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Original article

Influence of rumen protein degradability on productive and reproductive performance in buffalo cows

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Abstract — The present study aimed to ascertain the influence of crude protein (CP) digestibility in the rumen on the quantity and quality of milk production and reproductive performance, blood (BU) and milk (MU) urea, haematological profile and vaginal mucus urea, ammonia and potassium of buffalo cows. Lactating buffaloes (n = 84), 60 days in milk, were randomly subdivided into Group C (control, n = 42) and Group T (fed a diet supplemented with *Aspergillus oryzae,* n = 42). In three fistulated buffalo, the diet supplemented with *Aspergillus oryzae* showed a decrease ($P < 0.01$) in protein digestibility in the rumen (79.3 vs. 45.9%). No differences were registered in productive performance. Nine buffaloes not in oestrus during the dietary treatment (Groups T1 and C1), 30 days in milk, were used to study the haematological profile and to determine milk urea and ammonia in the vaginal mucus. The animals in Group T1 had higher ammonia values in the blood ($P < 0.01$) but not in the vaginal mucus than Group C1. A relationship was found between MU and BU. MU was influenced by CP intake and dry matter intake. No differences between the treatments were observed in reproductive performance and the conception rate and calving interval were 37.9% and 41.4% (90 trial-day) and 449 and 419 days respectively in Groups T and C. Reproductive performance was not influenced by high levels of BU nor by blood ammonia levels, although the latter were higher in the group fed the diet supplemented with *Aspergillus oryzae*.

buffalo cow / protein digestibility in the rumen / blood / milk / vaginal mucus

1. INTRODUCTION

Dietary proteins and their digestibility in the rumen affect blood (BU) and milk (MU) urea concentration [1–5]. Overfeeding proteins has been associated with a decline in fertility in most [6–9], but not all [10] studies. Jordan et al. [11] found an increase in urea-nitrogen (N) concentration in the uterine secretions and plasma of cows fed 23%

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crude protein (CP). Elrod et al. [7] showed that an excess of either digestible (RDP) or indigestible (RUP) protein, in the rumen increases BU and alters uterine pH to a similar degree, interfering with the normal inductive effects of progesterone on the microenvironment of the uterus, thereby providing suboptimal conditions to support embryo development.

In buffaloes a decline in circulating urea occurs after a sharp reduction in the protein content of the diet while a fairly long period of CP deficiency does not result in low MU levels; in addition, dietary protein characteristics and the protein/energy (P/E) ratio influence blood and milk urea [14].

To date, there have been few studies on the influence of dietary protein concentration on the fertility of female buffaloes [17–18].

The present study aimed to ascertain the influence of different CP digestibilities in the rumen obtained by supplementing the diet with *Aspergillus oryzae* on milk production and reproductive performance, BU, MU, haematological profile and vaginal mucus urea, ammonia and potassium levels of lactating buffalo cows.

2. MATERIALS AND METHODS

2.1. Animals and dietary treatments

The influence of dietary CP digestibility in the rumen on milk yield and quality was determined using 84 lactating buffaloes (60 ± 42 days in milk - DIM) of 129 kg metabolic weight (MW). In the previous lactation their average milk production over 270 days was greater than 2 500 kg. The cows were randomly assigned to Group C (control) and Group T (treated) and maintained in open yards that allowed 15 m^2 and a feeding trough space of around 1.0 m for each buffalo. Milk yield, at the beginning of the study, was 13.3 ± 3.3 $kg \cdot day^{-1}$ (Group C) and 13.0 ± 3.3 $kg \cdot day^{-1}$ (Group T).

The ingredients and chemical composition of the diets, administered as the total mixed ration (TMR), are shown in Table I. The T group diet was supplemented with 350 g of Millymix® (CLS, Zelo Buon Persico, MI, Italy) containing microelements and *Aspergillus oryzae* (125 g) in order to decrease the amount of RDP (Tab. I).

Two bulls of proven libido and fertility were kept with each group of buffalo cows for the duration of the study (90 days). Pregnancy was determined by rectal palpation at 30-day intervals. The day of conception was calculated by subtracting the average gestation length (308 days) from the day at calving. Days open and conception rate were calculated only for the animals that became pregnant during the experimental period.

Feed intake and orts for each group were measured daily. Individual feedstuff, TMR and orts were sampled once weekly on a random day. The analyses of individual feedstuff, TMR and orts were carried out as per AOAC [20] methods and energy values (Milk Forage Units –MFU = 1 700 kcal NEL) were calculated according to the INRA equations [21].

2.2. In sacco measurements

The protein digestibility in the rumen was evaluated by the in sacco method using three fistulated buffaloes. The diets were sampled and incubated as fed. For each time, 3 bags per animal and diet were used. After incubation the bags were hand-rinsed in cold tap water for 5 min, and dried at 60 °C for 72 h. The washing losses were determined by washing 5 non-incubated bags for each diet in cold water, following the same procedure adopted for the bags removed from the rumen at the end of each incubation period. During rumen incubation, the bags were fixed to teflon sticks anchored to the rumen valve plug. RDP and RUP were calculated according to ASPA [22].

The cannulated buffaloes were fed a diet (CP/DM 12%; 50 g $DM \cdot kg^{-1}$ of metabolic

Table I. Feed and chemical composition of the diets in the control group (C) and the group fed a diet supplemented with *A. oryzae* (T).

Group	T	C
Corn silage (kg)	28.0	28.0
Sugar dry beet pulp (kg)	5.5	5.5
Soybean meal (kg)	3.0	3.0
Fat (g)	300	350
$CaCO_3$ (g)	150	100
Millymix® (g)*	350	–
Vitamin (g)	40	40
Chemical composition		
Dry matter (kg)	17.9	18.0
Crude protein (%DM)	15.8	15.8
Ether extract (%DM)	5.0	5.0
UFL·kg^{-1} DM	0.9	0.9
Crude fiber (%DM)	20.6	20.6
Ash (%DM)	7.2	7.3
NDF (%DM)	41.1	41.0
ADF (%DM)	25.8	25.8
NSC (%DM)	29.5	29.4

* Containing *Aspergillus oryzae* (125 g) and following microelement (in 100 g): Fe (sulfate) 220 mg; Mn (oxide) 450 mg; Zn (oxide) 630 mg; I (iodide) 3 mg; Cb (sulfate) 0.5 mg; Se 0.2 mg; Sulphur 1.2 mg. UFL (energy), NDF (neutral detergent fiber), ADF (acid detergent fiber), NSC (non structural carbohydrates).

weight) with 75% forage and 25% concentrate.

2.3. Milk sampling, analytical methods, standard milk and calculation of differences between intake and requirements

Fortnightly, for 90 days, the milk samples from each buffalo were collected during the morning and afternoon milking in order to evaluate milk composition (fat and protein) using the IR spectroscopy (Milkoscan 139, Foss Electric, Hillerød, DK) calibrated with the appropriate buffalo standard.

Energy corrected milk (ECM = 740 kcal) was calculated using the formula for buffalo cows: ([{fat (g·kg^{-1}) – 40 + protein (g·kg^{-1}) – 31} × 0.01155] +1) × milk yield. The animals were weighed at the beginning and end of the trial.

Individual feed intake and differences (Δ) between nutritive intake and relative requirements were estimated as suggested by Campanile et al. [14]:

dry matter (DM) intake = 91g × MW + 0.27 kg × kg ECM;

ΔCP = g CP intake – (80 g CP × 100 kg live weight + 2.7 g CP × g milk protein yield);

ΔMFU = MFU intake – [(1.4 + 0.6 × 100 kg live weight) × 1.1 + 0.44 MFU × kg ECM] [21].

2.4. Blood and vaginal mucus samples and analytical methods; milk urea analyses; body condition score

Nine buffaloes, 31 ± 11 DIM, per dietary treatment (Groups C1 and T1) not in oestrus were used to study the metabolic profile and to determine milk urea and ammonia in the vaginal mucus. Every 20 days, for a total of three times, blood samples, vaginal mucus and milk samples were collected. Blood samples were collected at 08.00 h, before feeding, from the jugular vein in vacutainer tubes. The tubes were centrifuged at 3 000 *g* for 15 min. The recovered serum was stored at –18 °C until analysis for metabolic profile. Immediately after sampling, the ammonia (NH_3) was measured on whole blood with a rapid method (Cat. No. 29060, Ammonia kit, Menarini, Florence, Italy) that showed a high correlation (r = 0.988) with the enzymatic UV-method. Glycemia (Cat. No. 10011, SCM, Rome, Italy) was measured on plasma by means of a colorimetric method. Blood parameters were measured on serum using the enzymatic colorimetric method (SCM, Rome, Italy) for urea (BU)

(Cat. No. 10230), creatinine (Cat. No. 10073), total cholesterol (Cat. No. 10028), HDL cholesterol (Cat. No. 10176), triglycerides (Cat. No. 10163), ALT (Cat. No. 10122), AST (Cat. No. 10132), GGT (kinetic at 37 ºC) (Cat. No. 10228), β-hydroxybutyrate (BHBA) (Cat. No. 310-A, Sigma Diagnostic, St. Louis, MO, USA) and non-esterified fatty acids (NEFA) (Cat. No. AM0115, Randox Laboratories Ltd., Crumlin, UK). Colorimetric methods (SCM, Rome, Italy) were used to measure calcium (Cat. No. 10053), phosphorus (Cat. No. 10241), magnesium (Cat. No. 10410) and total proteins (Cat. No. 10031); the serum protein fractions were evaluated by the electrophoresis technique (Alfa Wasterman, Milan, Italy). Potassium was measured by means of an aphotometer with an air/acetylene flame (Cat. No. DV710, Giodevita, Rome, Italy). Insulin was measured by the automated fluoro-immunometric method (AIA 1200; TOSO, Eurogenetics, Milan, Italy). Vaginal mucus was aspirated with an insemination pipette and syringe, diluted 1:1 with a sterile physiological solution and frozen until analysis for urea and potassium performed according to Jordan et al. [11] and for NH_3 according to Rumello et al. [24]. Milk urea (MU) was measured spectrophotometrically (Cat. No. 535A, Sigma Diagnostic, St. Louis, MO, USA).

Body condition scores (BCS) were recorded at every milk collection assigned using a scale of 1 to 9 [25] modified for the buffalo.

2.5. Statistical analyses

The general linear model (GLM) [26] procedure for repeated measures was carried out for milk and blood parameters between dietary groups (T and C; T1 and C1) and between pregnant (P) and non-pregnant (NP) buffaloes at the end of the trial. In the latter two groups we excluded from the analysis buffaloes already pregnant at the beginning of the trial and, for each variable, the buffaloes that scored anomalous values. In addition for only milk parameters, the GLM [26] was performed between Groups C and T within productive levels (High: > 13 kg; Medium: 10–13 kg; Low: < 10 kg: groups H, M and L respectively).

Table II. Number of enrolled animals in the control and *A. oryzae* treated groups (C and T) and in the different subgroups: P (pregnant), NP (non-pregnant), H (high lactating cows, > 13 kg), M (medium lactating cows, 10–13 kg) and L (low lactating cows, < 10 kg).

Group	C	T
	n	*n*
P	13	11
NP	18	18
H	15	12
M	12	12
L	15	18
Total	73	71

Correlation analyses [26] were performed between blood, milk, and mucus parameters and dietary characteristics. Linear regression analyses were performed for blood NH_3 and MU as dependent variables using BU as the independent variable. Multiple linear regression was performed with the SPSS 10.0 [26] stepwise procedure in order to evaluate the relationship between blood NH_3, BU, MU and the dietary characteristics. The conception rate was analyzed by the Chi-square test.

The number of animals in the various groups and subgroups are reported in Table II.

3. RESULTS

Diet supplemented with Millymix® containing microelements and *Aspergillus oryzae* showed a decrease in protein digestibility in the rumen (79.3% ± 1.7 vs. 45.9% ± 4.5; $P < 0.01$).

Table III. Mean and standard error (m ±se) of energy corrected milk (ECM) and milk protein yields; differences between energy (MFU) and crude protein (CP) intakes with their respective requirements, rumen digestible proteins (RDP) and rumen indigestible proteins (RUP) intake in the control (C) and *A. oryzae* treated groups (T) and in pregnant (P) and non-pregnant animals (NP).

Group	**C**	T	P	NP
n	42	42	24	36
ECM·d^{-1} (kg)	19.1 ± 0.5	19.1 ± 0.4	19.6 ± 0.6	18.8 ± 0.4
Milk protein·d^{-1} (g)	525 ± 11	534 ± 13	543 ± 19	509 ± 12
DMI (kg)	16.1 ± 0.1	16.6 ± 0.1	16.6 ± 0.2	16.4 ± 0.2
MFU intake	14.7 ± 0.1	15.1 ± 0.1	15.5 ± 0.2	15.3 ± 0.1
ΔMFU	+0.53 ± 0.04	+0.84 ± 0.06	+1.00 ± 0.06	+1.20 ± 0.14
CP intake (g)	2512 ± 23	2556 ± 21	2669 ± 22	2644 ± 37
ΔCP (g)	+575 ± 14	+594 ± 12	+683 ± 23	+750 ± 19
RDP intake (g)	1992 ± 19	1173 ± 34	1743 ± 54	1629 ± 44
RUP intake (g)	520 ± 49	1382 ± 46	926 ± 67	1015 ± 45

Total dry matter (DM) intake (Tab. III) was similar for buffaloes in Group T (16.6 kg·day^{-1}) and Group C (16.1 kg·day^{-1}).

No differences between dietary treatments (C and T) were observed in milk and ECM yield (Fig. 1) and milk quality (Fig. 2).

The intake of DM, CP, RUP and RDP did not differ between Groups P and NP. Similarly, no differences were found for milk yield and quality (Tab. III).

No difference emerged between Group C1 and T1 in MU (8.8 vs. 8.4 mmol·L^{-1}) and BU (Tab. IV) values. However, Group T1 had higher ($P < 0.01$) blood ammonia (Tab. IV). The relationship between MU and BU is expressed by the following equation:

MU (mmol·L^{-1}) = 1.02 + 0.802 BU (mmol.L^{-1}); $n = 51$; mse = 0.213; $r^2 = 0.814$.

CP intake and days in milk influenced MU levels:

MU (mmol·L^{-1}) = –12.475 + 0.008 CP intake (g) + 0.029 days in milk; $n = 51$; mse = 0.473; $r^2 = 0.587$.

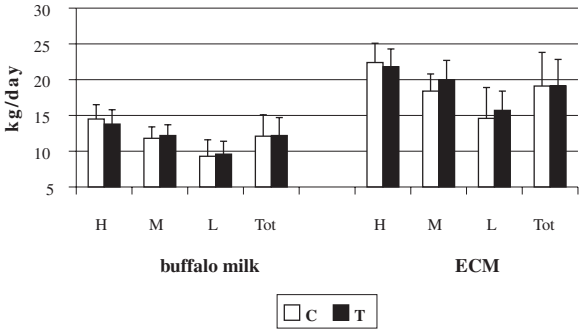

Figure 1. Mean values and sd values of milk and energy corrected milk (ECM) yields of the groups H (high lactating cows, > 13 kg·day^{-1}), M (medium lactating cows, 10–13 kg·day^{-1}), L (low lactating cows, < 10 kg·day^{-1}) within control group (C) and *A. oryzae* treated group (T).

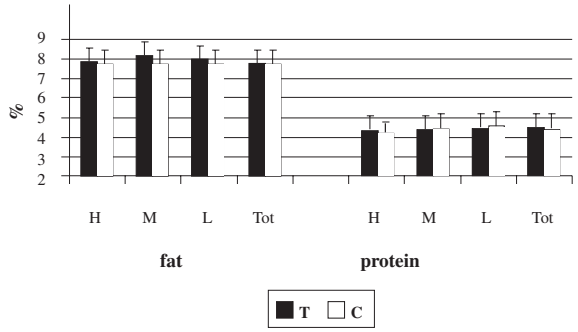

Figure 2. Mean values and sd values of fat and protein percentages of the groups H (high lactating cows, > 13 kg·day^{-1}), M (medium lactating cows, 10–13 kg·day^{-1}), L (low lactating cows, < 10 kg·day^{-1}) within control group (C) and *A. oryzae* treated group (T).

No relationship emerged between blood urea and ammonia levels. The latter was negatively influenced by RDP intake:

NH_3 (μmol·L^{-1}) = 202.65 – 0.055 RDP intake (kg); n = 53; mse = 2 958; r^2 = 0.483

Within the 18 subjects that were 30 days in milk (T1 and C1 groups), no differences were observed between P and NP buffaloes in MU (8.5 ± 0.3 mmol·L^{-1} vs. 8.8 ± 0.3 mmol·L^{-1}), BU or blood ammonia (Tab. IV).

Except for the BU, whose levels were higher than normal, the other haematological parameters examined were in the physiological range for buffaloes [28–31] and did not differ between Group C1 and Group T1. Considering the P and NP groups, the latter showed higher (P < 0.05) values for K (3.9 ± 0.1 vs. 4.3 ± 0.1 mEq·L^{-1}) and total protein (7.8 ± 0.2 vs. 8.5 ± 0.3 g·L^{-1}).

The BCS values were lower (P < 0.05) in Group T than in Group C at 80 and 100 days in milk (Fig. 3). The BCS value was influenced by non-structural carbohydrates (NSC) and RDP intake and by DIM: BCS = –0.402 + 0.001 NSC intake (g) + 0.0006 RDP intake (g) + 0.012 DIM; n = 51; mse = 0.095;

Table IV. Estimated means (m ± s.e.) of the urea, ammonia (NH3) and potassium (K) in blood and vaginal mucus in the control (C) and *A. oryzae* treated gropups (T) and in pregnant (P) and non-pregnant animals (NP) at the end of the trial (Groups P1 and NP1).

Group	Blood			Mucus		
	Urea (mmol·L^{-1})	NH3 (μmol·L^{-1})	K (mEq·L^{-1})	Urea (mmol·L^{-1})	NH3 (μmol·L^{-1})	K (mEq·L^{-1}l)
C1	9.1 ± 0.2	103.0 ± 10.3 B	4.2 ± 0.1	5.2 ± 0.7	17.0 ± 7.0	6.4 ± 1.9
(*n*)	(8)	(9)	(7)	(7)	(7)	(6)
T1	9.6 ± 0.3	146.0 ± 10.0 A	4.0 ± 0.1	5.7 ± 0.9	18.0 ± 9.0	8.0 ± 2.1
(*n*)	(7)	(8)	(8)	(5)	(4)	(5)
P1	9.2 ± 0.3	117.0 ± 11.0	4.0 ± 0.1 a	6.5 ± 0.8	17.0 ± 8.0	4.5 ± 2.1
(*n*)	(9)	(10)	(9)	(6)	(6)	(5)
NP1	9.6 ± 0.4	132.0 ± 9.0	4.3 ± 0.1 b	4.4 ± 0.8	18.0 ± 9.0	9.9 ± 1.9
(*n*)	(6)	(7)	(6)	(6)	(5)	(6)
Tot.	9.4 ± 0.2	124.0 ± 7.0	4.1 ± 0.1	5.5 ± 0.6	17.0 ± 16.0	7.2 ± 1.4
(*n*)	(15)	(17)	(15)	(12)	(11)	(11)

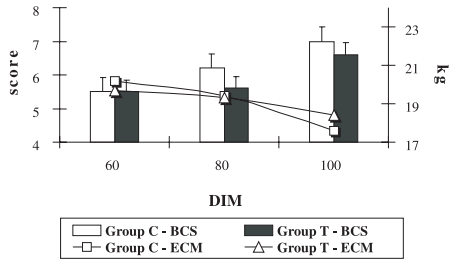

Figure 3. Patterns (m ± sd) of body condition scores (BCS) and energy corrected milk (ECM) in the control (C) and the group fed a diet supplemented with *A. oryzae* (T) during the trial. DIM: days in milk.

$r^2 = 0.828$. The trend and differences between Groups T1 and C1 were similar. No differences in BCS were observed between P and NP buffaloes.

In the 90 days of the trial, the conception rate and calving interval were 37.9% and 41.4% and 449 d and 419 d respectively in buffaloes of Group T and Group C. There were no significant differences in urea, ammonia and K measured in the vaginal mucus, neither between dietary treatment (Groups T1 and C1) nor between pregnant and non-pregnant cows amongst the eighteen buffaloes (Tab. IV). The urea values of vaginal mucus were related to BU ($r = 0.373$; $P < 0.05$); furthermore a negative relation ($r = –0.389$; $P < 0.05$) was found between the ammonia level of the vaginal mucus and ΔMFU. Potassium values in the vaginal mucus were related to CP intake ($r = 0.525$; $P < 0.01$).

4. DISCUSSION

In the present study *Aspergillus oryzae* did not influence milk yield during the first 150 days of lactation in pluriparous buffaloes. DM intake·kg^{-1} ECM was similar to that observed in cattle [21] and in buffalo [32] at the same stage of lactation.

According to Campanile et al. [14], energy requirements were met while CP intake of all the subjects was 26% higher than that required for their milk production. Supplementing the diet with microelements and *Aspergillus oryzae* resulted in a higher RUP intake in Group T, exceeding the requirements (+80%) suggested by NRC [27] for cows yielding less than 600 g milk protein·day^{-1}. By contrast, the RDP intake of Group C was 29% lower than required. However, differences in milk yield and quality were not observed even in high producing buffaloes. In contrast, Infascelli et al. [15] found a significant increase in milk yield by supplementing the diet for buffalo cows with *Aspergillus oryzae*; the different findings could be due to the very high productive level of the buffalo cows used by these authors.

In the 18 subjects that were 30 days in milk, MU and BU were correlated ($r = 0.902$) as shown by other studies in dairy cows [5, 35, 36] and buffalo cows [14]. Urea levels in milk and blood were influenced by CP intake and days in milk, while protein digestibility in the rumen did not influence both parameters. In mid-lactation [14] MU and BU were influenced by the P/E ratio. Carruthers et al. [37] found that in cows in late lactation the P/E ratio increased microbial protein synthesis in the rumen. Urea levels in the blood and milk increased according to the days open due to the higher DM and hence CP intake [28].

The blood ammonia level was negatively affected by RDP intake and, as found in dairy cows, it was not influenced by BU [38]. The latter author attributed this result to the fact that urea peaks 4 to 8 h after feeding [38]. Ammonia values in buffalo cows were higher than those reported by Elrod and Butler [7] in dairy cows, and McEvoy et al. [39] in sheep, and lower compared with the results of Jordan et al. [11] and Garcia-Bojalil [40] in cattle.

The higher ammonia values in Group T may have resulted from the high level of RUP which, not being completely digested in the jejune may pass into the large

intestine where it should be degraded by microbial flora and transformed into ammonia which circulates again [41]. The low level of diet fermentable carbohydrates (NSC/DM 25.9%) in the present trial could have further promoted the phenomenon as suggested by NRC [41].

Group T showed a lag phase in recovering BCS; which appears difficult to explain. However, considering the higher blood ammonia values of Group T, the delay in recovering the BCS could be due to the lower protein availability as an energy source and to the higher energy loss in order to eliminate the excess ammonia.

Protein digestibility in the rumen did not influence reproductive activity in buffalo cows. Indeed, it is important to underline that feeding both groups with the experimental diet throughout the mating period (March-September) resulted in an 80% pregnancy rate; this percentage is normally observed in southern Italy for the buffalo. The urea values measured in the vaginal mucus were similar [11] or higher than those reported respectively in the dairy cow [24] and sheep [39]. By contrast, the ammonia level in the vaginal mucus was considerably lower than those found by Rumello et al. [24] in the dairy cow and by McEvoy et al. [39] in sheep. High levels of ammonia in the uterus affect fertility in dairy cows due to the detrimental effect on embryo development [39]. It is possible that in the buffalo, independently of the BU, a lower diffusion of ammonia occurs in the uterus, reducing the detrimental effect on reproductive efficiency. The high values of fertility registered during the trial and at the end of the mating period (March-September) show that urea levels in the blood and in the vaginal mucus do not have a negative effect on reproductive performance.

It is widely reported that the negative effect of excess protein on fertility, in cattle, may be due to the RDP intake [6, 11, 38, 39]. In the present trial the negative effect of RDP excess was not observed in buffalo cows since this species uses nitrogen better than cattle, also with NSC deficiency [43]. In buffalo, the intraruminal environment is more favorable for NPN-using bacteria [43].

Energy deficiency decreases the ability of the liver to transform ammonia into urea. This results in an increase in blood ammonia levels and, by diffusion, in the vaginal mucus ammonia level. In addition Schepers and Meijer [44] found a relationship between diet energy and MU.

According to Jordan et al. [11], K values in the vaginal mucus are conditioned by CP intake irrespective of protein digestibility in the rumen.

5. CONCLUSION

Dietary protein digestibility in the rumen did not influence buffalo milk yield or quality, probably due to its low yield potential.

Reproductive performances were not influenced by high levels of BU nor by blood ammonia levels, although the latter were higher in the group fed the diet supplemented with *Aspergillus oryzae*.

In the buffalo, a lower diffusion of ammonia occurs in the uterus, reducing the detrimental effect on reproductive efficiency. The high values of fertility registered during the trial and at the end of the mating period (March-September) appear to confirm that urea levels in the blood and in vaginal mucus do not have a negative effect on reproductive performance. This result may well reflect the tropical (North of the Equator) origin of buffalo which modifies diet protein use according to season and feed availability. Indeed, in tropical regions, forage availability occurs only for a few months.

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