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# Effect of fermentation and enzyme treatment of mulberry leaf meal on enzymatic pre-digestion in the chicken simulated digestive system

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**Abstract**
This study examined the effect of (Natuzyme®) enzyme and fermentation treatments on *in-vitro* dry matter digestibility (IVDMD) of mulberry leaf meal (MLM). Pepsin-pancreatin hydrolysis was used in this experiment to mimic the chicken stomach. The MLM was subjected to four treatments replicated 3 times in a completely randomized design (CRD), Treatment 1: enzyme-treated MLM, Treatment 2: untreated MLM, Treatment 3: MLM fermented with *Bacillus coagulan* and Treatment 4: Naturally fermented MLM. Data on proximate analysis and digestibility were analysed using Statistical Analysis Software SAS version 9.4, general linear model (GLM). Tukey's test at ($p<0.05$) was used to do means separation where means were significantly different. This study's findings showed that the treatment method had a significant effect ($p<0.05$) on the *in-vitro* dry matter digestibility (IVDMD). MLM treated with enzyme (Natuzyme®) and *Bacillus coagulan* had improved IVDMD by 7.31% and 3.99% respectively compared to untreated ($p<0.05$). Natural fermentation significantly differed from enzyme treatment ($p$<0.05). Based on the outcome of this research pre-treatment of MLM with enzyme (Natuzyme®) enhanced *in-vitro* digestibility as compared to untreated, natural, and *Bacillus coagulan* induced fermentation.

**Keywords:** Chicken, enzyme, *in- vitro* digestibility, Mulberry leaf meal

## 1. Introduction
Millions of Kenyans depend on Kenya's livestock subsector for food, income, and employment, and indirectly for raw materials for the country's agro-processing industry. However, the GDP contribution of livestock to agriculture has been declining [26]. Feed prices affect the poultry sector significantly since feed costs account for 60–70% of poultry production costs [20]. Owing to the rising costs of traditional poultry feeds such as maize, soybean meal, and fish meal, consumers are looking for less expensive local alternatives [27]. Among the possible feed subtitute is mulberry leaf meal (*Morus Alba* [2]. Due to its high crude protein content (22-29.8%), balanced amino acid composition, and abundance in vitamins, trace elements, phytosterols, flavonoids, alkaloids, polysaccharides, and other bioactive compounds, its leaves are regarded as a high-quality forage plant resource [31, 11]. Despite the high protein content in mulberry leaves, it has high content of crude fibre which limits the high levels of inclusion in poultry feeds. The gastrointestinal tract (GIT) of chicken lacks the necessary microbes (fungi and bacteria) to digest fibre into products that are utilizable, in contrast to ruminants (cattle, sheep, and goats). Additionally, they lack the ability to use non-starch polysaccharides and oligosaccharides as well as cellulase, an enzyme that breaks down cellulose [6]. Feed and forages with more fibre content, in general, is detrimental to poultry. Plant cell walls are composed of cellulose, non-starch polysaccharides (NSP), pectin, and lignin [8]. The plant's NSP component is associated with antinutritive factors that can make it difficult for chickens to digest nutrients [8]. Thus, this study evaluated the impact of mulberry leaf meal's enzyme and fermentation treatment on *in-vitro* dry matter digestibility.

## 2. Materials and methods
### 2.1 Area of study
The experiment was conducted at the Animal Nutrition laboratory, department of animal sciences, Egerton University, which is located in the Njoro Sub-County of Nakuru County at

0° 23 S and 35° 55 N. The region is 1800 meters above sea level with an average annual rainfall of 900-1,200 mm and temperatures ranging from 17 °C to 22 °C.

### 2.2 Experimental layout and samples preparation

The *in-vitro* digestibility trial consisted of four treatments replicated 3 times, in a completely randomized design (CRD). The treatments were; $T_1$: Enzyme-treated MLM, T2: Untreated MLM, $T_3$: *Bacillus coagulan* fermented MLM and $T_4$: MLM treated using natural fermentation. In preparation of enzyme- treated MLM, the powdery enzyme (Natuzyme®) containing (12,000 parts/g of xylanase, 6,000 parts of cellulase, 1,500 parts/g of phytase 700 parts/g of beta-glucanase, 700 parts/g protease and 400 parts/g of alpha-amylases) was utilized. It was incorporated in triplicate and thoroughly mixed with the MLM samples. Based on the guidelines and instructions from the manufacturer, 350 mg/kg of dry feedstuff was used as the enzyme inclusion rate. An inoculant containing a single strain of *Bacillus coagulan* powder sourced from the feed biotechnology laboratory, China Agricultural University with a concentration of $2.0\times10^4$ CFU/g was utilized as the starting culture. The culture was added to three separate samples of 1 kg of MLM and then mixed with distilled water at a ratio of 1:2.50 (wt/vol) [7]. The inoculated MLM was cultured in the laboratory at ambient temperature in tightly wrapped 2kg plastic bottles for 7 days. The natural fermentation was prepared by incubating a mixture of 1 kg MLM and 1:2.75 (wt/vol) distilled water in triplicate for seven days at room temperature (22 °C) in two 2 kg sealed plastic bottles [19]. The pH of each sample was determined using a portable pH meter (pH/ORP/Temperature Combo Tester - HI98121 HANNA instruments) after 7 days, whereupon a sample was collected for proximate analysis.

### 2.3 Proximate analysis

During the dry matter determination, samples were dried in a hot air oven at 105 °C for 24 h [3] method.930.15, ash content was determined by burning samples in a muffle furnace at 550°C for 8h [3] method.942.05 and ether extract Soxhlet method (using ether) [3] method 920.39. Total nitrogen for crude protein (N x 6.25) was determined by Kjeldahl method [3] method 984.13. Neutral Using the Van Soest method, neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined [36]. The difference regarding the neutral detergent fibre (NDF) and the acid detergent fibre (ADF) was used to identify hemicellulose.

### 3. Enzymatic pre-digestion of mulberry leaf meal

The experiment was conducted to mimic the digestion in the chicken digestive system as described by [41]. The treatments were; Treatment 1: enzyme-treated MLM, Treatment 2: untreated MLM, Treatment 3: Fermented MLM with *Bacillus coagulan* and Treatment 4: Naturally fermented mulberry replicated three times**.**

A ground MLM sample (0.4g) was weighed and put in a 100 ml digestibility test tube before the addition of simulated gastric fluid. To mimic the *in vivo* activity of pepsin in chicken stomach fluid, a fluid containing 1,550 U/mL of pepsin (Sigma 10070; Sigma-Aldrich Co., St. Louis, MO) was used [33]. The gastric buffer solution contained 16.9 mmol/L of NaCl, 9.6 mmol/L of KCl, and 10 mmol/L of HCl to match the *in vivo* ionic concentration of gastric fluid from roosters [33]. The pH was raised to 2.0 at 41 °C by adding 200 mmol/L of HCl. Each digestibility test tube was put 2ml Chloramphenicol C-0378; Sigma-Aldrich Corp., St. Louis, MO, USA (0.5 g/100 ml 19 ethanol) to prevent bacterial growth. The test tubes were then sealed and incubated in a water bath at 39°C with continuous stirring for 2 h.

The first step's mixture was combined with 20 ml of 0.6M NaOH and 80 ml of phosphate buffer (0.2M, pH 6.8). The pH was brought down to 6.8 using 1M HCl or 1M NaOH to establish a stable environment for intestinal enzymes to perform well. 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 constant stirring for 4 h. Remains were put in 1.5ml centrifuge tubes and centrifuged (12700×g) for 2 min. The mixture was carefully withdrawn, washed twice with 20 ml of 95% ethanol and 99.5% acetone, and then rinsed with distilled water. Those that remained were then dried for 12 h at 70°C in the oven prior to weighing.

### 3.1 Computation of dry matter digestibility

The following formulae were used to determine the *in-vitro* digestibility (IVDMD) of dry matter (DM) as per [4].

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

Where,

The initial (DM) and residual (DM) are denoted by DMIn and DMRS, respectively

### 3.2 statistical analysis

Data on proximate analysis and digestibility were analysed using Statistical Analysis Software SAS version 9.4, general linear model (GLM). Tukey's test at ($p<0.05$) was used to do means separation where means were significantly different.

### 3.3 Statistical model

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$$

where;

$Y_{ijk}$ = overall effect of treatment

$\mu$ = overall mean

$\alpha_i$ = effect due to fermentation

$\beta_j$ = effect due to enzyme

$\varepsilon_{ij}$ = error term component

### 4. Results

**Table 1:** Nutrient composition of treated MLM

| Parameters | $T_1$ | $T_2$ | $T_3$ | $T_4$ | p-value |
|---|---|---|---|---|---|
| Moisture | $7.16^{c}$ ±0.07 | $6.92^{a}$ ±0.14 | $11.14^{b}$ ±0.03 | $15.48^{c}$±0.13 | <.0001 |
| DM | $92.84^{b}$ ±0.07 | $93.07^{a}$±014 | $93.07^{a}$±0.03 | $84.52^{c}$ ±0.13 | <.0001 |
| Ash | $11.34^{a}$±0.05 | $11.09^{a}$ ±0.33 | $10.75^{ab}$ ±0.03 | $10.11^{b}$±0.03 | 0.0309 |
| CF | $10.58^{b}$ ±0.09 | $12.05^{a}$ ±0.04 | $11.24^{ab}$ ±0.03 | $12.02^{a}$ ±0.33 | 0.0123 |
| CP | $28.42^{a}$ ±0.2 | $28.42^{a}$ ±0.2 | $26.94^{b}$ ±0.08 | $24.43^{c}$ ±0.16 | <.0001 |
| EE | $11.91^{b}$ ±0.12 | $14.18^{a}$ ±0.05 | $2.53^{d}$ ±0.13 | $9.75^{c}$ ±0.06 | <.0001 |
| NDF | $39.00^{a}$ ±1.00 | $25.00^{b}$ ±1.00 | $33.00^{ab}$ ±3.00 | $30.00^{ab}$ ±2.00 | 0.0484 |

| | | | | | |
|---|---|---|---|---|---|
| ADF | 25.58$^{a}$ ±0.32 | 20.48$^{b}$ ± 0.32 | 23.34$^{a}$ ±0.28 | 20.00$^{b}$ ± 0.32 | 0.0063 |
| ADL | 2.10$^{b}$ ±0.10 | 5.39$^{a}$ ±0.21 | 2.09$^{b}$ ±0.30 | 3.69$^{ab}$ ± 0.51 | 0.0281 |
| Hemicellulose | 13.75 | 4.52 | 9.66 | 10.00 | |

Means $^{abcd}$ within a row with different superscripts differ significantly ($p$<0.05). $T_1$: Enzyme-treated MLM, $T_2$: Untreated MLM, $T_3$: *Bacillus coagulan* fermented MLM $T_4$: MLM treated using natural fermentation.DM= Dry matter, EE= Ether extract, NDF= Neutral Detergent Fibre, ADF= Acid Detergent fibre, ADL= Acid Detergent lignin.

### 4.1 Nutritional quality of treated MLM

The moisture content of MLM in the four treatments were significantly different ($p$<0.05). The moisture content ranged between 6.92- 15.48%. DM content differed significantly in the four treatments at ($p$<0.05). Crude protein content differed significantly. Enzyme-treated MLM had the highest crude protein content of 28.42% while naturally fermented MLM had the least protein content of 24.43%. The crude fat content of the four treatments varied significantly from one another. The *Bacillus coagulan-treated* MLM contained crude fat of 2.53% while the Untreated MLM had 14.98%. The ash content varied from 10.11 to 11.34%. The highest ash content (11.34%) was recorded in enzyme-treated MLM. The NDF, ADF and ADL of MLM differed significantly among various treatments.

### 4.2 The enzymatic pre-digestion of untreated, enzyme-treated, and fermented mulberry leaf meal

The highest digestibility was achieved with enzyme treatment (65.78±0.22), while natural fermentation had the lowest digestibility (52.98±0.75). Natural fermentation and enzyme treatment were significantly different ($p$<0.05). In contrast to natural fermentation, IVDMD was enhanced by *Bacillus coagulan* inoculation. The IVDMD increased by 7.31% (Fig. 1) following the MLM's treatment with the enzyme (65.78±0.22), relative to the untreated (58.47±0.29).

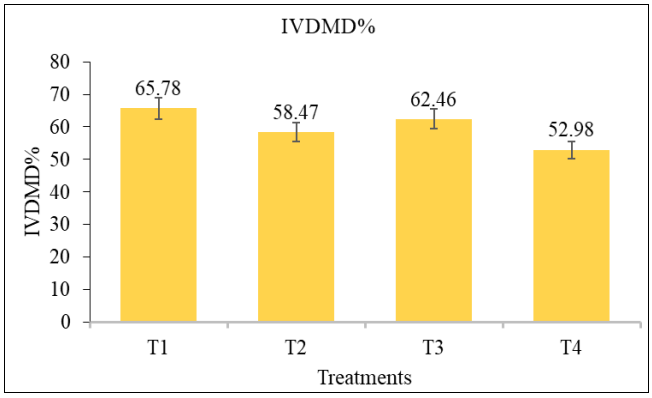


**Fig 1:** The *in-vitro* digestibility of enzyme-treated, fermented and untreated MLM. Error bars represent the Mean (±SE) IVDMD of MLM

### 5. Discussion

Since chickens are single-stomach, they lack the enzymes needed to break down complex carbohydrates like cellulose, hemicellulose, and lignin [34]. Because complex carbohydrates make up a sizable portion of fibrous by-products, it is essential to develop methods to make better use of these materials so that they can be added to poultry feed without negatively affecting the health and productivity of the birds. Utilizing *in-vitro* digestibility and fermentation techniques offers methods for assessing various alternate feed resources that can be used to enhance their digestibility and utilization.

Several variables, including the feed-to-water ratio, the inoculant used in the fermentation process, and temperature changes between the environment and the fermentation process, can affect how feed material ferments [22]. After the 7$^{th}$ day of fermentation, there was no significant difference ($p$>0.05) in pH results for naturally and *Bacillus coagulan* fermented MLM. Many researchers believed that additions of Lactic acid bacteria inoculant could improve the quality of silage fermentation. The pH value is regarded as a critical component influencing and improving the degree of fermentation quality of ensiled forage [10]. In both the control and treated silages, the final pH (4.5 or less) promotes optimal fermentation [5]. The pH for *Bacillus coagulan* and natural fermentation in this study varied between 4.6 and 5.67, which is higher than the pH of silage. The possible explanation for the disparency in pH could be differences in DM contents of mulberry leaves. Therefore, the reason for the rise in the pH value of mulberry leaf meal in this research could be due to the high dry matter content and less fermentable carbohydrates. According to many researchers, the population of LABs reduces because bacterial development slows down in low water activity due to the high dry matter content of ensiled forages [38, 30]. According to Hristov and McAllister [17], grasses and forages with high dry matter contents ferment more slowly than those with lower dry matter contents. The MLM treated with *Bacillus coagulan* however had a lower pH (4.6) compared to natural fermentations. In ensiled plants, water-soluble carbohydrates are utilized by epiphytic lactic acid bacteria (LAB), which then transforms them into lactic acid with a lesser proportion of acetic acid. This lowers the pH of the silage and inhibits unfavourable microbial growth, allowing it to be kept for a long period [32].

Mulberry leaf meal's nutritional content increased following fermentation, while the proportion of fibre decreased. In this research, the CF for untreated MLM was 12.05, naturally fermented MLM 12.02%, and *Bacillus coagulan* inoculant 11.24% while for enzyme treatment the CF was 10.58%. respectively. This could be due to the ability of *Bacillus coagulan* to lower fibre constituents, dietary anti-nutritional factors and improve feed nutritional quality in fermented feeds [39]. The findings of this study are consistent with [40, 11] who reported that unfermented and fermented mulberry leaf meal had a crude fibre range between 11-12.30%. Furthermore, [9] found reduced NDF and ADF contents in treated silages after *Lactobacillus plantarium* and *Bacillus coagulan* were added because of the acid breakdown of hemicellulose during ensiling. Additionally, *Bacillus coagulan* could produce the enzymes cellulase and feruloyl esterase, which may account for the pH drop caused by the released fermentable carbohydrates. NDF and ADF levels dropped indicating the destruction of plant structural carbohydrates, which may be connected to the production of fibrinolytic enzymes by *Bacillus coagulan* during the fermentation of silage [14]. This discovery might help to explain why NDF and ADF levels dropped after fermentations.

According to research reports, fermenting feeds lowers the quantity of anti-nutrients in the feed, increases the protein content (lysine, histidine, and methionine), improves the bioavailability of minerals (such as P, Ca, Mg, and Cu), and breaks down indigestible carbohydrates [26]. LAB fermentations have been proven to increase the amount of key amino acids like lysine, methionine, and tryptophan in cereals, hence enhancing their nutritional value [23].

Mulberry leaves can be conserved effectively by ensiling and

the addition of additives could enhance the silage's quality [12, 35]. In the present study, fermentations of mulberry leaves with *Bacillus coagulan* and natural fermentations led to the loss of organic matter, which was in line with [2] who reported decreased DM losses due to LAB inoculation in the combined silage of sorghum and soybean. The soluble carbohydrates are converted during LAB fermentation into lactic acid, acetic acid, ethanol, $CO_2$, and water, which represents a modest loss of DM and energy [28, 42]. Reported that additives (hemicellulose and *Lactobacillus plantarum*) induced a rapid pH decline at the early stage of ensiling and significantly decreased DM loss. In the present study, *Bacillus coagulan* inoculation, increased acid production and lowered the pH, inhibiting protein hydrolysis. It could be that inoculation with *Bacillus coagulan* increased the initial LAB load, accelerating fermentation, promoting acid production and pH decline and preventing the functioning of spoilage organisms and protein hydrolysis [15]. The crude protein of inoculated MLM increased in comparison to naturally fermented MLM. This could be explained by the efficient bioconversion of highly polymerized carbohydrates into microbial protein and the resulting production of several enzymes, many of which are proteinaceous in nature, could be responsible for the fermentation's notable increase in protein content [18, 37]. The increase in protein content in this study is consistent with the findings of [13] who reported that following fermentation, the crude protein of mulberry leaves increased.

In this study, both enzyme and *Bacillus coagulan* treatment improved *in-vitro* dry matter digestibility of MLM by 7.31 and 3.99% respectively while natural fermentation decreased it by 5.49% compared to untreated at ($p$<0.05). The increased IVDMD in enzyme treatment could be because of the (Natuzyme®) enzyme that contains cellulase, xylanase and phytase that decreases the anti-nutrient factors and crude fibre [1, 16]. The (Natuzyme®) enzyme also contains xylanase and cellulase, which have both been shown to hydrolyze plant cell wall polysaccharides and degrade their structure, permitting the release of oligosaccharides and other desirable compounds [25]. This could explain the improved digestibility of enzyme-treated MLM. While the digestibility of *Bacillus coagulan* treated MLM might be explained by reducing the main fermentation phase and achieving the expected fermentation quality. *Bacillus coagulan*, can consume oxygen sources to generate an anaerobic environment, which fastens the growth of lactic acid bacteria [29] and the acidity of silages, reducing nutrient loss by limiting the evolution of spoilage microbes. Additionally, increased IVDMD could be explained by lower fibre content in *Bacillus coagulan* fermentations compared to natural fermentation.

## 6. Conclusion

Based on the findings of this study, mulberry leaf meal treated with an exogenous enzyme (Natuzyme®) had better enzymatic pre-digestion compared to untreated, natural, and *Bacillus coagulan* induced fermentation.

## 7. Acknowledgements

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