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Ruminal and morphometric parameters of rumen and intestine in lambs fed guava (*Psidium guajava* L.) agroindustrial waste

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ABSTRACT - The objective of this study was to evaluate the effect of guava agroindustrial waste (GAW) on ruminal parameters (pH, N-NH_3, and microbial protein), volatile fatty acid (VFA), and morphometry of the rumen and intestine of sheep. A total of forty Santa Inês sheep (120 days old and 21.3±2.62 kg) were used. The animals were fed diets with 0, 7.5, 15, 22.5, and 30% inclusion of GAW. The pH, ammonia nitrogen, microbial protein, and VFA were evaluated in the ruminal fluid, alongside the morphometric characteristics of the rumen and intestine. The inclusion of GAW linearly increased fasting and postprandial pH, N-NH_3 only showed a quadratic effect for fasting animal, whereas MP presented a quadratic effect for pre- and post-prandial animals. There was a quadratic effect for papilla width, with a maximum value of 393.33 µm at the level of 34.43% GAW in the diet. The papilla absorption area showed a linear effect, in which increasing levels of GAW in the diet had a smaller area of papillae absorption. The inclusion of GAW in the diet of Santa Inês sheep favored pH neutrality, reduced N-NH_3 and ruminal MP concentrations, decreased the thickness of the rumen muscular layer, and increased the intestinal mucosa, favoring greater absorption of nutrients.

Keywords: microbial protein, pH, ruminal ammonia, volatile fatty acid

Introduction

The low-cost feed ingredients based on agroindustrial byproducts are potential alternatives that can reduce the cost of mixed feed, but the effects of these ingredients on animal performance and final products need to be further considered (Whitney and Smith, 2015). Some of these discarded wastes are considered pollutants, but most can be used to feed ruminants, reducing production costs by turning low-nutrient residues into high-value products such as meat and milk (Geron et al., 2015; Hassan et al., 2016). Among the fruits processed by agribusiness, guava (*Psidium guajava* L.) is widely used in the manufacture of beverages, syrup, ice cream, jams, jellies, caramel, juice, and dehydrated and canned products (Denny et al., 2013). After processing (pulping and washing with chlorinated water), a residue is obtained, consisting of bark, pulp, and mainly seeds, which, according to Uchôa-Thomaz et al. (2014), represents between 4 and 12% of the total mass of the fruits.

The composition of guava agroindustrial waste (GAW) contains crude protein (CP; 39.5 g/kg dry matter [DM]), neutral detergent fiber (NDF; 761.8 g/kg DM), acid detergent fiber (ADF; 453.2 g/kg), and ash (10.0 g/kg DM) (Oliveira et al., 2018). Moreover, its lipid fraction is predominantly composed of unsaturated fatty acids, especially linoleic acid (77.35% of all fatty acids) (Uchôa-Thomaz et al., 2014). Additionally, GAW is rich in polyphenols, such as tannins (2-4%), which have a great antioxidant activity and may provide beneficial effects on protein metabolism in ruminants (Khalifa et al., 2016; Costa et al., 2018). The antioxidant properties of polyphenols have been extensively studied (Lorenzo et al., 2018; Pateiro et al., 2018). In this regard, polyphenols added to the feed of ruminant animals are subjected to the effect of chewing, rumen bacteria, and microbial gut metabolism before being absorbed in the small intestine, metabolized in the liver, and finally deposed in the tissues (Vasta and Luciano, 2011). The GAW has low degradability in the ruminal environment, which may interfere with the availability of nutrients (carbohydrates and proteins) for the production of microbial protein due to the high contents of anti-nutritional components such as tannin and lignin, thereby reducing the digestibility of the feed (Chang et al., 2014).

Diets with high levels of rapidly fermentable carbohydrates tend to produce high levels of volatile fatty acids (Costa et al., 2008), thereby providing greater development of the ruminal epithelium, and more specifically the papillae. On the other hand, GAW contains a high content of ether extract (EE; 10.8%) in the seeds (Lira et al., 2011), which is digested and absorbed in the small intestine, thus altering the structure of the intestinal mucosa.

The hypothesis of our study is that increasing levels of guava agroindustrial waste in sheep feed modifies ruminal parameters as well as the morphometry of the rumen. Therefore, objective of this study was to evaluate the effect of different inclusion levels of GAW on the ruminal parameters, pH, N-NH_3, microbial protein, and morphometry of the rumen and intestine of lambs.

Material and Methods

Research on animals was conducted according to the institutional committee on animal use (case no. 2305/14). The trial was conducted in Bananeiras, Paraíba state, Brazil (6°41'11" latitude, 35°37'41" longitude, and 552 m altitude). The air temperature (black globe temperature, BGT) was 24.97 °C, and relative humidity was 76.4 8% in the stalls.

Forty non-castrated male animals of the Santa Inês breed were used, with an average initial weight of 21.3±2.62 kg and an average age of 120 days. The animals were divided into individual stalls (1.50 m^2) with slatted and suspended floors. The animals had free access to feed and water, and were distributed in a completely randomized design with four inclusion levels (0, 7.5, 15, 22.5, and 30% in dry matter) of GAW.

The experiment lasted 63 days, 15 of which were for adaptation to the feeds, facilities, and management. The diet was provided with a forage:concentrate ratio of 50:50, to provide a gain of 250 g day^{-1}, as recommended by the NRC (2007). Tifton 85 hay (*Cynodon dactylon* L.) was replaced with dehydrated and ground GAW at levels of 0, 7.5, 15, 22.5, and 30% in the dry matter of the diets that contained ground corn, soybean meal, and a vitamin and mineral supplement (Table 1). The experimental diet was offered *ad libitum* at 07:30 and 16:30 h as a complete mixture.

The samples taken from the piles were immediately frozen in a refrigerator for further analysis. They were thawed and pre-dried in forced air at 55 °C for 72 h and was ground to a mesh size of a 1-mm sieve knife mill and packed into plastic bags. We estimated the DM (method 934.01; AOAC, 2005), CP (Kjeldahl method, method 984.13; AOAC, 2005), EE (method 920.39; AOAC, 2005), crude fiber (method 978.10; AOAC, 2005), ash content (method 942.05; AOAC, 2005), NDF (method of Holst, 1973), and ADF (method 973.18; AOAC, 2005). The total carbohydrate (TC) content was estimated using the equation proposed by Sniffen et al. (1992): TC = 100 − (%CP + %EE + %ash). Non-fibrous carbohydrates (NFC) were estimated using the equation proposed by Mertens (1997): NFC = 100 − (%CP + %EE + %DM+ %NDF). Estimation of total digestible nutrients (TDN) was based on the equation described by Weiss (1999): TDN = CPD + EED × 2.25 + NFCD + NDFcpD; in this equation, CPD = (CP ingested − CP feces), EED = (EE ingested − EE feces), NFCD = (NFC ingested − NFC feces), and NDFcpD = (NDFcp ingested − NDFcp feces). To calculate the metabolizable energy (ME) (kcal ME/kg DM), the digestible energy (DE) was initially calculated as the product between the content of total digestive nutrients (TDN) and the factor 4.409/100, considering the ME concentration of 82% of DE (Silva and Leão, 1979).

To determine the concentration of total tannins (TT), the butanol-HCl method described by Terrill et al. (1992) was used; the result was converted to % relative to the black jurema tannin, based on the regression equation of the standard curve made from the purified black jurema condensed tannin according to the methodology proposed by Guimarães-Beelen et al. (2006).

The samples were taken from 40 animals. For the determination of pH, N-NH_3 concentration and microbial protein (MP) of rumen liquid were assessed. Samples were collected manually through the esophageal tube and filtered through gauze. The collection times were 0 (before morning feed) and 4 h after the morning feed. The pH of the ruminal liquid was measured immediately after collection using a portable digital potentiometer. The ruminal liquid samples were placed in three 1.5-mL Eppendorf tubes, freezing them shortly afterwards for further analysis.

The concentrations of N-NH_3 were determined according to the method of Chaney and Marbach (1962). Both methods are based on colorimetry, using a spectrophotometer with the wavelengths of 630 and 660 nm, respectively. For the analysis of ruminal liquid samples frozen in Eppendorf tubes, they were thawed, then centrifuged in the Eppendorf tubes of 1.5 mL at 12,000 rpm (161 × *g*) for 10 min, and the supernatant was transferred to a new Eppendorf tube and frozen for the further analysis of ammonia and soluble protein (peptides and amino acids). The sediments resulting from the above procedure were resuspended in saline (0.9% NaCl) and centrifuged at 12,000 rpm (161 × *g*) for 10 min, twice consecutively. Finally, they were resuspended in distilled water up to the volume of 0.6 mL, homogenized, and frozen for the later analysis of microbial protein, by means of the Bradford (1976) method.

Table 1 - Percentage and chemical composition of experimental diets

Item	Inclusion level (%)				
	0.0	7.50	15.0	22.5	30.0
Ingredient (g kg^{-1} DM)					
Guava agroindustrial waste (GAW)[1]	0.0	75.0	150	225	300
Tifton hay	500	425	350	275	200
Ground corn	310	310	310	310	310
Soybean meal	170	170	170	170	170
Mineral supplement[2]	10.0	10.0	10.0	10.0	10.0
Calcitic limestone	5.00	5.00	5.00	5.00	5.00
Chemical composition					
Dry matter (DM; g kg^{-1} as fed)	888	888	889	889	890
Crude protein (CP; g kg^{-1} DM)	154	154	154	154	154
Ethereal extract (EE; g kg^{-1} DM)	31.3	37.9	44.5	51.1	57.7
Neutral detergent fiber (NDF; g kg^{-1} DM)	489	484	479	474	468
Acid detergent fiber (ADF; g kg^{-1} DM)	249	262	276	290	304
Ash (g kg^{-1} DM)	64.5	60.3	56.2	52.0	47.8
Total carbohydrates (TC; g kg^{-1} DM)	748	744	741	766	734
Non-fibrous carbohydrates (NFC; g kg^{-1} DM)	258	260	262	292	265
Total tannins (g kg^{-1} DM)	0.00	5.00	9.90	14.9	19.8
Lignin[3] (g kg^{-1} DM)	33.7	44.4	55.0	65.7	76.3
Metabolizable energy (ME; Mcal/kg DM)	2.48	2.44	2.39	2.34	2.30

[1] GAW composition (g kg^{-1}): DM, 908.0; CP, 91.8; EE, 107.5; NDF, 730.7; ADF, 620.7; ash, 21.3; TC, 779.4; NFC, 48.7; tannins, 6.6%.
[2] Composition of mineral supplement per kg: P, 70 g; Ca, 140 g; Na, 148 g; S, 12 g; Mg, 1.320 mg; F, 700 mg; Zn, 4.700 mg; Mn, 3.690 mg; Fe, 2.200 mg; Co, 140 mg; I, 61 mg; Se, 15 mg; sodium monensin, 100 mg.
[3] Lignin (19.7%)

For the analysis of volatile fatty acids (VFA), 2.0 mL of culture medium sample was removed from all experimental units after 48 h of incubation and placed in Eppendorf tubes, which were centrifuged at 5,200 × *g* for 10 min; the supernatant was then frozen for VFA on a High-Performance Liquid Chromatograph (HPLC) (brand SHIMADZU, model SPD-10A VP), coupled to an ultra violet (UV) detector, using a wavelength of 210 nm. A SHIMADZU C18 column with a diameter of 30 cm × 7.9 mm was used, with a flow in the column of 0.6 mL/min. At a pressure of 69 kgf, 20 µL of the mobile phase of water in 1% orthophosphoric acid was injected. Concentrations of VFA acetate, propionate, and butyrate were analyzed.

For the morphometric analyses of the rumen papillae length (RPL = from base to apex), rumen papillae width (RPW = in the middle region of the papilae), rumen papillae area (RPA = RPL × RPW), rumen muscle thickness (RMT), and intestinal mucosal height (IMH), six animals per treatment were used. Five photomicrographies per animal were scanned with a 5X objective on an Olympus Cellsens Dimension BX-60 microscope and Zeiss AxioCam camera coupled with a Motic Image Plus 2.0 digital image capture program for each organ analyzed. In each photomicrograph, two measurements were performed for each variable and organ, with a "n" of 60 per treatment (six animals × five photomicrographies × two measurements) for each variable (Lima et al., 2019).

The N-NH_3, protein, pH, and VFA data were evaluated by means of analysis of variance; the means were compared by Tukey's test at 5% probability using the General Linear Model (GLM) procedure, and regression analysis were undertaken using the REG procedure of SAS (Statistical Analysis System, version 9.2). The following mathematical model was used:

$$Y_{ijk} = \mu + Z_i + \beta_j + Z\beta_{ij} + \mathcal{E}_{ijk}, \quad (1)$$

in which Y_{ijk} is the dependent variable; µ is the overall mean; Z_i = is the effect of level i of factor Z (i = 1, 2, ..., I); β_j = is the effect of level j of factor β (j = 1, 2, ..., J); $Z\beta_{ij}$ = is the effect of the interaction between level i of factor Z and level j of factor β; and $\mathcal{E}_{ijk}$ is the random error, considering mean 0 and variance σ^2.

For the morphometric variables, the Graph Prisma 5.0 program was used.

Results

The ruminal pH, N-NH_3, and MP parameters (Table 2) showed a significant effect of the treatments. The GAW levels in the diets linearly influenced the ruminal pH, both for fasting animals (P = 0.0445), and for postprandial animals (P = 0.0244). In relation to the ruminal N-NH_3, a difference was observed only for fasting animals (N-NH_3 mM 0 h), showing a quadratic tendency (P<0.0001), with a maximum point of 40 mM, for the level of 12.34%.

Table 2 - pH, concentration of ammonia nitrogen (N-NH_3), and microbial protein (MP) of rumen liquid of Santa Inês sheep fed guava agroindustrial waste (GAW)

Variable	Inclusion level (%)					SEM	P-value	
	0.0	7.5	15.0	22.5	30.0		L	Q
pH 0 h	6.89	6.90	6.96	7.03	7.10	0.24	0.044[1]	0.126
pH 4 h	6.51	6.46	6.56	6.69	6.77	0.29	0.024[2]	0.060
N-NH_3 mM 0 h	30.74*	41.31	39.33	32.71	24.21	10.46	0.030	<0.0001[3]
N-NH_3 mM 4 h	28.63	26.60	38.49	27.21	28.80	8.59	0.901	0.197
MP mg dL^{-1} 0 h	76.42*	179.07	112.21	121.91	94.27	59.37	0.709	0.020[4]
MP mg dL^{-1} 4 h	247.24	281.16	250.53	226.60	188.85	50.07	<0.0001	<0.0001[5]

SEM - standard error of the means.
* Orthogonal contrast = control vs. GAW inclusion levels; [1] $y = 6.86750 + 0.00717x$ ($R^2 = 0.96$); [2] $y = 6.44825 + 0.01010x$ ($R^2 = 0.85$); [3] $y = 31.88176 + 1.34048x - 0.05431x^2$ ($R^2 = 0.94$); [4] $y = 94.78043 + 6.72434x - 0.23369x^2$ ($R^2 = 0.41$); [5] $y = 253.62698 + 2.92043x - 0.17350x^2$ ($R^2 = 0.92$).

The results regarding ruminal fermentation parameters (Table 3) showed that, even in the fasting animals (0 h), there was a linear regression (P<0.05) of acetic and butyric acid concentrations. In the samples of ruminal liquid from the animals after 4 h of feeding, where the maximum production of VFA in the rumen was found, only the propionic acid presented a dietary effect (P<0.05), showing that there was a higher proportion of NFC in the diets at from 15% GAW.

In the morphometric characteristics of the rumen (Figure 1), a quadratic effect was observed for the width of the papilla (P<0.05), estimating a maximum value of 393.33 µm for the level of 34.4% GAW in the diet. For the papilla length, no significant differences were observed (P>0.05). However, the area of papilla absorption had a linear decreasing effect (P = 0.0193), in which increasing levels of GAW in the diet represented a smaller absorption area.

The thickness of the muscular layer of the rumen was higher in the control treatment; however, a quadratic effect was observed with negative inflection when GAW was included in the diet (P<0.0001), obtaining the lowest layer thickness of 850.87 µm at 28.87%.

Table 3 - Parameters of ruminal fermentation in Santa Inês sheep fed guava agroindustrial waste

Acid		Inclusion level (%)					SEM	P-value	
		0.0	7.5	15.0	22.5	30.0		L	Q
Acetate	0 h	122.4a*	86.2ab	73.6ab	84.3ab	43.9b	31.63	0.004[1]	0.805
	4 h	154.2	121.9	187.4	171.4	136.7	47.37	0.853	0.370
Propionate	0 h	32.8	35.3	32.7	35.2	19.2	12.35	0.178	0.174
	4 h	64.3ab	49.9b	99.2a	82.7ab	92.6ab	22.22	0.033[2]	0.717
Butyrate	0 h	9.6a*	7.2ab	6.2ab	5.9ab	2.2b	2.88	0.001[3]	0.705
	4 h	16.1	9.5	14.8	12.3	13.8	5.20	0.839	0.400

SEM - standard error of the means.
* Orthogonal contrast = control vs. GAW inclusion levels; [1] Y = 113.81281 – 2.11552x (R^2 = 0.59); [2] Y = 59.83938 + 1.19252x (R^2 = 0.48); [3] Y = 9.42607 – 0.21440x (R^2 = 0.48).

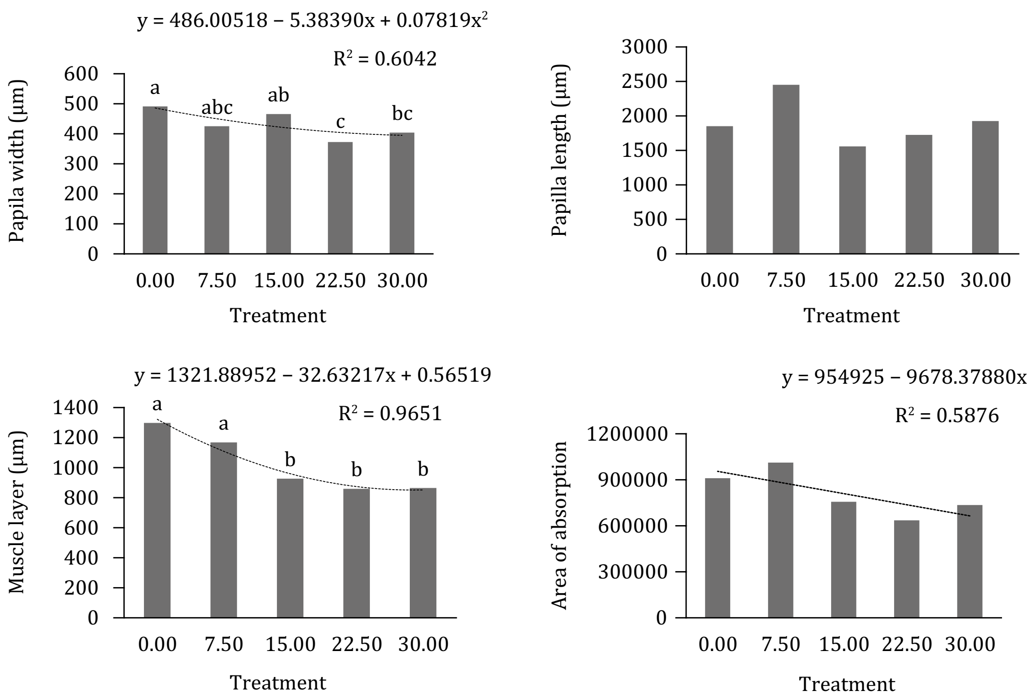

Figure 1 - Width, length, absorption area of rumen papillae, and thickness of muscle layer of ovine rumen as a function of inclusion levels of guava agroindustrial waste in experimental diets.

The thickness of the intestinal mucosa (Figure 2) had a quadratic effect with negative inflection (P = 0.0012), in which the increase started at the level of 7.13%, obtaining a mucosal thickness of 810 µm. In this way, the largest contact surface with feed found in this research was at the level of 30% (2158.9 µm); that is, 142% increase from the minimum point.

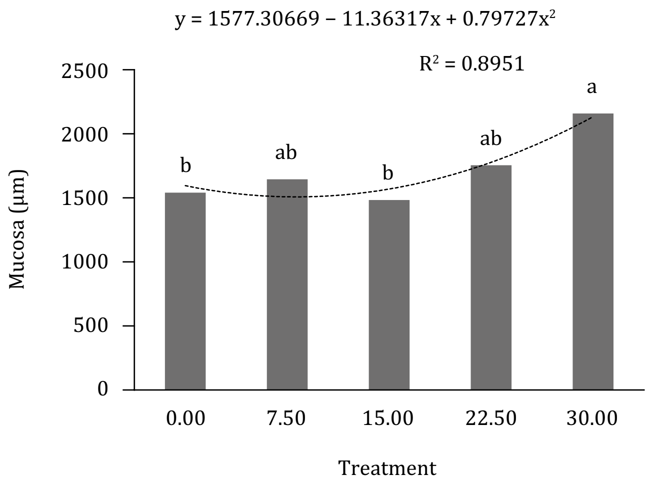

Figure 2 - Thickness of the intestinal mucosa of ovine rumen as a function of inclusion levels of guava agroindustrial waste in experimental diets.

Discussion

The pH is a parameter that has a close relationship to the type of feed consumed by the animal, i.e., diets with a larger particle size results in higher rumen pH (Beharka et al., 1998). In the diets used, GAW has lower fiber effectiveness than Tifton hay. This waste presents 0.58 g/100 g of pectin (Uchôa-Thomaz et al., 2014), which provides a healthier ruminal environment than starch by not producing lactic acid, whose fermentation pattern lowers pH. Ruminants are prone to metabolic disorders such as acidosis and bloat in intensive systems (Peixoto et al., 2015).

The sources and amounts of carbohydrate and nitrogen in the diet can determine N-NH_3 concentrations since the ability of bacteria to synthesize proteins and the use of ammonia depends on the fermentation rate of carbohydrates (Van Soest, 1994).

It was observed that the concentration of MP mg/dL after 4 h in the GAW diets is practically double the MP mg/dL at time 0 h, whereas the concentration of N-NH_3 did not show a big difference between the pre- and postprandial periods. The concentrations of MP at the fasting time were higher in the treatments with the inclusion of GAW. However, a quadratic effect with a maximum point at the level of 14.39% GAW was observed when MP was 143.15 mg/dL, showing that the amount of MP began to decrease gradually from this level with the inclusion of GAW in the diets. Postharvest animals presented the same behavior, with a quadratic positive effect with maximum points at the level of 8.42% GAW and MP of 265.92 mg/dL. It is important to emphasize that the diets were isoproteic and, therefore, this decrease in MP with the inclusion of GAW in the diet is justified due to the low degradability of GAW, since it consists of high levels of anti-nutritional factors such as tannin (6.6%) and lignin (19.5%), which hinders fiber degradability. In addition, the GAW has a smaller particle size and a higher density of specific mass than Tifton hay, thus increasing the rate of passage and decreasing the retention time of the feed in the digestive tract.

Acetic acid ranged from 122.35 mmol/L in the control treatment to 43.99 mmol/L in the treatment with 30% GAW in the diet, and butyric acid from 9.58 mmol/L in the control treatment, to 2.17 mmol/L at the level of 30% GAW. The type of VFA produced in the rumen is related to the composition of the diet. Regardless of the pre- or postprandial collection period, acetic acid has always been shown to be at a higher concentration than the other short chain fatty acids studied.

The reduction in the concentration of butyric acid, when GAW is included in the diet, may be related to the increase of fat in the diets. These results are in agreement with Patra (2014) meta-analysis, which showed a negative effect of increasing fat levels on sheep diets in the proportion of butyrate, probably due to the inhibition of microorganisms (protozoa and *Butyvibrio fibrisolvens*) involved in their production (Hristov et al., 2009).

This is due to changes in the acetate:rumen propionate ratio (Firkins et al., 2006). In addition, the presence of unsaturated lipids in rations, as in the case of the treatments with higher GAW content, can stimulate the ruminal bacteria to produce propionate (Van Nevel and Demeyer, 1988).

The energy intake of experimental diets may explain this decrease in absorption area. Diets with higher levels of GAW contain lower energy levels, and the development of ruminal papillae are directly related to the total production of volatile fatty acids. In the present study, the use of propionate (Gálfi et al., 1993) and butyrate stimulate papillary growth, a fact that does not occur with acetate (Tamate et al., 1962). This corroborates the results of this research, since the pectin present in GAW provided increased acetate production (Liu et al., 2015), interfering significantly with the development of the papillae.

This decrease in the muscular layer is probably directly related to the nature of the dietary fiber since the diet NDF did not differ. The control treatment consists exclusively of hay as a source of forage, and this feed has a greater amount of effective fiber than GAW, with a particle size that has a direct relation to the ruminoreticular motility. That is, the larger the food particles, the greater the ruminal motility, which in turn increases the development of the muscular layer; therefore, the decrease of hay in the feed reduced the muscular layer of the rumen.

Increasing levels of lipids (Table 3), according to the increase in GAW in the diet, favored this increase in the intestinal mucosa, since mucosal increases are associated with a greater supply of energy (Montanholi et al., 2013), providing longer villi and resulting in increased capacity of nutrient absorption by the intestine (Wang et al., 2009).

Conclusions

The inclusion of guava agroindustrial waste in the diet of Santa Inês sheep favors pH neutrality, increases the concentration of propionic acid and $N\text{-}NH_3$, decreases the concentration of microbial protein, and reduces the thickness of the muscular layer, but increases the intestinal mucosa, allowing a greater nutrient absorption.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: P.T. Nobre. Formal analysis: R.R. Guerra, J.S. Oliveira and N.L. Ribeiro. Investigation: P.T. Nobre. Methodology: P.T. Nobre, R.R. Guerra, J.S. Oliveira, N.L. Ribeiro, A.N. Medeiros and E.O.S. Saliba. Project administration: R.G. Costa, F.F.R. Carvalho and A.N. Medeiros. Software: N.L. Ribeiro. Supervision: R.G. Costa, F.F.R. Carvalho and A.N. Medeiros.

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