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Abstract- Using rice bran in broiler diets has limitation due higher content of fiber and lower availability of few micronutrients including phosphorus. So, they were fermented anaerobically using 10% *Ruminococcus albus* isolated from rumen of cattle to get fermented value-added feed ingredient. It was fermented for 48 hours at 39°C giving 60% moisture at different conditions like FRB (Rice Bran treated with *Ruminococcus* sp.), UFRB (Rice Bran treated with 2.0% urea using *Ruminococcus* sp.), MFRB (Rice Bran treated with 5.0% molasses using *Ruminococcus* sp.), UMFRB (Rice bran treated with 2% urea & 5% molasses using *Ruminococcus* sp.). The protein content was increased in UFRB (18.43%), UMFRB (17.19%) in comparison to RB group (14.42%) where UFRB showed highest crude protein ($p<0.05$). The crude fiber was decreased in FRB (11.64), UFRB (9.92), MFRB (11.67), and UMFRB (10.83) in comparison to RB (12.57%). Phytate-P was also decreasing in UFRB (1.00%), MFRB (1.00%), UMFRB (0.82%) then to RB (1.13%). So, in vitro fermentation using *Ruminococcus spp.* reduces phytate-P and fiber content (CF and ADF) and increase crude protein of RB and UFRB.

Keywords: in vitro, fermentation, ruminococcus spp. rice bran, feed.

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In Vitro Fermentation of Rice Bran by *Ruminococcus* Sp. for Desirable Chemical Changes as Feed for Livestock

M A Alam [α], M S R Khan [σ], K M S Islam [ρ], U Dickhöfer [ω] & M A Grashorn[¥]

Abstract- Using rice bran in broiler diets has limitation due higher content of fiber and lower availability of few micronutrients including phosphorus. So, they were fermented anaerobically using 10% *Ruminococcus albus* isolated from rumen of cattle to get fermented value-added feed ingredient. It was fermented for 48 hours at 39˚C giving 60% moisture at different conditions like FRB (Rice Bran treated with *Ruminococcus* sp.), UFRB (Rice Bran treated with 2.0% urea using *Ruminococcus* sp.), MFRB (Rice Bran treated with 5.0% molasses using *Ruminococcus* sp.), UMFRB (Rice bran treated with 2% urea & 5% molasses using *Ruminococcus* sp.). The protein content was increased in UFRB (18.43%), UMFRB (17.19%) in comparison to RB group (14.42%) where UFRB showed highest crude protein ($p<0.05$). The crude fiber was decreased in FRB (11.64), UFRB (9.92), MFRB (11.67), and UMFRB (10.83) in comparison to RB (12.57%). Phytate-P was also decreasing in UFRB (1.00%), MFRB (1.00%), UMFRB (0.82%) then to RB (1.13%). So, in vitro fermentation using *Ruminococcus spp.* reduces phytate-P and fiber content (CF and ADF) and increase crude protein of RB and UFRB.

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I. Introduction

Rice bran is a major cereal agricultural by-products in rice-based agricultural countries like Bangladesh and has the potential as a feed ingredient. However, its utilization, especially for poultry is limited. The limitation of its use was due to its high fiber content, low protein and antinutritional factors such as Phytic acid as phytate. These antinutritive factors have been reported by Khalique *et al*., (2003) cause reduction of feed intake and depressed performance of broiler.

Nutritionally, several factors limited its use in poultry, especially broiler chicken diet. Almost half of phosphorous are in phytates form. Hull adulteration is a factor reducing the quality of rice bran (Farrell, 1994). High level of ash content indicates high level of hull (Warren and Farrell, 1990). Previous researches had attempted to use different techniques like fermentation (wizna *et al*., 2012), enzyme supplementation (Tirajoh *et al*., 2010) and the inclusion of fermented product (Kompiang *et al*., 1995) in increasing rice bran utilization for poultry feed.

Fermentation is one of the most advantageous approaches to improve the nutritive value of rice bran (Hardini, 2010). Microorganisms induced fermentation processes transformations of their metabolic activity and also increase the availability of nutrients in raw materials (Pelizer, Pontieri, & Moraes, 2007) which has been widely adopted to develop novel functional ingredients because this process may promote their functional quality such as antioxidant (Lee *et al*., 2008; Hardini, 2010; Wang *et al*., 2011; Cao *et al*., 2012; Kim *et al*., 2012) and optimize the use of rice bran in poultry feeding. Bidura *et al*., (2012) found that inclusion of yeast (*Saccharomyces cerevisiae*) increases the bioavailability of minerals and nutrients of rice bran and increase growth performance of male bali duckling. Also, fermentation of rice bran with *Aspergillus niger* caused change of nutrient content as poultry feed (Hardini, 2010).

Rice bran consisting of cellulose as the major component composed of cellulose, hemicellulose and lignin are coarse fiber which has some the limitations of the use of rice bran as feed in the broiler due to lack of lignocellulosic enzymes producing by digest tract but enzymes can be aided to hydrolyze the cellulose. This is different to ruminants (cattle, sheep, goats), rumen microbes producing lignocellulosic enzymes help the degradation of cellulose and hemicellulose (Muthukrishnan, 2007) by the species of cellulolytic bacteria which are *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *R. albus* (Julliand *et al*., 1999; Koike *et al*., 2000; Chen and Weimer, 2001; Koike and Kobayashi, 2001). Cellulolytic ruminococci play a major role in the breakdown of plant cell wall material in the rumen (Bryant *et al.,* 1958; Dehority *et al.,* 1967; Sijpesteijn *et al.,* 1951; Flint *et al.,* 2008) that effectively reduced fiber and increased crude protein from corn stacks with the supplementation of Urea (3% w/w) and Molasses (5% w/w) (Gado *et al*., 2007; Supyiyati., 2012) due to effect of the non-protein nitrogen contribution from urea (Fontenot *et al*., 1983) also serves an important role in the metabolism of nitrogen-containing

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compounds by animals (Wizna *et al*., 2012) that increases the crude protein content of feed materials including rice milling waste (Amaefule *et al*., (2003).

In this present study, a fermentation technique was used in an attempt to improve the quality of rice bran. *Ruminococcus* sp. was used as the inoculum since it had been reported to produce the various cellulosomal types of enzyme complex which possesses a potential to degrade fiber (Flint *et al.,* 1997) supplementation with urea (3% w/w) and molasses (5% w/w) which supports fermentation media and stimulate the growth of microorganisms to change the nutritional value of rice bran.

II. Materials and Methods

The present study was carried out at the Department of Microbiology and Hygiene, Faculty of Veterinary Science and Department of Animal Nutrition, Faculty of Animal Husbandry, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

a) Bacterial Culture

Rumen ingesta was obtained through a permanent rumen fistula from the Sahjalal Animal Nutrition Field Laboratory to the analytical laboratory of the Department of Animal Nutrition Bangladesh Agricultural University, Mymensingh-2202 in strictly anaerobic conditions within half an hour for further processing.

Rumen liquor was obtained approximately 8hr. after feeding, strained through two thicknesses of cheesecloth, and collected in a 500 ml. centrifuge bottle. Air was excluded by completely filling the bottle, and closing it with a rubber stopper. The bottle was then held overnight at 2°C and centrifuged at *1200 g* for 10 min. before use.

Samples of rumen contents were 10 fold serial diluted in pre-reduced anaerobic diluents solutions (ADS) in the serum bottle with rubber stopper by anaerobic techniques up to 10^{-8} dilution (Hungate, 1966) then samples were cultured into the pre-reduced specific media contained serum bottle for rumen bacteria using 1mL syringe; Rumen Fluid Glucose Cellobiose agar (RGCA) medium which was prepared under continuous CO_2 flow and incubate in Anaerobic Jar (OXOID, England) at 39°C for 48 hours. Commercial CO_2 was freed from 0_2, by passing it over heated reduced copper gauze. The RGCA growth media contained: 15 mL Mineral Solution I (KH_2PO_4 3.0g; $(NH_4)_2SO_4$ 6.0g; NaCl 6.0g; $MgSO_4$ 0.6g; $CaCl_2{\cdot}2H_2O$ 0.795g p/L), 15 mL Mineral Solution II (K_2HPO_4 0.3 g/L), 0.25g Yeast Extract, 1g Tryptone, 0.1mL Resazurine (0.1%), 0.2mL Hemin (0.05%), 0.5g Microcrystaline Cellulose, 0.1g Cellobiose, 0.4g Sodium Carbonate, 20 mL Clear Rumen Fluid, 50mL Distilled Water and 50mg Cysteine Hydrochloride. Adjust pH to 6.7 with NaOH.

Morphological characteristics of the bacteria was cocci, single or pair, (figure-2) always gram-positive, non-motile. Some were rod shape. Short chain also found. None produced catalase, indole. Those bacteria were fermented cellulose and cellobiose. The acid produced from glucose, d-xylose and cellobiose. Hydrolysis of starch and gelatin liquefaction occurred. Presence of zone of clears around the colony and produce yellow pigment. Positive biochemical test samples were kept for DNA preparation and PCR.

The ADS media contained; 350 mL distilled H_2O, 0.1349g K_2HPO_4, 0.1349g KH_2PO_4, 0.2697g NaCl, 0.02697g $MgSO_4$, 0.0357g $CaCl_2{\cdot}2H_2O$, 0.2697g $(NH_4)_2SO_4$, 3 drops of 0.1% resazurin. After boiling and cooling, slowly add 0.9g Na_2CO_3. Bubble overnight (until color turns pink). Then add 5 mL of 3% (w/v) L-cysteine hydrochloride. Continue bubbling until colorless (usually requires 1 to 4 h). Dispense to serum bottle and autoclave.

DNA Isolation and PCR Amplification: Total DNA extraction was performed with the QIAamp DNA Stool Kit (QIAGEN, Germany). Species-specific primer sets that amplify 16S rRNA of *Ruminococcus albus,* available to detect these species in rumen microbial ecosystems (Tajima *et al*., 2001; Koike and Kobayashi, 2001). The PCR mixture was performed using 1X PCR buffer (60 mM Tris-SO_4 pH 8.9, 18mM ammonium sulphate), 0.25mM dNTPs, 2mM $MgSO_4$, 0.2 mM primer, 1U of Platinum Taq High Fidelity (Invitrogen, USA), 20ng of genomic DNA and DNA/RNA free water adjusted to a total volume of 50µL. The PCR condition was 95°C for 5 min followed by 30 cycles of 94°C for 30 sec/cycle for denaturing, annealing at 60°C(Table 1) for 30 sec and finally 68°C 45sec for elongation, using a PxE 0.2 thermal cycler (Thermo electron corporation, USA). The PCR products were separated by 2% agarose gel electrophoresis using the molecular weight marker 100bp Ladder (Promega, USA) and the image was captured with a gel image analyzer. The purified PCR product was stored and will be sent for sequencing. The isolates were again confirmed by using the specific primer of bacteria.

The DNA fragments of the expected size (Table 1) were amplified from all the samples tested a representative image of the amplification after gel electrophoresis is shown in Figure 1.

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Table-01: Species-Specific primers sequences for 16S RNA genes used in this study

Bacterium	Primer name	Sequence (5´-3´)	Annealing temp. (°C)	Product size (bp)	Ref.
Ruminococcus albus	Ra1281 f	CCCTAAAAGCAGTCTTAGTTCG	60	175	Koike and Kobayashi, 2001
	Ra1439 r	CCTCCTTGCGGTTAGAACA			

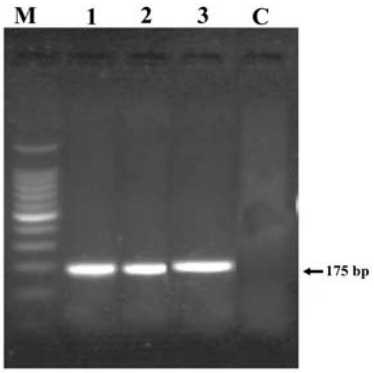


Figure 1: PCR detection of cellulytic bacteria from the rumen contents using species-specific primers (175bp) for *Ruminococcus albus* (1 & 2) MWM: 100bp ladder molecular weight marker.

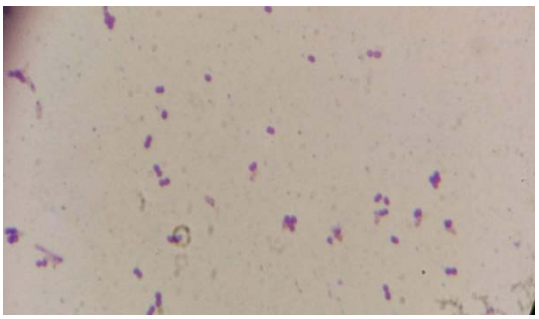


Figure 2: *Ruminococcus spp x 100*

b) Fermentation of Rice Bran

Rice bran was used throughout the study and was gathered from a local market and screened to remove any impurities and dirt through a sieve. It was kept in a clean polythene bag in the laboratory until used. Rice bran was diluted using carbonated water to get different moisture content at 60% level. 10% bacterial inoculum (on DM basis) were added in the diluted rice bran mixed with 2% urea (UFRB), 5% molasses (MFRB) and 2% urea plus 5% molasses (UMFRB) separately or combinedly. Anaerobic fermentation continued for a period of 48 hours at 39°C in sealed serum bottle. After fermentation of fermented rice bran was immediately transferred to the refrigerator to stop further fermentation. pH, Proximate components (CP, CF, ADF, NDF and Ash), Total-P and Phytate-P were determined before and after fermentation of rice bran in accordance with AOAC (2005). These are the fermentated groups; *RB*: Rice Bran (control), *RBB*: Rice Bran treated with *Ruminococcus* sp. *UFRB*: Rice Bran treated with 2% urea using *Ruminococcus* sp. *MFRB*: Rice Bran treated with 5% molasses using *Ruminococcus* sp. *UMFRB*: treated with 2% urea & 5% molasses using *Ruminococcus* sp.

c) Chemical analysis

The proximate analysis of ingredients was measured by AOAC (2005). The crude protein content was measured by macro Kjehdahl digestion unit using Kjeltec 1030 and Auto analyzer procedure using autoanalyzer. Total phosphorus was measured according to AOAC (1980) and Phytate-phosphorus was determined according to Latta and Eskin, (1980).

d) Statistical analysis

All variables were subjected to analysis of variance (ANOVA) (Duncan, 1955) in a completely randomized design (CRD) by the statistical package using statistical computer package program (SPSS). Tukey pairwise comparisons were used to compare treatment means (Steel and Torrie, 1980).

III. Results and Discussion

According to Morphological characteristics they were all gram positive coccoid and showed catalase & indole negative, cell arrangement were single or diplococci belong to the genus *Ruminococcus* sp. (Bryant *et al*, 1959) (figure-2). This bacterium including species *(R. albus*) was confirmed identified by molecular techniques (Koike and Kobayashi, 2001) and used for the fermentation of rice bran.

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Table-02: The composition of different Fermented Rice Bran

Parameters	Fermented groups				
	RB	RBB	UFRB	MFRB	UMFRB
pH	*6.62^{a}±0.03	5.44^{cd}±0.01	6.16^{b}±0.04	5.35^{d}±0.01	5.62^{c}±0.18
Crude Protein (CP)	14.42^{bc}±0.21	13.99^{c}±0.50	18.43^{a}±3.30	13.20^{c}±0.29	17.19^{ab}±0.44
Crude Fiber (CF)	12.57^{a}±0.22	11.64^{ab}±0.41	9.92^{b}±1.38	11.67^{ab}±0.79	10.83^{b}±0.09
Total Phosphorus	3.29±08.	2.99±0.30	3.36±0.34	3.28±0.55	2.95±0.29
Phytate-P	1.13^{a}±0.03	1.21^{a}±0.20	1.00^{ab}±0.07	1.00^{ab}±0.05	0.82^{b}±0.05
Ash	12.08±0.80	11.96±0.30	11.01±2.11	11.43±0.28	10.58±0.62
ADF	24.07±4.75	20.60±2.37	18.52±0.48	17.75±1.37	20.23±2.17

RB: Rice bran (control), RBB: Rice bran treated with *Ruminococcus* sp, UFRB: Rice bran treated with 2% urea using *Ruminococcus* sp. MFRB: Rice bran treated with 5% molasses using *Ruminococcus* sp.UMFRB: Rice bran treated with 2% urea & 5% molasses using *Ruminococcus* sp.

*Mean±SD; ^{abc}Means with dissimilar superscripts are significantly different ($p<0.05$)

Our Study observed that pH changes from 6.62 to 5.35 which were decreased. Results indicate that the phytate degrading enzymes from rice bran were active in the first six hours of the process. The pH changes during production of phytase in the rice bran media over 10 weeks were observed. Initial 3 weeks, a reduction in pH from pH 6 to pH 4.2 (Abd-ElAziem Farouk, 2017). The optimum initial pH for phytase production of *B. cereus* was pH 7.2 (Vohra and Satyanarayana, 2003). pH changes are considered to be due to the production of sugar molecule to an equimolar mixture of organic acids, ethanol and carbon dioxide by fermentation and the period of microbial growth during fermentation (Mackenzie, *et al*., 1965; Prabhu, *et al*., 2014).

In this study after 48 hours anaerobic fermentation of rice bran with *Ruminococcus albus*, the data of Table-2 showed that the crude protein was significantly increased in UFRB (18.43%), UMFRB (17.19%) than control RB (14.42%) but decreased in RBB (13.99), MFRB (13.20%). The highest crude protein was found in UFRB (17.19%) ($p<0.05$). On another hand, the data of table-02 clearly showed that crude fiber and phytate-P content was significantly decreased in all the treated groups RBB, UFRB, MFRB and UMFRB than RB control. The lowest crude fiber was found in UFRB (9.92%) ($p<0.05$).These results indicate that the cellulytic bacteria of rumen can improve the quality of rice bran that increased the CP with the addition of urea and molasses. The result also supported that rice bran contain cellulose as the major component, which is best for the growth of microorganisms and the production of single cell protein biomass (Yunus *et al*., 2015; Khin *et al*., 2011) which increase the crude protein content of rice bran (Sukaryana, 2001) with the addition of urea in the UFRB using cellulolytic bacteria *B. amyloliquefaciens* as an inoculum improved fermentation and its microbial population (Wizna *et al*. 2012). Protein content was also increased after fermentation of cassava waste Supriyati (2002) that agree with the result of the present experiment as protein content was increase when urea and molasses were added during fermentation (Supriyati and Kompiang, 2002). In this study, UFRB showed highest CP (18.435). *Ruminococcus* sp. produces the various cellulosomal type of enzyme complex which possesses a potential to degrade fiber (Flint, 2008). In this study, crude fiber was decreased in rice bran using *R. albus* which supports the results of Galil (2008), using bacterial treatments (*Ruminococcus albus and Cl. cellulovorans*) caused increases crude protein (from 1.45 to 15.16) and decreases in crude fiber (from 44.08 to 28.44%) of rice straw. Wizna *et al.,* (2009) also found that is inoculation of *B. amyloliqfacience* was increased enzymes activities during fermentation of cassava waste that produces many kinds of enzymes to decrease crude fiber.

On the other hand, MFRB and RBB could not increase crude protein due to lack of additional nitrogen source to grow microbes that nitrogen was a crucial component needed by ruminal microbes after carbon and oxygen (Griffin, 1991) which need a much amino acid higher.

There was a decrease in phytate-P in a definite order in UFRB (1%), MFRB (1%), UMFRB (0.82%) than RB (1.12%) control but increase in RBB (1.21%) ($p<0.05$). Ravindran (1995) reported that among the common feedstuff sesame meal and rice bran have the highest level of phytate but after fermentation by *Ruminococcus albus*, phytate-P was decreased. Yanke *et al.,* (1998) reported that the presence of phytase activity was investigated in 334 strains of 22 species of obligatory anaerobic bacteria that decrease phytate phosphorus in fermentation of rice bran by using rumen liquor. this results also agreed with hungate (1966) that Phytate phosphorus degrades by rumen microbes.

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After fermentation of rice bran, there was no significant difference in total-P, ADF and ash content ($p<0.05$) but the difference in numerically. However, Total phosphorus content were within the range of 1.26-1.79% reported by Ukil (1999) and 1.62-1.81% reported by Warren and Farrel (1990c). The variations in nutrient composition might be due to the sources from which the bran was obtained. The chemical composition of rice bran varies due to the variation in the milling process and adulteration with hull (Warren and Farrel, 1990a). In this study, Total phosphorus was higher than that report.

IV. Conclusions

It can be concluded that under this study fermentation of rice bran using *Ruminococcus albus* isolate from rumen liquid from cattle might improve nutritional value i.e. increase crude protein and decrease crude fiber, Phytate-phosphorus. However, animal experiments are required to confirm the effectiveness of fermented rice bran using *Ruminococcus albus*.

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