DIGESTIBILITY, URINARY EXCRETION OF PURINE DERIVATIVES AND RUMINAL CILIATE PROTOZOA IN BUFFALO ON DIETS CONTAINING RAW OR ROASTED SOYBEAN

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ABSTRACT

The aim of this study was to evaluate the apparent digestibility, excretion of purine derivatives (PD) as well as the rumen protozoa population (density and genus composition) in buffaloes on two comparative diets containing raw or roast soybean in concentrate. Four buffaloes with fistula in the rumen were fed with roughage grass hay (70%) and concentrate (30%) consisting ground grain corn and ground grain raw soybean (RAS treatment) or roasted soybean (RSB Treatment). Total collection of feces and urine were performed to estimate the apparent digestibility of dry matter, crude protein and neutral detergent fiber; urinary excretion of purine derivatives (PD), and rumen contents sampling to identification and counting of ciliate protozoa population. The apparent digestibility of the crude protein was higher in the animals receiving diets with roasted soybean (63.45%) than with raw soybean (47.16%). But, no significant differences were observed in the DM and NDF digestibility. Purine derivatives excretion did not differ between treatments, maintaining relative proportion of allantoin: uric acid of 89:11%. The average synthesis of microbial nitrogen was estimated in 33.88 g/d. We have concluded that substitution of the raw by roasted soybean in the diet for buffalo has promoted increase in the apparent digestibility of crude protein, and reduction of total number and *Entodinium* protozoa population in the rumen, but had no influence in the composition of the fauna, excretion of PD, and microbial protein synthesis.

Keywords: buffalo, *Bubalus bubalis*, digestibility, excretion, ruminal, soybean

INTRODUCTION

Buffalo in Brazil is a relevant animal involved in the agribusiness with milk and meat productions. Basically they are fed on grazing system. However, fibrous roughages are not able to meet the animal requirements for a good production due the limited feeding intake and low digestibility. Then, energy-protein supplements are fundamentals specially on milking specialized herds to supply the nutritional deficiencies and improve the animal efficiency. Brazil is the second world producer of soybean grains with 81.7 million tons (FAOSTAT, 2013), showing a big potential fou use in animal feeding as soybean meal as well

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as full grain.

Soybean extrusion or roasted allows a greater passage of nitrogen through the rumen as undegradable protein to the lower gastrointestinal tract, where amino acids of good quality can be absorbed with animal gain. However, no differences have been observed between diets with raw or roasted soybean in the milk, protein and fat productions, feeding intake and digestibility in lactating crossbred cows (Sirohi *et al.*, 2011).

Differences in urinary excretion of purine derivatives have been observed among different ruminant species, such as cattle, buffalo, sheep and goats (Chen *et al.*, 1990; Chen *et al.*, 1996; Thanh and Orskov, 2006; Jetana *et al.*, 2013) and even between animals of the same species from different groups, such as European and zebu cattle (Bowen *et al.*, 2006). But data from specific buffalo on several feeding condition are still deficient to define a real standard of purines derivatives excretion.

Then, the aim of this study was to evaluate the effects of the use of raw and roasted soybean on feed intake, tropical grass digestibility, urinary excretion of purine derivatives and rumen protozoa population in buffaloes.

MATERIALS AND METHODS

The experiment was conducted according to rules of Ethics Committee on Animal Welfare from the FZEA/USP. Four buffaloes (*Bubalus bubalis*) of Mediterranean breed with rumen fistula, weighting average 360 kg and 14 months age were maintained in appropriate individual facilities with free access to water and feed intake of diet has been daily controlled.

The experimental treatments consisted of two diets with 70% coastcross hay (*Cynodon dactylon*) coarsely ground, 8.6% of grain corn ground 21.4% of raw soybean (RAS) or 21.4% of roasted soybean (RSB). The final diets showed with 14.1% of crude protein (CP) and 61.1% of neutral detergent fiber (NDF).

Switchback or doubled reversal design experimental model was used with two periods (23 days each) and two animals in each treatment on reversal schedule of the treatments, i.e., the animals from the first to the second period (Lindahl, 1959). The last eight days of each period, the animals were moved to metabolism cages with three days of adaptation and the others as sampling period with total collection of feces and urine were. Food was offered twice daily at 8:00 and 16:00 h. The total daily feces was weighed, homogenized and samples of 10% of the total was frozen (-20^{o}C) for subsequent chemical analysis.

Apparent digestibility of dry matter, crude protein and neutral detergent fiber were determined by the difference between the amount ingested and excreted in proportion to the total intake. Urine was collected in plastic containers with a capacity of 20 L with addition of 100 ml of H_2SO_4 (20% v/v) maintaining the pH around 3. After sampling a total of 24 h, the volume of urine produced was measured, homogenized and diluted to a constant volume. Then 100 ml aliquots were stored at freezer (-20^{o}C) for quantification of purine derivatives.

Purine derivatives (PD) and creatinine were analyzed by high performance liquid chromatography (HPLC) on Packard Model 1100 Series according to a modified methodology described by (Czauderna and Kowalczyk, 2000). Purine derivatives were quantified by integrating peak, fluorescence detectors, refractive index and UV-PAD/Visible, using ChemStation software to obtain the final concentration (FAO/IAEA, 2003).

Rumen samples were obtained from fistula last three consecutive days just before morning feeding for identification and counting the protozoa ciliate population. The samples were preserved with formaldehyde solution (1:2) and after staining with brilliant green were analyzed in optical microscopy in 100 times magnitude according to technique describe by (Dehority, 1993).

Statistical analysis was performed with a linear model of variance (main effects ANOVA) with three factors: animal, period and treatment. The data were processed using statistical package software (StatSoft, 2012).

RESULTS AND DISCUSSION

Dry matter, and crude protein intakes (kg/d) were higher ($P<0.05$) in animals receiving RSB (Table 1). They also had higher DMI and CPI as percentage of body weight (1.34% and 0.24%) than those receiving RAS (1.24 and 0.21%, respectively). The mean values obtained were lower than values observed in buffaloes fed diets roughage with different levels of concentrate (Delgado *et al.*, 2006; Maeda *et al.*, 2007). The low DM intake may be related to intrinsic buffalo feeding behavior on experiment controlled and feed ingredients of the diet. The roughage had low quality with high levels of DM and NDF. In this case, it generally takes longer retention of food in the rumen and limits the feed intake. Furthermore, fibrous roughages cause a considerable flow of saliva with little mucin, which makes mastication and deglutition labored reducing dry matter intake (Maeda *et al.*, 2007). No differences in NDF intake were observed between treatments, representing 54% of dry matter ingested by animals.

No significant difference ($P>0.05$) were observed in the DM and NDF digestibility between the treatments, but the mean values have reached above 60% and 56% respectively, indicating in despite of the low DM intake, a good efficiency of diets utilization by buffalo. (Itavo *et al.*, 2002) observed in growing cattle, lower DM digestibility (53%) for diets with the same grass utilized in this study. Then, as discussed below, the low intake in this experiment is probably seems to be more related to the physical structure of forage and intrinsic ruminant species. In the other hand, low fiber quality spends more time in the rumen allowing larger action of the microorganism on fiber digestion, as signaled in this experiment with small increasing of the NDF digestibility on RSB diet, even though no significative difference was detected.

The extruded soybean (RSB) promoted improves in the digestibility of crude protein of the diet than whole raw soybean (RAS) with marked difference of 16.29 percentage units, representing a 34.5% greater digestibility. (Stern *et al.*, 1985); (Aldrich *et al.*, 1995) demonstrated *in vivo* experiments, the amino acid absorption in the intestine increased in diets containing 17% roasted soybean compared with the raw soybean. Also, study in vitro indicated that heat treatments of soybean increased the undegradable N fraction and the N digestibility for ruminants (Turner and McNiven, 2011). Then, this result indicates that the heat effect of extrusion of soybean has greater significance for protein digestion in the lower gastrointestinal tract, where the buffalo can best use this fraction.

Urinary excretion of creatinine was not affected by treatments, maintaining a mean value of 20.1 mmol/d (Table 2). This finding agrees with the observation has done by different researchers that creatinine excretion on ruminants of varying body weights, is excreted at a constant rate with low

Table 1. Feed intake and coastcross grass digestibility in buffaloes fed with concentrate using raw or roasted soybean.

Treatments[1]	RAS	RSB	SE[2]
Feed intake			
Hay (kg/d)	3.15	3.51	0.10*
Total DM (kg/d)	5.65	4.93	0.12**
Hay (% BW)	0.87	0.96	0.03*
Total DM (% BW)	1.24	1.34	0.03*
CP (kg/d)	0.75	0.85	0.02**
CP (% BW)	0.21	0.24	0.005**
NDF (kg/d)	2.44	2.63	0.09
NDF (% BW)	0.67	0.73	0.02
Apparent digestibility			
DM (%)	61.58	63.28	1.26
CP (%)	47.16	63.45	1.73**
NDF (%)	56.20	59.26	1.8

[1]Concentrate rations with raw soybean (RAS) or with roast soybean (RSB).

[2]SE = Standard error with differences between the treatment *P<0.05 and **P<0.001.

Table 2. Urine output of creatinine and purine derivatives in buffaloes fed with concentrate using raw or roasted soybean.

Treatments[1]	RAS	RSB	Mean	SE[2]
Urine output (L/d)	9.11	9.78	9.45	1.14
Creatinine (mmol/d)	22.00	18.23	20.12	2.69
Allantoin (mmol/d)	41.19	40.48	40.84	4.54
Uric acid (mmol/d)	5.08	6.44	5.76	0.53
PD (mmol/d)	46.27	46.92	46.60	4.75
N microbian (g/d)	33.64	34.11	33.88	3.45
Allantoin: uric acid	8.38	7.09	7.74	0.67

[1]Concentrate rations with raw soybean (RAS) or with roast soybean (RSB).

[2]SE = Standard error;

No significative difference was detected between the treatments (P>0.05).

daytime variability, regardless of diet (Lindberg *et al.*, 1989; Lindberg and Jacobsson, 1990). Also, no differences (P>0.05) were observed in the urinary excretion of purine derivatives between the both treatments studied (Table 2). Allantoin was the principal purine derivative excreted in the urine of buffaloes with an average of 40.84 mmol/day, representing 87.6% of total excretion of purine derivatives along with 12.4% of uric acid excretion. These excretion data were consistent with values obtained with cattle (Vagnoni *et al.*, 1997; Oliveira *et al.*, 2001) and with swamp buffalo (Pimpa *et al.*, 2003).

The relative proportion of allantoin:uric acid was 89: 11% and was constant for the two treatments. This relationship had a higher proportion of allantoin and lower of uric acid than the data cited by (FAO/IAEA, 2003) which is 80 to 85: 20 to 15%. However, it was similar to that found with buffalo (90: 10%) (Chen *et al.*, 1996; Moscardini *et al.*, 1999).

Xanthine and hypoxanthine were not observed along the curve of urinary excretion of purine derivatives on chromatography analysis (Figure 1). This is probably due to the high activity of the enzyme xanthine oxidase present in the blood and tissues of large ruminants (cattle, buffalo, yaks) that converts these metabolites in uric acid and other metabolites, which, in turn, are transformed into allantoin by the action of uricase (Chen *et al.*, 1996). However, this metabolic step needs to be confirmed and clarified.

Urinary excretion of purine derivatives overall did not differ between treatments, with a mean value of 46.6 mmol/d or 0.56 mmol/kg$^{0.75}$. This value is higher that raging values of 0.20 to 0.31 mmol/kg$^{0.75}$ obtained from buffaloes fed mainly straw and concentrates based diets by researches conducted in Asia (Chen *et al.*, 1996; Dipu *et al.*, 2006; Thanh and Orskov, 2006). Thus, this finding does not confirm the information that the excretion of PD in buffaloes is about half that

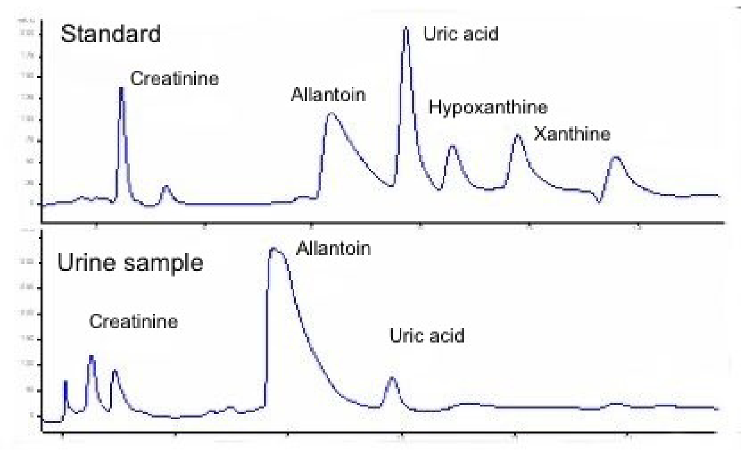

Figure 1. Cromatogram of excretion of purine derivatives in the urine of buffalo.

seen with cattle (Liang *et al.*, 1994; Moscardini *et al.*, 1999; Thanh and Orskov, 2006; Vo *et al.*, 2009), since the observed value of 0.56 mmol/kg$^{0.75}$ is close to values obtained with bovine in some feeding systems, such as diet of tropical grass hay, of 0.53 mmol/kg$^{0.75}$ (Chen *et al.*, 1990) and of 0.41 mmol/kg0$^{.75}$ (Bowen *et al.*, 2006)

The explanation for the variation values of urinary excretion of PD in buffaloes needs to be clarified. The metabolism of purine derivatives has been found to be similar in lactating cow and buffalo, but very low in buffaloes after the development of the rumen. These studies indicated that glomerular filtration rate is lower in buffalo promoting more time of the PD in blood and recycled to the rumen where are metabolized by bacteria or the permeability from blood into the rumen is much larger in buffaloes than cattle (Thanh and Orskov, 2006; Vo *et al.*, 2009). However, urinary excretion of PD appears also to be related to several factors, including: dry matter intake, type of foods and feeding behavior (Dipu *et al.*, 2006; Jetana *et al.*, 2009), geographic environment and ruminant species. It is noteworthy that the animals in present study were young (14 months) with a mean weight of 360 kg, while the buffaloes from the experiments of (Chen *et al.*, 1996) were 2.5 years old and only between 188 to 262 kg, probably reflecting in different forms in the PD metabolism.

The estimation of microbial nitrogen production from purine derivatives excreted by buffalo was mean of 33.9 g/day and did not differ between treatments (Table 2). This value was lower than found in Nellore consuming 25% concentrate (40.28 g/day) (Barbosa *et al.*, 2006), but similar to that obtained with zebu cattle studies (Renno *et al.*, 2000). The results in the production of microbial nitrogen obtained in this study should be confirmed, since in buffalo, urinary PD excretion has been found to be less than half, compared with other ruminant species.

The rumen protozoa ciliate density (number per ml of rumen contents) was changing according to treatment, but no difference was observed on the composition (% of total) of the fauna, except a lower percentage of *Dasytricha* was noted when roasted soybean was included in the diet (Table 3). Reducing number of protozoa species of genus *Entodinium* (P=0.011), *Epidinium* (P=0,072), *Dasytricha* (0.024) and in the total (P=0.059) occurred in buffaloes receiving roast soybean comparing to raw soybean. Rumen protozoa have preference to utilize insoluble proteins, and they are not able to synthetize protein from ammonia (Jouany and Ushida, 1999). Since the heat process of soybean promoted more insoluble protein in the rumen we were expecting to increase the protozoa concentration with RSB concentrate diet, seeing that both diets had the same energy level. Our results seem to have been contradictory considering the reduction of total protozoa and *Entodinium* on RSB diet. However, this nitrogen metabolism of rumen protozoa could be related to fauna composition, since no difference was observed in the ciliates of subfamily Diplodiniinae between both diets. Furthermore, the type of protein, the interactions with others nutrients and the predominant microbial population are the most important factors affecting the microbial nitrogen metabolism (Bach *et al.*, 2005). Therefore, the heat treatment of soybean could has changed the form of protein, decreasing the N availability to protozoa species in the rumen, and the density population, especially *Entodinium* species. As a consequence, the bacterial population could be increased, due the ability of protozoa to engulf bacteria (Williams and Coleman, 1992), and more protein passage out from the rumen, explaining the higher protein digestibility apparent obtained (Table 1).

The average fauna composition has indicated high percentage protozoa species belong to subfamily Diplodiniinae (37.6%) and reduce *Entodinium* (58.6%) comparing to other domestic ruminants in which the ciliates of genus *Entodinium* has been identified as around 90% of the total rumen population in various types of diets (Dehority, 2003). But this findings agree with the composition fauna observed in rumen of buffalo on different diets (Franzolin *et al.*, 2010) (Table 3).

It is interesting to note that PD and allantoin excretions have been increased in defaunated compared with faunated lambs (Fujihara *et al.*, 2003a), while the PD excretion in goats has been decreased in defaunated regarding faunated animals (Fujihara *et al.*, 2003b). In our findings no differences were observed on PD urinary excretion (Table 2) even thought there were reduced of total protozoa concentration (Table 3). However, no difference was observed in the Diplodiniinae concentration. These data support the hypothesis that different composition of protozoa population in the rumen can influence the density and composition of bacteria, and contribute to promote variation of rumen nitrogen metabolism among the ruminant species.

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Table 3. Concentration of rumen protozoa and genus composition in buffalo fed with concentrate using raw or roasted soybean.

Treatments[1]	**RAS**	**RSB**	**P-Value**
Number (x 10^5/mL ruminal contents)			
Entodinium	1.55	1.09	0.011
Diplodiniinae	0.96	0.86	0.674
Epidinium	0.02	0.00	0.072
Isotricha	0.04	0.03	0.480
Dasytricha	0.16	0.04	0.024
Total	2.73	2.02	0.059
Composition (% of total/mL ruminal contents)			
Entodinium	59.49	57.65	0,778
Diplodiniinae	35.62	39.47	0.563
Epidinium	0.30	0.00	0.074
Isotricha	1.03	1.47	0.048
Dasytricha	3.56	1.41	0.005

[1]Concentrate rations with raw soybean (RAS) or with roast soybean (RSB)

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