

Ruminal and Intestinal Morphometric Parameters of Lambs Fed Diets Containing Mimosa Tenuiflora (Willd.) Hay Replacing Buffel Grass Hay

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Abstract

The objective in this study was to evaluate morphometric parameters of the ruminal papillae and intestinal villi of lambs fed diets containing *Mimosa tenuiflora* (Willd.) hay replacing Buffel grass (*Cenchrus ciliaris* L.) hay. Twenty-eight Santa Inês lambs with initial body weight of 20.3 ± 1.49 kg were used, being distributed in a completely randomized design with four treatments (0, 333, 670 and 1000 g/kg DM). The width of the ruminal papillae was not affected ($P > 0.05$), while their area linearly decreased ($P = 0.01$) as the level of *M. tenuiflora* hay increased. In the duodenum, the height ($P < 0.0001$) and the surface ($P = 0.01$) of the villi linearly decreased with the increase in the level of *M. tenuiflora*, while the width ($P = 0.0001$) and the area ($P = 0.03$) quadratically decreased with minimum values of 132.95 and 50,625.53 μm at the levels of 436 and 575 g/kg, respectively. There was a quadratic increase in the height of the jejunum villi ($P < 0.0001$) with a maximum value of 623.22 μm at the level of 333 g/kg, while the width quadratically decreased with a minimum value of 143.33 μm at the level of 526 g/kg and the area linearly decreased ($P = 0.02$). The height of the ileum villi ($P = 0.0003$) as well as the depth of the crypts in all segments ($P < 0.001$) linearly decreased. Therefore, it is suggested the substitution of *C. ciliaris* hay by *M. tenuiflora* hay up to 333 g/kg DM.

Introduction

In semiarid regions, such as the Northeast of Brazil, producing feed in the required amount and quality for the maintenance of animals throughout the year is a constant challenge for the farmers. Thus, it is necessary to store forage as hay or silage to be offered to the animals throughout the year, especially in the dry period, which is predominant. In this context, there is a wide variety of native forages that can be used for this purpose, among them there is the *Mimosa tenuiflora* (Willd.) which assumes an important role because it is easily found in native pastures and for presenting good acceptability by the animals (Bakke et al. 2010). Studies have shown that *M. tenuiflora* has crude protein content between 100 and 122 g/kg of DM, however, it has high fiber values, as well as anti-nutritional factors (tannins) that can negatively influence the intake and digestibility of nutrients when offered alone in large proportions (Cordão et al. 2008; Bandeira et al. 2017).

Besides native forages, several other grasses that present good productivity and resistance to the edaphoclimatic conditions of the Brazilian semiarid region have been evaluated for pasture formation, among them there is the Buffel grass (*Cenchrus ciliaris* L.) which represents one of the main cultivated grasses available for the herds in the region (Moreira et al. 2007). However, the low levels of crude protein observed in this grass during the dry period give it limited potential to meet the nutritional requirements of the animals throughout the year, which suggests that it should be associated with legumes such as *M. tenuiflora* (Monção et al. 2001). Thus, the association of hay of adapted grasses, such as Buffel grass, with a native legume such as *M. tenuiflora* can lead to improvement in the quality of the diet, in addition to contributing as a source of roughage for feeding small ruminants. With this in mind, some studies (Lins et al. 2012; Bandeira et al. 2017) have been developed in order to demonstrate the feasibility of the association of these forages as a viable low-cost alternative and available throughout the year.

It is known that the type and composition of the feed can cause changes in the characteristics of the animals' digestive tract. The presence of antinutritional factors, such as the tannins in *M. tenuiflora* can lead to changes in these structures that hinder the use of the feed. Segments such as the rumen and small intestine are especially vital in this process since these parts have important physiological functions, including the digestion and absorption of nutrients that are related to the development of the papillae and villi in the rumen and intestine segments, respectively (Xu et al. 2009). Thus, we hypothesize that lambs fed diets containing *M. tenuiflora* hay can present changes in the structures of the ruminal papillae, as well as the small intestine villi. Therefore, the objective in this study was to evaluate the morphometric characteristics of the ruminal papillae and intestinal villi of Santa Inês lambs, fed diets containing increasing levels of *M. tenuiflora* hay replacing Buffel grass hay (*Cenchrus ciliaris* L.).

Material And Methods

Local, animals and management

The experiment was carried out at the Center for Health and Rural Technology of the Federal University of Campina Grande (UFCG), Patos city, Paraíba State, Brazil.

Twenty-eight castrated Santa Inês lambs, with initial body weight of 20.3 ± 1.49 kg (mean \pm SD) and 120-day were used. The experiment lasted 76 days, with 15 days of adaptation of the animals to the environment and diets. For the adaptation period, the animals were previously treated against internal and external parasites (Ivomec gold®) and vaccinated against clostridiums (Sintoxan®). The animals were distributed in a completely randomized design with four levels of substitution of Buffel grass hay by *M. tenuiflora* hay [0 (control), 333, 670 and 1000 g/kg DM] and seven replications. During the experimental period, the animals were kept in individual stalls (1.6 \times 0.8 m), provided with feeders and drinkers, distributed in a shed covered with ceramic tiles and with wooden floors. There was natural light during the day and artificial light at night. The stalls were cleaned daily, with feces removed to be used as organic fertilizer of pasture areas.

The experimental diets were formulated according to the recommendations of the NRC (2007) for a gain of 200 g/day. The roughages offered were Buffel grass hay and *M. tenuiflora* hay and the concentrate had ground corn, soybean meal, urea, soybean oil, calcitic limestone and mineral supplement (each 100 g of the mineral supplement contained: 15.3 g Ca; 7.0 g P; 14.8 g Na; 0.13 g Mg; \leq 1.20 g S; 14.0 mg Co; and 6.10 mg I). Fresh *M. tenuiflora* and buffel grass were harvested and processed at the Nupeárido/UFCG farm. The *M. tenuiflora* plants were in the middle of their vegetative stage, with an average height of 3 meters and their branches (< 10 mm \varnothing) were harvested, cut and sundried. The Buffel grass was subjected to a standardization cut to supply biomass for the production of hay. The hays were milled and passed through a 2 mm mesh, and then they remained stored in raffia bags until being used.

Feed Chemical Analyses

The ingredients were analyzed for the determination of the contents of DM (Method 967.03 - AOAC 1990), ash (Method 942.05 - AOAC 1990), crude protein (Method 981.10 - AOAC 1990), and ether extract (EE) (Method 920.29 - AOAC 1990). Neutral detergent insoluble fiber (NDF) and acid detergent insoluble fiber (ADF) were analyzed according to the methodology described by Van Soest et al. (1991) with the changes proposed in the Ankom guide (Ankom Technology Corporation, Macedonia, NY, USA). NDFap and ADFap were corrected for ash (a) and protein (p). The NDF and ADF residues were incinerated in an oven at 600°C for 4 hours and the protein correction was determined by subtracting the neutral detergent insoluble protein (NDIP) and acid detergent insoluble protein (ADIP). The total carbohydrates (TC) and levels of non-fiber carbohydrates (NFC) were determined according to the methodology proposed by Sniffen et al. (1992): $TC = 100 - (CP + EE + ash)$ and $NFC = 100 - (NDFap + CP + EE + ash)$. The quantification of condensed tannins is detailed in Bandeira et al. (2017).

Slaughter procedures and morphometric evaluation

At the end of the experimental period, all animals were slaughtered after a feed fasting of 24 hours, then they were slaughtered by cranial stunning by sectioning of the blood vessel (the jugular veins and carotid arteries), followed by skinning and evisceration. The fragments of rumen (dorsal sac) and small intestine (duodenum, jejunum and ileum) were collected immediately after slaughter and emptying of the viscera. The samples were immediately immersed in buffered formaldehyde and fixed for a period of 24 hours. Three-millimeter tissue fragments were dehydrated in an increasing concentration of alcohol (70% – 100%), diaphanized in xylol and, soon after, inserted in paraffin at 58–60 °C. After being made, the paraffin blocks were taken to the rotating microtome to obtain histological sections with 5 µm of thickness. Then they were stained using the hematoxylin-eosin (HE) technique, and the slides mounted with a coverslip on entellan, according to Samuelson (2007). Height, width and area of the papillae were measured in the rumen sections and villous height, villous width, villous surface, villous area and crypt depth were measured in the small intestine.

The heights of the villi were defined from their tip to the base and the widths were measured at the half height point. Calculations using villous height and width at half height gave the villus area. The depth of intestinal crypts was measured as the distance from top of villus crypt to muscularis mucosa. It was taken 10 measurements on each sample and the value shown is an average value of these measurements. The capture and analysis of the images were performed using the software Leica LAS Interactive Measurements coupled to a bench microscope (Leica DM 2500), with objective lenses of 4× and 10×, equipped with a color digital camera system (Leica DFC 70000 T), for the rumen and intestine cuts, respectively. The relationship villi area: crypts depth was determined by the ratio between the area of the crypt and the depth of the intestine crypts.

Statistical analyses

The design adopted in the experiment was a completely randomized design. The data were analyzed through the MIXED procedure of the SAS software (SAS Inst. Inc., Cary, NC), after verifying the residue normality through the Shapiro-Wilk test (PROC UNIVARIATE). The model included the fixed effect of the

diet and experimental error as a random effect. Orthogonal polynomial contrasts were used to determine the linear and quadratic effects of the treatments (option CONTRAST) and $P < 0.05$ was considered statistically significant. All means are presented as least square means (LSMEANS). And the Pearson's correlation analysis was performed according to the procedure PROC CORR of SAS.

Results

According to our findings, the substitution of Buffel grass hay by *M. tenuiflora* hay quadratically influenced the height of the ruminal papillae ($P = 0.001$); the maximum height (2,086 μm) was found at the level of 380 g/kg DM (Table 2). On the other hand, the width of the ruminal papillae was not affected by the substitution ($P > 0.05$). However, the area of the ruminal papillae, which was positively correlated with their height ($P < 0.01$; $r = 0.74$), linearly decreased ($P = 0.01$) as the level of *M. tenuiflora* increased in the diet.

Table 1
 – Proportion of ingredients and chemical composition of the experimental diets

Ingredient (g/kg DM)	Substitution level (g/kg DM)			
	0	333	670	1000
Buffel grass hay	600	400	200	000
Mimosa tenuiflora hay	000	200	400	600
Ground corn	243	251	246	268
Soybean meal	136	128	136	115
Urea	5.00	5.00	1.70	4.60
Soya oil	2.40	2.30	2.30	2.10
Calcitic limestone	3.60	3.80	3.80	0.41
Mineral mixture	10.0	10.0	10.0	10.0
Chemical composition				
Dry matter (DM, g/kg as feed)	917	915	912	910
Crude protein (g/kg DM)	128	129	128	129
Neutral detergent fiber _{ap} (g/kg DM) ^a	518	496	478	452
Acid detergent fiber _{ap} (g/kg DM) ^a	330	329	330	327
Ash (g/kg DM)	74.8	62.0	46.3	35.6
Ether extract (g/kg DM)	28.7	31.0	32.3	35.3
Total carbohydrates (g/kg DM)	787	794	797	809
Non-fibrous carbohydrates (g/kg DM)	269	298	319	357
Condensed tannins (g/kg DM)	0.00	43.4	88.1	132
^a Corrected for ash and protein				

Table 2

– Histomorphometry of the gastrointestinal tract of lambs fed diets containing *Mimosa tenuiflora* hay as a replacement for Buffel grass hay

Variables	Substitution level (g/kg DM)				SEM	p -value	
	0	333	670	1000		L	Q
Ruminal papillae (µm)							
Height	1847.29	2002.61	2021.79	1393.85	114.88	0.01	0.001
Width	313.69	365.78	308.41	358.69	12.04	0.15	0.94
Area (µm ²)	642338.85	700639.55	601106.57	526000.38	37955.38	0.01	0.08
Villi of the duodenum (µm)							
Height	491.79	481.71	481.19	362.78	16.45	< 0.0001	0.001
Width	151.03	140.80	132.30	169.50	6.03	0.08	0.0001
Area (µm ²)	61450.46	51288.79	52136.83	55870.76	3271.05	0.28	0.03
Surface	1231.74	1172.77	1211.68	1067.73	40.00	0.01	0.28
Villi of the jejunum (µm)							
Height	488.30	623.22	520.11	469.57	16.21	0.28	< 0.0001
Width	161.12	148.55	141.40	159.16	6.40	0.65	0.02
Area (µm ²)	62209.35	72659.63	65321.92	63856.14	3299.82	0.87	0.07
Surface	1240.82	1505.29	1330.08	1254.59	37.91	0.43	< 0.0001
Villi of the ileum (µm)							
Height	456.72	428.55	444.23	377.60	13.44	0.0003	0.15
Width	140.33	155.22	128.63	144.38	4.83	0.50	0.93
Area (µm ²)	54791.50	54354.35	50883.15	49028.74	2586.31	0.07	0.78
Surface	1136.56	1089.76	1116.59	1078.77	33.47	0.32	0.90
Crypts depth (µm)							
Duodenum	118.49	108.98	125.98	86.06	4.12	< 0.0001	0.0003
SEM = standard error mean; L = linear; Q = quadratic; VAD = villi area of the duodenum; CDD = crypt depth of the duodenum; VAJ = villi area of the jejunum; CDJ = crypt depth of the jejunum; VAI = villi area of the ileum; CDI = crypt depth of the ileum.							

Variables	Substitution level (g/kg DM)				SEM	p - value	
	0	333	670	1000		L	Q
Jejunum	130.20	137.86	102.53	106.37	5.25	< 0.0001	0.72
Ileum	136.66	133.60	126.74	86.63	5.63	< 0.0001	0.001
Villi area / Crypts depth							
VAD/CDD	4.20	5.10	4.02	4.69	0.35	0.81	0.74
VAJ/CDJ	3.93	4.97	4.95	5.02	0.30	0.01	0.09
VAI/CDI	3.49	3.50	3.95	4.70	0.23	0.0001	0.20
SEM = standard error mean; L = linear; Q = quadratic; VAD = villi area of the duodenum; CDD = crypt depth of the duodenum; VAJ = villi area of the jejunum; CDJ = crypt depth of the jejunum; VAI = villi area of the ileum; CDI = crypt depth of the ileum.							

The height ($P < 0.0001$) and contact surface ($P = 0.01$) of the duodenal villi linearly decreased as the level of substitution of Buffel grass hay by *M. tenuiflora* hay increased (Table 2). On the other hand, the width ($P = 0.0001$) and the area ($P = 0.03$) of the duodenal villi were quadratically affected by the level of substitution; the minimum width ($132.95 \mu\text{m}$) and the minimum area ($50625.53 \mu\text{m}^2$) were found at the level of 436 and 575 g/kg of substitution, respectively (Table 2). In the duodenal villi, the height was positively correlated with their contact surface ($P < 0.01$; $r = 0.78$) and with their area ($P < 0.01$; $r = 0.60$). A similar response was observed for the width of the duodenal villi which was positively correlated with their contact surface ($P < 0.01$; $r = 0.50$) and with their area ($P < 0.01$; $r = 0.75$); as for the surface of the duodenal villi, it was correlated with their area ($P < 0.01$; $r = 0.83$).

The substitution of Buffel grass hay by *M. tenuiflora* hay did not affect the villi area of the jejunum ($P = 0.07$), but quadratically affected the height ($P < 0.0001$), width ($P = 0.02$) and contact surface ($P < 0.0001$; Table 2), with maximum height ($623.22 \mu\text{m}$) and maximum surface area ($1505.29 \mu\text{m}$) observed at the level of 333 g/kg DM and minimum width ($143.33 \mu\text{m}$) at the level of 526 g/kg DM, respectively. In the jejunum villi, the height was positively correlated with their surface ($P < 0.01$; $r = 0.90$) and with their area ($P < 0.01$; $r = 0.62$). A similar response was observed for the width and surface of the villi which were positively correlated with their area, ($P < 0.01$; $r = 0.65$) and ($P < 0.01$; $r = 0.77$), respectively.

The height of the ileum villi linearly decreased ($P = 0.0003$) due to the increase in the level of substitution of Buffel grass hay by *M. tenuiflora* hay. On the other hand, the width, area and contact surface of the ileum villi were not affected ($P > 0.05$) by the level of substitution, presenting mean values of $144.38 \mu\text{m}$, $49028.74 \mu\text{m}^2$ and $1078.77 \mu\text{m}$, respectively (Table 2). The height of the ileum villi was positively correlated with their surface ($P < 0.01$; $r = 0.78$) and with their area ($P < 0.01$; $r = 0.60$). The width and surface of the ileum villi were also positively correlated with their area, ($P < 0.01$; $r = 0.63$) and ($P < 0.01$; $r = 0.84$), respectively.

Overall, the depth of the intestinal crypts (duodenum, jejunum and ileum) linearly decreased ($P < 0.0001$) due to the increase in the level of substitution of Buffel grass hay by *M. tenuiflora* hay (Table 2). The ratio between the villi area and depth of the crypts in the duodenum was not affected by the diets ($P > 0.05$). However, this ratio linearly increased in the jejunum ($P = 0.01$) and in the ileum ($P = 0.0001$) with the increase in the level of substitution (Table 2).

Discussion

The ruminal epithelium is sensitive to the quality of the diet, mainly in response to increased production of short-chain fatty acids such as propionate, butyrate (Baldwin et al. 2004) and acetate (Ragionieri et al. 2016). The substitution of Buffel grass hay by *M. tenuiflora* hay caused increase in the height of the ruminal papillae up to the level of 380 g/kg and, greater levels of *M. tenuiflora* hay promoted reduction in the height of these papillae. That reduction was possibly a reflection of a lower ruminal digestive efficiency caused by the higher concentrations of condensed tannins with the greater levels of *M. tenuiflora* which increased from 0.00 to 132 g/kg DM. Similar results have been demonstrated in studies carried out with animals fed forages containing high concentrations of tannins, which promoted reduction of ruminal degradability of DM, CP and NDF (Ramirez et al. 2000) and, consequently, in the feed efficiency (Cordão et al. 2016).

Negative effects on protein intake and digestibility in ruminants are mentioned in the literature due to the formation of protein-tannin complexes, which interfere in the microbial adhesion, with negative consequences on the performance of goats and sheep (Bueno et al. 2015; Silva et al. 2016). Tannins exert this effect mainly on proteins, but they also affect other nutrients such as minerals and polysaccharides to varying degrees. Tannins can interact with the cell wall and secrete catabolic enzymes and then inhibit cellulolytic microorganisms in the rumen and ultimately reduce cellulase secretion (McSweeney et al. 2001), as a result decreasing fiber digestion (Olsen et al. 2011).

The lower availability of energy in the rumen, due to the adverse effects of tannins not only on the degradation of proteins, but also of carbohydrates, possibly explains the decrease in the height of the ruminal papillae, since maintaining the height of the papillae demands a large amount of energy as it is a continuous cell renewal process obtained through mitosis, characterizing a very expensive process energetically and protein wise, whose intensity is due to the nutritional contribution to these cells, as demonstrated by Sakata and Tamate (1979). Unlike the response observed for the height of the ruminal papillae, the width of those papillae was not altered by the substitution levels. In some studies, width is more influenced by factors such as age at weaning than by chemical and physical factors in the diet (Zitnan et al. 1999). As the area of the papilla is directly correlated with its height, it was negatively affected by the substitution of Buffel grass hay by *M. tenuiflora* hay.

Studies have shown that the morphology of the intestinal villi changes due to the availability of nutrients, especially in diets that contain a greater amount of energy (Montanholi et al. 2013). In the present study, the height and surface of the villi in the duodenum decreased with the increase in the inclusion of *M.*

tenuiflora hay in the diets. These results are possibly associated to the lower availability of nutrients reaching this organ, due to the presence of tannins. This hypothesis is supported by the findings of Patra and Saxena (2011), who found a reduction in the energy released in the small intestine and increase in the net energy released in the feces, in a study carried out with cows receiving tannin extract from *Acacia mearnsii*.

Tannin-protein complexes are stable at ruminal pH and dissociate when the pH drops below 3.5 or when it is greater than 8.0. As a result, condensed tannins decrease protein degradation in the rumen, causing a greater flow of post-ruminal amino acids, but the effects on intestinal availability of amino acids vary widely (Norton 2000; Min et al. 2003; Carula et al. 2005). These responses are sometimes dependent on the level, type and origin of the tannins, as well as on the animal species, animal health and the composition of the feed (Nawab et al. 2020). In this context, Naumann et al. (2017) hypothesized that, when condensed tannins are not degraded in the rumen or abomasum, it is possible that they can re-complex with any undigested proteins, peptides or amino acids, thus preventing the absorption of these compounds in the small intestine, thereby reducing the potential increase in the absorption of amino acids by the animal.

In non-ruminants, the negative effect of tannins on intestinal amino acid absorption has been attributed to the reduced action of the aminopeptidase enzyme and excessive mucus production (Alpers 1994; Van Leeuwen et al. 1995). According to Walton et al. (2001), the impact of reduced cell turnover on nutrient digestibility in the small intestine in sheep remains unknown and requires further investigation.

The results of the present study suggest that, to compensate for the decrease in the height of the duodenal villi, there was an enlargement of these villi, in an attempt to increase the absorptive area, since the area was positively correlated with the villi width. A similar behavior was observed in the jejunum villi, as with a level greater than 333 g/kg of substitution, the height and surface of the villi in the jejunum decreased and there was an enlargement of these villi at a level greater than 523 g/kg of substitution. Therefore, it was observed that higher levels of participation of *M. tenuiflora* hay compromised the jejunum epithelium, which may affect the nutrient absorption capacity in this segment of the gastrointestinal tract.

Studies have shown that high levels of digestible fiber in the diet are associated with an increase in the depth of the crypt and in the relationship between the height of the villus and the depth of the crypt (Jin et al. 2018). In the present study, the depth of the crypts decreased as the level of inclusion of *M. tenuiflora* hay increased in all sections of the small intestine. However, it was expected that the crypts would have an opposite effect due to the action of the tannins present in the *M. tenuiflora* hay. In this case, there are two supposed hypotheses: a) high proportions of *M. tenuiflora* hay in the diet do not seem to directly affect the crypts and only the intestinal villi or b) this variable changes more slowly and requires more time to manifest negative effects.

In non-ruminants, studies have shown that the depth of the crypts is related to the need for cell renewal due to lesions caused in the mucosa of these organs by pathogens, toxins and antinutritional factors

(Silva et al. 2010). The deeper the crypt, the greater the aggression suffered and the need for renewal. However, such crypts did not demonstrate this effect, showing themselves to be more superficial as the Buffel grass hay was substituted by *M. tenuiflora* hay.

The relationship between the depth of the crypts and the villi area in the duodenum was not affected by the diets in their most proximal portion (duodenum). However, there was an effect of the treatments on the jejunum and ileum. That variable is usually used to demonstrate intestinal health as well as its digestive capacity (Montagner et al. 2003). The greater this ratio, the better the intestinal health as well as the digestion capacity. In this sense, since in the present study this relationship increased, it is assumed that the health of these segments, as well as, their digestive capacity remains viable in the different proportions of *M. tenuiflora* hay.

In this study, in general, it was found that the duodenal villi were more sensitive to the diets when compared to the villi in the jejunum and ileum. These results are consistent with those reported by Jackson and Diamond (1996), who reported that the rate of intestinal development is not uniform across their segments and that the duodenum variables were also most affected by the treatments. According to Starck (1996) this can be explained by the fact that the more proximal segment (duodenum) has larger cells and lesser quantities when compared to the more distal segments of the intestine, jejunum and ileum, which have smaller cells and larger quantities. Therefore, the duodenum may change more quickly and might have reflected more pronounced behavior when compared to the other segments of the intestine.

In view of these results, our study suggests that the supply of *M. tenuiflora* hay as a single roughage should be avoided. A similar study carried out by Bandeira et al. (2017) recommended the inclusion of *M. tenuiflora* hay up to the level of 20 g/100 g of the total DM of the diet, as it resulted in greater average daily weight gain, feed efficiency, carcass weight and better carcass yield of Santa Inês lambs.

Conclusion

Based on the results, it is recommended that the Buffel grass (*Cenchrus ciliaris*) hay be substituted by *Mimosa tenuiflora* hay up to 333 g/kg of the total DM of the diet.

Declarations

Availability of data and material

Not applicable for that section.

Code availability

Not applicable for that section.

Authors' contribution

MLDLT conducted experiments, analyzed data and wrote the manuscript. LHOT and JBGD conducted experiments, collected and analyzed data. LMFG laboratorial analysis and revised the manuscript. OBS, JMPF, and MJA contributed to the study conception, supervision, and methodology. All authors read and approved the final manuscript.

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Ethics approval

Ethics approval The Animal Ethics Committee approved this study of the Federal University of Campina Grande (UFCG), Brazil (protocol no. 26/2012).

Consent participate

All the authors approved the final manuscript.

Consent for publication

Not applicable

Conflict of interest

The authors declare that there are no conflicts of interest in this research paper.

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