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The Effects of *Lactobacillus Plantarum* and Cellulase on Mixed Silages of Amaranthus Hypochondriacus and Corn Meal: Fermentation Characteristics, Nutritional Value and Aerobic Stability

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Abstract: The aim of this experiment was to investigate the effects of moisture content, additives and their interactions on the fermentation quality, aerobic stability and *in vitro* digestibility of mixed silage of amaranth and corn meal. Amaranths and corn meal were mixed in mass ratios of 69:31, 76:24 and 84:16, and the silage water content was adjusted to 60% (W1), 65% (W2) and 70% (W3), respectively. Silage treatments included no additives (U), the addition of *Lactobacillus plantarum* (L), the addition of cellulase (E), and the addition of *Lactobacillus plantarum* + cellulase (M) mixed reagents. The results showed that pH and ammonia nitrogen (NH_3-N/TN) levels were significantly lower ($p < 0.05$) in W1 compared to W2 and W3. At the same time, dry matter (DM), organic matter (OM), *in vitro* dry matter digestibility (*iv*DMD), *in vitro* dry matter digestibility (*iv*DMD), *in vitro* organic matter digestibility (*iv*OMD), and *in vitro* crude protein digestibility (*iv*CPD) were significantly increased ($p < 0.05$). Meanwhile, the aerobic stability of mixed silage of seed amaranth and corn meal decreased with increasing water content. The aerobic stability of the L, E, and M treatment groups was improved by 15, 105, and 111 hours, respectively, compared to the control group at W1. pH and NH_3-N/TN levels were lower in the addition of E (E and M) than in the absence of E (U and L) (3.73, 20.1 g kg^{-1}DM vs. 3.64, 22.9 g kg^{-1} DM, $p < 0.05$).NDF and ADF were significantly lower with the addition of E than without the addition of E (598 g kg^{-1} DM, 145 g kg^{-1} DM vs. 632 g kg^{-1} DM, 160 g kg^{-1} DM, $p < 0.05$). However, CP, *iv*DMD, *iv*OMD, and *iv*CPD were significantly higher ($p < 0.05$). AA and NH_3-N/TN were significantly reduced ($p < 0.05$) with the addition of L (L and M) compared to without the addition of (U and E). In conclusion, the best fermentation quality, *in vitro* digestibility and aerobic stability of amaranth and cornmeal mixed silage treated with *Lactobacillus plantarum* + cellulase (M) were achieved at 60% water content.

Keywords: amaranth; fermentation quality; nutritional value; *in vitro* digestibility; aerobic stability

1. Introduction

With the development of livestock and poultry farming and the increased demand for feed resources, traditional silage cannot fully meet the needs of the farming industry.Therefore, the pursuit of reasonably priced, highly productive and efficient novel protein feeds to completely or partially replace traditional protein feeds is of great significance in improving the quality of animal products [1]. In such context, amaranth, as a plant-based protein feed with rich nutrients and unique properties, is considered as a feed resource with great potential.Amaranth can be grown in many areas of China, and the yield is generally 75,000-150,000 kg per hectare of fresh weight, with some varieties yielding as much as 225,000 kg, which has been considered to be a promising feed resource [2].

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Amaranth belongs to the genus Amaranthus of the family Amaranthaceae and is used as both human food and animal feed. It is rich in vitamins and minerals, with high protein content, high resistance and high yield, making it a high-quality feed resource for ruminants [3,4]. In addition, the dry matter of amaranth has good degradability and fermentation properties which can add value to ruminant feed. The addition of amaranth silage has been reported to contribute to increase body weight gain and reduce rumen methane emissions in male lambs [5-8]. However, amaranth cannot be preserved for a long period of time by conventional method due to its high protein content, high water content and thick stalks that cannot be easily dried into hay [3,4]. Therefore, silage is a good way to improve the utilization of amaranth, which can not only prolong the preservation time, but also improve the palatability of the feed. Amaranth is characterized by high moisture and low soluble carbohydrate content.Thus, preserving amaranth directly though conventional silage methods are considered difficult. The soluble carbohydrate content of amaranth was determined to be only 50.46 g kg^{-1} DM, which only meets the requirement of the 50 g kg^{-1} soluble carbohydrate content threshold for the preparation of superior silage [9]. It has been reported that the addition of 10% cornmeal improves the fermentation quality and apparent digestibility of silage [10]. Moreover corn meal, is characterized by high contents of soluble carbohydrates (WSC) and dry matter, is a good mixed silage auxiliary, which can directly increase the fermentation substrate, make up for the lack of fermentable carbohydrates in amaranth, and reduce the water content to improve the fermentation success of silage. However,to the best of our knowledge, few studies had been conducted to detect the effect of mixed amaranth silage with corn meal during ensiling.

The success of silage also depends on appropriate biological and chemical conditions that allow a rapid and sufficient decrease in the pH of the silage. Therefore, silage additives are recommended to manipulate fermentation and prolong aerobic stability [3]. Microbial additives such as lactic acid bacteria and cellulase can lead to a rapid drop in pH, facilitating the silage process and improving the fermentation quality [11].

Therefore, the aim of this experiment was to explore the effects of moisture and additives on the fermentation quality, aerobic stability and *in vitro* digestibility of mixed silage of amaranth and corn meal by adding lactobacilli and cellulase individually or in a mixture. This study can provide more feed choices and ways to utilize the resources in the farming industry, and promote the sustainable development of the livestock and poultry farming industry.

2. Materials and Methods

2.1. Experimental Materials and Design

The cultivation experiment was conducted in the experimental field of Jilin University (123.3°E, 44.1°N), China. Planting was carried out in June 2020, and harvest was in September.Amaranth is in full maturity at the time of harvesting.

Amaranth was chopped into approximately 1 to 2 cm lengths before ensiling. The mixing ratios (w/w) of amaranth and corn meal ingredients were 69:31, 76:24, and 84:16 with the moisture content of the silage ingredients was 60%, 65%, and 70%, respectively. For each silage moisture, the silage treatment was designed as follows: no additive (U), lactic acid bacteria inoculant (L), cellulase (E), and a mixed preparation of lactic acid bacteria and cellulase (M). For the lactic acid bacteria inoculant, Chikusou-1 (*Lactobacillus plantarum*) was obtained from Snow Brand Seed Co. Ltd. (Tokyo, Japan). Cellulase, Acremonium cellulase, was provided by Meiji Seika Pharma Co. Ltd., Tokyo, Japan (Lot No.: ACCF-6940). The dosage of lactic acid bacteria was 4.7×10^6 colony-forming units (cfu) per gram of fresh weight (FW). Meanwhile, Acremonium cellulase was added at a dosage of 50 mg kg^{-1} FW, with a measurable activity level of 4.2×10^{-3} U per gram of FW. Then, the mixed silage was loaded into a 5-litre plastic silo. The silage density was 550.1 ± 20.0 kg m^{-3} FW, and the silo was kept at room temperature (21°C-25°C) for anaerobic fermentation. After 60 days of fermentation, three repeating silos were opened for the determination of silage's chemical composition, fermentation quality and *in vitro* digestibility. The remaining silage was repeatedly mixed for the aerobic stability tests.

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2.2. Fermentation Quality Analysis

Once opened, silage samples were taken by the "tetrad" method. Subsequently, thoroughly mixed 20 g of silage with 180 mL of distilled water, and homogenized in a polyethylene vacuum bags for 1 minute. The sample was then extracted in a refrigerator at a constant temperature of 4°C for 24 hours, filtered through 4 layers of gauze and qualitative filter paper [15]. A portion of the resulting extract was used to measure pH using a pH meter (PHSJ-4F, Yidian Scientific Instruments Co., Ltd., Shanghai, China). The other part was frozen and stored at -20°C for the determination of organic acids and ammonia nitrogen (NH_3-N) contents. NH_3-Ncontentwas determined by the Robinson method [16]. Lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA) were determined by using high-performance liquid chromatography(column: ShodexRspak KC-811s-DVB gel column, Japan; detector: SPD-M10AVP; mobile phase: 3 mmol L^{-1} perchloric acid; flow rate: 1 mL min^{-1}; column temperature: 50°C; detection wavelength: 210 nm; injection volume: 5 μL).

2.3. Chemical Composition, Energy, and In Vitro DegradabilityAnalysis

The dry matter (DM) content of fresh samples and silages were determined in a 65°C oven for 48 hours. The dried samples were grounded and passed through a 1.0 mm sieve for chemical analysis. The contents of organic matter (OM) and crude protein (CP)were determined by the method of Official Association of Analytical Chemists [16]. The neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) contents were determined ac-cording to the methods reported by Van et al. [18]. The water-soluble carbohydrate (WSC) was measured using the anthrone-sulfate colorimetric method [19]. Buffering capacity (BC) was measured using the method of Playne and McDonald [20]. The gross energy (GE) content was determined by an oxygen cartridge calorimeter (SDAC1000, Sundy, Changsha, China).

In vitro degradability experiment was conducted according to the principles of the Laboratory Animal Guidelines for Ethical Review of Animal Welfare. The protocol of the study was reviewed and approved by the Animal Ethics and Welfare Committee of Jilin University (Jilin, China; Approval Number: SY202009600). The dried silage sample (0.5 g) was placed in a filter bag (ANKOM F57; diameter of hole 25 μm; Ankang Technology; Macedon, NY, USA) and sealed by hand pressure sealing machine (PFS-400; Zhejiang dongfeng packing machine co.ltd. Wenzhou, China) for later *in vitro* incubation. Before placing the samples, the fibre bags were rinsed with acetone and then thoroughly air-dried for 5 hours at 105°C in a forced convection drying oven (VL 115, VWR, Shanghai, China). A total of 196 fibre bags were prepared (48 silage silos × 4 parallel samples + 4 blank controls). The filter bag was processed and then loaded into a 130mL serum bottle. Four little-tailed billy goats were fed a mixture consisting of 40% corn silage, 20% alfalfa hay, and 40% concentrate (DM-based) twice daily. Ruminal fluid was collected from these animals. The rumen fluid was maintained at a temperature of 39°C under an atmosphere of carbon dioxide. The medium was filtered through four layers ofcoarse cotton cloth, and then the filtrate was mixed with McDougall artificial saliva at a 1:4 (v/v) ratio. Each serum vial was supplemented with a 60 ml mixture followed by incubation in a CO_2 atmosphere at 39°C. The incubation was performed in a water bath. After 72 hours of incubation, the filter bag was re-moved from the serum vial and gently rinsed with cold distilled water until the water becomes clear. The fibre bags are gently squeezed to remove excess moisture and then dried in a forced convection oven at 100°C for 24 hours. The residue is then weighed and measured for *in vitro* dry matter digestibility (*iv*DMD) [22]. *In vitro* neutral deter-gent fiber digestibility (*iv*NDFD) was determined by analyzing the residual NDF [23]. The formulas for *iv*DMD, *iv*NDFD, *in vitro* crude protein digestibility (*iv*CPD), and *in vitro* organic matter digestibility (*iv*OMD) are as follows: (respective weights of DM, NDF, CP, and OM before digestion-respective residual weight of DM, NDF, CP, and OM)/(respective weights of DM, NDF, CP, and OM before digestion).

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2.4. Microbiological Analysis

Fresh samples (20 g) were mixed uniformly with 180 mL of a sterile saline solution (0.85% NaCl), shaken on a shaker for 30 min. Then, 1 mL of the homogenate was taken for 10 × serial dilutions. Each gradient was prepared as 3 parallel replicates and poured into dishes. Finally, 100 μL of the dilutions at various concentrations were evenly applied on an agar medium as described below with coated rods. Lactobacillus bacteria were cultivated using De Man, Rogosa and Sharp agar media (Budweiser Technology Co, Ltd, Shanghai, China) via incubation for 48 hours under anaerobic conditions at 37°C. Aerobic bacteria were cultivated using nutrient agar medium via incubation at 37°C (Hope Biotechnology Co., Ltd., Qingdao, China). Yeast and moulds were grown using potato glucose agar medium at 28°C for 48 hours (Budweiser Technology Co., Ltd., Shanghai, China). The numbers of microorganisms were counted on plates of 20-200 cfu. All microbial data were converted to log10 cfu g^{-1}. The results are reported as fresh weight basis.

2.5. Aerobic Stability Analysis

Amaranth silage from each treatment was placed in a clean 1 L plastic bucket. A thermocouple wire was placed in the centre of the amaranth silage, and the ambient temperature was recorded by the thermocouple line in the empty bucket using a data recorder (OHR-G100T; Hongrun Company, Ltd., Fujian, China). Silage temperature was recorded at 1-hour intervals. Ambient temperature was also recorded every hour as a blank. Aerobic stability is the time taken for silage to reach a temperature 2°C higher than the ambient temperature.

2.6. Statistical Analysis

The data were analysed usingthe GLM program of SPSS statistical software (version 26; International Business Machine Corporation; Armonk, NY, USA)for each indicator according to the model:

$$Y_{ijk} = a + W_i + E_j + L_k + (W \times E)_{ij} + (W \times L)_{ik} + (L \times E)_{jk} + (W \times E \times L)_{ijk} + b_{ijk}.$$

In the above model, Y_{ijk} is the response variable, a is the overall mean, and W_i is the fixed effect of the moisture content of silage material i (i= 1, 2, 3), $E_j$is the fixed effect of cellulase j (j = 1, 2), L_k is the fixed effect of lactic acid bacteria k (k = 1, 2). $(W \times E)_{ij}$ is the interaction of silage feedstock moisture content i and cellulase j. $(W \times L)_{ik}$ is the interaction of silage feedstock moisture content i and lactic acid bacteria k. $(L \times E)_{jk}$ is the interaction of cellulase j and lactic acid bacteria k. $(W \times E \times L)_{ijk}$ is the interaction of moisture i, cellulase j, and lactic acid bacteria k. b_{ijk} is the residual error.

Multiple comparisons were made using Tukey's test based on the results of significance tests for water content, enzyme treatment, bacterial treatment and interaction.

3. Results

3.1. Chemical Composition and Microbial Counts of Fresh Materials

The chemical composition, gross energy, buffering capacity, and microbial counts of amaranth and corn meal mixed silage are shown in Table 1. The DM, CP, and WSC contents of amaranth were 185 g kg^{-1}, 124 g kg^{-1} DM, and 50.46 g kg^{-1} DM, respectively. The bufferingcapacity value of amaranth was 340 mEq kg^{-1} DM, which was 4.0 times higher than corn meal (mEq kg^{-1} DM). The number of lactic acid bacteria, yeast and mould adhering to the surface of amaranth was 2.42 log_{10} cfu^{-1}, 2.00 log_{10} cfu^{-1}, and 0.41 log_{10} cfu^{-1}, respectively.

Table 1. Chemical composition, energy, buffering capacity, andmicrobial countsof amaranth and corn meal.

Item	Amaranth	Corn meal
Chemical composition,energy, and buffering capacity		
Dry matter (g kg^{-1}FW)	185	873

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Organic matter (g kg^{-1} DM)	876	981
Crudeprotein (g kg^{-1} DM)	124	91.4
Neutral detergentfiber (g kg^{-1} DM)	651	303
Acid detergentfiber (g kg^{-1} DM)	377	85.0
Acid detergentlignin (g kg^{-1} DM)	111	16.7
Water-soluble carbohydrate (g kg^{-1} DM)	50.46	103.32
Gross energy (MJ kg^{-1} DM)	18.0	19.5
Buffering capacity (mEq kg^{-1} DM)	340	85.9
Microbial counts		
Lactic acid bacteria (log_{10} cfu^{-1} FW)	2.42	ND
Yeast (log_{10} cfu^{-1} FW)	2.00	ND
Mould (log_{10} cfu^{-1} FW)	0.41	ND

DM, dry matter; FW, fresh weight; cfu, colony-forming units; ND, not detected.

3.2. Fermentation Quality of AC-silage

Table 2 showed the fermentation quality of AC-silage. All treatment groups had pH values less than 4.0 after 60 days of silage. The addition of L (L and M groups) and E (E and M groups) significantly ($p < 0.05$) decreased the pH of silages. In addition, there was an interaction effect of W×E on pH ($p < 0.001$). The without the addition of E (U and L groups) significantly increased pH compared to the addition of E (3.74, 3.72 vs. 3.64, 3.63, $p < 0.05$). However, this effect was greater in W2 compared to W1 and W3. In contrast, the addition of L led to a decrease in pH (3.72, 3.63 vs. 3.74, 3.64), but L and W did not interact.

Water content significantly affected LA and PA content, with W2 having significantly higher LA content than W1 and W3 (20.3 g kg^{-1} DM vs. 16.9 g kg^{-1} DM, 14.6 g kg^{-1} DM, $p < 0.05$) and significantly lower PA content (0.00 g kg^{-1} DM vs. 0.03 g kg^{-1} DM, 7.34 g kg^{-1} DM, $p < 0.05$). The AA content of AC-silage was significantly lower (16.3 g kg^{-1} DM, vs. 17.8 g kg^{-1} DM) in group L compared to group U. The AA content of silage was significantly lower in group L compared to group U. The AA content of silage was significantly lower in group L compared to group U.

There was an interaction between W×E and W×L on BA content ($p < 0.05$). The BA content of the addition of E was significantly higher than that of BA without E (1.26 g kg^{-1} DM, 1.81 g kg^{-1} DM vs. 0.649 g kg^{-1} DM, 0.246 g kg^{-1} DM). With the addition of E, W3 had the lowest BA content. However, without the addition of E, W1 had the lowest BA content. The BA content with L addition was significantly lower than that without the addition of L (0.246 g kg^{-1} DM, 1.26 g kg^{-1} DM vs. 0.649 g kg^{-1} DM, 1.81 g kg^{-1} DM). Without the addition of L, BA content decreased with increasing water content. However, with the addition of L, the change in BA content with water content was not significant ($p > 0.05$).

There was an interaction between W × E, W × L, and L × E on NH_3-N/TN ($p < 0.05$). NH_3-N/TN decreased with decreasing water content (25.1 g kg^{-1} DM, 20.0 g kg^{-1} DM, and 19.3 g kg^{-1} DM, $p < 0.05$). However, without the addition of E, the mean NH_3-N/TN was higher than that of the E-added group (22.9 g kg^{-1} DM vs. 20.1 g kg^{-1} DM, $p < 0.05$). Meanwhile, the NH_3-N/TN with the addition of L was significantly lower than that without addition of L (20.6 g kg^{-1} DM vs. 22.4 g kg^{-1} DM, $p < 0.05$). With the addition of L, W1 has the lowest NH_3-N/TN. And without the addition of L, W2 has the lowest NH_3-N/TN. The addition of L significantly reduced NH_3-N/TN, but with the addition of E, the mean NH_3-N/TN was lower compared to without the addition of E (19.9 g kg^{-1} DM vs. 21.3 g kg^{-1} DM, $p < 0.05$).

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Table 2. Fermentation quality of amaranth and corn meal mixed silage prepared with lactic acid bacteria and cellulose.

Item‡	Moisture	Average	Additives†				SEM	Significance of main effects and interactions (*p*-value)						
			U	E	L	M		W	E	L	W×E	W×L	L×E	W×L×E
pH value	W1	3.66	3.69^{Ab}	3.63^{a}	3.69^{Ab}	3.62^{a}	0.002	<0.001	<0.001	0.001	<0.001	0.179	0.217	0.093
	W2	3.70	3.77^{Bb}	3.64^{a}	3.73^{Bb}	3.64^{a}								
	W3	3.70	3.77^{Bc}	3.64^{a}	3.73^{Bb}	3.62^{a}								
	Average	3.68	3.74	3.64	3.72	3.63								
LA (g kg^{-1} DM)	W1	16.9	16.6^{Aab}	20.6^{b}	12.2^{a}	18.1^{b}	0.078	0.020	0.811	0.338	0.116	0.599	0.953	0.455
	W2	20.3	22.4^{B}	17.7	21.1	19.8								
	W3	14.6	14.3^{A}	16.3	15.7	12.0								
	Average	17.2	17.8	18.2	16.3	16.6								
AA (g kg^{-1} DM)	W1	17.1	13.7	18.3	9.10^{A}	10.1	0.090	0.041	0.941	0.047	0.090	0.199	0.514	0.126
	W2	18.3	22.0	13.7	20.4^{B}	17.2								
	W3	17.5	16.5	24.2	15.0^{AB}	14.1								
	Average	16.2	17.4	18.7	14.8	13.8								
PA (g kg-^{1}DM)	W1	0.03	0.11	ND	ND	ND^{A}	0.131	0.002	0.082	0.681	0.053	0.836	0.535	0.671
	W2	ND	ND	ND	ND	ND^{A}								
	W3	7.34	ND	19.3	8.30	1.76^{B}								
	Average	2.46	0.37	6.43	2.77	0.59								
BA (g kg^{-1} DM)	W1	1.67	0.902^{a}	3.85^{Cb}	0.152^{a}	1.78^{a}	0.009	<0.001	<0.001	0.006	<0.001	0.008	0.433	0.139
	W2	1.06	0.721	1.59^{B}	0.330	1.58								
	W3	0.198	0.323	ND^{A}	0.255	0.017								
	Average	0.990	0.649	1.81	0.246	1.26								
NH_3-N (g kg^{-1} TN)	W1	19.3	21.0^{Ab}	19.9^{Aab}	18.1^{Aa}	18.3^{Aa}	0.016	<0.001	<0.001	<0.001	<0.001	0.036	<0.001	<0.001
	W2	20.0	21.4^{Ab}	19.2^{Aab}	21.1^{Bb}	18.4^{Aa}								
	W3	25.1	30.7^{Bc}	21.9^{Ba}	24.7^{Cb}	22.9^{Bab}								
	Average	21.5	24.4	20.3	21.3	19.9								

$^{A-C}$ Means of water contents within a column with different superscripts differ in the same additive treatment ($p < 0.05$). $^{a-c}$ Means of additives treatments within a row with different superscripts differ on the same water content ($p < 0.05$). SEM, standard error of the mean; W,moisture; A, additive. † L, lactic acid bacteria; E, cellulase; M, the mixture of lactic acid bacteria and cellulase. ‡ DM, dry matter; LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid; NH_3-N, ammonia nitrogen; TN, total nitrogen; ND, not detected; NA, not applicable.

3.3. Chemical Composition of AC-silage

Table 3 showed the chemical composition of AC-silage. The effect of water content and additives on the *in vitro* digestibility of mixed with silage is shown in Table 4. DM content increased with decreasing water content (287 g kg^{-1}, 330 g kg^{-1}, 389 g kg^{-1}, $p < 0.05$). There was an interaction effect of L × E on DM content ($p = 0.010$). The addition of L reduced the DM content of silage. However, the effect of E addition was greater compared to without the addition of E (329 g kg^{-1} vs. 340 g kg^{-1}, $p < 0.05$). There was an interaction effect of W × E on OM content ($p < 0.001$). With the addition of E, OM content was significantly lower than without the addition of E (940 g kg^{-1} DM, 941 g kg^{-1} DM vs. 942 g kg^{-1} DM, 942 g kg^{-1} DM, $p < 0.05$). However, this effect was minimized for W2 compared to W1 and W3.

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There was an interaction between W × E and W × L on CP content ($p < 0.05$). With the addition of E, the CP content was higher than that of the group without E (121 g kg^{-1} DM vs. 118 g kg^{-1} DM, $p < 0.05$). However, this effect is smaller for W1 compared to W2 and W3. In the addition of L, CP content increased ($p < 0.05$) with increasing water content. However, it was unaffected ($p > 0.05$) in silage without the addition of L.

There was an interaction of W × L on NDF content ($p = 0.024$). The NDF content of the addition of L treatment was significantly lower than that without the addition of L (631 gkg^{-1} DM, 588 g kg^{-1} DM vs. 632 g kg^{-1} DM, 607 g kg^{-1} DM, $p < 0.05$). With the addition of L, W1 had the highest NDF content, whereas without the addition of L, W3 had the highest NDF content.

There was an interaction effect of W × E on ADF content ($p = 0.033$). ADF content decreased with decreasing water content (190 g kg^{-1} DM, 153 g kg^{-1} DM, 115 g kg^{-1} DM, $p < 0.05$). Without the addition of E, ADF content was significantly higher than with E addition (160 g kg^{-1} DM, 160 g kg^{-1} DM vs. 146 g kg^{-1} DM, 143 g kg^{-1} DM, $p < 0.05$). However, this effect was smaller for W1 compared to W2 and W3.

There was an interaction between W × E and W × L on GE ($p < 0.05$). The addition of E increased GE content under W2 (18.9 MJ kg^{-1} DM, 19.0 MJ kg^{-1} DM vs. 18.7 MJ kg^{-1} DM, 18.8 MJ kg^{-1} DM, $p < 0.05$), but there was no significant difference between W1 and W3 conditions with the addition of E. The effect of W × E on the GE content of silage with the addition of L was not significant ($p < 0.05$), and the effect of W × L on the GE content of silage with the addition of L was not significant ($p < 0.05$). In silage with L addition, GE decreased ($p < 0.05$) with increasing water content, but was unaffected ($p > 0.05$) in silage without L addition.

Table 3. Chemical composition*and energy* of amaranth and corn meal mixed silage prepared with lactic acid bacteria and cellulose.

Item‡	Moisture	Average	Additives†				SEM	Significance of main effects and interactions (*p*-value)						
			U	E	L	M		W	E	L	W×E	W×L	L×E	W×L×E
DM (g kg^{-1})	W1	389	400^{Cb}	382^{Ca}	394^{Cb}	381^{Ca}	0.050	<0.001	<0.001	0.074	0.492	0.468	0.010	0.971
	W2	330	338^{Bb}	323^{Ba}	335^{Bb}	325^{Ba}								
	W3	287	296^{Ab}	280^{Aa}	292^{Ab}	280^{Aa}								
	Average	336	345	328	340	329								
OM (g kg^{-1} DM)	W1	952	954^{Cb}	951^{Ca}	953^{Cb}	951^{Ca}	0.015	<0.001	<0.001	0.832	<0.001	0.108	0.924	0.957
	W2	941	940^{B}	941^{B}	941^{B}	942^{B}								
	W3	931	933^{Ab}	929^{Aa}	933^{Ab}	929^{Aa}								
	Average	941	942	940	942	941								
CP (g kg^{-1} DM)	W1	118	119	118^{A}	116^{A}	118^{A}	0.022	<0.001	<0.001	0.766	0.002	0.017	0.773	0.035
	W2	120	117^{a}	122^{Bb}	118^{ABa}	121^{Bb}								
	W3	122	119^{a}	122^{Bb}	121^{Bab}	124^{Cb}								
	Average	120	118	121	118	121								
NDF (g kg^{-1} DM)	W1	627	643^{B}	608	644	613^{B}	0.368	0.004	<0.001	0.190	0.924	0.024	0.248	0.553
	W2	595	598^{A}	584	623	575^{A}								
	W3	621	654^{Bb}	628^{ab}	625^{ab}	576^{Aa}								
	Average	615	632	607	631	588								
ADF (g kg^{-1} DM)	W1	115	114^{A}	115^{A}	121^{A}	110^{A}	0.151	<0.001	<0.001	0.828	0.033	0.900	0.655	0.508
	W2	153	162^{B}	145^{B}	159^{B}	144^{B}								
	W3	190	204^{Cb}	177^{Ca}	201^{Cb}	176^{Ca}								
	Average	152	160	146	160	143								

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ADL (g kg^{-1} DM)	W1	19.9	19.1^{A}	21.1^{A}	19.4^{A}	19.8^{A}	0.033	<0.001	0.478	0.713	0.393	0.854	0.193	0.110
	W2	25.5	25.5^{B}	25.9^{BC}	24.3^{B}	26.1^{B}								
	W3	34.0	35.7^{C}	32.1^{C}	33.2^{C}	35.2^{C}								
	Average	26.5	26.8	26.4	25.6	27.0								
GE (MJ kg^{-1}DM)	W1	18.7	18.7^{B}	18.7^{B}	18.7^{B}	18.6^{B}	0.009	<0.001	0.274	0.467	<0.001	0.017	0.734	0.920
	W2	18.9	18.7^{B}	18.9^{C}	18.8^{B}	19.0^{C}								
	W3	17.9	18.0^{Ac}	17.9^{Aab}	18.0^{Abc}	17.8^{Aa}								
	SEM	18.5	18.5	18.5	18.5	18.5								

$^{A-C}$ Means of water contents within a column with different superscripts differ in the same additive treatment ($p < 0.05$). $^{a-c}$ Means of additives treatments within a row with different superscripts differ on the same water content ($p < 0.05$). SEM, standard error of the mean; W, moisture; A, additive. † L, lactic acid bacteria; E, cellulase; M, the mixture of lactic acid bacteria and cellulase. ‡ DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; GE, gross energy.

3.4. In Vitro Digestibility of AC-silage

There was an interaction effect of W×E on *iv*DMD, *iv*OMD, and *iv*CPD ($p<0.05$). *iv*DMD, *iv*OMD, and *iv*CPD were significantly lower in the without the addition of E than the addition of E (715 g kg^{-1} DM, 755 g kg^{-1} DM, 585 g kg^{-1} DM vs. 703 g kg^{-1} DM, 742 g kg^{-1} DM, 578 g kg^{-1} DM, $p < 0.05$). However, this effect was smaller in W1 compared to W2 and W3. There was an interaction effect of W×L on *iv*NDFD ($p = 0.024$). The addition of L increased *iv*NDFD content under W1 (595 g kg^{-1} DM, 565 g kg^{-1} DM vs. 594 g kg^{-1} DM, 560 g kg^{-1} DM, $p < 0.05$), but there was no significant difference between W2 and W3 conditions with the addition of E.

Table 4. *In vitro* digestibility of amaranth and corn meal mixed silage prepared with lactic acid bacteria and cellulase.

Item‡	Moisture	Average	Additives†				SEM	Significance of main effects and interactions (p-value)						
			U	E	L	M		W	E	L	W×E	W×L	L×E	W×L×E
*iv*DMD (g kg^{-1})	W1	737	738^{C}	737^{C}	732^{C}	741^{C}	0.117	<0.001	<0.001	0.828	0.033	0.900	0.655	0.508
	W2	709	701^{B}	715^{B}	703^{B}	715^{B}								
	W3	680	669^{Aa}	690^{Ab}	671^{Aa}	690^{Ab}								
	Average	709	703	714	702	715								
*iv*OMD (g kg^{-1} DM)	W1	777	778^{C}	777^{C}	772^{C}	781^{C}	0.118	<0.001	<0.001	0.828	0.033	0.900	0.655	0.508
	W2	748	740^{B}	754^{B}	742^{B}	754^{B}								
	W3	719	708^{Aa}	729^{Ab}	710^{Aa}	729^{Ab}								
	Average	748	742	753	741	756								
*iv*CPD (g kg^{-1} DM)	W1	589	590^{Cb}	589^{Bab}	586^{Ca}	591^{Bb}	0.040	<0.001	<0.001	0.733	0.001	0.204	0.585	0.078
	W2	582	577^{Ba}	586^{Bb}	578^{Ba}	585^{ABb}								
	W3	574	568^{Aa}	578^{Ab}	571^{Aa}	580^{Ab}								
	Average	581	578	584	578	585								
*iv*NDFD (g kg^{-1} DM)	W1	579	594^{B}	560	595	565^{B}	0.355	0.004	<0.001	0.190	0.924	0.024	0.248	0.553
	W2	548	550^{A}	537	575	528^{A}								
	W3	572	605^{Bb}	579^{ab}	576^{ab}	529^{Aa}								
	Average	567	583	559	583	541								

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[A-C] Means of water contents within a column with different superscripts differ in the same additive treatment ($p < 0.05$). [a-b] Means of additives treatments within a row with different superscripts differ on the same water content ($p < 0.05$). SEM, standard error of the mean; W, moisture; A, additive. [†] L, lactic acid bacteria; E, cellulase; M, the mixture of lactic acid bacteria and cellulase. [‡]*iv*DMD, *in vitro* dry matter digestibility; *iv*OMD, *in vitro* organic matter digestibility; *iv*CPD, *in vitro* crude protein digestibility; *iv*NDFD, *in vitro* neutral detergent fibre digestibility.

3.5. Aerobic Stability of AC-silage

The aerobic stability of AC-silage based on water content and additives is shown in Figure 1. The aerobic stability decreased significantly ($p < 0.05$) with increasing water content. The aerobic stability of the E and M treatments was significantly higher than that of the U treatment at all water contents.

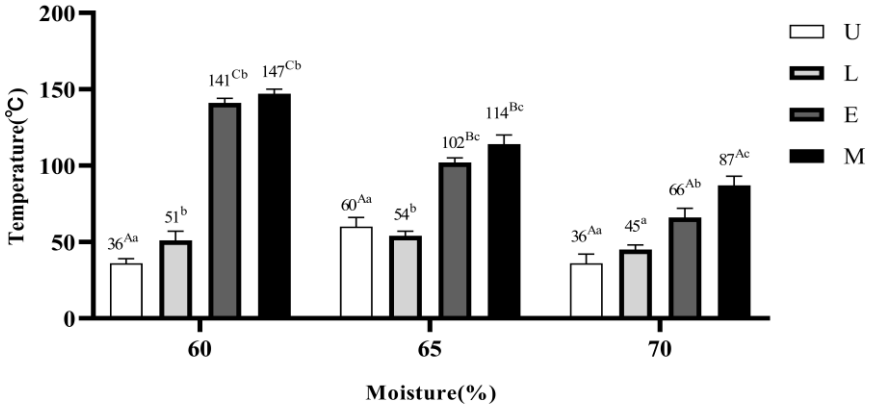

Figure 1. Time required for the temperature of amaranth and corn meal mixed silage to exceed room temperature by 2°C after exposure to air. U, control; L, lactic acid bacteria; E, cellulase; M, mixture of lactic acid bacteria and cellulase. [A–C] Means of water contents with different superscripts differ under the same additive treatment ($p < 0.05$). [a–c] Means of additives treatments with different superscripts differ under the same water content.

4. Discussion

In our experimental hypotheses, we speculated that the addition of lactic acid bacteria and cellulase at W1content could induce earlier lactic acid fermentation in the mixed silage and improve fermentation quality, *in vitro* digestibility, as well as aerobic stability. As shown in Table 2, The M treatment had the best quality. According to Table 4, *iv*DMD and *iv*OMD were the highest in M treatment at all water contents. As shown in Figure 1, the M treatment had the highest aerobic stability, which is consistent with our previous speculation.

4.1. Effect of Moisture and Additives on the Fermentation Quality of AC-silage

Water content is the main factor affecting silage quality. When silage water content is too high, it can lead to a negative quality in silage [20]. However, when the water content is too low, there will be more porous in the silage silos compared to silages with higher water content. Moreover, the low content of organic acids with antifungal activity (acetic acid) is not sufficient to inhibit the growth of yeasts, which can deteriorate quickly after opening [21]. Muck et al. showed that fermentation quality was improved and nutrient losses were reduced at silage moisture of about 65% [22]. Therefore, three moistures levels of 60%, 65%, and 70% were set in current study. The decrease in pH was more pronounced at lower water content conditions.This may be due to the higher DM content in low water content which provides more fermentation substrate. It enables lactic acid bacteria to produce large amounts of lactic acid while inhibiting the respiration of plant cells and reducing glycogen consumption and protein degradation. The experiment of Yahaya et al. found that high moisture

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silage with a high pH value was not as effective in fermentation as low moisture fermentation, which is consistent with the results of the present experiment [23]. In the present study, W×E had an interaction effect, and the E addition treatment further reduced the pH of the silage. This is due to the addition of cellulase, which breaks down the plant cell wall during ensiling and provides soluble sugars to lactic acid bacterial. Increased sugar content during the early stages of ensiling, promoted lactic acid bacteria colonization. This leads to a rapid increase in lactic acid and a decrease in pH, which in turn inhibits the protein hydrolysing activity of harmful microorganisms and plant enzymes [24, 25].Generally, the low pH means high lactic acid concentration, and the typical concentration of lactic acid in silage ranging from 2% to 4% of the DM. Interestingly, although the pH in this experiment was below 4.0, the lactic acid content was not high. This may be due to the fact that enterobacteriaceae could convert nitrate to nitrite, which then was converted to NO and NO_3 in a 2:1 ratio under acidic conditions, resulting in a lower pH [21, 26]. The BA content of E-added or L-added treatments ranged from 0.00 to 1.78 g kg^{-1} DM, which was low, indicating that lactic acid bacteria and cellulase preparations can reduce clostridial fermentation [27].

The NH_3-N/TN ratio is an indicator of protein hydrolysis activity, amino acid deamination, and decarboxylation. This is mainly due to the fact that protein hydrolysing Clostridium perfringens ferments amino acids through valine and leucine deamination and redox reactions between alanine and glycine. This usually indicates the degradation of nutrients in mixed silage [28]. The NH_3-N/TN ratiosof all silages in this experiment were within satisfactory limits (<10% TN), indicating that extensive protein hydrolysis did not occur [29]. Li et al. found that the addition of cellulase to cassava leaf silage significantly reduced NH_3-N, supporting the results of the present treatment. In addition, it has been shown that the addition of lactic acid bacteria can reduce the microbial diversity of clover, annual ryegrass, and their mixed silage and improve silage quality [30]. This may be due to the addition of exogenous lactic acid bacteria, shifting fermentation towards lactic acid with homofermentative lactic acid bacteria, or towards acetic acid with fermentative lactic acid bacteria. It also reduces the growth of clostridia and molds in silage, which reduces the degradation of proteins via the silage process and retains more nutrients in the silage, which is consistent with the results of this experimental [31]. The combined action of lactic acid bacteria and cellulase improved fermentation quality, reduced plant cell wall fraction and protein loss, provided more digestible substrate for rumen microbial fermentation, and promoted rumen digestion. The combined treatment of lactic acid bacteria and cellulase may have beneficial synergistic effects on the fermentation quality of *AC-silage* [25].

4.2. Effect of Moisture and Additives on the Chemical Composition and In Vitro Digestibility of AC-silage

The DM, GE, and *in vitro* digestibility of silage tended to decrease with increasing moisture. These results indicated that high moisture mixed silage had high loss of WSC and hemicellulose and low digestibility. This is in line with the results of Yahayaet al.'s study on orchard grass [32]. At the same time, we found an interesting phenomenon:

Numbered lists can be added as follows:There was the highest DM in all treatment groups at W1, but the CP content was the lowest. This may be because the addition of corn meal to regulate the moisture. However, the protein content of corn meal was 32.6 g kg^{-1} DM lower than the amaranth, thereby resulting in the lowest CP content in all treatment groups at W1. This is similar to the study of Mehrangiz et al., where it was found that the addition of molasses could lead to amaranth fermentation, increasing DM concentration [33]. In the report of Mehrangiz et al., it was shown that soluble and degradable CP fractions of amaranth as well as effective CP degradability were not affected by wilting or any additives to silage [33]. However, the addition of E treatment significantly increased the CP content in mixed silage compared to no E treatment. This may be because cellulase disrupts the plant cell wall and releases more plant proteins. The plant proteins continue to synthesize new bacterial proteins that are more easily digested and absorbed by the animals, which in turn promotes digestion and degradation and improves the *iv*CPD. This was also indicated by the results of a previous study on the mixed silage of soybean residue and corn stover by Zhao et al. [34]. W1 reduced the levels of ADF and ADL compared to W2 and W3. This is due to the increase of raw

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material leading to higher WSC content, and lower NDF and ADF levels in silage [35]. It was demonstrated that high-moisture silage tends to have higher cellulose digestibility. Morrison reported a similar increase in cellulose digestibility due to the action of extracellular cellulase, which leads to the shortening of the cellulose chain length and makes it more susceptible to enzymatic attack [36]. The addition of E treatment significantly reduced NDF and ADF content compared to no E treatment, which is similar to the findings of Lynchet al. on corn silage [37]. This may reflect the fact that the added fibrocystic enzymes increased the hydrolysis of cell wall carbohydrates, decreased their fiber content, and increased the WSC content. This result is in agreement with the findings of Foster et al. that the addition of cellulase to warm-season legumes and Bahia grass silage increased WSC content [35, 38].

*iv*DMD and *iv*OMD of mixed silage under M treatment were higher than the other groups, which may be due to the reduction of DM loss from silage with the addition of L and E treatments. As a result, *iv*DMD and *iv*OMD in the rumen were elevated. Low *iv*NDFD was observed in mixed silage under M treatment. This result may have two reasons. One is related to the hydrolysis of hemicellulose due to silage fermentation. Hemi-cellulose is acid-unstable under strong acidic conditions, and silage fermentation leads to hydrolysis of the most readily available feed structural carbohydrate [39]. Secondly, the addition of lactic acid bacteria enhanced NDF fermentation and increased hydrolysis. At the same time, cellulase treatment reduced the amount of available NDF degraded by rumen microorganisms in mixed silage [40]. At W3, *in vitro* digestibility was significantly increased in mixed silage under E and M treatments compared with U and L treatments. So we can infer that *in vitro* digestibility and NDF and ADF contents were negatively correlated, and our conclusions were the same as those of Bao et al. [35].

4.3. Effect of Moisture Content and Additives on the Aerobic Stability of AC-silage

Aerobic instability is the underlying cause of the loss of nutrients and DM, and mycotoxins produced from undesirable microorganisms also lead to health risks in people and animals. Therefore, aerobic stability is an important factor affecting the nutritional quality and subsequent feeding value of silage in ruminants [29]. Aerobic microorganisms metabolize and consume nutrients, and a change in silage temperature is usually used as an important parameter to evaluate the aerobic stability of silage [41].

In this experiment, the aerobic stability AC-silage decreased with increasing water content (Fig. 1). This may be due to the fact that a moist environment is more favorable for the growth of microorganisms such as yeasts, acetic acid bacteria and, acid-tolerant yeasts can survive in silage [42]. Increased yeast growth rate in high moisture treatments was also demonstrated in a study of total mixed ration by Hao et al [43].

The aerobic stability of L, E, and M treatments was improved at all water contents in this experiment. This is due to the fact that the inoculated lactic acid bacteria have anisolactic acid metabolic pathway that capable of producing acetic acid during fermentation after opening the silos. Thus, effectively controlling the yeast and filamentous fungi could improve aerobic stability [44]. In addition, according to Kaewpila et al., the addition of cellulase can improve the aerobic stability of Napier Pakchong grass, which was consistent with our experimental results [18]. The exposure time of all of the M treatment groups was longer than the other groups, which may be due to the synergistic effect of lactic acid bacteria and cellulase when used together. Many studies have shown that lactic acid bacteria or cellulase can have a positive effect on improving the aerobic stability of mixed silage by lowering pH and NH_3-N content, and reducing yeasts and clostridia [25]. As a result, the M treatment group was more stable during aerobic exposure and reduced spoilage losses in silage fermentation.

5. Conclusions

In summary, silage water content, lactic acid bacteria and cellulase affect the fermentation quality, nutrient content, *in vitro* digestibility and aerobic stability of mixed amaranth and corn meal silage. In this study, the simultaneous addition of Lactobacillus and cellulase at 60% water content

had the lowest pH, PA, AA, and NH_3-N/TN and therefore the best fermentation quality. At the same time, mixed silage under the above conditions had the lowest content of ADF, the highest content of *iv*DMD, *iv*OMD, and *iv*CPD, and higher content of DM and OM, thus providing higher nutritional value and digestibility. However, further *in suit* experiments are needed in this experiment to evaluate the effect of seed amaranth and cornmeal silage mix on rumen growth performance.

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