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Determination of *in-vitro* dry matter digestibility and condensed tannins of probiotics-treated molm

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Abstract
This study investigated the impact of three fermentation methods (*Clostridium butyricum*, *Bacillus coagulan*, and spontaneous fermentation) on MOLM's nutritional and anti-nutritional changes. Three fermentation methods were used *Clostridium butyricum Bacillus coagulan* and spontaneous fermentation, each in three replicates. All the fermentation methods had a positive impact on the measured parameters. For the *in-vitro* digestibility to match the digestive system in the chicken stomach and intestines, a trial was conducted following the methodology outlined by Gabler *et al*. (2015). Results indicated a significant difference in *in-vitro* DMD%. Fermentation with *Bacillus coagulan* had the highest digestibility (66.24±0.76%) compared to the rest, while untreated had the lowest (42.20±0.78%). *Clostridium butyricum* fermentation resulted in a higher *in-vitro* DMD (57.24±0.74%) than Spontaneous fermentation (48.23±0.57%). There was an increase in *in-vitro* DMD% after treating the leaf meal with the *Bacillus coagulan* (66.24±0.76%) relative to the untreated (42.20±0.78). There was a significant improvement in the C.P. content of 33.41±0.57 compared to the untreated 27.48±0.25. The anti-nutritive (tannins and phenolics) compounds were reduced in the fermented substrate; *Bacillus coagulan* fermented substrates had higher nutritional value and increased *in-vitro* DMD, improved C.P. and decreased C.F. and tannins in fermented substrates. Among the three fermentations, *Bacillus coagulan* proved best in all the parameters evaluated. It was therefore used for the fermentation of MOLM for feeding trials.

Keywords: Anti-nutritive compounds, fermentation, *in-vitro*, Moringa, probiotics, proximate analysis

1. Introduction
Poultry production is highly beneficial for the economy, society, and culture. It plays a significant role in providing nutrition to families worldwide and contributes significantly to household food security in developing countries (Hinsemu *et al*., 2018) [14]. Despite being one of the fastest-growing animal sectors globally, the poultry industry faces challenges due to the limited availability of feed components, especially in developing countries. It is essential to assess non-conventional feed sources locally available and can be used in chicken feed production (Mahfuz *et al*., 2019) [20]. It's worth noting that Moringa, a non-legume tree, makes for fantastic animal feed. Its nutritional value is unparalleled, and it's readily available. As Okiki *et al*. (2015) [26] discovered, Moringa leaves are bursting with vitamins, minerals, amino acids, and phytochemicals. Mahfuz *et al*. (2019) [20] found that Moringa leaves can be a dietary supplement for layers and broilers, resulting in improved egg quality and high production performance. Studies have shown the viability of adding MOLM to animal feed for livestock and poultry. Research has found that including 2 to 25% of this meal in chicken diets can improve growth rates and egg production for broilers and layers (Banjo, 2012; AbouSekken, 2015) [3, 2].
Additionally, studies have suggested that Moringa might positively impact the health and performance of chickens (Mahfuz *et al*., 2019) [20]. According to recent research (Ijarotimi *et al*., 2013) [16], fermenting moringa seeds in banana leaves can significantly boost their protein content, essential amino acid profiles, and polyunsaturated fatty acid profiles. Additionally, studies have shown that Moringa leaves fermented in methanol with lactic acid bacteria can successfully treat obese mice's hyperglycemia and hepatic steatosis. A different study investigated the use of fermented moringa leaf extracts on mice with atopic dermatitis. The *in-vitro* dry matter digestibility and condensed tannins of MOLM treated with probiotics are assessed in this study.

2. Material and Methods

2.1 Research Area

An *in vitro* trial study was conducted at Egerton University's Animal Nutrition department in Njoro Sub-County, Nakuru County. The laboratory is located at 0° 23' S and 35° 55' N, with an elevation of 1800 meters above sea level and an annual average rainfall of 900-1200 mm. Meteorological data from Egerton University's Department of Agricultural Engineering's Metrological Station in 2018 (obtained through personal communication) revealed that the area experiences an average daily temperature range of 17 °C to 22 °C.

2.2 Collection and processing of MOLM

The dried Moringa leaves were sourced from Meru County in Kenya, located along the eastern side of the Mount Kenya region. Local farmers in Meru were contracted to obtain fresh leaves by cutting the tree branches and stripping leaves off the tips of the branches by hand (manually). To prepare the Moringa leaves for analysis and feed compounding, they were air-dried under a shady area until they became crisp while maintaining their green colour. The dried leaves were then milled with a 5 mm sieve using a hammer mill. Finally, they were stored in airtight bags until needed.

2.3 Preparation of experimental diets

There were four experimental diets with three replicates each; T_1 MOLM fermented with *Clostridium butyricum*, T_2 MOLM treated *Bacillus coagulan*, T_3 MOLM fermented using Spontaneous fermentation, and T_4 untreated MOLM (control).

2.3 Fermentation of MOLM

With some adjustments, a method similar to Ogodo *et al*. (2018) was used to prepare the Moringa leaf meal samples. *Moringa oleifera* leaf meal was mixed with distilled water in a 1:0.5 ratio (weight to volume) in a 500-ml beaker and stirred thoroughly using a hand mixer. To ensure the samples were uncontaminated, they were sterilised in an autoclave at 121°C for 10 minutes. After cooling for 30 minutes at room temperature (25±2 °C), Moringa was mixed with distilled water in a 1:0.5 ratio and sterilised the samples. The samples were added with 0.0025 grams of 2.0× 104 CFU/g of *Clostridium butryricum and Bacillus coagulan* strains samples, ensuring they were adequately inoculated. The samples were left to ferment in a solid state for 48 hours, allowing the fermentation process to occur.

2.4 Moringa leaf meal treated with *Clostridium butyricum*

Clostridium butyricum powder was acquired from Feed Biotechnology Laboratory, China Agricultural University Ninja, and its concentration was 2.0×10^4 CFU/g. According to the instructions and recommendations of the manufacturer, 0.0025 grams of Clostridium butyricum in its dry state were added to the feedstuff and mixed thoroughly. The inoculated MOLM were incubated in the laboratory at 37 °C in tightly sealed 750ml plastic bottles for 48 h. Samples for proximate analysis were taken after 48 hours. HANNA instruments' digital hand-held pH meter, which also measures ORP and temperature, was used to determine the pH level of each sample. Afterwards, these readings were written down.

2.5 Fermentation of MOLM with *Bacillus coagulan*

A single strain of commercial *Bacillus coagulan* powder was acquired from Feed Biotechnology Laboratory, China Agricultural University Ninja, and its concentration is 2.0×10^4 CFU/g. The initial culture for the procedure was *Bacillus coagulan*. At a ratio of 1:2.50 (wt/vol), three samples were combined with 600 mg of MOLM and distilled water before the culture was added. 48 hours were spent incubating the fermented MOLM at 37 °C in tightly closed 750ml plastic bottles. A digital hand-held pH meter (pH/ORP/Temperature Combo Tester - HI98121 HANNA instruments) was used to test the pH of each sample once the incubation process was complete. The samples were then collected for proximate analysis.

2.6 Spontaneous fermentation of MOLM

The spontaneous fermentation was carried out by incubating 600 g of MOLM at 22 °C for seven days while it was combined with distilled water in a 1:2.75 (wt/vol) ratio. To maintain anaerobic conditions, the containers were firmly sealed. A sample was taken for proximate analysis after seven days, and the pH of each sample was determined using a portable pH meter (HI98121 HANNA devices). The experiment was carried out in triplicates in accordance with the procedure by Marii's (2021).

2.7 Proximate analysis

Various standard procedures were employed to analyse the elements of a sample. Dry matter was estimated by drying the sample for 24 hours in a hot oven at 105 °C. The ash content was evaluated by burning the sample for eight hours in a muffle furnace at 550 °C. The ether extract was made using the Soxhlet technique and ether. The micro-Kjeldahl method was used to calculate total nitrogen in crude protein. The Van Soest method examined the cell wall components, including neutral detergent fibre, acid detergent fibre, and acid detergent lignin. Hemicellulose was estimated by subtracting the neutral detergent fibre, acid detergent fibre, and acid detergent lignin from the total amount of fibre.

2.8 Determination of condensed tannins and total phenolics

Extraction of tannins

The samples were extracted using 70% aqueous acetone. First, a 25 mL glass beaker was filled with 200 mg of each dried and finely ground material. 10 mL of 70% aqueous acetone was added to the sample before the beaker was suspended in an ultrasonic water bath for 20 minutes at room temperature. The beaker's contents were then transferred to centrifuge tubes and subjected to centrifugation using a chilled centrifuge for 10 minutes at about 3000 g and 4 °C (Rahman & Lamara, 2023) [29]. Then, gather the supernatant and store it on ice. The pellet still in the tube was then transferred back to the beaker using two volumes of 5 mL of 70% aqueous acetone each, and the contents were then treated with ultrasonic treatment for 20 minutes. Finally, collect the supernatant once more, as previously instructed.

Determination of condensed tannins (*Proanthocyanins*)

The Porter *et al*. (1986) method was used to quantify the condensed tannins in the extracts. First, 950 mL of n-butanol and 50 mL of concentrated hydrochloric acid (37% concentration) were combined to create the butanol-HCl reagent. Then, 2.0 g of ferric ammonium sulphate was dissolved in 2N HCl to create the ferric reagent. Both reagents were kept in dark vials. After that, 0.5 mL of the 70% acetone-diluted tannin extract was pipetted into a glass test tube measuring 100 mm by 12 mm. Then added, 0.1 mL of the ferric reagent to the tubes and 3 mL of the butanol-HCl reagent. Finally, put a glass of marble on top of the tubes and

give them a Vortex shake. The tubes were then placed on a heating block and set to 97 to 100 °C for 60 minutes. The tubes were then allowed to cool, and the Absorbance was measured at 550 nm. Condensed tannins were calculated at 550 nm by subtracting the heated combination's Absorbance from the unheated mixture's Absorbance, which was employed as a suitable blank. The amount of acetone was sufficient to keep the assay's Absorbance (550 nm) from surpassing 0.6. Without being heated, the sample will turn pink, indicating the presence of flavanols. To determine the proportion of condensed tannins in dry matter as leucocyanidin equivalent, each sample was made up of 0.5 mL extract, 3 mL butanol, and 0.1 mL ferric reagent in a heated blank. The calculation uses the assumption that the effective El%, 1 cm, 550 nm of leucocyanidin is 460 (Porter, 1986) using the formula (A 550 nm x 78.26 x Dilution factor), (% dry matter). When no 70% acetone is used, the dilution factor is 1, and the extract was produced using a 200 mg sample in 10 mL of solvent. The dilution factor was 0.5 mL, (Volume of extract collected) in the current if 70% acetone was added (to avoid the Absorbance from surpassing 0.6).

2.9 Two-step procedures of *in-vitro* digestibility of dry matter determination (*IVDMD*)

To simulate the chicken's digestive system, a study was conducted following Gabler *et al*.'s (2015) [21] protocol. Four treatments, each with three replicates, were used: T_1 involved treating Moringa with Clostridium butyricum, T_2 involved treating Moringa with Bacillus coagulan, T_3 involved spontaneous fermentation, and T_4 was the untreated control group using MOLM**.**

Step one (poultry stomach simulation phase)

To conduct this step, a 0.4g sample of pulverised feed was weighed and put into a 100 ml conical flask. The flask was then filled with 200 ml of a sodium phosphate buffer solution (0.1 M, pH 6.0), and the mixture was agitated. 80 ml of 0.2 M HCl was added to replicate the stomach's digestion process, and the pH was raised to 2.0 by adding either 1 M HCl or 1 M NaOH solutions. Then, 5 mL of a pepsin porcine grade enzyme (Pepsin from porcine Gastric Mucosa Powder, 250 units/mg Solid Sigma-Aldrich Corp., St. Louis, MO, USA) containing 1 mg of pepsin per ml 0.02 M HCl, with 4x USP activity was added to each flask. 2ml of Chloramphenicol C-0378 from Sigma-Aldrich Corp. in St. Louis, Missouri, USA (0.5g/100ml ethanol) was added to stop bacterial growth. The flasks were sealed and constantly swirled for 2 hours in a water bath at 39 °C.

Step two: Poultry intestines simulation

In this step, the procedure was modelled after the digestion of chicken in the intestine. Then, combine the first-step mixture with 20 ml of 0.6 M NaOH and 80 ml of phosphate buffer (0.2 M, pH 6.8). According to Ramaswamy (2001) [29], the pH was then corrected to 6.8 using 1M HCl or 1M NaOH to guarantee that the intestinal enzymes could work properly. 10.6 ml of synthetic pancreatin P-1750 (porcine grade enzyme with three times the USP activity) from Sigma-Aldrich Corp. in St. Louis, Missouri, USA was added to the mixture, and it was held at 39 °C with constant stirring for four (4) hours. The residues were then rinsed with distilled water, washed twice with 20 ml each of 95% ethanol and 99.5% acetone, and filtered through a nylon bag with pores measuring 42 m. The leftovers were then weighed after being dried in an oven at 70 °C for 12 hours.

Dry matter digestibility calculations

The *in-vitro* digestibility (*IVDMD*) of dry matter (D.M.) was calculated using the following formulae (Boisen & Fernandez, 1997).

$$DM\ digestibility = \left(\frac{DM_{In} - DM_{RS}}{DM_{In}}\right) \times 100$$

Where:

DM_{In} and DM_{RS} are the initial (D.M.) and residual (D.M.), respectively.

3 Statistical analysis

To analyse the data, IBM SPSS Statistics version 22 was used. Then checked data normality assumption using the Shapiro-Wilk test and the homogeneity of variance assumption using Levenes test statistics. Data was considered normal if $p<0.05$. GLM model procedures were used to analyse the *in-vitro* DMD. The Mean was separated using Tukey's HSD test at a 0.05 significance level to determine significant differences. Additionally, curve fitting and analysis of *in-vitro* DMD% were performed using Excel solver in Microsoft Excel.

4 Results

4.1 pH of fermented MOLM

The fermentation method had an effect on the pH of the MOLM (Table 3.1).

Table 1: pH of treated and fermented MOLM

Treatments	pH	P-Value
T_1	3.63^b±.0.98	< 0.0001
T_2	3.57^b±0.98	
T3	3.89^a±1.04	

The means within a column with the same letter are not significantly different (at a 5% significance level). T_1 was treated with *Clostridium butyricum*. T_2 was treated with *Bacillus coagulan* - T_3 spontaneous fermentation, and T_4 untreated MOLM.

4.2 Determination of the *in-vitro* dry matter digestibility and condensed tannins of probiotics-treated MOLM

Treatment 2 with *Bacillus coagulan* had the highest digestibility (66.24±0.76%) compared to the rest, while the untreated fermentation recorded the lowest (42.20±0.78%). *Clostridium butyricum* resulted in a higher *in-vitro* DMD% (57.24±0.74%) than spontaneous fermentation (48.23±0.57%). There was an increase in *in-vitro* DMD% (Fig. 1) after treating the MOLM with the *Bacillus coagulan* (66.24±0.76) relative to the untreated (T_4), (42.20±0.78%).

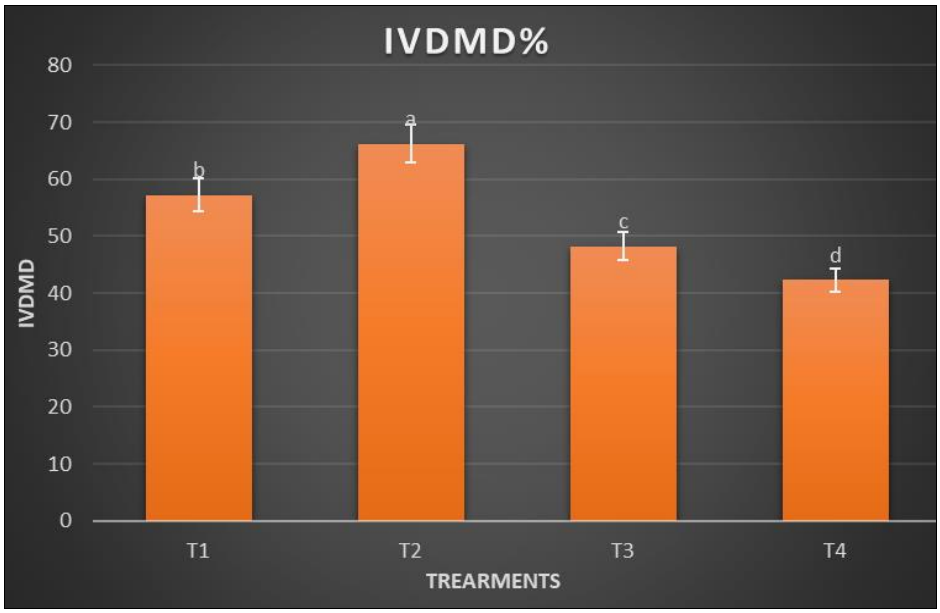

Fig 1: This study examines the digestibility of probiotics, fermented, and untreated moringa leaf meals through *in-vitro* testing. The error bars indicate the average IVDMD of MOLM with standard error

Table 2: Chemical composition (Mg/100g) of moringa leaf meal on (D.M. basis %)

		Treatments			
Parameters %	**T_1**	**T_2**	**T_3**	**T_4**	**P-Value**
Moisture	$7.86^{b}\pm0.26$	$6.7^{b}\pm0.31$	$12.83^{a}\pm0.28$	$8.71^{b}\pm0.16$	0.0128
DM	$92.14^{a}\pm0.26$	$93.30^{a}\pm0.31$	$87.17^{c}\pm1.98$	$91.28\pm^{ab}0.17$	0.0129
Crude Protein	$29.31^{b}\pm0.29$	$33.41^{a}\pm0.57$	$27.18^{c}\pm0.55$	$27.48^{bc}\pm0.25$	<.0001
Crude fibre	$7.90^{c}\pm0.01$	$6.61^{d}\pm0.16$	$8.61^{b}\pm0.08$	$9.54^{a}\pm0.09$	<.0001
Ash	7.75 ± 0.05^{c}	9.28 ± 0.03^{a}	8.63 ± 0.2^{b}	8.55 ± 0.71^{b}	0.2170
Crude fat	$8.73^{b}\pm0.05$	$7.35^{c}\pm0.01$	$10.4^{a}\pm0.25$	$6.73^{c}\pm0.11$	<.0001
ADL	$1.41^{ab}\pm0.2$	$1.06^{b}\pm0.07$	$1.73^{a}\pm0.06$	$1.78^{a}\pm0.01$	0.0052
ADF	$6.63^{b}\pm0.37$	$5.01^{c}\pm0.28$	$8.05^{a}\pm0.25$	$8.45^{a}\pm0.34$	0.0002
NDF	$9.95^{ab}\pm0.13$	$9.73^{b}\pm0.09$	$10.6^{ab}\pm0.12$	$10.9^{a}\pm0.38$	0.0160
TANNINS mg	$2.33^{b}\pm0.14$	$2.09^{b}\pm0.14$	$3.10^{a}\pm0.21$	$3.10^{a}\pm0.16$	0.0040
Phenolics mg	$1.13^{b}\pm0.01$	$1.04^{c}\pm0.01$	$1.16^{b}\pm0.01$	$1.26^{a}\pm0.01$	<.0001

If the p-value falls below 0.05, it unequivocally indicates that the means within a row are dissimilar and must be identified by unique superscript letters (ABCD). T_1 = *Clostridium butyricum* treated moringa leaf, T_2= *Bacillus coagulan* treated moringa leaf, T_3 spontaneous fermentation of moringa leaf, T_4 untreated moringa ± represents standard error of the Mean.

5. Discussion

Solid-state fermentation (SSF) requires various important biochemical processes to achieve the best outcomes (Dhiman *et al*., 2018) [7]. Culturing specific bacterial strains can enhance the nutritional quality of non-traditional feed resources (Yao *et al*., 2018) [37]. Wang *et al*. (2018) [37] found that SSF using lactic acid bacteria significantly Improves the nutritional quality and digestibility of MOLM. However, the application of multiple bacterial strains in fermentation remains relatively unexplored, and the SSF of MOLM using *Clostridium butyricum* and *B. Coagulans* has not been studied yet. In this research, microorganisms were used to ferment MOLM, and the success of MOLM fermentation can be attributed to the fact that *Bacillus coagulan* has more significant cellulose, pectinase, and amylase activities, which aid in the degradation of cellulose and starch, and consequently increase the available carbon. *Bacillus coagulans* can transform non-protein nitrogen into microbial protein. It also secretes enzymes that aid in the breakdown of cellulose and starch, promoting mycelium's growth. They can also improve the palatability of MOLM for livestock. It appeared that There was no significant difference (p>0.05) between *Bacillus coagulan* and *Clostridium butyricum* in the fermented substrate's pH Table (1). During fermentation, all methods used showed a significant decrease in pH compared to untreated substrates. This is due to the high concentration of lactic acid produced by microorganisms. Lactic acid is about 10 to 12 times stronger than other acids, such as acetic acid and propionic acid, making it the main contributor to the decrease in pH. The pH measurement of the growth medium showed a reduction of 4.4 to 3.2 by all strains of lactic acid bacteria within 48 hours. This reduction can help prevent spoilage and pathogenic organisms that may be present in food products.

In-vitro dry matter digestibility

In the study, Treatment 2 had the highest digestibility rate at 66.24±0.76%, while untreated T4 had the lowest at 42.20±0.78%. *Bacillus coagulan* proved more effective than untreated MOLM, resulting in a significant difference (p<0.05). *Clostridium butyricum* had a higher *in-vitro* DMD% rate at 57.24±0.74% compared to spontaneous fermentation at 48.23±0.57%. The use of probiotics, specifically *Bacillus coagulan*, showed an increase in *in-vitro* DMD% (as shown in Fig 1), indicating its potential to aid in protein and carbohydrate digestion in the gastrointestinal tract of poultry. These results suggest a promising future for using *Bacillus*

coagulan to improve poultry nutrition. This result agrees with Sui *et al*. (2020) [33] finding, who report that *B. Coagulans* showed good assimilation potential in *in-vitro* dry matter digestibility. According to the study conducted by Igwe *et al*. (2012), the Moisture value of MOLM was higher during spontaneous fermentation (12.83±0.28%).

In contrast, the lowest Moisture value was recorded in *Bacillus coagulan* (6.6±0.31%). This increase in moisture can be attributed to the addition of water to the substrate before fermentation. The quality of the feed is determined by moisture, which is essential for the body's rate of feed absorption and assimilation. Furthermore, the biomass of the microorganisms may be the cause of the rise in the DM content of the fermented substrates in this investigation. The presence of water-soluble carbohydrates in the leaves can be easily consumed by lactobacillus bacteria, leading to high losses in dry matter due to fermentation towards the ethanol pathway. To avoid this, reducing the moisture content can improve the longevity of fermented MOLM and protect it from mycotoxin contamination. This, in turn, can improve the shelf life of fermented leaves and ensure their suitability for large-scale feed manufacturing. (Silva *et al*., 2017; Gillis *et al*., 2016) [35, 13].

The study showed that all fermented substrates had increased crude protein. The treatment with *Bacillus coagulan* had the highest C.P. (33.41±0.57) compared to the untreated sample (27.48±0.25). There was a difference between *Bacillus coagulan* and the untreated sample (p<0.05). *Clostridium butyricum* had a higher C.P. (29.31±0.29) than spontaneous fermentation (27.18c±0.55). According to Corona *et al*. (2020), the rise in C.P. may be caused by the proteolytic enzymes produced by lactic acid bacteria during fermentation or by increased microorganisms and their activities. Another possible reason is protein synthesis by fermenting substrates, leading to higher production of amino acids. The fermentation conditions in MOLM, which lie within the ideal pH range for lactic bacteria and produce greater biomass, are responsible for the increase in protein content. This result is consistent with Léopold *et al*. (2013) [36], who observed that while fermenting Moringa leaves at 37 C, the crude protein concentration increased from 38 g/100g D.M. to 44 g/100g D.M. Similarly, the crude protein content of fermented MOLM leaves increased from 33 g/100g D.M. to 39 g/100g D.M for the fermented MOLM at 37˚C. Because MOLM contains a high amount of crude protein, moringa leaf protein concentrates could be employed as a nutritious, healthy element to reduce protein deficiency in the diet of chicken. Shi *et al*. (2015) [31] reported that food's protein value increased during fermentation. The study found that Bacillus coagulan had a larger ash content than spontaneous or untreated growth, which did not differ significantly (p>0.05), as shown in Table 2. The changes in ash content during fermentation indicate the transformation of substrates from one form to another with no significant loss in volatile compounds, as noted by Ntuli *et al*. (2013). The ash content reported in this study (9.28±0.03) was lower than the one reported by Thierry *et al*. (2013) [36]. (12.68±0.51) in MOLM. Ash in the diet contributes to the residue left after removing all the moisture and incinerating organic materials such as fat, protein, carbohydrates, vitamins, and organic acids (Salma Sultana, 2020) [34]. This study found that increased ash content may indicate a higher mineral composition in the substrates. The results in this study showed that crude fat (ether extract) of MOLM was high in spontaneous (10.4^{a}±0.25) compared to untreated (6.73^{c}±0.11), (Table 3.2). In contrast, *Clostridium butyricum* (8.73^{b}±0.05) significantly differed (p<0.05) from *Bacillus coagulan* (7.35^{c}±0.01), (Table 2). These figures concur with Salma Sultana's (2020) [34] research, which claimed that the fat content of moringa leaf meal was in the optimum range (4.03, 9.51%). More dietary polyunsaturated fatty acids (PUFAs) than saturated fatty acids (SFAs) can be found in moringa leaf meal. The increase in lipids in fermented leaves may be attributed to the microbial transformation of carbohydrates to lipids. Bairagi *et al*. (2004) and Ramachandran *et al*. (2005) have reported that fermenting moringa leaf meal and grass pea seed meal with *Bacillus sp*. can increase the level of lipids, specifically free fatty acids. The findings of this study agree with those of Madukwe *et al*. (2013) [19]. MOLM's crude fibre level mainly consists of cellulose and some lignin, which poultry cannot digest. Compared to other forage plants, MOLM has a low fibre content, which affects how easily and quickly the feed can be digested. Even though excessive amounts of crude fibre might irritate the intestines and reduce nutritional uptake, they can also aid in digestion and the absorption of microelements, glucose, and fat. Sultana *et al*.'s (2020) [34]. The crude fibre content of MOLM in this study shows that T_2 had lower crude fibre compared to the rest; untreated T_4 recorded the highest CF, and Treatment 2 significantly differed from T_4 in the (p<0.05). Treatment1 had lower C.F. than T_3; this finding agrees with Sultana *et al.* (2020) [34], who reported that the crude fibre content of MOLM (6.00-9.60%) recorded was considered acceptable. Compared to the spontaneously fermented and untreated substrate, the crude fibre content of MOLM of the fermented substrate after microbial inoculation was lower. This could be attributed to the microorganism's ability to degrade fibre, thereby freeing the nutrients in MOLM, allowing for rapid microbial growth and enzyme synthesis, and hence the prompt and rapid breakdown of crude fibre (Hu *et al*., 2011) [15]. Fermentation techniques had a favourable effect on the acid detergent lignin (ADL), neutral detergent fibre (NDF), and acid detergent fibre (ADF) fractions. Plant cell materials can be divided into two categories: highly digestible cell contents comprised of starch and sugars and less digestible cell walls made of hemicellulose, cellulose, and lignin. It is believed that non-ruminants cannot digest hemicellulose, cellulose, and lignin, although hemicellulose and cellulose can be partially digested by ruminants. Neutral detergent fibre has been used to forecast feed intake since it indicates bulk fibre. After fermentation, the amount of ADL, NDF, and ADF fibre was significantly reduced as a result of the probiotic fermentation of MOLM. *Bacillus coagulan* probiotics were reported to degrade crude fibre, cellulose and hemicellulose levels in *Leucaena leucocephala* leaf meal, wheat bran and grass pea seed meal (Ghosh *et al*., 2017) [12]. Plants can produce anti-nutritional factors, compounds that can lower the amount of nutrients available for animals that consume them. These factors can impact the choice of plants used for animal feed (Gemede & Ratta, 2014) [11]. Plants produce secondary metabolites as a defence mechanism created through normal metabolic pathways. These metabolites impact the digestibility, bioavailability, and utilisation of nutrients in food, such as proteins, minerals, and vitamins, and lower their nutritional value. Moringa leaf meal specifically contains 21 g/kg of phytate, 10.5 g/kg of oxalates, and minimal amounts of tannins, saponins, trypsin, and amylase, according to Teixeira *et al*. (2014) [35]. Fermentation methods significantly decreased tannin and phenolic contents, with notable differences in phenolic content (p<0.05). The untreated

MOLM meal had the highest phenolic content (1.26^{a}±0.01), while *Bacillus coagulan* fermentation had the lowest (1.04^{c}±0.01). Fermentation using *Clostridium butyricum* (1.13^{b}±0.01) did not differ significantly (p>0.05) from spontaneous fermentation. The condensed tannins showed no significant differences (p>0.05) between untreated MOLM meal and spontaneous fermentation. However, *Bacillus coagulan* fermentation and *Clostridium butyricum* did not differ significantly (p>0.05). Interestingly, tannin levels were below detectable in MOLM fermented with *Bacillus coagulan* and *Clostridium butyricum* compared to untreated. This agrees with the finding of Amita *et al*. (2014) [30], who reported that tannin and phytic acid had been observed in all the fermented groups with B.S. *(Bacillus subtilis)*, B.C. (*Bacillus coagulan*) and S.C. *(Saccharomyces cerevisiae*), and the phytic acid level was reduced to below detectable levels in pods fermented for 96 h with *Bacillus sp*. At the same time, there was no significant difference between spontaneous and untreated, respectively. From Table (2), the finding shows that there was a significant difference (p<0.05) between T_2 and T_4, *Clostridium butyricum* T_1 and T_2 did not differ from (p<0.05) in the phenol content of fermented MOLM. Cellulolytic, ligninolytic, and pectinolytic enzymes are produced by bacteria during fermentation in order to disassemble plant wall constituents and hydrolysed ester bonds holding phenolics to the cell wall. According to research by Ajila *et al*. (2012) and Dulf *et al*. (2018) [8], this mechanism causes the release of certain phenolic chemicals from the matrix.

6. Conclusion

From the study's findings, it was determined that fermentation with *Bacillus coagulan* (T_2) had the highest *in-vitro* digestibility, while *Clostridium butyricum* was significantly higher (57.24±0.74%) than spontaneous (48.23±0.57%). Untreated MOLM recorded the lowest (42.20±0.78%) *in-vitro* DMD%. Condensed tannins levels were also lower in *Bacillus coagulan* (2.09^{b}±0.14) and *Clostridium butyricum* (2.33^{b}±0.14) than in untreated and spontaneous (2.33^{b}±0.14, 1.26^{a}±0.01) respectively.

7. References

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