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The *In-Vitro* Digestibility, Proximate Analysis, Condensed Tannins, and Phenolics of Probiotics-Treated *M. Oleifera* Leaf

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Abstract
This study evaluated the in-vitro dry matter digestibility and nutritional and anti-nutritional changes that occurred in Moringa oleifera leaves on fermentation. Four fermentation methods were used spontaneous (S) fermentation, probiotic fermentation using clostridium butyricum, and Bacillus coagulans, with three replicates, each in a completely randomised design (CRD). All the fermentation methods used affected the parameters measured positively. An IVDM trial was performed by adding 5 mL pepsin porcine grade enzyme with 4x USP activity (Pepsin from porcine gastric mucosa powder, ≥250 units/mg solid Sigma-Aldrich Corp., St. Louis, MO, USA) containing 1 mg pepsin per ml 0.02 M HCl method. Results indicated a significant difference in IVDMD%. Fermentation T2 (bacillus coagulans) had the highest digestibility (66.24±0.76%) relative to the rest, while untreated fermentation T4 had the least (42.20±0.78%). Treatment 4 significantly differed from T2 ($p<0.05$). Inoculation using clostridium butyricum (T1 57.24±0.74%) resulted in a higher IVDMD than spontaneous fermentation (T3 48.23±0.57%). There was an increase in IVDMD (Fig3.1) after treating the leaves with the probiotics Bacillus coagulans (66.24±0.76%) relative to the untreated (T4) (42.20±0.78). There was a significant improvement in the value of the CP 33.41±0.57 compared to untreated 27.48±0.25. Furthermore, anti-nutritive (tannins and phenolics) compounds were reduced in the fermented substrate; probiotics fermented substrates had higher nutritional value. It can therefore be used as a supplementary feed ingredient in a layer's diet.

Keywords: ***Probiotics, in-vitro, fermentation, Anti-nutritive compounds.***

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I. INTRODUCTION

Poultry production has significant economic, social, and cultural benefits. It plays a significant role in family nutrition globally, substantially contributing to household food security throughout developing countries (Hinsemu *et al.,* 2018)**.** Even though it is one of the animal sectors with the most remarkable rate of growth globally, the poultry industry is constrained by a severe lack of feed components, particularly in emerging nations investigating non-traditional feed sources that could be used in chicken feed formulas is therefore crucial (Darboe *et al.,* 2022)**.**

Moringa is a non-legume tree with the potential for use as animal feed due to its high availability and excellent nutritive value (Beyene, 2021). Its leaves have been found to contain phytochemicals, vitamins, minerals, and amino acids (Okiki *et al.,* 2015). Mahfuz *et al.* (2019) stated that Moringa leaf could be applied as a dietary supplement in layers and broilers due to high production performance and improved egg quality in several studies, demonstrating the feasibility of using Moringa in livestock and poultry diets (Abbas *et al.,* 2013). It has been shown that the dietary inclusion of 2 to 25% of moringa leaf meal in broiler and layer chicken diets improved growth rate and egg production (Banjo, 2012; Gadzirayi et al., 2012; Moreki & Gabanakgosi, 2014; AbouSekken, 2015) while the feeding of moringa foliage replaced up to 75% of conventional concentrate (i.e., sunflower seed cake, cotton seed cake, Etc.) in the diets of goat and sheep (Briones *et al.,* 2015) research have shown that Moringa plant might have a positive role in improving chickens' production performance and health status.

Previous studies have revealed that the natural fermentation of cooked moringa seeds in banana leaves significantly increased protein content, essential amino acids and polyunsaturated fatty acid profiles (Ijarotimi *et al.,* 2013). Furthermore, methanolic Moringa leaves fermented by lactic acid bacteria were used to treat hyperglycemia and hepatic steatosis in obese mice (Joung *et al.,* 2017). Moringa leaf extracts fermented by *Rhizopus oligosporus* were applied in mice to treat atopic dermatitis. This objective describes a novel means of

evaluating *In-vitro* digestibility, Proximate analysis, condensed tannins, and Probiotics-treated M. oleifera leaf phenolics.

3.2 MATERIAL AND METHODS

3.2.1 Study site

An *in-vitro* experiment was conducted at Egerton University Animal Nutrition laboratory. The University is situated at 0° 23' S, 35° 55' N within Njoro Sub-County, Nakuru County. The altitude is 1800 meters above sea level with an average annual rainfall of 900- 17 1,200 mm. The area has average daily temperatures ranging from 17°C- 22°C (Egerton University Department of Agriculture Engineering, Metrological Station 2018).

3.2.2 Collection and Processing of Moringa Leaves

The Moringa leaves were sourced from Meru**;** Meru is a county in Kenya located along the eastern side of the Mount Kenya region. Where local farmers in Meru were contracted to obtain the fresh leaves by cutting the tree branches and stripping the leaves off the tips of the branches by hand (manually), air dried them under a shade until they were crisp to the touch retaining their greenish colouration. The leaves were then milled using a hammer mill with a 5 mm sieve size. They were stored in airtight sacs for laboratory analysis.

3.2.3 *Preparation of experimental treatments*

There were four treatments with three replicates each; T1: MOLM treated with *clostridium butyricum* T2 MOLM treated *Bacillus coagulans*: T3: MOLM fermented using natural fermentation T4: untreated MOLM (control).

3.2.3 *Fermentation of Moringa oleifera*

The Moringa leaf meal samples were fermented with four fermentation set-ups according to the method described by Ogodo *et al*. (2018) with slight modifications. The MOLM was mixed with distilled water in the ratio of 1:0.5 (w/v) in a 500-ml beaker and mixed thoroughly with a hand mixer. The samples were sterilised in an autoclave at 121°C for 10 min to minimise the risk of contamination and allowed to cool for 30 min at room temperature (25 ± 2°C). The samples were inoculated with 0.00025 grams of 2.0×10^4 CFU/g of *Clostridium butryricum* and *Bacillus coagulans* strains and allowed to ferment in a solid state.

3.2.4 *Moringa oleifera leaf meal treated with Clostridium butyricum*

Clostridium butyricum powder was acquired from Feed Biotechnology Laboratory, China Agricultural University, and its concentration was 2.0×10^4 CFU/g. The *Clostridium butyricum* was included at 0.00025 grams mg/kg of feedstuff in the dry state per the manufacturer's instructions and recommendations and mixed thoroughly.

3.2.5 *Spontaneous fermentation of MOLM*

A mixture of 600 g MOLM with distilled water at a ratio of 1:2.75 (wt. /vol) in triplicate was incubated at 22°C for seven days using 250 ml plastic bottles (Jørgensen *et al.,* 2010). The plastic containers were sealed tightly to create anaerobic conditions. After seven days, the pH of individual samples was recorded using a portable pH meter (pH/ORP/ Temperature Combo Tester - HI98121 HANNA instruments), and a sample was obtained for proximate analysis.

3.2.6 *Fermentation of MOLM with Bacillus coagulans*

A single strain of commercial *Bacillus coagulans* powder was acquired from Feed Biotechnology Laboratory, China Agricultural University, and its concentration is 2.0×10^4 CFU/g. *Bacillus coagulans* were used as the starter culture. Three samples of 600mg of MOLM were mixed with distilled water at a ratio of 1:2.50 (wt. /vol), and the culture was added to the mixture (Chuah *et al*., 2021). The inoculated MOLM were incubated in the laboratory at 37°C in tightly sealed 750ml plastic bottles for 48 h. After 48 h, Samples were collected for proximate analysis. The pH of individual samples was measured and recorded using a digital hand-held pH meter (pH/ORP/Temperature Combo Tester - HI98121 HANNA instruments).

3.2.7 *Proximate analysis*

Dry matter (DM) was determined by drying in a hot air oven at 105°C for 24 h following standard methods (AOAC, 2006), ash by burning the samples in a muffle furnace at 550°C for eight hours following standard methods 923.03 (AOAC, 2006), ether extract by the soxhlet method (using ether) following standard methods 920.39 (AOAC, 2006). Total nitrogen for crude protein (N x 6.25) determination was obtained using the micro-Kjeldahl method following standard methods 920.87 (AOAC, 2006). Constituents of the cell wall, neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid lignin fibre (ADL) were determined using the Van Soest method (Van Soest *et al.,* 1991). Hemicellulose was determined as a difference between the neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid lignin fibre (ADL).(Nur-Nazratul *et al.,* 2021).

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

For the in-vitro digestibility to match the digestive system in the chicken stomach and intestines, a trial was carried out as described by Gabler *et al.* (2015). Four treatments with three replicates each were used: T1: Moringa treated with *Clostridium butyricum*, T2: Moringa treated with *Bacillus coagulans*, and T3 using natural fermentation.: T4: Untreated MOLM (control).

Step one (poultry stomach simulation phase)

A ground feed sample (0.4g) was weighed and placed in a 100 ml conical flask. A sample of 0.4 g was weighed and placed in a 100 ml conical flask. Sodium phosphate buffer solution, 200 ml (0.1 M, pH 6.0), was added to the flask and carefully mixed with the sample by stirring. To simulate the stomach digestive process (Sakamoto *et al.,* 1980), 80 ml of 0.2 M HCl was added, and the pH was adjusted to 2.0 with 1M HCl or 1M NaOH solutions (Park *et al.,* 2016). This was followed by adding prepared 5 mL pepsin porcine grade enzyme with 4x USP activity (Pepsin from porcine gastric mucosa powder, ≥250 units/mg solid Sigma-Aldrich Corp., St. Louis, MO, USA) containing 1 mg pepsin per ml 0.02 M HCl. To each conical flask, 2ml Chloramphenicol C-0378; Sigma-Aldrich Corp., St. Louis, MO, USA (0.5g/100ml ethanol) was added to inhibit bacterial growth. The flasks were closed and incu, incubated in a water bath at 39° C,red continuously for 2 hours.

Step two: Poultry intestines simulation

This step simulated the intestinal digestion of the poultry. The mixture from step one was mixed with 80 ml of phosphate buffer (0.2M, pH 6.8) and 20 ml of 0.6 M NaOH. The pH was adjusted to 6.8 using 1M HCl or 1M NaOH to provide a stable environment for intestinal enzymes to thrive, as described by (Ramaswamy, 2001). To the mixture, 10.6 ml of artificial pancreatin P-1750 Sigma-Aldrich Corp., St. Louis, MO, USA (porcine grade enzyme with 3 x USP activities) containing 100 mg/1 litre buffer was added and incubated at 39°C with continuous stirring for 4 hours. The residues were filtered through a nylon bag (pores size of (42 µm) , washed with distilled water, then washed twice using 20 ml, 95% ethanol, and 20 ml, 99.5% acetone. The residues were dried in an oven at 70°C for 12 hours and weighed**.**

Dry matter digestibility calculations

The *in-vitro* digestibility (IVDMD) of dry matter (DM) 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 (DM) and residual (DM), respectively.

3.2.9 Determination of condensed tannins and total phenolics

Extraction of tannins:

For the extraction process, aqueous acetone (70%) was used. Each of the dried (finely ground) samples (200 mg) was taken in a glass beaker of approximately 25 mL capacity. Ten mL of aqueous acetone (70%) was added, and the beaker was suspended in an ultrasonic water bath and subjected to ultrasonic treatment for 20 minutes at room temperature. The contents of the beaker were then transferred to centrifuge tubes and subjected to centrifugation for 10 min at approximately 3000 g at 4°C using a refrigerated centrifuge (Rahman & Lamara, 2023). The supernatant was collected and kept on ice. The pellet left in the tube was transferred to the beaker using two portions of 5 mL each of 70% aqueous acetone and again subjected the contents to ultrasonic treatment for 20 min. The supernatant was again collected as described above.

***Determination of condensed tannins (Proanthocyanins*)**

The method described by Porter *et al.* (1986) was followed for the determination of condensed tannins in the extracts. Briefly, Butanol–HCl reagent (butanol–HCI 95:5 v/v) was prepared by mixing 950 mL of n–butanol with 50 mL concentrated HCl (37%). Ferric reagent (2% ferric ammonium sulfate in 2N HCl) was prepared by dissolving 2.0 g of ferric ammonium sulfate in 2N HCl (16.6 mL of concentrated HCl was made up to 100 mL with distilled water to make 2N HCl). The reagents were stored in dark bottles. In a 100 mm x 12 mm glass test tube, 0.5 mL of the tannin extract diluted with 70% acetone was pipetted. The quantity of acetone was large enough to prevent the Absorbance (550 nm) in the assay from exceeding 0.6. Three mL of the butanol–HCl reagent and 0.1 mL of the ferric reagent were added to the tubes. The tubes capped with a glass marble were shaken using a Vortex and then placed on a heating block adjusted at 97 to 100°C for 60 min. After cooling the tubes, Absorbance was recorded at 550 nm. The Absorbance of the unheated mixture (considered as a suitable blank) was subtracted from the Absorbance of the heated mixture, which was the actual reading at 550 nm to be used for the calculation of condensed tannins.

The development of pink colour without heating the sample indicates the presence of flavanols. If this happened, one heated blank for each sample, comprising 0.5 mL of the extract, 3 mL of butanol and 0.1 mL of the ferric reagent, was used. Condensed tannins (% in dry matter) as leucocyanidin equivalent was calculated by the formula: (A 550 nm x 78.26 x Dilution factor)/(% dry matter). This formula assumes that the effective El%, 1 cm, 550 nm of leucocyanidin is 460 (Porter *et al.,* 1986). Here, the dilution factor is equal to 1 if no 70% acetone was added and the extract was made from a 200 mg sample in 10 mL solvent. Where 70% acetone was added (for example, to prevent the Absorbance from exceeding 0.6), the dilution factor was 0.5 mL/(volume of extract taken) in the current.

3.3 Statistical analysis

Data were analysed with IBM SPSS Statistics version 22. The assumption for normality and homogeneity of variance of the data was checked using Shapiro-Wilk and Levenes test statistics, respectively, with data assumed to be normal when (p>0.05). The IVDMD was analysed using the GLM model procedures. Mean separation was conducted using Tukey's HSD (Honest Significant Difference) test at a 0.05 level of significance. Application of Excel solver in Microsoft Excel was used in curve fitting and in the IVDMD

$$\boldsymbol{y_{ijk}} = \mu + \boldsymbol{\alpha_i} + \boldsymbol{\beta_j}\, \boldsymbol{\varepsilon_{ij}k}$$

where,

$\boldsymbol{y_{ijk}}$ = Observation on the dependent variables.

μ= overall population mean.

$\boldsymbol{\alpha_i}$= Fixed Effect due to the ith treatment.

$\boldsymbol{\beta_j}$=Random Effect due to trial**.**

$\boldsymbol{\varepsilon_{ij}}$ = random error term.

3.4 RESULTS

3.4.1 pH of fermented MOLM

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

Table 3.1 pH of treated and fermented MOLM

TREATMENTS	pH	p. Value
T1	3.63^{b}±.0.98	<.0001
T2	3.57^{b}±0.98	
T3	3.89^{a}±1.04	
T4	4.733^{a}±1.18	

This means within a column with the same letter are not significantly different (at a 5% level of significance). T1 was treated with *Clostridium butyricum*. T2 treated with *bacillus coagulans*—T3 natural fermentation. T4 Untreated MOLM.

3.4.2 The *in-vitro* digestibility of untreated, Probiotics treated, and natural fermented Moringa leaves

Treatment 2 resulted in the highest digestibility (66.24±0.76%) relative to the rest, while untreated fermentation T4 had the least (42.20±0.78%). T4 significantly differed from T2 ($p<0.05$). Inoculation using *Clostridium butyricum* (T1 57.24±0.74%) resulted in a higher IVDMD compared to spontaneous fermentation (T3 48.23±0.57%). There was an increase in IVDMD (Fig. 1) after treating the MOLM with the *Bacillus coagulans* (66.24±0.76) relative to the untreated (T4) (42.20±0.78%).

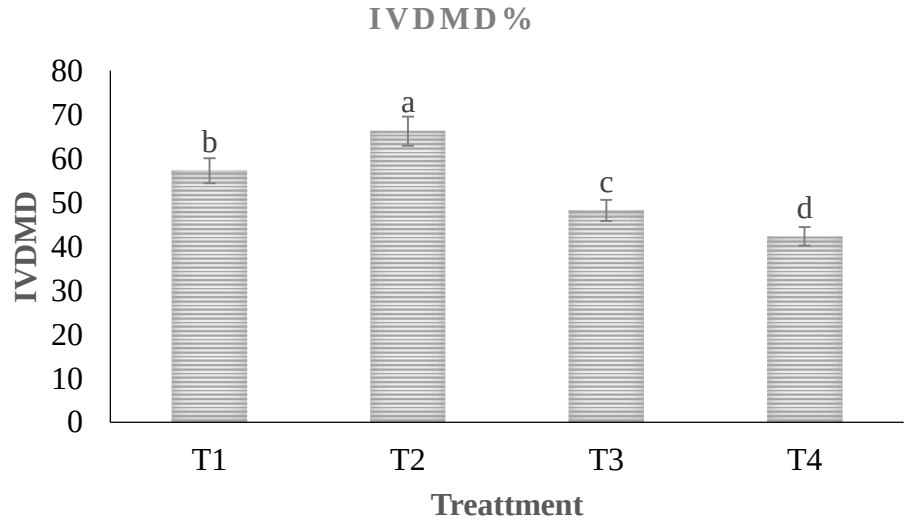

Figure 3.1 The *in-vitro* digestibility of probiotics, fermented and untreated moringa leaf meal. Error bars represent the mean (±SE) IVDMD of MOLM**.**

Table 3:2 Chemical composition of *Moringa oleifera* leaf meal concentration Mg/100g

	TREATMENTS				
Parameters	**Trt 1**	**Trt 2**	**Trt 3**	**Trt 4**	P-value
Moisture	7.86 ^{b}±0.26	6.7^{b}±0.31	12.83^{a}±0.28	8.71^{b}±0.16	0.0128
DM	92.14^{a}±0.26	93.30^{a}±0.31	87.17^{c}±1.98	91.28±^{ab} 0.17	0.0129
Crude Protein	29.31^{b}±0.29	33.41^{a}±0.57	27.18^{c}±0.55	27.48^{bc}±0.25	<.0001
Crude fibre	7.90^{c}±0.01	6.61^{d}±0.16	8.61^{b}±0.08	9.54^{a}±0.09	<.0001
Ash	9.28^{a}±0.03	7.75^{a}±0.05	8.63^{a}±0.2	8.55^{a}±0.71	0.2170
Ether Esther	8.73^{b}±0.05	7.35^{c}±0.01	10.4^{a}±0.25	6.73^{c}±0.11	<.0001
ADL	1.41^{ab}±0.2	1.06^{b}±0.07	1.73^{a}±0.06	1.78^{a}±0.01	0.0052
ADF	6.63^{b}±0.37	5.01^{c}±0.28	8.05^{a}±0.25	8.45^{a}±0.34	0.0002
NDF	9.95^{ab}±0.13	9.73^{b}±0.09	10.6^{ab}±0.12	10.9^{a}±0.38	0.0160
TANNINS mg	2.33^{b}±0.14	2.09^{b}±0.14	3.10^{a}±0.21	3.10^{a}±0.16	0.0040
Phenolics mg	1.13^{b}±0.01	1.04^{c}±0.01	1.16^{b}±0.01	1.26^{a}±0.01	<.0001

Means within a row with the different superscript letters ^{abcd} are statistically different p<0.05. T1= *Clostridium butyricum* treated moringa leaf, T2= *Bacillus coagulans* treated moringa leaf, T3 spontaneous fermentation of moringa leaf, T4 untreated Moringa ± represent standard error of the mean.

3.5 DISCUSSION

Recent evidence suggests that several important biochemical processes are used for Solid state fermentation, and that is necessary to achieve optimal SSF outcomes (Dhiman *et al.,* 2018). Several bacterial strains are able to grow as cultures to improve the nutritional quality of unconventional feed resources (Yao *et al.,* 2018). As reported by Wang *et al.* (2018), the nutritional quality and digestibility of MOLM were significantly improved via SSF using lactic acid bacteria. The application of several strains of bacteria in fermentation is relatively unexplored, and SSF of MOLM using *Clostridium* and *B. coagulans* has not yet been reported. In the present study, these microorganisms were used to ferment MOLM, which the success of MOLM fermentation can be explained in part by the fact that *Bacillus* has greater cellulose, pectinase, and amylase activities; this facilitates the degradation of cellulose and starch, which in turn increases the available carbon. *Bacillus* can also convert non-protein nitrogen into microbial protein and secrete enzymes that accelerate cellulose and starch degradation, thereby promoting mycelial growth; they can also produce additional functional compounds, thus improving the palatability of MOLM for livestock.

There was no significant difference between T1 and T2 in pH Table (3.1) of the fermented substrate. There was a significant decrease in the pH of the fermenting substrates in all the probiotics fermentation methods used. Compared to untreated, this is attributed to the highest concentration of lactic acid found in the microorganism during fermenting. This contributes most to the decline in pH during fermentation because it is about 10 to 12 times stronger than any of the other significant acids [e.g., acetic acid and propionic acid] (Kung *et al.,* 2018). The result of pH measurement in the growth medium indicated a reduction ranging between 4.4 and 3.2 by all the strains of LAB within 24 h; this could be a useful factor in the exertion of antagonism against spoilage and pathogenic organisms that may be associated with food products.

In-vitro dry matter digestibility

Treatment 2 had the highest digestibility (66.24±0.76%) relative to the rest, while untreated fermentation T4 had the least (42.20±0.78%). T4 significantly differed from T2 (p<0.05). Inoculation using *Clostridium butyricum* (T1 57.24±0.74%) resulted in a higher IVDMD than spontaneous fermentation (T3 48.23±0.57%). There was an increase in IVDMD (Fig. 1) after treating the leaves with the probiotics *Bacillus coagulans* (66.24±0.76%), relative to the untreated (T4) (42.20±0.78%). These characteristics showed that *B. coagulans* (Table 3.1) could be used to predict the survival of probiotic bacteria in the poultry intestinal tract. Therefore, the results obtained in the current study point in the direction of a good survival of *Bacillus coagulans* in the GI tract of poultry, as well as the potential to aid in the digestion of protein carbohydrates. This result agreed with (Lin sui *et al.* (2020), who report that *B. coagulans* showed good cholesterol assimilation potential in *in-vitro* dry matter digestibility.

From (Table 3.2.) all fermentation methods used positively affected the proximate, starch, NDF and ADF fractions. Moisture decreased, leading to increased DM, and Ash content increased, indicating mineral enrichment during fermentation. Spontaneous and probiotic fermentations improved the crude protein content of *Moringa oleifera* leaf meal.

Results revealed that the Moisture value of MOLM was higher in spontaneous fermentation, 12.83±0.28%, while the lowest was recorded in *Bacillus coagulans* (6.6±0.31), respectively. Moisture in feed determines the rate of feed absorption and assimilation within the body. It also determines the keeping quality of feed. Also, the increase in DM content of the fermented substrates in this study could be due to the microorganisms' biomass. (Igwe *et al.,* 2012). Fermentation, therefore, reduced the moisture content and improved the keeping quality. This can protect the fermented MOLM from mycotoxins attack, improve the keeping quality of fermented leaves and ensure longevity in case of use for large-scale feed manufacturing (Gillis *et al.,* 2016).

The results revealed that all the fermented substrates exhibited an increase in crude protein, where T2 *Bacillus coagulans* had the highest CP (33.41±0.57) compared to T4 untreated (27.48±0.25). T2 significantly differed from T4 ($p<0.05$). *Clostridium butyricum* (T1) resulted in a higher CP (29.31±0.29) than spontaneous fermentation (T3 27.18^{c}±0.55). The evolution of crude proteins during fermentation is represented in Table (3.2). There was a general increase in crude protein content ($p < 0.05$). However, this agrees with Léopold *et al.* (2013), who fermented Moringa leaves and he noticed an Increase from 38 g/100g DM to 44 g/100g DM for the MOLM powders fermented at 37˚C and from 33 g/100g DM to 39 g/100g DM for the young MOLM powders fermented at 37˚C were noticed. However, a significant quantity of crude protein in Moringa means that Moringa leaf protein concentrates could be used as a nutritionally valuable healthy ingredient to improve protein deficiency in the poultry diet. The improvement in the protein value of food during fermentation has been reported by several authors (Shi *et al.,* 2015). The increase in the protein content would be due either to the increase in the biomass supported in this case by the pH of the powders during the fermentation, which is in the interval of optimal pH of the lactic bacteria, or the reduction of the amount of dry matter with consumption of component such as sugars. (Noumo *et al.,* 2013).

Ash in feed contributes to the residue remaining after all the Moisture has been removed and after the organic materials (fat, protein, carbohydrates, vitamins, organic acid etc.) have been incinerated (Salma Sultana, 2020). The results finding in this study revealed that there was no significant difference ($p<0.05$) between T1, T2, T3, and T4 ((7.75^{a}±0.05, 9.28^{a}±0.03, 8.63^{a}±0.2, 8.55^{a}±0.71) respectively. in ash content in MOLM (Table 3.2); these values were lower (12.68 ± 0.51) than the ash reported by Thierry *et al.* (2013). Ash on feed determines largely the extent of mineral matters likely to be found on feed substance. Thus, the ash content of the dried MOLM is considered to be a measure of the mineral content. The results indicate that the dried MOLM have high deposits of mineral elements, which is in agreement with the previous findings (Moyo *et al*., 2011). Calcium is required for the formation and maintenance of bones and teeth, thus preventing osteoporosis. Calcium is also needed for normal blood clotting and nerve function.

From Table (3.2) the results revealed that Crude fat of MOLM was high in Spontaneous T3 (10.4^{a}±0.25) compared to T4 untreated (6.73±0.11) T1 and T4 did not differ from ($p < 0.05$) (Table 3.2), while T3 significantly differed from T2 (Table 3.2) these values agrees with the finding (Salma Sultana, 2020) who reported that the fat content is in the range (4.03,9.51%), which is desirable. Moreover, Moringa contains more dietary polyunsaturated fatty acids (PUFAs) than saturated fatty acids (SFAs). A higher content of PUFAs and a lower amount of SFAs is desirable as such, the inclusion PUFAs in the diet is recommended, as they can prevent the occurrence of diseases, thereby promoting good health in poultry. Madukwe *et al.* (2013) also reported that the fat content of dry Moringa leaves was 9.21%. The decrease observed in fat content may also be attributed to the breakdown of fat into free fatty acids by the Lactobacillus spp., some of which might have been used in flavour and aroma generation; such may be due to reaction with other components of the mash to form esters which produced the characteristic aroma of the beverage. This agreed with the work of Ouoba *et al.* (2005), who reported on the beneficial effects of lipase in the development of characteristic flavours and aromas.

The value of crude fibre obtained for MOLM is primarily composed of cellulose with small amounts of lignin, which is indigestible for poultry. The low fibre content in the study compared with most forage plants is interesting because the fibre fraction defines the extent and rate of feed digestibility. Although appropriate Crude fibre enhances digestibility and aids in the absorption of microelements, glucose and fat, its presence in high levels can cause intestinal irritation, lower digestibility and decreased nutrient usage (Sultana *et al.*, 2020). The crude fibre content of MOLM obtained from this study revealed that T2 *Bacillus coagulans* have lower crude fibre (6.61±0.16) relative to the rest, T4 Untreated record the highest CF(9.54^{a}±0.09), T2 significantly differ from T4 in the ($P>0.05$). T1 records lower CF (7.90^{c}±0.01) than T3 (8.61±0.08), respectively; this finding is in line with (Sultana *et al.,* 2020), who claimed that the crude fibre content of MOLM (6.00–9.60%) obtained was considered to be at the acceptable level, making MOLM a promising ingredient for animal diet, Crude fibre content of the fermenting substrate with microbial inoculation was lower compared to spontaneously fermented substrate and untreated substrate. This could have been due to the microorganism's ability to degrade fibre, hence unlocking the nutrients in MOLM, allowing for rapid microbial growth and enzymatic production, therefore, immediate and fast breakdown of Crude Fibre (Hu *et al.,* 2011).

Using different methods of fermentation are shown in (Table 3.2.) All fermentation methods affected the ADL, NDF and ADF fractions positively; plant cell substances can be divided into less digestible cell walls (made of hemicellulose, cellulose and lignin) and highly digestible cell contents (containing starch and sugars). Hemicellulose, cellulose and lignin are indigestible in non-ruminants, while hemicellulose and cellulose are partially digestible in ruminants. NDF is a good indicator of the "bulk fibre and has been used to predict feed intake; the probiotic fermentation of Moringa leaf has a significant effect on ADL, NDF and ADF fibre, thereby reducing the fibre contents after fermentation. *Bacillus* probiotics were reported to degrade crude fibres, cellulose and hemicellulose levels in *Leucaena leucocephala* leaf meal, wheat bran and grass pea seed meal (Ghosh *et al.*, 2017).

Anti-nutritional factors are compounds synthesised by the plant that reduce nutrient utilisation from plants or plant products and determine the use of particular plants as animal feed (Gemede & Ratta, 2014). They are synthesised through normal metabolism pathways in the plant as secondary metabolites intended for their plant defence (Itkin *et al.*, 2013). They affect the digestibility, bioavailability and utilisation of nutrients, mainly proteins, minerals and vitamins in food and reduce their nutritive values (Akinrinde & Adisa, 2014). Moringa leaves contain 21 g/kg phytate, 10.5 g/kg oxalates and a negligible amount of tannins, saponins, trypsin and amylase inhibitors (Teixeira *et al.*, 2014).

All fermentation methods significantly decreased ($p<0.05$) in tannins and phenolics contents. There was no significant difference ($P>0.05$). Between T1 *and* T2 Table (3.2), T1 and T2 differ from T3 and T4 tannin levels (Table 3.2), while There was no significant difference ($P>0.05$). from T3 and T4 (Table 3.2). Tannins levels were reduced to below detectable levels in fermented with *Bacillus* sp *Clostridium spp*. This agrees with (Amita *et al.*, 2014), who reported that tannin and phytic acid had been observed in all the fermented groups with BS, BC and SC, 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 (3.2), the finding shows that There was a significantly different ($P>0.05$). From T2 and T4 Table (3.2), T1 and T2 did not differ from ($p<0.05$) in phenol content of fermented MOLM (Table 3.2). This can be explained by the fact of cellulolytic, ligninolytic and pectinolytic enzymes produced by Microorganisms (bacteria) during the fermentation to break down the plant wall components and to hydrolyse the ester bonds which link phenolics to the cell wall, contributing to the release of the individual phenolic compounds from the matrix (Ajila *et al.*, 2012; Dulf *et al.*, 2018).

3.6 CONCLUSION

Fermentation with *Bacillus coagulans* resulted in improved *in-vitro*-dry matter digestibility compared to untreated; there was a significant increase in CP. CF, tannins and phenolics decreased in fermented substrates.

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