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EFFECT OF VARIOUS NON PROTEIN NITROGEN SOURCES ON *IN VITRO* DRY MATTER DIGESTIBILITY, AMMONIA PRODUCTION, MICROBIAL GROWTH AND pH CHANGES BY RUMEN BACTERIA

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ABSTRACT

Four slow releasing non-protein nitrogen (NPN) sources viz. urea-straw (US), urea-starch (UST), biuret-straw (BS) and biuret-starch (BST) were compared with cotton seed meal (CSM) as control nitrogen source. In all experiments, 2% nitrogen level on DM basis in contents of each fermentation flask were maintained through replacing 30, 50 and 70% of CSM by respective NPN source. Four *in vitro* experiments were `conducted to see the effects of various NPN sources on dry matter digestibility (DMD), microbial growth, ammonia nitrogen release and pH changes. The effects of NPN sources CSM, BST, UST, BS and US on DMD were significant (P<0.001) and the mean DMD values for the five NPN sources were 13.65, 31.76, 28.81, 27.14 and 22.39%, respectively. The UST resulted in maximum bacterial count of 290.0 X 10^7. The bacterial counts with US, CSM, BST and BS were significantly lower (P< 0.001) than that of UST. The NH_3-N released due to NPN sources, levels, time of incubation and interactions between sources x levels, sources x time and levels x time were significant (P<0.001). The release of NH_3-N by BST was maximum (17.77 mg/dl), followed by UST (17.35 mg/dl), BS (15.85 mg/dl) and US (15.67 mg/dl). The effect of various NPN sources on pH were also significant (P<0.01). The mean pH values were 7.13, 7.15, 7.08, 7.13 and 7.15 for US, BS, UST, BUST and CSM, respectively. The UST containing cultures had lowest pH values compared to other NPN sources.

Key words: Non protein nitrogen, in vitro, dry matter digestibility, microbial growth, ammonia nitrogen.

INTRODUCTION

The value of dietary protein in ruminants depends upon its chemical nature and solubility. The plant protein sources i.e. oil seed cakes are becoming expensive due to their short supply and, therefore, use of non protein nitrogen (NPN) is imperative in animal diets. The dietary supply of nitrogen must be sufficient enough to support a dense population of bacteria in the rumen. The animal itself assists this process through urea recycling and its delivery in the rumen. The rumen microorganisms convert NPN to ammonia (NH_3) and ultimately to bacterial mass.

The microbial protein is produced by multiplication of a single or multiple microbes of the ecosystem in the rumen (Hungate, 1966). The efficiency of microbial fermentation depends mainly upon the availability of essential nutrients. There is much evidence that efficient utilization of NH_3 for microbial protein synthesis in the rumen occurs at relatively low concentration i.e., 5-8 mg NH_3-N/100 mL (Mercer and Annison, 1976). Urea serves as a substrate for urease enzyme in the rumen to liberate NH_3 (Sundstol, 1984). The efficiency of NH_3 utilization depends on rumen environment and energy availability (Hungate, 1966; Ali *et al.*, 2007).

Excessive production of NH_3 in the rumen may be toxic for the animal; therefore, efforts to produce slow NH_3 releasing products for animal production are being made. The present project was planned with an aim to fix urea and biuret with wheat straw and corn starch and compare their efficiency as NPN sources for animal production.

MATERIALS AND METHODS

NPS sources

Following four products were made and compared as slow releasing NPN sources: (1) Ten grams of fertilizer grade urea was dissolved in 50 ml of water and 10 grams of fine wheat straw was mixed in it homogenously and kept in an oven at 40°C for 48 h in tightly closed container. (2) Ten grams of biuret was dissolved in 50 ml of water and 10 grams of fine wheat straw was mixed in it homogenously and kept in an oven at 80°C for 48 h in tightly closed container. (3) Ten grams of urea was dissolved in 50 ml of water and 10 grams of corn starch was mixed in it homogenously and stored in an oven at 40°C for 48 h. (4) Ten grams of biuret was dissolved in 50 ml water and 10 grams of

corn starch was mixed in it homogenously and kept in an oven at 80°C for 48 h in a tightly closed container. These four NPN sources were named as US, BS, UST and BST, respectively and were compared with cotton seed meal (CSM) as nitrogen source. In all experiments, 2% nitrogen level on DM basis in each flask contents was maintained by replacing 30, 50 and 70% of CSM by respective NPN source.

Artificial rumen and inoculums

The artificial rumen comprised of conical glass flasks of 250 ml with rubber stoppers fitted with Bunsen valves, CO2 gas cylinder and water bath at 39°C with shaker (Johnson, 1966). Two cannulated buffalo bulls were used as donor of the rumen inoculums. The bulls were fed seasonal green fodder and 70 g of urea solution was infused intra-ruminally at least 10 days prior to the commencement and during the period of collection of inoculums. A plastic bottle with its mouth secured by thumb was introduced in the ventral sac of the rumen. The contents of rumen were shaken well with hand movements and bottle filled with fluid was drawn out. The fluid was strained through four layers of muslin cloth and kept in glass vacuum container till use.

Buffer mineral solution

Artificial saliva (McDougall, 1948) was used to maintain the aqueous environment and regulate pH as well as to provide nutrients to the microbes (Singh and Pradhan, 1981). Composition of artificial saliva is given in Table 1.

Table 1: Composition of the artificial saliva

S. No.	Ingredients	Quantity (g)
1.	$NaHCO_3$	9.80
2.	Na_2HPO_4 $2HO_2$	4.65
3.	KCl	0.57
4.	$MgSO_4$ $7H_2O$	0.12
5.	NaCl	0.47
6.	$CaCl_2$	0.04

First five ingredients were dissolved in one liter of distilled water and stored as stock solution. Four percent solution of $CaCl_2$ was prepared and just before use one ml of this solution was added to the one liter of the stock buffer solution to provide 0.04 g $CaCl_2$ per liter. The solution was bubbled with CO_2 until the pH of 6.9 was achieved. The *in vitro* environment included carbonic atmosphere with pH 6.7-7.0 and temperature 39°C with gentle shaking.

Triplicate samples were placed in 250 ml glass flasks each containing one gram of dried wheat straw, 50 ml of strained rumen liquor and 50 ml of McDougall's buffer ((Johnson, 1966). The flasks were capped with rubber stopper equipped with Bunsen valve for gas escape. The flasks were incubated at 39°C in a water bath fitted with a shaker. Following four experiments were conducted:

Experiment 1

In this experiment, the effects of CSM and NPN sources on *in vitro* day matter digestibility (DMD) were investigated. Four NPN sources were US, BS, UST and BST and four levels of the NPN were 0, 30, 50 and 70%. After 48 h of incubation, the microorganism activity was stopped by immediate cooling of the flasks in ice cold water. The contents of the flasks were strained through 4 layers of muslin cloth and the filtrate was used for microbial count. The residue was dried at 70°C for 24 h for the determination of DMD according to the following formula:

$$DMD = \frac{\text{Sample dry matter - residue dry matter}}{\text{Sample dry matter}} \times 100$$

Experiment II

The effect of replacement of CSM with four levels (0, 30, 50, and 70%) of four NPN sources on microbial counts were studied in this experiment. The bacterial counts were studied according to the modified technique of Knaysi and Ford (1938). One ml of strained rumen fluid from each treatment was diluted serially in distilled water to obtain final dilution of 10^2. All dilutions were shaken vigorously to break the bacterial clumps, if any. From the final dilution, 0.01 ml fluid was transferred to a clean slide upon which 1 cm^2 area had previously been marked. The sample was spread evenly over the marked area, air dried, fixed over a flame, stained with Gram's stain and examined under oil immersion (100 X and 6.8 X eye piece). The counts were made from 5 randomly selected microscopic fields using a stage micrometer with 12 divisions, with each division of 10 u. Total microbial counts were computed by the following formula:

Microorganism /ml = MF x N x 100 x dilution factor, where

MF (microscopic factor) = 8846.43 (10 *8*/11304),

N (total organisms/field) = Total organisms in 5 fields/5

Experiment III

Effects of four NPN sources e.g. US, BS, UST and BST and their four levels 0, 30, 50 and 70% on NH_3-N concentration were evaluated. For this purpose, eight sets of duplicate flasks were fermented. The fermented samples were centrifuged for 5 minutes at 4000 rpm and supernatant was used for NH_3-N measurement at 10, 20, 40 minutes and 1, 2, 4, 24 and 48 h of fermentation. One milliliter (ml) of supernatant was added to 9 ml of distilled water, thoroughly mixed and

to one ml of this mixture 3.6 ml of distilled water and 0.04 ml of Nesseler reagent were added.

A standard curve was made to determine whether a linear relationship existed between varying concentrations of ammonium sulphate standard solution and intensity of color produced by Nesselerizatoin. For this purpose, 10 test tubes containing 0.10 to 1.0 ml of standard solution were prepared. To each test tube, 0.04 ml Nessler's reagent was added and volume was made up to 5 ml with distilled water. The intensity of color thus developed was measured at 420 nm within 5 to 10 minutes after setting it at 0 absorbance with blank. The NH_3-N concentration was calculated as below:

$$NH_3\text{-N (mg/dl)} = \frac{\text{Absorbance of samples}}{\text{Absorbance of standard}} \times \text{conc. of standard}$$

Experiment IV

The effects of replacement of CSM with various levels of NPN sources on *in vitro* pH changes were measured at 0 and 48 h post fermentation with a pH meter. The pH meter was standardized with standard buffer solutions at the room temperature. Then the pH of all the culture flasks was measured with glass electrode at the temperature of the flask contents.

Statistical analysis

The data were analyzed in factorial design by using analysis of variance (Steel and Torrie, 1981) to find th**e e**ffect of four nitrogen sources, four levels of NPN and sources X levels of NPN interactions. The significance of differences between means was tested by Duncan Multiple Range Test (Duncan, 1955). For bacterial counts and NH_3-N concentrations, the effects of fermentation hours and their interactions with nitrogen sources X corn starch levels were also tested.

RESULTS

Digestibility

The effect of CSM and NPN sources (BST, UST, BS and US) on DMD was significant (P<0.001). The DMD was minimum with CSM as protein source and maximum with BST, followed by UST, BS and US (Table 2). There was a significant (P<0.001) effect of different levels of NPN on DMD. The mean DMD values for CSM with O, 30, 50 and 70% NPN levels were 13.65, 20.30, 26.05 and 27.91%, respectively. The results revealed that maximum DMD was achieved with 70% NPN level. There were significant interactions between NPN sources and NPN levels. Maximum DMD of 38.44% was achieved with BST X 70% NPN, followed by UST X 50%, UST X 70%, BST X 50% and BS X 70% NPN. The differences among the last four interactions were non significant.

Table 2: *In vitro* DMD as influenced by replacement of CSM with various levels of NPN sources (values are presented in ranked order)

NPN sources*	DMD (%)	NPN level (%)	DMD (%)
BST	31.76[a]	70	29.91[a]
UST	28.81[b]	50	26.05[b]
BS	27.14[c]	30	20.30[c]
US	22.39[d]	0	13.65[d]
CSM	13.65[e]		

*US= Urea straw, BS= Biuret straw, UST= Urea starch, BST= Biuret straw, CSM= Cotton seed meal.
abc= Means in the same column with different superscripts differ significantly (P<0.001).

Microbial growth

The effect of NPN sources revealed that UST resulted in maximum bacterial count of 290.0 X 10^7. The bacterial counts with US, CSM, BST and BS were significantly lower (P<0.001) compared to UST. However, the differences among the latter four sources were non significant (P>0.05, Table 3). The influence of CSM with varying levels of NPN on bacterial counts was non significant. The bacterial counts measured at 0, 4 and 48 h of incubation revealed that there was a significant increase at 4 (T_2) and 48 hours (T_3) compared to that of 0 hour (T_1). However, the bacterial counts at 4 and 48 hrs were non significantly different from each other (Table 3).

Table 3: Bacterial counts (X 10^7) by replacement of CSM with various NPN sources during *in vitro* fermentation (values are presented in ranked order)

NPN sources*	Bacterial count	Fermentation time (hrs)	Bacterial count
UST	290.0[a]	48	283.3[a]
US	206.0[b]	4	270.0[a]
CSM	206.7[b]	0	103.1[b]
BST	197.6[b]		
BS	193.0[b]		

*US= Urea straw, BS= Biuret straw, UST= Urea starch, BST= Biuret straw, CSM= Cotton seed meal.
abc= Means in the same column with different superscripts differ significantly (P<0.001).

The interactions revealed that UST X T_3 yielded 390.0 X 10^7 bacteria which were highest compared to all other interactions. However, the differences among UST X T_3, UST X T_2, US X T_2, BST X T_2 and CSM X T_3 were non significantly different from each other.

Ammonia nitrogen release (NH_3-N)

The differences in NH_3-N released due to NPN sources, levels, time of incubation and interactions between sources x levels, sources x time and levels x time were significant (Table 4, P<0.001). Further comparison revealed that release of NH_3-N by BST was maximum (17.77 mg/dl), followed by UST, BS and US (Table 4). However, ammonia release was minimum (8.94 mg/dl) when CSM was used as the only source of nitrogen. The three levels of NPN had significant (P< 0.001) effect on the rate of NH_3-N release. The maximum NH_3-N of 17.09 mg/dl was released by 70% NPN, followed by 15.24 and 13.03 mg/dl by 50 and 30% NPN levels, respectively (Table 4).

Table 4: Ammonia nitrogen as influenced by replacement of CSM with various levels of NPN sources (values are presented in ranked order)

NPN sources*	NH_3-N (mg/dl)	NPN Level (%)	NH_3-N (mg/dl)	Fermentation periods	NH_3-N (mg/dl)
BST	17.77[a]	70	17.09[a]	T_5	17.10[a]
UST	17.35[b]	50	15.24[b]	T_8	16.50[b]
BS	15.85[c]	30	13.03[c]	T_6	15.77[c]
US	15.67[d]			T_4	15.60[c]
CSM	8.94[e]			T_3	15.07[d]
				T_7	14.70[e]
				T_2	13.40[f]
				T_1	12.60[g]

*US= Urea straw, BS= Biuret straw, UST= Urea starch, BST= Biuret straw, CSM= Cotton seed meal.
**T_1 = 10 min, T_2 = 20 min, T_3 = 40 min, T_4 = 60 min, T_5 = 2 hrs, T_6 = 4 hrs, T_7 = 24 hrs, T_8 = 48 hrs post-fermentation.
abc= Means in the same column with different superscripts differ significantly (P<0.001).

The NH_3-N release gradually increased from 12.67 mg/dl at T_1 (10 minutes) post incubation to 17.10 mg/dl at T_5 (2 hours). The increases in NH_3-N at each interval were significant except at T_4 and T_6 (Table 4). The interactions indicate that NH_3-N production during incubation was highly related with source and levels of NPN, as well as the time of incubation. The maximum NH_3-N releases were observed with UST x 70% NPN level, BST x T_8 incubation period, NPN level 70% x T_8 incubation period.

pH changes

The effects of various NPN sources on pH were significant (P<0.001). The mean pH values were 7.13, 7.15, 7.08, 7.13 and 7.15 for US, BS, UST, BST and CSM, respectively (Table 5). Further analysis revealed that UST containing culture had minimum pH compared to all other cultures. The differences between other NPN sources and CSM containing cultures were not statistically different from each other (P>0.05). The increase in levels of the NPN sources in CSM containing culture did not affect the pH compared to the control (CSM containing cultures). However, the difference in pH at 0 and 48 h post culture was significant (P<0.001). The pH of 7.05 at 0 h increased to 7.2 after 48 h fermentation.

The interaction revealed that fixing urea and biuret dropped the pH to the lowest level. This was seen in cases of BST x 50%, UST x 30% and UST x 70% NPN.

The interaction between NPN sources and fermentation time showed that pH after 48 h fermentation irrespective of nitrogen source used was higher than the pre-fermentation period. However, highest pH of 7.26 was in cultures containing NPN fixed with straw (BS), followed by US. No difference in pH was observed due to levels of the NPN at the 48 h post-fermentation.

Table 5: pH changes as influenced by replacement of CSM with various levels of NPN sources during in vitro fermentation (values are presented in ranked order)

N sources*	pH	NPN level (%)	pH	Times (hours)	pH
BS	7.15[a]	30	7.130[a]	0	7.05[a]
CSM	7.15[a]	50	7.140[a]	48	7.21[b]
US	7.13[a]	70	7.120[a]		
BST	7.13[a]				
UST	7.08[b]				

*US= Urea sraw, BS= Biuret sraw, UST= Urea sarch, BST= Biuret sraw, CSM= Cotton seed meal.
ab= Means in the same column with different superscripts differ significantly (P<0.001).

DISCUSSION

Dry matter digestibility

In the present study, CSM was replaced with rumen degradable NPN sources i.e. urea and biuret. The substrates contained 2% nitrogen which was expected to be sufficient to meet nitrogen requirements of bacteria. The results indicated that CSM resulted in significantly lower (P<0.001) DMD compared to NPN sources. Replacement of CSM with NPN significantly increased the DMD and combination of NPN with starch (UST and BST) further enhanced DMD over that of NPN–straw combinations (US and BS). This can be explained in two ways: (a) starch is non-structural carbohydrate and easily fermentable by the rumen

inoculums to release required energy and (b) the rumen inoculums obtained from urea adapted cannulated buffalo bulls preferred NH_3-N from NPN than amino acids or peptides from CSM. With increasing level of NPN from 30 to 70%, there was a progressive increase in DMD which supports previous report of this laboratory that increasing NPN during *in vitro* cultures of rumen inoculums enhanced the DMD (Ali *et al.*, 1993). The BST X 70% NPN interaction yielded maximum DMD, indicating efficiency of biuret than other NPN combinations. The results of DMD indicate that with poor quality roughages like wheat straw, the combination of starch and biuret would enhance the digestibility of the feeds in ruminants.

Bacterial counts

The bacterial counts were highest with UST compared to other NPN sources. The differences among other NPN sources `were, however, non significant ($P>0.05$). The bacterial counts obtained with CSM were not different from other NPN sources except UST. The bacterial counts did not change when CSM was replaced by NPN up to 70% level, thus supporting the logic presented in previous section that NH_3-N was favorite for the rumen bacteria used in these cultures and the amount of NH_3-N released from 30% NPN was sufficient for bacterial requirement and increase in NPN levels up to 70% was not worth if bacterial population was the only parameter in consideration. However, during *in vivo* use of NPN for enhanced bacterial harvest, the nitrogen source shall be considered on cost effective basis. In a feeding trial in cattle, effect of increasing levels of urea by replacing CSM apparently increased duodenal microbial nitrogen flow, microbial efficiency g N/kg digestible organic matter when 25% nitrogen with urea replaced CSM (Koster *et al.*, 1997).

The bacterial counts at 4 and 48 hours post-fermentation were higher than that of 0 hour but were not different from each other. This indicates that within first four hours of fermentation, the bacterial population was capable to use NPN and maximize their population. Measurement of ruminal microbial protein is necessary to quantify ruminal escape of microbial yields *in vivo*. Different methods, markers (internal and external), isotopic techniques (^{15}N, ^{35}S) and high pressure liquid chromatography (HPLC) are used to estimate rumen microbial yields *in vivo*. The reports of bacterial protein yield (Broderick and Merchen, 1992; Dell'anno *et al.*, 1998) appear to be flawed and criticized for efficiency. In the present study, however, we agreed to use total microbial count in spite of its inherent weaknesses. Keeping in view the design of the experiment, relative variations in bacterial yields for different substrates are expected to be constant.

Ammonia nitrogen (NH_3-N)

The NH_3-N differed ($P<0.001$) for NPN-sources, as well as three levels of NPN. It was maximum for BST and minimum for CSM. As expected, it was highest with 70% and lowest for 30% NPN. The interactions revealed that UST x 70% NPN ranked top, followed by BST x 70% NPN. The NH_3-N gradually increased during fermentation for all three NPN levels from 10 minutes to 2 hours. Thereafter, it showed a decline at 4 and 24 hours. However, at 48 hours it again surged. These changes can be explained on the basis that during first 2 hours of fermentation, the bacterial growth continues using the substrate nutrients which probably declined to the level which could not support further bacterial growth.

Earlier *in vivo* studies with dairy cattle showed that supplementation of urea and starch in the rations resulted in increased rumen fluid NH_3-N which was increased by additional urea feeding but the same declined by starch supplementation (Cameron *et al.*, 1991). Decline of nutrients resulted in sustaining the population till 24 hours post fermentation but thereafter death and lysis of the microbes released NH_3-N. Since this was a closed system, this pattern is not expected in continuous or *in vivo* where a constant inflow of nutrients, as well as outflow of bacteria to the next chamber/duodenum takes place. Ammonia is the main nitrogen source for growth of rumen bacteria (Bryant and Robbinson, 1961), however, the pattern of its use is much more variable when different cultures are compared (Bryant and Robbinson, 1963). Russel *et al.* (1992) concluded that mixed bacteria fermenting soluble carbohydrates derived 60% of their nitrogen from peptides when both were available.

Rumen pH

Significantly lower pH was obtained in UST (urea-straw) media compared to other NPN sources and CSM containing medias. Increase in NPN levels upto 70% did not affect the pH ($P>0.05$) which was within normal range of 7.12 to 7.14. This indicates that ammonia released from NPN sources was not sufficient to upset the pH in these cultures. It further shows that combination of NPN with straw and starch provided suitable environment for efficient bacterial growth, as is evident from higher bacterial number. This bacterial efficiency is expected to be due to suitable NH_3-N and energy release in these cultures. This possibility has been explained by the interaction UST X T_3 and UST X T_2, indicating that bacterial counts of 390×10^7 and 353×10^7 at 48 and 24 hours post fermentations were higher than others. The interaction shows that 7.2 pH in BST X 70% NPN was higher that 7.033 pH in media containing UST X 70% NPN. Other interactions

between sources X levels of NPN did not show a regular pattern. In spite of the significant variations in pH of the cultures, it was within normal functioning of rumen.

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