PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
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Pakistan Journal of Nutrition 15 (3): 299-304, 2016
ISSN 1680-5194

The Effects of P, S and Mg Supplementation of Oil Palm Fronds Fermented by *Phanerochaete chrysosporium* on Rumen Fluid Characteristics and Microbial Protein Synthesis

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Abstract: The objective of this study was to evaluate the effects of sulfur, phosphorus and magnesium supplementation of oil palm fronds fermented by *Phanerochaete chrysosporium* on rumen fluid characteristics and microbial protein synthesis. This research was carried out using a randomized block design with 4 treatments and 4 replications. The following treatments were performed: A = fermented oil palm fronds (FOPFs); B = FOPFs+0.4% P; C = FOPFs+0.4% P+0.3% S and D = FOPFs+0.4% P+0.3% S+0.1% Mg. The data were subjected to analysis of Variance (ANOVA) and differences between treatments were tested using Duncan's Multiple Range Test (DMRT). The observed parameters were microbial protein synthesis and rumen fluid characteristics. Supplementation of FOPFs with P, S and Mg resulted in the highest microbial protein synthesis and VFA concentration.

Key words: Oil palm frond, *Phanerochaete chrysosporium*, P, S, Mg

INTRODUCTION

Oil palm fronds (OPFs) are one of the main by-products of the palm oil industry and have great potential as a source of roughage or as a component of a complete feed for ruminants (Zahari *et al*., 2003). OPFs are limited as a feedstuff due to their high lignin content (30.18%) (Febrina *et al*., 2014; Zain *et al*., 2014). The optimization of OPFs as feed has focused on efforts to reduce their lignin content. Related physical, chemical, biological or physical-chemical-biological treatments aim to break lignocellulose-lignocellulose bonds. However, the use of chemicals pollutes the environment and reduces the ability of microorganisms to degrade lignin by inhibiting their ability to break lignocellulosic bonds.

Lignin is a natural phenolic polymer that is commonly found in plant cell walls and it is a limiting factor in animal feed due to its low digestibility (Imsya *et al*., 2013). Lignin is degraded by Basidiomycete fungi, which are its most efficient digesters (Rabinovich *et al*., 2004) and the species *Phanerochaete chrysosporium* has been the most extensively studied due to its ability to degrade a wide range of organic substrates (Dorado *et al*., 1999; Fragoeiro and Magan, 2005; Wen *et al*., 2009; Yu *et al*., 2009). *Phanerochaete chrysosporium* strains simultaneously degrade cellulose, hemicellulose and lignin, whereas other species (such as *Ceriporiopsis subvermispora*) tend to degrade lignin prior to cellulose and hemicellulose (Sanchez, 2009). *Phanerochaete chrysosporium* growth affects the availability of minerals in the substrate. The biodelignification of OPFs using *Phanerochaete chrysosporium* degrades 27.34% and 29.89% of lignin when Ca (Febrina, 2014) and Mn (Febrina *et al*., 2014) are added, respectively.

Improving the quality of OPFs through a biodelignification process should be integrated with the optimization of rumen bioprocessing by increasing the population of rumen microbes; the digestibility of the fibre is dependent on rumen microbial activity (Zain *et al*., 2010a,b,c). Increasing the digestibility of the fibre should be approached in terms of providing the nutrients required for rumen microbial growth (Leng, 1990). Microbial protein synthesis can be optimized through the addition of these components.

The elements P, S and Mg have low bioavailability but are essential for microbial growth; additionally, these elements are often deficient in fibrous low-quality feed (Preston and Leng, 1987; Komisarczuk and Durand, 1991; Little, 1986). Phosphorus is important for the metabolism and maintenance of cell membranes and cell walls and is a component of nucleic acids and high-energy molecules (ATP, ADP and AMP) (Komisarczuk and Durand, 1991; Bravo *et al*., 2003; Rodehutscord *et al*., 2000; Zain *et al*., 2010a,b,c). Sulfur supports the formation of sulfur-containing amino acids (Slyter *et al*., 1986) and aids in the synthesis of microbial proteins (Zain *et al*., 2010a,b,c), vitamins (thiamine and biotin) and coenzyme A (CoASH) (Komisarczuk and Durand, 1991). Sulfur also supports crude fibre degradation in the rumen and the stimulation of cellulolytic bacterial growth (Bal and Ozturk, 2006). Magnesium is important

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for microbe growth and functions in enzyme systems (Little, 1986; Komisarczuk and Durand, 1991); however, magnesium is often deficient in low-quality forage (Leng, 1990). The biodelignification of OPFs with *Phanerochaete chrysosporium* plus the elements Ca and Mn can break down lignocellulose and lignin hemicellulose bonds. P, S and Mg were added to fermented oil palm fronds (FOPFs) to optimize the bioprocessing of the rumen. This study aimed to determine the effects of P, S and Mg on fermented OPFs and their effects on microbial protein synthesis and the characteristics of rumen fluid.

MATERIALS AND METHODS

OPF fermentation: The OPFs used in this study were cut, dried and finely milled. Ca was obtained from $CaCl_2$ and Mn was obtained from $MnSO_4.H_2O$. *Phanerochaete chrysosporium* was maintained on potato dextrose agar (PDA) slants at 4°C, transferred to PDA plates at 37°C for 6 days and subsequently grown on rice bran. The fermentation process was initiated by adding water to the OPFs until the water level reached 70%; either Ca or Mn was then added depending on the treatment.

Rumen fluid characteristics: Rumen Fluid Characteristics (ruminal pH, NH_3-N and VFA concentration). The ruminal pH was measured using a pH meter. The concentration of ammonia-N was determined via the Conway microdiffusion method. VFA was analyzed using the steam distillation method. Buffalo rumen liquor was filtered through 4 layers of cheesecloth. One gram of sample was added to the 100-mL incubation tube containing 50 mL of mixed solution. Before the incubation tube was closed, it was purged with CO_2 gas for 30 sec and then incubated for 48 h. When each incubation had finished, 2 drops of $HgCl_2$ were added to the cultures. The incubation tubes were then centrifuged at 4000 rpm for 10 min. The supernatants were used for the determinations of pH, NH_3-N concentration and total VFA; the residue was added to 50 ml of a 0.2% pepsin HCl solution and incubated for 48 h. The residue was then filtered using Whatman no. 41 filter paper and dried at 60°C for 48 h.

Rumen protozoa population: Measurements were carried out using a counting chamber. Up to 0.5 mL of rumen solution was fixed with 0.5 mL methyl green formalin saline (MFS) solution in tubes and thoroughly mixed (Ogimoto and Imai, 1981). Up to 0.1 ml of this sample was dropped on the counting chamber (haemocytometer) using a pipette and a coverslip was placed over the sample. The protozoa were quantified on the counter under a microscope at 40x magnification. According to the number of protozoa obtained by the above counting procedure, the number per 1 mL of rumen contents can be calculated using the following formula:

Protozoa population/mL = (1/0.1 x 0.065 x 5 x 16) x n x d

where n = the number of protozoa in the counting chamber and d = the sample dilution.

Total bacterial population: Brain-heart infusion (BHI) medium consisting of BHI powder, glucose, cellulobiose, starch, cysteine, hemin and resazurin was used to calculate the total bacterial population. It was heated until its colour changed from yellowish brown to reddish brown and back to yellowish brown. The substance was then cooled and the CO was drained. Anaerobic BHI medium (4.9 mL) was added to Hungate tubes filled with 0.150 g of bacto. Using a pipette, 0.05 mL sample volumes of rumen fluid that had undergone treatment and incubations of 4 h were added to the diluent media. The following dilution process was used: 0.05 mL of bacterial culture was added to 4.95 mL of diluent media and then 0.05 mL of the diluted bacterial culture was added to 4.95 mL of diluent media; this process was continued until dilutions of 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} were achieved. Volumes of 0.1 ml of each dilution were removed from the tube, added to the media, rotated on a roller drum. The bacteria were then incubated for 24 h.

$$\text{Bacteria/mL} = \frac{\text{No. of colonies}}{0.05 \times 0.1 \times 10}$$

where x is the tube's serial dilution to 10^{-x}

Experimental design and statistical analysis: The research was carried out using a randomized block design (4 x 4) with four replications (the addition of P, S and Mg as treatments). Differences between treatments were analyzed using Duncan's Multiple Range Test.

The following treatments were carried out:

A = FOPFs without the addition of minerals (control)
B = FOPFs+P (0.4% DM)
C = FOPFs+P (0.4% DM)+S (0.3% DM)
D = FOPFs+P (0.4% DM)+S (0.3% DM)+Mg (0.1% DM)

RESULTS AND DISCUSSION

The influence of P, S and Mg on palm frond fermented by the fungus *Phanerochaete chrysosporium* and their effects on rumen fluid characteristics are shown in Table 1.

The addition of P, S and Mg had no effect ($p>0.05$) on the rumen fluid, i.e., 6.80-6.87 is a normal pH for optimal rumen microbial activity. The same results were reported by Suyitman *et al.* (2013), who found that the pH of rumen fluid was 6.60-6.84 when S and P were supplemented during the ammonization of palm leaves. Van Soest (1994) determined that the optimal pH for the maintenance of cellulolytic activity was 6.6±0.5; Keidane

and Birgele (2003) found that the pH ranges for the optimal activities of proteinases plus peptidases, cellulases and deaminases were 5.5-7.0, 6.2-7.0 and 6.5-7, respectively; they also found that the optimal pH for VFA synthesis was 6.0-8.0. Chiba (2009) determined that the rumen pH was 6-7 and Kamra (2005) determined that a pH of 6-6.9 was optimal for rumen microbial growth. The pH values of all treatments were nearly the same due to the balance between VFA and NH_3 in rumen fluid. The rumen pH represents a balance between its buffering capacity and the acidity or alkalinity of fermentation products (VFA and NH_3) and pH is affected by the products of fermentation (such as NH_3-N). In Church's study, the pH value was directly proportional to the concentration of NH_3-N (Church, 1988).

The VFA concentration was lowest after treatment A at 95.791 mM, which indicates a low fermentation ratio. Supplementation with P, P and S and P, S and Mg (treatments B, C and D, respectively) increased the concentration of the total VFA in the rumen fluid; this was also reported by Zain *et al.* (2010a,b,c). These findings indicate that mineral supplementation can improve the growth and proliferation of rumen microbes, thereby increasing fermentation. VFA production is affected by the type and amount of forage, the rumen pH and the rate of passage through the rumen (Peters *et al*., 1989); the type of carbohydrate, the physical form of the feed, intake, the feeding frequency and the use of additives (France and Dijkstra, 2005) and the substrate, rumen microbial populations and ecology (Bannink *et al*., 2008).

Treatment D (supplementation with P, S and Mg) produced the highest concentration of VFA at 111.007 mM. Supplementation with P, S and Mg thus encourages rumen microbe growth. The nutrient content of FOPFs is not sufficient for rumen microbes and supplementation with P, S and Mg encourages optimal growth. In the rumen, sulfur is critical for the synthesis of S-containing amino acids used in microbial protein synthesis and livestock are frequently deficient in S. P supplementation is essential for the animal and the microbes and sufficient Mg is a precondition for the optimal utilization of low-quality forages (Leng, 1990; Zain *et al*., 2010a,b,c). P deficiency is widespread in tropical countries (McDowell *et al*., 1983). Normal levels of Ca, P, Mg and Mo in the rumen increase the digestion of fibre (especially cellulose) via microbial activity. This increases the digestibility of cellulose and the quantity of volatile fatty acids produced (Adriani and Mushawwir, 2009).

The NH_3 concentration was 4.136-4.895 mM, which is within the normal range for supporting the growth of microbial protein synthesis. Paengkoum *et al.* (2006) found that a 5-20 mg/dL concentration of N-NH_3 was optimal for digestion, which is equivalent to 3.57-14.28 mM. Microbial growth is not optimal if NH_3 levels are less than 3.6 mM (McDonald *et al*., 2010). In our study, the lowest NH_3 concentration was 4.136 mM (treatment A); a decrease in the concentration of NH_3 with increasing concentrations of VFA was reflected in a higher content of total microbes, which increased microbial protein synthesis after treatment D due to the increased use of nitrogen by the rumen bacteria. Similar results were reported by Zain *et al.* (2010 a,b,c).

Rumetor (2009) argued that a decrease in N-NH_3 resulted in a reduction in protein digestibility, whereas increased levels of VFA are due to an increase in the digestibility of carbohydrates. This mechanism suggests that the types of microbes change with decreasing NH_3-N; the population of proteolytic microbes is reduced while the population of cellulolytic microbes increases. Microbial protein synthesis is affected by NH_3 availability in the rumen because it is a major source of nitrogen and essential for microbial protein synthesis. An environment containing approximately 80% ammonia is required for the growth of rumen bacteria (Baldwin and Allison, 1983).

The effects of the addition of the minerals P, S and Mg on palm frond fermentation by the fungus *Phanerochaete chrysosporium* and their effects on microbial protein synthesis are shown in Table 2.

Supplementation with P, S and Mg had no effect ($p>0.05$) on the rumen bacterial and protozoan populations. This is because the addition of P, S and Mg did not affect the pH of the rumen fluid. The protozoan population in this study ranged from 5.851-5.864 cells/mL (log 10). The same results were reported by Badarina *et al.* (2013); in their study, coffee husks fermented by *Pleurotus ostreatus* had a protozoa count of 5.69-5.71 cells/mL (log 10) following supplementation. In this study, the population of bacteria was 6.843-6.987 cells/mL (log 10), which was in agreement with Stewart (1991), who found that bacterial counts were 10^9-10^{12} cells/mL in rumen contents. Supplementation with P, S and Mg had no effect on the total bacteria because supplementation with P, S and Mg did not affect the number of protozoa. This result shows that the numbers of protozoa and bacteria are in equilibrium due to a decreased number of protozoa, which increased the number of total bacteria.

Supplementation with P, S and Mg during the fermentation of OPFs did not affect microbial protein synthesis. This can be concluded because all treatments (with the same nutrient content) allowed rumen microbial degradation to occur. Microbial protein synthesis (MPS) rates ranged from 36.878-46.816 mg/ml. The lowest MPS, 36.878 mg/ml, was found for treatment A (without the addition of minerals). The highest MPS was obtained for treatment D (with supplementation of fermented OPFs with P, S and Mg). This indicates that the minerals P, S and Mg are required for rumen microbial growth and is in agreement

Table 1: Supplementation of P, S and Mg in the oil palm fronds fermented and their effects on rumen fluid characteristic

	Treatments			
Measured parameters	A	B	C	D
pH	6.875±0.05	6.875±0.05	6.800±0.08	6.875±0.05
VFA (mM)	95.791±11.47^{b}	104.823±2.41^{ab}	102.050±6.52^{ab}	111.007±8.98^{a}
NH_3 (mM)	4.895±0.28^{ab}	4.817±0.47^{a}	4.773±0.48^{ab}	4.136±0.17^{b}

Means in the same row with different letters (a and b) are significantly ($p<0.05$)
A: Oil palm frond fermented without the addition of mineral (control)
B: Oil palm frond fermented+mineral P (0.4% DM)
C: Oil palm frond fermented+mineral P (0.4% DM)+S (0.3% DM)
D: Oil palm frond fermented+mineral P (0.4% DM)+S (0.3% DM)+Mg (0.1% DM)

Table 2: Supplementation of P, S and Mg in the oil palm fronds fermented and their effects on Microbial Protein Synthesis (MPS)

	Treatments			
Measured parameters	A	B	C	D
Protozoa (cell/mL) (log 10)	5.851±0.05	5.855±0.02	5.854±0.07	5.864±0.01
Bakteri (cell/mL) (log 10)	6.987±0.04	6.866±0.12	6.852±0.12	6.843±0.12
MPS (mg/ml)	36.878±14.23	43.938±7.63	44.067±9.64	46.816±10.85

Means in the same row with different letters (a and b) are significantly ($p<0.05$)
A: Oil palm frond fermented without the addition of mineral (control)
B: Oil palm frond fermented+mineral P (0.4% DM)
C: Oil palm frond fermented+mineral P (0.4% DM)+S (0.3% DM)
D: Oil palm frond fermented+mineral P (0.4% DM)+S (0.3% DM)+Mg (0.1% DM)

with Slyter *et al.* (1986), who showed that sulfur stimulates rumen microbial growth. Mg and Co also play roles in the growth of microbes and in the functioning of enzyme systems (Little, 1986); these minerals are often deficient in low-quality forage (Leng, 1990). The optimal rumen microbial growth leading to high microbial protein synthesis in treatment D increased the fermentability of fermented OPFs in the rumen. This was accompanied by the highest total VFA of 111.007 mM. This result agrees with those of Zain *et al.* (2010 a,b,c).

Sanchez (2009) argued that it is difficult to determine the proportion of substrate degradation that contributes to energy production and the proportion that contributes to microbial protein synthesis because microbial growth is supported by the fermentation of substrate and the fermentation of the substrate occurs via microbial growth. Microbial protein synthesis depends on determining the correct amount of nitrogen in the feed, the absorption of ammonia and amino acids, the flow of material out of the rumen, the amino acids required by the microbes and rumen fermentation according to the type of feed.

Conclusion: Supplementation of fermented OPFs with P, S and Mg to optimize the rumen bioprocess results in the highest concentration of VFA and the greatest amount of microbial protein synthesis.

ACKNOWLEDGEMENTS

This research is part of the Ph.D. dissertation of Dewi Febrina in Animal Nutrition, Faculty of Animal Science Andalas University. We thank the undergraduate students who collected the data and analyzed samples at the Laboratory of Ruminant Nutrition in the Faculty of Animal Science at Andalas University.

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