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Biological enrichment of mustard oil cake by solid-state fermentation using lactic acid bacteria and its impact on rumen fermentation

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Abstract

Mustard oil cake (MOC) is a protein-rich agro-industrial by-product with potential as an animal feed ingredient; however, its utilization is limited by anti-nutritional factors and reduced nutrient availability. The present study evaluated the effect of solid-state fermentation (SSF) using a *Lactobacillus* consortium (*Lactobacillus plantarum* and *Pediococcus acidilactici*) on the nutritional quality and *in vitro* rumen fermentation characteristics of MOC. Fermentation was carried out at 37 °C for 72 h, and fermented mustard oil cake (FMOC) was analysed for pH, lactic acid production, chemical composition, and rumen fermentation parameters. The pH decreased significantly from 5.66 to 4.35, while lactic acid concentration increased from 47.18 to 667.04 µg/mL, confirming successful fermentation. Fermentation significantly increased crude protein content (30.55% to 35.96%) and reduced crude fibre (5.86% to 4.72%) and ether extract (8.52% to 7.16%). *in vitro* evaluation revealed significantly higher net gas production (34.00 ml), dry matter digestibility (66.08%), organic matter digestibility (68.27%), ammonia nitrogen (10.20 mg/dL), and total volatile fatty acids (89.87 mM/L) in TMR containing 100% FMOC compared to control. These improvements indicate enhanced microbial fermentation, nutrient availability, and substrate utilization. The results demonstrate that solid-state fermentation using lactic acid bacteria effectively improves the nutritional value and rumen fermentability of mustard oil cake, making it a promising strategy for sustainable utilization of oilseed by-products in ruminant feeding.

Keywords: Mustard oil cake, solid-state fermentation, lactic acid bacteria, rumen fermentation, digestibility, volatile fatty acids

1. Introduction

Mustard oil cake (MOC) is a by-product of mustard oil extraction and is a nutritionally rich agro-industrial residue with appreciable levels of protein, fibre, and minerals. However, its direct use in animal feed or value-added products is limited by the presence of anti-nutritional factors, such as glucosinolates, phenolic compounds, and complex fibre fractions, which reduce digestibility and palatability (Singh *et al.*, 2025) ^[24]. In recent years, there has been growing interest in biotechnological approaches to enhance the nutritional quality and bioavailability of such agro-residues, promoting sustainable feed resources and reducing environmental burden (Barcelos *et al.*, 2020) ^[2]. Solid state fermentation (SSF) is a cost-effective bioprocess technique that involves the growth of microorganisms on moist solid substrates in the absence or near-absence of free water. SSF has shown considerable promise in improving the nutritional profile of various agro-wastes by degrading complex polymers, increasing protein content, and reducing anti-nutritional compounds (Pandey, 2003; Soccol *et al.*, 2017) ^[21, 25]. The unique ecological niche provided in SSF closely mimics natural decomposition and allows selected microbial strains to produce extracellular enzymes such as cellulases, proteases, and phytases, which enhance substrate digestibility (Llimós Turet, J., 2022) ^[14]. Lactic acid bacteria (LAB), particularly strains of the genus *Lactobacillus*, are widely recognized for their safe status (GRAS) and their ability to ferment a broad range of substrates, producing organic acids, vitamins, and antimicrobial compounds (Hammes & Vogel, 1995) ^[11]. Their application in SSF of oilseed cakes has been associated with improved nutrient release, reduction of anti-nutritional factors, and increased probiotic potential (Barcelos *et al.* 2020; Bintsis *et al.*, 2018) ^[2, 4].

Despite extensive studies on bacterial and fungal fermentations of various oilseed cakes, there is limited research specifically targeting SSF of mustard oil cake using *Lactobacillus* strains for nutrient enhancement.

In this context, the present study investigates the potential of selected *Lactobacillus* strains to biologically enrich mustard oil cake through solid state fermentation. We hypothesise that SSF with *Lactobacillus* will improve the nutritional availability, decrease anti-nutritional components, and enhance the overall suitability of MOC for use in animal feed and other value-added applications. Findings from this study may contribute to more sustainable utilization of oilseed residues and offer insights into microbial treatments that enhance agro-industrial by-products. Solid-state fermentation (SSF) can be used to valorize agro-industrial by-products such as brewer's spent grains, olive pomace, grape mark, vine-shoot trimmings, wheat bran, rice bran, using an eco-friendly approach with low environmental impact compared to conventional submerged fermentation (Zhao *et al.*, 2017; Ferreira *et al.*, 2020) [31, 10]

2. Materials and Methods

The current research was conducted at the Rumen Biotechnology Laboratory, Animal Nutrition Division, ICAR-National Dairy Research Institute, Karnal, Haryana. All media, chemical ingredients, and reagents utilized during the investigation were procured from M/S Hi Media Laboratories Private Ltd. (Mumbai, India).

2.1 Mustard oil cake fermentation

2.1.1 Inoculum preparation

The inoculum preparation method described by Zhang *et al.*, (2014) [30], with slight modification was used. The selected strain of *Lactobacillus* consortia which contain *Lactobacillus plantarum* and *Pediococcus acidilactici* was grown in MRS broth with (peptone 10 g/L, yeast extract 5 g/L, beef extract 10 g/L, sodium acetate anhydrous 5 g/L, ammonium citrate tribasic 2 g/L, K₂HPO₄ 2g/L, MgSO₄·7H₂O 0.1 g/L, MnSO₄·H₂O 0.05 g/L, tween-80 1 g/L, pH 6.8) at 37 °C for 20 h. The cells were then harvested and resuspended in sterilized physiological saline and adjusted to 10⁸ CFU/mL. This cell suspension was ready to serve as an inoculum for MOC fermentation.

2.1.2 Preparation of fermented mustard oil cake

Fermented mustard oil cake was prepared using a method adapted from Dai *et al.*, (2017) [8] with slight modifications. Mustard oil cake was first ground into a powder using a 40-mesh sieve before fermentation. About 50 g of each mustard oil cake was mixed with 50 mL of water and autoclaved at 121 °C for 15 minutes. After cooling, the mixtures were inoculated with *Lactobacillus* at a 2 McFarland concentration and incubated at 37 °C for 72 hours to obtain the fermented mustard oil cake.

2.1.3 Preparation of fermented meal hydrolysate extract

Fermented feed extract was prepared according to the method described by Ye *et al.*, (2003) [29] with slight modifications. About 5 g of fermented feed sample was mixed with 50 ml of distilled water, homogenized for 1 minute and incubated at 37 °C for 60 minutes. The incubated mixture was centrifuged at 10,000 rpm for 4 minutes, and the residue was washed with 20 ml distilled water, centrifuged again at the same speed and time, and the

combined supernatant was freeze-dried and stored at (-) 20 °C until further use.

2.1.4 The determination of pH

Approximately 10 g of fermented mustard oil cake was homogenized in a blender with 90 mL of distilled water for 30 seconds. The pH value of the resulting suspension was then measured using an EUTECH pH meter.

2.1.5 Determination of lactic acid in fermented feed

The estimation of lactic acid in mustard oil cake and fermented mustard oil cake samples was done as per the method of Barker and Summerson (1941) [3]. In all the test tube containing samples and standards, 0.05 mL 4% CuSO₄ was added, subsequently 6 mL concentrated H₂SO₄ was added drop by drop at the center of the liquid with continuous shaking. The tubes were kept in boiling water bath for 5 min cooled down to room temperature, and added with 0.1 mL p-hydroxy diphenyl reagent drop by drop. The contents were mixed immediately with vigorous shaking followed by incubation at 30 °C for 30 min with periodic shaking. Again, the tubes were kept in boiling water bath for 90 seconds, remove and cooled to room temperature. Finally, the colour of the solution was read at 560 nm against a reagent blank. The value obtained from the standard curve is multiplied by the dilution factor 10 to get the lactic acid content in µg/mL water extract of fermented feed.

2.2 Chemical analysis of collected samples (AOAC, 2005)

2.2.1 Dry matter (DM)

A measured quantity of sample in a dry aluminum moisture cup or aluminum tray was taken and placed in hot air oven to dry at 100±5 °C for 24 h. The loss in moisture content after drying was estimated and the dry matter was calculated as follows:

$$\text{DM (\%)} = \frac{(\text{wt. of moisture cup} + \text{sample after drying} - \text{Wt. of moisture cup})}{\text{wt. of sample}} * 100$$

2.2.2 Total ash (TA)

3g sample was taken in pre-weighed silica crucible. After charring the sample on heater (till the smoke disappeared), the crucible was kept in the muffle furnace for ignition at 550 °C for 2-3 h. The crucible was removed on cooling and kept in a desiccator and weighed again to find out weight of ash. The ash content was calculated as given below.

$$\text{Total ash (\%)} = \frac{(\text{wt. of crucible} + \text{ash after cooling} - \text{wt. of crucible})}{\text{Wt. of fresh sample}} * 100$$

2.2.3 Organic matter (OM)

The organic matter was determined by subtracting the total ash content from 100 i.e.;

$$\text{OM (\%)} = 100 - \text{Total ash (\%)}$$

2.2.4 Crude Protein (CP)

1.0 g sample was taken in Kjeldahl flask and digested with 30 mL concentrated H₂SO₄ in the presence of 2-3 g of digestion mixture, till the solution became colourless. After digestion, the contents were cooled and volume was made to 100 mL, 10 mL of aliquot was distilled in Kjeldahl distillation apparatus (KELPLUS Nitrogen Analyzer) after

adding 10-15 mL of 40% NaOH solution. About 60-75 mL of distillate (light green colour) was collected into an Erlenmeyer flask containing 10 mL of 2% boric acid indicator solution. The distillate was then titrated against N/100 H₂SO₄ solution, and the end point was recorded when the colour changed to a slight pinkish. The volume of N/100 H₂SO₄ solution used in titration was measured and recorded.

$$N (\%) = \frac{(0.014 \times \text{volume of N H}_2\text{SO}_4 \times \text{volume made (ml)})}{(\text{Aliquot taken} \times \text{wt. of sample})} \times 100$$

The crude protein (%) of the sample was calculated by multiplying the N-content with factor 6.25. This was based on the principle that protein contains 16% nitrogen.

2.2.5 Ether extract (EE)

For ether extract analysis 3g sample was taken in a cellulose thimble and extracted for 8 h with petroleum ether (40-60 °C) in Soxhlet's extraction apparatus attached to a pre weighed oil flask. The oil flask was removed, and after evaporating the excess of ether, it was dried overnight in a hot air oven (temperature 100±5 °C). The flask was cooled in a desiccator and weighed to a constant value. The ether extract was estimated as the difference in the weight of oil flask with and without oil.

$$EE (\%) = \frac{(\text{wt. of oil flask with ether extract} - \text{wt. of oil flask})}{\text{wt. of sample}} \times 100$$

2.2.6 Neutral detergent fibre (NDF) (McQueen and Nicholson, 1979)

1g of sample was taken in the 1L capacity spoutless beaker. To this, 100 mL of neutral detergent solution (Sodium lauryl sulphate 30.00g, Disodium ethylene diamino tetra-acetate (EDTA) 18.61 g, Sodium borate decahydrate 6.81 g, Disodium hydrogen phosphate (anhydrous) 4.56, Triethylene glycol 10 mL, Distilled water 990 mL) was added. Afterwards, 0.5 g of anhydrous Sodium sulphite was added. The contents of the spoutless beaker were refluxed for an hour. One hour after the initial onset of boiling, the sample was filtered through pre-weighed 50 mL sintered glass crucible grade-1 using an oil-free vacuum pump. The material was washed with hot boiling water and then acetone to remove all salts. The sintered crucible containing residue was dried in a hot air oven (100±5 °C) and weighed again. The NDF was calculated as follows:

$$NDF (\%) = \frac{(\text{wt. of crucible with residue} - \text{wt. of empty crucible})}{\text{wt. of sample taken}} \times 100$$

2.3 Measurement of *in vitro* Rumen Fermentation Parameters

2.3.1 Substrate Preparation and Experimental Treatments

The substrate feed was prepared containing 60% roughage and 40% concentrate mixture. The concentrate mixture was composed of maize grain (33%), cotton seed cake (11%), ground nut cake (11%), soybean meal (11%), wheat bran (20%), De-oiled rice bran (6%), Bajra (5%), mineral mixture (2%), and common salt (1%). Roughage feed contains green maize fodder and wheat straw fodder in a 60:40 ratio. Both components were thoroughly mixed. The

substrate feed was dried at 60 °C for 24 hours to ensure consistent dry matter content.

Four experimental treatments were evaluated: control T₀ (only TMR), T₁ (22% mustard oil cake in concentrate of TMR), T₂ (11% mustard oil cake +11% fermented mustard oil cake in concentrate of TMR), T₃ (22 % fermented mustard oil cake in concentrate of TMR). All treatments were conducted in triplicate.

2.3.2 Incubation Procedure

The incubations were performed in 100mL calibrated glass syringes, by the method given by Menke and Steingass (1988) [18] with slight modifications. For each incubation syringe, 200 mg of the T₀, T₁, T₂ and T₃ were precisely weighed into each syringe. The piston was coated with petroleum jelly and pushed inside the syringe. The syringes were incubated at 39 °C in a water bath after adding *in vitro* solution and removal of air from the syringe. The *in vitro* solution consisting of distilled water (500 mL), rumen buffer solution (250 mL), resazurin solution (1.25 mL), micro mineral solution (0.124 mL), and macro mineral solution (250 mL) was prepared separately. Rumen liquor was added once the mixture had turned colorless after being preheated to 39 °C and fizzed with CO₂. Following adequate mixing, 30 mL of this mixture was injected into syringes via an auto dispenser, gently shaken, the outlet closed, and then incubated for 24 hours at 39 °C for later use.

Syringes were shaken every 30 minutes, and the piston level was verified for the initial reading.

2.4 Measurements and Analytical Procedures

2.4.1 Total gas production

In vitro gas production was measured at 24 hours after incubation by reading the volume on the calibrated syringes. The total gas produced was expressed as the volume of gas accumulated (mL/200 mg DM).

2.4.2 Digestibility parameters

After end of 24 h of incubation, fermentation was arrested by chilling at 4 °C, followed by the collection of a suitable aliquot of gas for methane estimation; the syringe contents were then transferred to centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min, and the resulting pellets were used to determine *in vitro* dry matter (DM) and organic matter (OM) digestibility. Pellets were dissolved in 100 mL neutral detergent solution (NDS) in 500 mL spoutless beakers and refluxed at 100 °C for 1 h. The contents were vacuum-filtered through pre-weighed (G1) crucibles, rinsed with hot water, oven-dried at 65 °C to constant weight, and ashed at 550 °C. *in vitro* dry matter digestibility (IVDMD) and true organic matter digestibility (IVTOMD) were determined from DM and OM disappearance, respectively.

$$IVDMD = \frac{(\text{DM taken for incubation} - \text{NDF residue})}{(\text{DM taken for incubation})} \times 100$$

$$IVOMD = \frac{(\text{DM taken for incubation} - \text{residue OM})}{(\text{OM taken for incubation})} \times 100$$

Partitioning factor (PF) was calculated based on the *in vitro* organic matter (IVOMD) as described by Blummel *et al.* (1999).

$$\text{PF (mg/mL)} = \frac{\text{In vitro organic matter digestibility}}{\text{Net gas production (mL)}}$$

2.5 Estimation of Total and Individual Volatile Acid Production

For determining individual volatile fatty acids, 5 mL of the sample (from IVGPT) was taken in a beaker, and 1 mL of 25 % metaphosphoric acid (prepared in 5 N H₂SO₄) was added. The sample was kept overnight and centrifuged at 3500-4000 rpm for 15-20 minutes. The supernatant was injected in gas chromatograph (NUCON 5700, NUCON ENGINEERS, NEW DELHI) equipped with a flame ionization detector and stainless-steel column packed with Chromosorb 101 (length 4 inch; d 1/4"; d 3 mm; mesh range 80-100) to serve as a stationary phase. Analytical conditions of fractionation of volatile fatty acid were as follows: injection port temperature, 250 °C, column temperature 190 °C and detector temperature 260 °C. The flowrate of carrier gas (Nitrogen) was 40 mL/min, hydrogen 30 mL/min, air 300 mL/min. Injection volume was 3 µL. The injection was performed using 10 µL Hamilton syringe (Hamilton Nevada, USA). The different volatile fatty acid of the samples was identified based on their retention time and their concentration (mM/L) was calculated by comparing the retention time as well as the peak area of the standard after deducting the corresponding blank values. Calculation of individual volatile fatty acid was done as follows:

Area of the peak = 1/2 x height x weight

Area of the peak for acetate, propionate and butyrate was calculated, and the concentration of each acid was determined by comparing it with standard peaks.

For depicting the result in percentage, the following formula is adopted:

% of individual VFA = (Molecular weight x mmol IVFA per 100g DM)/1000

Molecular weight (g) of IVFA: Acetate = 60.05, Propionate = 74.07 and Butyrate = 88.105

2.6 Ammonia nitrogen

For estimation of ammonia-N, about 5 mL of acidified supernatant was mixed with 10 mL of NaOH (1N) and immediately steam distilled using KEL PLUS-N analyzer (Pelican, India). The NH₃ evolved was collected in boric acid solution (20% w/v) having mixed indicator and titrated against N/100 H₂SO₄.

$$\text{NH}_3\text{-N (mg/dL)} = \frac{0.14 \times \text{volume of acid used (N)}}{\text{Volume of supernatant taken}} \times 100$$

2.7 Statistical analysis

Statistical analysis of data was carried out with SPSS 26.0 (SPSS Inc., version, Chicago IL, USA). One-way analysis of variance (ANOVA) was used to study significant variation between means, with a significance level at $p < 0.05$. For multiple comparisons between means, two-sided Tukey test was performed. All the experimental data depicted are mean values of three replicates ($n = 3$). The principal component analysis (PCA) was used to obtain statistical differences and relationships among the isolates using XLSTATTM software (version 2017.3, Addinsoft

SARL, Paris, France).

3. Results and Discussion

3.1 Change in pH of fermented Mustard oil cake

The pH of mustard oil cake decreased significantly ($p < 0.05$) during solid-state fermentation with lactic acid bacteria. The initial pH of raw MOC was 5.66, which decreased to 5.31 in the control after 72 h and further declined to 4.35 in the fermented treatment. The significant reduction in pH in the fermented mustard oil cake indicates active fermentation and organic acid production by lactic acid bacteria.

The decrease in pH is primarily attributed to the production of lactic acid and other organic acids during microbial fermentation of soluble carbohydrates (McDonald *et al.*, 2011; Kung *et al.*, 2018) [17, 13]. Lactic acid bacteria convert fermentable sugars into lactic acid, resulting in acidification of the substrate and improved preservation and microbial stability (Soccol *et al.*, 2017) [25]. Lower pH also inhibits the growth of spoilage and pathogenic microorganisms, thereby improving feed safety and quality (Niba *et al.*, 2009) [20].

Similar reductions in pH during lactic acid bacterial fermentation of oilseed cakes and plant protein sources have been reported by Mukherjee *et al.* (2006) [19], indicating effective fermentation and improved feed quality. The observed decrease in pH in fermented mustard oil cake confirms successful lactic acid fermentation and enhanced preservation and nutritional value.

Table 1: pH activity of fermented and non-fermented MOC at 0 and 72 hours

MOC	pH
0 h	5.66 ^a ±0.03
Control (72 h)	5.31 ^b ±0.02
FSBM (72 h)	4.35 ^a ±0.03

3.2 Change in lactic acid of fermented Mustard oil cake

Lactic acid concentration increased significantly ($p < 0.05$) during solid-state fermentation of mustard oil cake with lactic acid bacteria. The initial lactic acid concentration in raw MOC was low (47.18 µg/mL at 0 h), which increased to 230.42 µg/mL in the control (72 h) and reached the highest level in the LAB-treated fermented mustard oil cake (667.04 µg/mL at 72 h). The substantial increase in lactic acid concentration in the treated group indicates active growth and metabolic activity of lactic acid bacteria, resulting in efficient fermentation of available carbohydrates into lactic acid.

Lactic acid bacteria utilize soluble carbohydrates as substrates and convert them into lactic acid through glycolysis, thereby lowering pH and improving preservation and nutritional quality of the substrate (Kung *et al.*, 2018; Soccol *et al.*, 2017) [13, 25]. The higher lactic acid production in fermented mustard oil cake reflects enhanced microbial fermentation and improved substrate utilization. Increased lactic acid concentration also helps reduce undesirable microorganisms and improves feed stability and safety (McDonald *et al.*, 2011) [17]. Similar increases in lactic acid production during LAB fermentation of plant-based feed substrates have been reported by Mukherjee *et al.* (2006) [19] and Niba *et al.* (2009) [20], indicating improved fermentation efficiency and feed quality.

Table 2: Lactic acid ($\mu\text{g/mL}$) of unfermented and fermented MOC at 0 and 72 hours

MOC	Lactic acid ($\mu\text{g/mL}$)
0 h	47.18 $\pm$ 0.54
Control (72 h)	230.42 $\pm$ 0.48
Treatment (72 h)	667.04 $\pm$ 7.75

3.3 Organoleptic evaluation of fermented MOC

After 72 hours of fermentation, mustard oil cake (MOC) exhibited a lighter texture, darker color, and a stronger sour aroma compared to its unfermented MOC. In a similar study, Ma *et al.* (2022) [15] observed that after 3 and 30 days of fermentation, the ZZUPF95 and ZZUPF95+protease groups consistently maintained a fluffy texture, brown coloration, and sour aroma, distinguishing them from other groups after just 3 days of fermentation.

The lighter texture of the fermented mustard oil cake (MOC) is likely a result of the breakdown of complex macromolecules into simpler forms, such as proteins and carbohydrates, during fermentation. The enzymatic activity of lactic acid bacteria (LAB), coupled with the production of organic acids, helps loosen the substrate structure, contributing to the fluffy appearance (Ma *et al.*, 2022) [15]. The darkening of color is primarily due to Maillard reactions and the oxidation of phenolic compounds during fermentation. Additionally, microbial growth and the accumulation of fermentation by-products, such as organic acids, contribute to these colour changes. The darker coloration enhances the visual appeal, particularly for animal feed formulations. The sour aroma is a consequence of the production of lactic acid, volatile organic compounds, and other metabolites by LAB.

3.4 Proximate analysis of fermented mustard oil cake

The proximate composition of mustard oil cake (MOC) before and after fermentation with lactic acid bacteria (LAB) is shown in Table 2. Solid-state fermentation significantly ($p < 0.05$) influenced the chemical composition of mustard oil cake. The crude protein (CP) content increased significantly from 30.55% in raw MOC and 33.36% in MOC control to 35.96% in fermented mustard oil cake (FMOC). The increase in CP may be attributed to microbial biomass synthesis and concentration effects due to utilization of carbohydrates during fermentation (Mukherjee *et al.*, 2006; Soccol *et al.*, 2017) [6, 25]. Microbial fermentation enriches protein content through microbial cell proliferation and synthesis of single-cell protein, thereby improving the nutritional quality of the substrate (Niba *et al.*, 2009) [20]. Crude fiber (CF) content decreased significantly from 5.86% in MOC to 4.72% in FMOC, indicating partial degradation of structural carbohydrates

during fermentation. Lactic acid bacteria and associated microorganisms produce fibrolytic enzymes that degrade cellulose and hemicellulose, resulting in reduced fiber content and improved nutrient availability (Van Soest, 1994; Kung *et al.*, 2018) [27, 13].

Table 3: Chemical composition of unfermented and fermented MOC

	DM %	CP%	CF%	EE%	TA%
MOC	88.64 $\pm$ 0.48	30.55 $\pm$ 0.16	5.86 $\pm$ 0.17	8.52 $\pm$ 0.01	7.41 $\pm$ 0.38
MOC control	49.76 $\pm$ 0.76	33.36 $\pm$ 0.24	5.55 $\pm$ 0.09	8.18 $\pm$ 0.06	5.31 $\pm$ 0.43
FMOC	47.13 $\pm$ 0.04	35.96 $\pm$ 0.26	4.72 $\pm$ 0.20	7.16 $\pm$ 0.14	5.17 $\pm$ 0.05

Ether extract (EE) content also decreased significantly in FMOC (7.16%) compared to MOC (8.52%), which may be due to microbial utilization of lipids as an energy source during fermentation (Soccol *et al.*, 2017) [25]. Similar reductions in ether extract and fiber content following microbial fermentation have been reported in fermented oilseed cakes and plant protein sources (Kumar *et al.*, 2015) [12]. Dry matter (DM) content decreased significantly in fermented samples compared to raw MOC, which may be attributed to moisture addition and microbial metabolism during solid-state fermentation (Mukherjee *et al.*, 2006) [6]. Total ash (TA) content did not differ significantly among treatments, indicating minimal effect of fermentation on mineral concentration. Overall, the results indicate that solid-state fermentation of mustard oil cake with lactic acid bacteria improved its nutritional quality by increasing crude protein and reducing fiber and ether extract content, thereby enhancing its suitability as a protein source for ruminant feeding.

3.5 *In vitro* gas production technique for evaluation of Total gas production, IVDMD, IVOMD, and partition factor of fermented soybean meal

The total gas production (TGP), neutral detergent fibre (NDF), *in vitro* dry matter digestibility (IVDMD), *in vitro* organic matter digestibility (IVOMD), and partitioning factor (PF) for the various samples are presented in Table 4. Net gas production differed significantly ($p < 0.01$) among treatments. The highest gas production was recorded in T₃ (34.00 mL), followed by T₂ (28.33 mL), whereas T₀ (25.33 mL) and T₁ (26.00 mL) showed significantly lower and statistically similar values. The increase in gas production with fermented mustard oil cake indicates enhanced microbial fermentation and improved availability of fermentable nutrients.

Solid-state fermentation by lactic acid bacteria improves substrate degradability by

Table 4: *In vitro* evaluation of fermented and unfermented SBM

Feed	NGP (ml)	NDF (%)	IVDMD (%)	IVOMD (%)	PF (mg/mL)
TMR (T ₀)	25.33 $\pm$ 0.88	38.61 $\pm$ 0.32	52.41 $\pm$ 0.12	54.47 $\pm$ 0.34	2.15 $\pm$ 0.05
TMR+M1 (T ₁)	26.00 $\pm$ 0.58	36.62 $\pm$ 0.25	55.35 $\pm$ 0.27	57.96 $\pm$ 0.47	2.29 $\pm$ 0.04
TMR+M2 (T ₂)	28.33 $\pm$ 0.67	34.25 $\pm$ 0.14	62.78 $\pm$ 0.13	64.30 $\pm$ 0.27	2.26 $\pm$ 0.02
TMR+M3 (T ₃)	34.00 $\pm$ 0.58	32.94 $\pm$ 0.31	66.08 $\pm$ 0.26	68.27 $\pm$ 0.35	2.00 $\pm$ 0.03
p-value	<0.01	<0.00	<0.05	<0.05	<0.00

producing organic acids and hydrolytic enzymes that break down complex carbohydrates and proteins into simpler compounds (Mukherjee *et al.*, 2006; Soccol *et al.*, 2017) [6, 25]. LAB fermentation also reduces anti-nutritional factors

such as glucosinolates present in mustard oil cake, thereby improving microbial accessibility and fermentation efficiency (Tripathi and Mishra, 2007) [26]. Increased gas production reflects enhanced microbial activity and

improved fermentability of fermented substrates (Menke and Steingass, 1988) [18].

Similar findings have been reported where microbial fermentation of oilseed cakes significantly increased *in vitro* gas production due to improved nutrient availability and microbial utilization (Kumar *et al.*, 2015; Denek and Can, 2006) [12]. The NDF content decreased significantly ($p < 0.01$) with increasing inclusion of fermented mustard oil cake. The highest NDF was observed in T₀ (38.61%), followed by T₁ (36.62%), while T₂ (34.25%) and T₃ (32.94%) showed significantly lower values. The reduction in NDF indicates partial degradation of structural carbohydrates during solid-state fermentation. Lactic acid bacteria and associated microbial populations produce cellulolytic and hemicellulolytic enzymes that degrade plant cell wall components, resulting in reduced fiber content (Sharma *et al.*, 2016; Soccol *et al.*, 2017) [25]. Fermentation also disrupts lignocellulosic bonds, increasing accessibility of carbohydrates to rumen microbes (Kung *et al.*, 2018) [13]. Reduction in NDF improves rumen fermentation efficiency by enhancing microbial colonization and digestion (Van Soest, 1994) [27].

Similar reductions in fiber fractions following microbial fermentation of oilseed cakes and agro-industrial by-products have been reported by Chiou *et al.* (2000) [7] and Xu *et al.* (2012) [28].

The IVDMD increased significantly ($p < 0.05$) with increasing inclusion of fermented mustard oil cake. The lowest digestibility was observed in T₀ (52.41%), followed by T₁ (55.35%), while T₂ (62.78%) and T₃ (66.08%) showed significantly higher digestibility. The highest digestibility observed in T₃ indicates superior nutritional quality of fully fermented mustard oil cake. The improvement in digestibility is attributed to microbial hydrolysis of complex organic compounds during fermentation. LAB fermentation converts complex proteins and carbohydrates into simpler forms such as peptides, amino acids, and soluble sugars, which are readily utilized by rumen microorganisms (Niba *et al.*, 2009) [20]. Fermentation also reduces anti-nutritional factors, improving nutrient availability and digestibility (Tripathi and Mishra, 2007) [26].

These results are consistent with previous findings where microbial fermentation improved dry matter digestibility of oilseed cakes and plant-based feed ingredients (Kumar *et al.*, 2015; Canibe and Jensen, 2012) [12, 6]. IVOMD increased significantly ($p < 0.05$) with increasing levels of fermented mustard oil cake. The values increased from 54.47% in T₀ to 68.27% in T₃. The significant improvement in organic matter digestibility indicates enhanced microbial degradation and fermentation efficiency.

Improved organic matter digestibility is associated with microbial breakdown of structural carbohydrates and increased availability of fermentable substrates (Van Soest, 1994) [27]. LAB fermentation enhances enzymatic hydrolysis and nutrient solubilization, resulting in improved rumen fermentation (Soccol *et al.*, 2017) [25]. Enhanced digestibility leads to increased production of volatile fatty acids, which are major energy sources for ruminants (McDonald *et al.*, 2011) [17].

Similar improvements in organic matter digestibility following microbial fermentation have been reported in fermented soybean meal and oilseed cakes (Chiou *et al.*, 2000; Xu *et al.*, 2012) [7, 28].

The partitioning factor differed significantly ($p < 0.01$) among treatments. The highest PF values were observed in T₁ and T₂, while T₃ showed the lowest PF value (2.00 mg/mL). The lower PF value in T₃ may be attributed to increased gas production relative to substrate degradation.

Partitioning factor reflects the efficiency of microbial biomass production relative to gas production (Blümmel *et al.*, 1997) [5]. Lower PF values in highly fermentable substrates indicate increased fermentation rate and gas production. Although microbial biomass production may be slightly reduced relative to gas production, overall fermentation efficiency and nutrient availability are improved (Makkar, 2004) [16].

3.6 Ammonia Nitrogen, Individual total volatile fatty acid and individual volatile fatty acid of fermented feed.

Ammonia nitrogen concentration increased significantly ($p < 0.01$) with increasing inclusion of fermented mustard oil cake, with the highest value observed in T₃ (10.20 mg/dL), followed by T₂ (8.52 mg/dL). In comparison, T₀ (6.79 mg/dL) and T₁ (6.96 mg/dL) showed lower and comparable values. The increase in NH₃-N indicates enhanced protein degradation and improved nitrogen availability for microbial protein synthesis. Solid-state fermentation by lactic acid bacteria partially hydrolyzes proteins into peptides and amino acids, increasing ruminal nitrogen availability (Mukherjee *et al.*, 2006; Niba *et al.*, 2009) [6, 20]. The NH₃-N values observed were within the optimal range required for efficient microbial growth and rumen fermentation (Satter and Slyter, 1974). Total volatile fatty acid (TVFA) concentration increased significantly ($p < 0.05$) with fermented mustard oil cake inclusion, with the highest concentration in T₃ (89.87 mM/L), followed by T₂ (85.75 mM/L), whereas T₀ (80.40 mM/L) and T₁ (82.12 mM/L) showed lower values. Individual VFA concentrations, including acetate, propionate, and butyrate, also increased significantly ($p < 0.05$) in fermented treatments. The increase in VFA concentration reflects improved microbial fermentation and enhanced degradation of organic matter. LAB fermentation improves nutrient availability by breaking down complex carbohydrates and reducing anti-nutritional factors, thereby promoting rumen microbial activity and VFA production (Van Soest, 1994; Soccol *et al.*, 2017) [27, 25]. Acetate concentration increased significantly from 52.51 mM/L in T₀ to 57.40 mM/L in T₃, indicating enhanced fiber fermentation, while propionate increased from 19.49 to 22.60 mM/L, suggesting improved fermentation of soluble carbohydrates. Butyrate concentration also increased significantly, indicating enhanced overall fermentation efficiency. Similar increases in VFA production following microbial fermentation of feed substrates have been reported by Kung *et al.* (2018) [13] and Kumar *et al.* (2015) [12].

The results indicate that solid-state fermentation of mustard oil cake using lactic acid bacteria significantly improved rumen fermentation characteristics by increasing NH₃-N and VFA production. This improvement can be attributed to enhanced protein degradation, increased nutrient availability, and improved microbial activity due to fermentation (Mukherjee *et al.*, 2006; Van Soest, 1994) [6, 27]. The highest improvement observed in T₃ suggests that complete fermentation of mustard oil cake maximizes rumen fermentation efficiency and nutrient utilization.

Table 5: *In vitro* evaluation of NH₃N, Individual volatile fatty acid and TVFA of fermented and unfermented SBM

Feed	NH ₃ N (mg/dL)	Acetate (mM/L)	Propionate (mM/L)	Butyrate (mM/L)	TVFA (mM/L)
TMR (T ₀)	6.79 ^a ±0.08	52.51 ^a ±0.14	19.49 ^a ±0.22	8.40 ^a ±0.13	80.40 ^a ±0.42
TMR+M1 (T ₁)	6.96 ^a ±0.29	53.50 ^a ±0.23	20.18 ^{ab} ±0.03	8.44 ^a ±0.07	82.12 ^a ±0.43
TMR+M2 (T ₂)	8.52 ^b ±0.16	55.35 ^{ab} ±1.20	21.14 ^b ±0.04	9.26 ^a ±0.05	85.75 ^a ±0.58
TMR+M3 (T ₃)	10.20 ^c ±0.20	57.40 ^b ±1.10	22.60 ^c ±0.11	9.87 ^b ±0.06	89.87 ^b ±0.45
p-value	<0.00	<0.00	<0.02	<0.05	<0.05

4. Conclusion

Solid-state fermentation of mustard oil cake using a *Lactobacillus* consortium significantly improved its nutritional composition and rumen fermentation characteristics. Fermentation reduced pH and increased lactic acid concentration, confirming effective microbial activity. Fermented mustard oil cake showed higher crude protein and lower fiber and ether extract, indicating improved nutrient availability. *In vitro* studies demonstrated significant increases in gas production, dry and organic matter digestibility, ammonia nitrogen, and volatile fatty acid concentrations, reflecting enhanced microbial fermentation and substrate utilization. The greatest improvement was observed with complete replacement of mustard oil cake by fermented mustard oil cake in the total mixed ration. Overall, lactic acid bacteria-mediated fermentation is an effective and sustainable strategy to enhance the feeding value of mustard oil cake and promote efficient utilization of agro-industrial by-products in ruminant nutrition.

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