic limb; DW, dorsal wall; AX, abaxial wall; SOL, sole; TC, transverse cut; LC, longitudinal cut; PIG, pigmented; DEPIG, depigmented.

compared to the findings regarding Girolando breed in this study (Assis, 2015). Such fact may explain the greatest resistance of the hoof capsule of buffaloes to several diseases that affect the hoof (Silva et al., 2015), emphasizing the importance of microstructural studies to better understand the etiopathogenesis and prevention of these severe diseases.

The helicoidal disposition of the corneal tubules, organized in parallel, is compatible with morphological findings in buffaloes (Assis, 2015). In the current investigation, the microtomographic examination represents advancement in the study of the hoof microstructure because it added important information, such as differences in the corneal tubules angulation in relation to the growth zone on the dorsal wall, abaxial wall and sole in the hoof capsule of Girolando cattle. Therefore, these findings are related to the resistance of the hoof, considering the angulation of corneous tubules in cattle of this breed revealed to be inferior to the one in buffaloes (Assis, 2015). It is possible to speculate that the smaller angulation of the corneal tubules may compromise the hoof capsule stability due to the challenge of absorbing the impact and supporting the overload of body mass in dorso-palmar and dorso-plantar directions. The distribution of the strength on the hoof capsule, whose corneal tubules angulation shows wider amplitude, as demonstrated in buffaloes (Assis, 2015), promotes greater stability and support capacity, leading to increased resistance to podal disease. Other researchers described microscopic structures of cattle hoof emphasizing the synthesis and the organization of keratin; however, they did not associate tubules angulation to the greater resistance of the different areas of the hoof (Tomlinson et al., 2004; Tombolato et al., 2010, Mckittrick et al., 2012).

Other researchers, who have used the tridimensional microtomography, also cited the absence of pores in the hoof tissue of Holstein cattle and buffalo (Assis, 2015; Rabelo et al., 2015). We may presume that the accentuated occurrence of infectious podal diseases in the rainy season due to the weakening of the hoof in this period does not happen because of the greatest water absorption by the pores, as some authors have suggested (Greenough, 2007; Belge et al., 2012; Mulling and Hagen, 2012), but by other mechanisms (Tomlinson et al., 2004). It is possible that the interaction between water and hoof occurs by means of the hydrogen bridges present in keratin molecules that are undone in water presence, causing microscopic lesions in the hoof tissue. Therefore, we cannot ignore the possibility of the interaction of the water with the hoof for extended time periods causing microfractures in keratin and the intracellular cement of the hoof capsule, predisposing to some podal disease. Ucko (1992) reported that the connections of hydrogen bridges are weak, and they can be undone in the presence of water, but they can be redone in its absence. Kester et al. (2014) related the origin of some podal diseases to microfractures in keratin and intracellular cement of the hoof capsule due to the management of cattle in high humidity environments.

The data of the current study suggests that there are similarities between the results found for Girolando cattle and reported for buffaloes (Assis, 2015). We verified Girolando cattle presented similar percentage of corneal tubules of 51 and 85 μm diameter distributed per mm^2. However, buffaloes presented higher values regarding the corneal tubules of 17, 119 and 153 μm diameter. We can infer that this characteristic may be related to the greater deposition of keratin in the hoof capsule of buffaloes hoof, offering to these animals bigger resistance to podal diseases. Other researchers have suggested that buffaloes are more resistant to digit diseases although they did not relate this characteristic to the higher number of corneal tubules (Campos, 2012; Sawad and Waad, 2012). Regarding the greater percentage of 85 μm-diameter corneal tubules observed in the abaxial wall of the thoracic limbs

in relation to the dorsal wall and sole of the cattle evaluated in this study, we can conclude that this finding is only related to the overload of body mass (Tomlinson et al., 2004). Based on these findings, this characteristic may be associated with the greater production and deposition of keratin in these digits, producing, thus, corneal tubules with bigger diameter.

The absence of statistical difference among the hoof capsule areas evaluated in this study by the nanoindentation test suggests that the dorsal wall, abaxial wall and sole of the hoof capsule present similar resistance. Nevertheless, the greater elastic module identified in the transverse cuts of the tubules when compared to the longitudinal cut seem to aid damping the impact during walking. By extrapolating these results to the study of the biomechanics of bovine locomotion, these tubules behave like a spring, damping the impact. The disposition of the helicoidal tubules formed from keratin synthesis and connected by sulphur bridges also minimizes the consequences of the impact (Tomlinson et al., 2004; Greenough, 2007). The bigger elasticity identified in the transverse cuts and the helicoidal disposition of tubules make the hoof less susceptible to microfractures, minimizing the mechanical and vascular pressure on the tissues that constitute the digit, preventing podal injuries.

The results of the microtomography and nanoindentation of the hoof capsule of pigmented and depigmented digits of Girolando cattle did not present differences regarding the parameters evaluated in this study. However, based on the results by other authors, the reports on this subject are controversial and need to be better analysed. Researchers have evaluated Holstein and Gir cattle and reported that depigmented hooves presented epidermal papilla with smaller diameter and length than the animals with pigmented hooves (Rabelo et al., 2015). For these researchers, this difference is related to a lower keratin synthesis, increasing the fragility of the depigmented hoof. Therefore, comparative studies between purebred and cross-bred animals are extremely relevant for the understanding of the real importance of pigmentation for the hoof capsule.

Finally, an analysis of the findings of this study and the confrontation with the information already published about the microtomographic examination (Assis, 2015; Rabelo et al., 2015) and the nanoindentation tests (Assis, 2015) of the hoof capsule of digits, we wonder whether multidisciplinary domains can benefit from the use of innovative techniques.

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